Fly in the coop

How black soldier fly larvae improve broiler and pig welfare

Allyson F. Ipema

Propositions

1. The potential of black soldier fly larvae to benefit broiler and pig welfare depends more on the provisioning method than on the amount provided.
   (This thesis)
2. For broilers, live larvae promote better welfare than dried larvae.
   (This thesis)
3. Incentives for reviewing scientific papers will significantly improve the speed of the reviewing process.
4. A “women quota” in science sends the message that a woman’s value is purely based on being female.
5. Patents on pharmaceutical research endanger human health.
6. Legislation to ban certain species as pets will lead to suboptimal care of these pets.

Propositions belonging to the thesis, entitled

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Part 3: The big picture

Chapter 1

General Introduction

Background

As the global population continues to expand, food systems are challenged to become more sustainable. This means that food systems must meet the needs of the current population without compromising the needs of future generations. Currently, poultry meat and pork provide the most consumed animal proteins worldwide (OECD/FAO, 2019). Moreover, many people, especially in developing countries, depend on poultry meat and pork for essential nutrients such as essential amino acids and most B vitamins (de Smet, 2012). As the global population is expected to rise to 9.7 billion people in 2050, the demand for poultry meat and pork is also expected to grow (OECD/FAO, 2019; United Nations Department of Economic and Social Affairs, 2022). Rearing meat-type chickens, also called broilers, and pigs puts extensive pressure on the environment, which is not sustainable. A major part of these environmental pressures are related to the production of livestock feed (de Vries and de Boer, 2010). Commercial pig and broiler diets often contain high amounts of human edible crops (e.g., soy, grains), and the production of these crops as feed requires the use of much land and water and competes with alternative uses such as the production of crops used as food (Gerber et al., 2013; Ran et al., 2017). Therefore, the largest possibilities to improve livestock production system sustainability lie in maximizing the use of by- and waste-products in livestock feed.

To address the need for sustainable livestock production, recent studies have pointed to insects as a feed ingredient that may be increasingly used in the coming years (e.g., Smetana et al., 2016; van Huis and Oonincx, 2017). Insects are part of the natural diet of both chickens (Savory et al., 1978) and pigs (Schley and Roper, 2003), and many insect species are suitable for inclusion in their commercial diets. Using insects as feedstuffs can have environmental benefits. For example, a livestock diet in which a 50:50 mixture of soybean meal and fishmeal was replaced with meal made from houseflies reared on organic waste streams required 98% less land use. This diet also had the potential to decrease energy use by 38% and to decrease the global warming potential by 61%, though these two factors depended highly on the housefly rearing substrate (van Zanten et al., 2015). Insects that can be reared on biological waste streams such as food waste or manure are expected to be most sustainable as livestock feed ingredients, because they can convert inedible waste to nutrients and thereby limit the environmental impact of this waste and minimize feed-feed competition (Bosch et al., 2019; Salomone et al., 2017).

Besides the need for livestock production systems to become more sustainable from an environmental perspective, there is also increasing societal concern about animal welfare. More than nine in ten EU citizens have the opinion that livestock welfare should be protected, and eight in ten EU citizens believe that livestock welfare should become better than it is now (European Commission, 2016). According to Fraser (2008), good animal welfare requires consideration of three interdependent pillars: 1) natural behaviour, 2) health and biological functioning, and 3) affective states. Natural behaviour considers behaviour that an animal is intrinsically motivated to perform, such as exploratory and foraging behaviour, and their environment must facilitate these behaviours to achieve good welfare. Good health and functioning signify that an animal's biological needs, such as an appropriate diet and possibilities to rest, are met, and that an animal does not experience substantial physiological disturbances including impaired health and stress. Finally, affective states encompass all emotions and feelings that an animal experiences, and for good welfare these should be balanced towards positive (e.g., pleasure, happiness, satisfaction) and away from negative (e.g., pain, suffering, hunger) affective states (Fraser, 2008). In the EU, several directives exist that set some minimum requirements for livestock rearing practices in an attempt to improve livestock welfare. For example, Directive 2001/93/EC states that pigs must have permanent access to some foraging materials to increase the opportunities to perform natural behaviour, and Directive 2007/43/EC limits broiler stocking densities to support better broiler health. However, scientific studies cannot confirm that implementation of these directives has actually improved broiler and pig welfare (European Commission, 2017; van de Weerd and Ison, 2019), suggesting that the requirements stated in the directives are insufficient and more changes to commercial rearing environments are needed.

Interestingly, insects potentially play a role in both reducing the environmental impact of poultry and pork production and improving broiler and pig welfare by meeting the need to forage, but this potential has not yet been explored. One promising insect species that could achieve both these goals is the black soldier fly (*Hermetia illucens*), specifically its larvae. To understand the potential of black soldier fly larvae to benefit broiler and pig welfare I will first discuss the relevant characteristics of these larvae. Subsequently, I will describe common welfare problems of broilers and pigs and suggest how black soldier fly larvae could potentially mitigate these problems, identifying key knowledge gaps. These knowledge gaps will be compiled into the overall aim and scope of this PhD thesis.

Black soldier fly larvae

Life cycle and composition

The black soldier fly is a true fly belonging to the Stratiomyidae family. Black soldier flies are found throughout the tropical and temporal regions of the world (Sheppard et al., 1994). They have a short life cycle, developing into fertile adults in approximately 5-6 weeks (Tomberlin et al., 2009). Adult flies deposit their eggs in cracks near larval feeding sites, where the eggs take about four days to hatch. Black soldier fly larvae, or BSFL, normally reach the prepupal stage in 2-4 weeks (Li et al., 2011), though under suboptimal rearing conditions (e.g., food shortage or low temperatures) they can prolong their developmental time for up to four months (Furman et al., 1959). BSFL can grow on a wide range of substrates, including fruit and vegetable waste, animal offal, manure, and human faeces (Diener et al., 2011; Lalander et al., 2013; Nguyen et al., 2015; St-Hilaire et al., 2007; Webster et al., 2016), making them a suitable species for inclusion in circular food production systems where waste is re-introduced into the system and converted into edible protein.

BSFL are generally rich in moisture (~55-65%), protein (~38-58% of dry matter (DM)), and fat (~15-49% of DM), while they are poor in fibre (~7% of DM) (reviewed by Barragan-Fonseca et al., 2017; Makkar et al., 2014). The protein content and amino acid profile of BSFL are similar to those of soybean (Veldkamp and Bosch, 2015) and are quite consistent across rearing substrates (Barragan-Fonseca et al., 2017). In contrast, the fat content and the fatty acid profile are more dependent on the fat content and composition of the diet, with generally high levels of lauric acid (35-50% of fatty acids) and palmitic acid (10-13% of fatty acids) (Cullere et al., 2019; B. Kim et al., 2020). The fibre fraction in BSFL consists only of chitin. Although both chickens and pigs produce chitin-degrading enzymes (Kawasaki et al., 2021; Tabata et al., 2017), the presence of chitin may impair nitrogen retention and DM digestibility (Khempaka et al., 2011, 2006), which may hinder nutrient utilization. For both broilers and pigs, the amount and the composition of BSFL protein and fat are such that they can replace part of their diet, though more studies are required to optimize the inclusion level (Makkar et al., 2014).

The short life cycle of black soldier flies and the wide range of possible rearing substrates and favourable composition of BSFL make *Hermetia illucens* a promising insect species to be used in broiler and pig diets.

BSFL and livestock welfare

Providing BSFL to broilers and pigs might influence all three pillars of welfare (i.e., natural behaviour, health and functioning, and affective states). There are two ways in which BSFL can influence the pillars of livestock welfare, namely through their nutritional properties and by functioning as environmental enrichment.

Concerning the nutritional properties, BSFL contain several compounds that have the potential to benefit broiler and pig health and functioning (reviewed by Koutsos et al., 2022). As mentioned before, lauric acid is abundantly present in BSFL fat (Cullere et al., 2019), and this acid has been found to inhibit the growth of several harmful bacterial species *in vitro* (Skřivanová et al., 2006; Suksathit and Tangwatcharin, 2013). Through this antimicrobial effect high levels of dietary lauric acid may benefit the gut microbiota composition of livestock, though results from *in vivo* studies are inconclusive. For example, one study found that lauric acid influenced the gut microbiota composition and immune function of broilers (Wu et al., 2021) but another study found no effects of esterified or free dietary lauric acid on the gut microbiota composition of broilers (Zeitz et al., 2015). BSFL also produce a broad spectrum of antimicrobial peptides, or AMPs (Vogel et al., 2018). AMPs have immunomodulatory properties, such as promoting T cell proliferation (Ren et al., 2015). Consuming AMPs can improve an animal's intestinal mucosal immunity, body weight gain, and nutrient digestibility, and it can benefit their intestinal morphology and microbiota (as reviewed by Wang et al., 2016). Finally, chitin and its derivates such as chitosan oligosaccharides may have anti-viral and anti-tumour activities (Nishimura et al., 1984; Suzuki et al., 1984). Including chitin or its derivates in feed was found to improve for example feed efficiency, immune organ development, and immune function of animals (Huang et al., 2005; Lee et al., 2008; Shenghe et al., 2017).

While some individual compounds in BSFL have the potential to benefit animal health and functioning, the effects of dietary BSFL on livestock health have been poorly investigated compared to their effects on livestock production performance. **Supplementary Tables S1A-B & S2** provide an overview of the studies on effects of dietary BSF (i.e., BSF larvae or pre-pupae meal (full-fat or defatted), BSFL oil, or whole BSFL) on the production performance (i.e., average daily gain, final weight, average daily feed intake, feed conversion ratio), gastro-intestinal tract development, blood composition, and health parameters of fast-growing broilers (**S1A**) and pigs (**S1B**). The main trends of these studies are summarized here. For broilers, BSFL inclusion in the diet often had positive or no effects on broiler production performance. However, at high inclusion levels (> 10% of the total DM intake) some negative effects on production performance were observed (**Table S1A**). The production performance of pigs also benefitted from or was not affected by dietary BSFL inclusion in all studied treatments except one (**Table S1B**). Dietary BSFL had inconsistent effects on the gastro-intestinal tract development of broilers and pigs. For example, the gizzard weight of broilers was reduced, increased, or unaffected by dietary BSFL in seven, two, and 14 of the treatments in which this was measured, respectively (**Table S1A**). Also, the villus height in the small intestine of pigs was increased (two treatments) or not affected (13 treatments) by dietary BSFL (**Table S1B**). For broilers and pigs, dietary BSFL either enhanced (three and two treatments for broilers and pigs, respectively) or did not influence (one treatment for both broilers and pigs) β-diversity of the intestinal microbiota (**Table S1A,B**). Several studies investigated a wide spectrum of blood components, where some dietary BSFL treatments caused no change in blood composition (six and two treatments for broilers and pigs, respectively) and some caused changes in the concentration of one or more components (three and nine treatments for broilers and pigs, respectively), though the type of affected components was not consistent across studies (**Table S1A,B**). Only two studies investigated the effect of dietary BSFL on broiler health after a health challenge (i.e., a *S. Gallinarum* or an infectious bronchitis challenge), and here dietary BSFL had positive effects on their recovery after the challenge (J. Lee et al., 2018; Zhang et al., 2021). One study investigated the effect of dietary

BSFL on pig health after *Escherichia coli* K88 infection and found that dietary BSFL improved the intestinal barrier function and regulated several immunological pathways (Tang et al., 2022).

Besides the nutritional effects, BSFL could benefit broiler and pig welfare by functioning as environmental enrichment. Environmental enrichment is defined as any addition to the environment of an animal that increases the biological relevance of the environment and thereby benefits welfare (Newberry, 1995; van de Weerd and Day, 2009). The potential function of BSFL as environmental enrichment for broilers or pigs has not been studied yet, and as this depends on and relates to species-specific biology and welfare problems, it will be discussed separately for broilers and pigs.

Broiler welfare

To understand how BSFL as environmental enrichment can benefit broiler welfare, I will first highlight the main welfare problems of broilers. Then, I will indicate why and how BSFL might function as environmental enrichment, and how this could potentially enhance broiler welfare.

Broiler welfare problems

Most welfare problems experienced by broilers are a result of their fast growth rate and are worsened by their commercial housing conditions. Broiler strains can be divided into slow-growing (< 50 g/day) and fast-growing (> 50 g/day) strains. Currently, fast-growing broilers can have an average daily body weight gain of more than 66 g/day, reaching a slaughter weight of 2.16 kg in 32 days (Singh et al., 2021). This fast growth rate results in growth imbalances where broilers' skeletal and visceral development cannot keep up with the rapid muscle gain. Compared to lighter broilers, heavier broilers have a displaced centre of gravity that requires them to place their feet further apart to remain balanced, causing excessive pressure on the leg bones (Corr et al., 2003a). As a result the leg bones of heavy broilers are relatively short and light, and their low concentrations of calcium and phosphorus suggests they are also fragile (Corr et al., 2003b; Güz et al., 2021). Impaired leg development often results in leg health problems such as tibial dyschondroplasia or varus/valgus deformities that can cause varying degrees of lameness (Granquist et al., 2019; Shim et al., 2012). Severe lameness, defined as having an obviously or severely abnormal gait that affects the ability to move (i.e., a gait score of 3 or higher on a scale of 0-5, Butterworth, 2009), affects between 8-58% of broilers across studies (Bassler et al., 2013; de Jong et al., 2019; Granquist et al., 2019; Knowles et al., 2008; Sanotra et al., 2001). Slower development of visceral organs, such as the lungs and heart, compared to the muscles can cause afflictions like hypoxia, ascites, and Sudden Death Syndrome (Gonzales et al., 1998; Julian, 2000). Oxidative stress as a consequence of unbalanced growth can lead to myopathies including wooden breast and white striping (Kuttappan et al., 2013; Norring et al., 2018). All these afflictions are problematic because they can be painful, limit the expression of natural behaviour, hinder the broiler's ability to access feed and water resulting in starvation or dehydration, and even result in death (Bradshaw et al., 2002; Danbury et al., 2000; ESFA, 2010; Knowles et al., 2008; Weeks et al., 2000).

Concerning behaviour, fast-growing broilers already show relatively low levels of active behaviour at a young age compared to slower-growing broilers (Bizeray et al., 2000), and near the end of the rearing period fast-growing broilers spend between 60-80% of their time sitting without performing any active behaviours (Baxter et al., 2018a; de Jong and Gunnink, 2018). Activity supports proper leg development, therefore the high inactivity of broilers can contribute to leg health problems (Reiter and Bessei, 2009). Furthermore, the body and legs of inactive broilers are in long-term contact with the wet and soiled litter, resulting in occurrences of contact dermatitis such as foot pad dermatitis, hock burn, and breast blisters (de Jong et al., 2014; Kaukonen et al., 2016). The prevalence of contact dermatitis varies across rearing systems. One study found that from the observed broilers 67.1% suffered from foot pad dermatitis and 52.6% of broilers showed signs of hock burn (de Jong et al., 2019). A large-scale study in the Netherlands showed that up to 38% of broilers had mild to severe foot pad lesions (de Jong et al., 2012). Overall, broiler health problems are prevalent in many rearing systems, and are identified as a major concern for broiler welfare (ESFA, 2010).

The housing conditions of broilers, specifically the high stocking densities and barren, stimulus-poor pens, further limit their behavioural expression. This has a negative impact on broiler activity and, by extension, welfare. EU-regulated stocking densities can be up to 42 kg/m^2 according to Directive 2007/43/EC. High stocking densities can restrict broiler movement and decrease broiler activity (Hall, 2001), although other conditions such as temperature, humidity, and litter and air quality may be even more important for broiler welfare (Dawkins et al., 2004). Barren broiler pens further limit activity as they do not facilitate active natural behaviours that broilers are highly motivated to perform, such as foraging behaviour and dust bathing (Bessei, 2006). Semi-wild red junglefowl spend approximately 60% of their active time ground pecking and 34% ground scratching in search for feed items (Dawkins, 1989), and the high motivation to perform foraging behaviour remains in broilers even when they have continuous access to feed (Bokkers and Koene, 2004, 2003a). Also, broilers regularly perform dust bathing when they have access to clean and preferred substrates (Baxter et al., 2018b; Bokkers and Koene, 2003a). Under commercial housing conditions, foraging behaviour is not reinforced as all feed is localized in feeders (Jordan et al., 2011), and dust bathing is impeded by the absence of a suitable substrate (Baxter et al., 2018a). Performing these behaviours satisfies intrinsic motivations and is therefore emotionally rewarding (Franks and Higgins, 2012) and not being able to perform them can cause frustration and stress, indicated for example by an increase in corticosterone production when dust bathing substrate is removed (Bradshaw et al., 2002; Vestergaard et al., 1997). Barren environments also limit perceptual learning, memory abilities, and habituation to novelty, and this can cause broilers to have excessive fear and stress responses when they are disturbed, for example by a visit from the farmer (Altan et al., 2013; Newberry, 1995). Stress, in turn, can result in a more pessimistic affective state (Anderson et al., 2021a; Iyasere et al., 2017).

It is abundantly clear that the high growth rate of broilers combined with their rearing conditions diminishes broiler welfare regarding natural behaviour, health and functioning, and affective states. One possible solution to improve broiler welfare is to reduce broiler growth rates. While there are initiatives to reduce broiler growth rates in the future (e.g., by using slow-growing strains or feed restriction), a recent report indicates that currently in the EU up to 95% of broilers are still fast-growing (European Commission, 2017) and in other regions this may be even higher. These broilers suffer from welfare problems now and providing BSFL could improve their welfare at present. Furthermore, reducing growth rates will not solve all welfare problems, as a large-scale study on slow-growing broilers housed at 34 kg/m^2 found that approximately 40% of these broilers had abnormal gaits and approximately 18% showed signs of hock burn (Rayner et al., 2020). Therefore, BSFL could also positively impact the welfare of slower growing broilers.

Providing BSFL to improve broiler welfare

BSFL might benefit broiler welfare as they could potentially enrich the environment of broilers by facilitating natural, active foraging behaviours. The effects of providing BSFL as environmental enrichment to broilers have not yet been studied. It is known that mealworms are attractive to broilers, as they are often used as a reward in broiler behavioural tests (Bokkers and Koene, 2004; Simon et al., 2015) and a short (i.e., 9 days) study found that scattering mealworms through the pen elicited more broiler activity than scattering whole wheat or wood shavings (Pichova et al., 2016). Insect larvae such as mealworms and BSFL are likely attractive because their high fat content makes them very palatable (Makkar et al., 2014). Foraging behaviour is reinforced by consuming feed items as the behaviour is extrinsically rewarded, and more palatable feed items are expected to reduce the rate of habituation and thus maintain high levels of foraging behaviour for a longer period of time (Tarou and Bashaw, 2007). Indeed, scattering mealworms was described to greatly increase the frequency of foraging behaviours in broilers, compared to broilers without scattered feed items (Pichova et al., 2016), although this was only investigated for short periods (up to 30 min). Both dried and live insect larvae may facilitate foraging behaviour. However, live larvae can move around, and as moving insects are more attractive to broilers than stationary insects (Clara et al., 2009; Jones et al., 1998), live larvae are expected to be more attractive and thereby more suitable for this purpose.

By facilitating active natural foraging behaviours, it is expected that BSFL provisioning can also improve broiler leg health and promote more positive affective states. So far, it has proven difficult to stimulate broilers to be active, as perches, platforms, straw bales, brightly coloured moving lights, strings, oat hulls as dustbathing substrate, and scattering whole wheat through the pen did not significantly increase broiler activity throughout the rearing period (Bailie et al., 2013; Bailie and O’Connell, 2015; Baxter et al., 2018c; Bizeray et al., 2002a; Norring et al., 2016). Strategies that did promote activity include increasing the distance between feed and water or placing a barrier between feed and water (Bizeray et al., 2002a; Reiter and Bessei, 2009). These strategies force broilers to be physically active to reach sustenance, which can impair their welfare if they suffer from health problems that make activity painful and/or difficult. While some materials did not stimulate activity on their own, combinations of enrichment materials, such as a combination of perches, ramps, platforms, and a dustbathing area (de Jong et al., 2021), or straw bales, platforms, and laser projectors (da Silva et al., 2021) did promote activity, though these may be too expensive and labour-intensive to apply commercially. Several studies indicate that increasing broiler activity for longer periods of time (e.g., by forcing broilers to walk to obtain feed and water) improved leg development, reflected by higher leg bone densities, reduced bending and twisting of leg bones, and reduced severity of lameness and contact dermatitis (da Silva et al., 2021; Reiter and Bessei, 2009; Ruiz-Feria et al., 2014; Ventura et al., 2012). Therefore, if BSFL stimulate active foraging behaviours, this is expected to also improve broiler health. BSFL can be provided in the litter, and if this facilitates litter scratching then this might promote drying of the litter, further reducing the risk of contact dermatitis (de Jong and van Harn, 2012).

Offering combinations of enrichment materials has benefitted the affective state of broilers. For example, providing broilers with plastic balls, plastic bottles, toys, and mirrors reduced broiler fearfulness (Altan et al., 2013). Also, providing broilers with wooden boxes containing peat, platforms, ramps, peat bales, and lucerne hay bales increased the occurrence of comfort behaviours such as body shaking, wing stretching, and ground pecking, which may indicate a more positive affective state (Vasdal et al., 2018a). Finally, broilers with access to pecking stones, dust baths, perches, and toys had a more optimistic state of mind than barren-housed broilers (Anderson et al., 2021a). As foraging for and consuming the highly palatable BSFL is expected to be very rewarding for broilers, this may have a positive influence on their affective state without requiring extensive environmental modulations, though this remains to be studied.

Pig welfare

As for broilers, providing BSFL might benefit pig welfare. Commercial pig production has several stages, including mating, gestation, farrowing, weaning, and rearing, and each stage imposes distinct welfare challenges on pigs (as reviewed by Pedersen, 2017). There are two rearing practices that can have major and long-lasting effects on pig welfare throughout multiple production stages, namely early and abrupt weaning, and barren housing. I will discuss how these two practices may impair pig welfare, followed by how BSFL could potentially mitigate these effects.

Pig welfare problems

Early and abrupt weaning

Weaning encompasses the transition from being dependent on the sow's milk for nutrients to independently consuming solid feed items. In nature, weaning is a gradual process that can take several months (Jensen and Recén, 1989; Newberry and Wood-Gush, 1985), during which piglets are slowly introduced to a wide range of solid feed items including plants, nuts, seeds, insects, and small animals (Schley and Roper, 2003). In this setting, piglets start to learn how to forage and sample feeds by observing the sow and other piglets already from the first day after birth, and from approximately 4 weeks of age they start to consume substantial amounts of solid feed items (Petersen, 1994). In contrast to natural weaning, commercial weaning in Europe generally takes place abruptly when piglets are between 3-4 weeks of age. From birth until weaning, piglets are typically housed in barren pens with their (crated) sow and siblings, where they have access to one type of pelleted feed ("creep feed") provided *ad libitum* in a feeder. These conditions limit the possibility to develop and perform foraging behaviours such as sniffing, nosing, rooting, and chewing feed items (Hötzel et al., 2004). Foraging behaviour precedes eating behaviour (Berridge, 2004), therefore the inability to perform extensive foraging behaviours may limit solid feed intake before and directly after weaning (Kuller et al., 2010). Indeed, the pre-weaning intake of creep feed is generally low and variable within and between commercial litters (Collins et al., 2013; Hedemann et al., 2007; Middelkoop et al., 2020a), and studies indicate that 30-66% of piglets have not sampled any solid feed when they are weaned at 4 weeks old (Bruininx et al., 2004; de Ruyter et al., 2017; Shea and Beaulieu, 2014).

Because of this, many 4-week-old piglets have little experience with behaviours that are essential after weaning when only solid feed is available, such as chewing hard pellets (Cox and Cooper, 2001; Middelkoop et al., 2019a). Furthermore, the gastro-intestinal tract (GIT) of these piglets is often unprepared for solid feed items, as solid feed intake supports, among others, the shift in microbial enzymes from lactases to (glycol)amylases and the development of a more suitable gut morphology (Heo et al., 2013) and microbiota (Choudhury et al., 2021). Therefore, low solid feed intake before weaning hinders digestion and nutrient utilization directly after weaning (Choudhury et al., 2021).

The abrupt feed transition during commercial piglet weaning is achieved by instant removal of the sow, and this is often combined with other stressful events such as transferral to unfamiliar and barren weaner pens and grouping with piglets from other litters. When several stressors related to weaning occur simultaneously this intensifies the overall stress experienced after weaning (Weary et al., 2008). This stress combined with the inexperience with solid feed items often results in a drop in feed intake directly after weaning (Bruininx et al., 2002; Colson et al., 2006), and it can take up to five days after weaning for piglets to reach the feed intake level that is required for physiological maintenance (Bruininx et al., 2001). The drop in feed intake and the subsequent consumption of novel feed items can impair GIT development and functioning, for example by increasing intestinal pH, decreasing villous height, increasing crypt depth, and impairing mucosal barrier functioning (Hedemann et al., 2007; Hu et al., 2013; Lee et al., 2021; Smith et al., 2010; Verdonk et al., 2007). These changes result in malabsorption of nutrients and allow for easier passage of pathogens, which can have short- and long-term ramifications for piglet production performance (e.g., diminished growth rates) and health (e.g., diarrhoea) (Cabrera et al., 2010; Lallès et al., 2004; Moeser et al., 2017), impairing pig welfare.

Early and abrupt weaning does not only have feed-related consequences. Mixing of different litters during the weaning transition often causes high levels of aggressiveness and fighting to establish ranks (D'Eath, 2005; Hayne and Gonyou, 2006). Aggressive behaviours such as biting and head knocking can cause painful lesions and wounds (Melotti et al., 2011), and receivers of aggressive behaviour can exhibit signs of chronic stress (de Jonge et al., 1996). Pigs housed in barren weaner pens exhibit more aggressive behaviours directly after weaning than pigs housed in more complex environments, likely due to the absence of any relevant distractions (Melotti et al., 2011; Oostindjer et al., 2011b). Besides aggression, manipulation of pen mates, including mounting, (belly) nosing, sucking, nibling, or chewing part of the other piglet's body, also increases after weaning. For example, one study found that in barren pens the occurrence of these behaviours more than doubled after weaning compared to before weaning (Oostindjer et al., 2011b). These behaviours can be a result of trying to cope with weaning stress and can be harmful to the receiving piglets, causing obvious welfare concerns (Camerlink et al., 2012; Oostindjer et al., 2011b). It is known that aversive and/or stressful experiences in the early life of an animal can result in chronic stress and impaired emotional development later in life (Holmes et al., 2005; Munsterhjelm et al., 2010). As such, the commercial weaning transition may have long-lasting negative consequences for a pig's affective state.

Barren housing

Despite Directive 2001/93/EC that indicates that pigs must have continuous access to appropriate foraging materials, in practice pigs are generally housed in barren pens that contain no such materials, or only simple objects that do not stimulate exploratory behaviour (van de Weerd and Ison, 2019). Like broilers, pigs are also highly motivated to perform exploratory and foraging behaviour, because in nature these behaviours provide critical information about the environment necessary for survival (Stolba and Wood-Gush, 1989; Studnitz et al., 2007). One study found that the frequency of foraging behaviour of piglets provided with peat during the pre-weaning period was 23.8 times higher than that of piglets without access to peat, despite all pigs having *ad libitum* feed access (Vanheukelom et al., 2011). This indicates that domestic pigs are still highly motivated to perform foraging behaviour. Insufficient behavioural stimulation in barren pens can lead to frustration, stress, impaired health (e.g., wounds caused by tail biting), and pessimistic affective states (Douglas et al., 2012; Ernst et al., 2006; Munsterhjelm et al., 2010; Schrøder-Petersen and Simonsen, 2001). Barren housing was also found to diminish pig production performance compared to enriched housing, resulting in for example a lower feed intake and body weight gain (Luo et al., 2020b; Oostindjer et al., 2010), though some studies found no such difference (Camerlink et al., 2014; Morrison et al., 2007). A reduced production performance might be attributed to the reduced opportunity to develop feeding-related behaviour under barren conditions, and/or to the enhanced stress experienced in such environments (Luo et al., 2020b).

To appease their exploratory motivations and to try to cope with the stress of barren housing, pigs will often redirect exploratory behaviour such as rooting and chewing towards pen fixtures or other pigs, for example by bar chewing, tail biting, or belly nosing (Dybkjær, 1992; Schrøder-Petersen and Simonsen, 2001; Widowski et al., 2008). Performing and receiving oral manipulative behaviours has been associated with stress, lesions, inflammation, and diminished production performance (Camerlink et al., 2012; Munsterhjelm et al., 2013; Torrey and Widowski, 2006), all indicating impaired pig welfare. Especially tail biting is very prominent in pigs, with some studies showing that up to 23-94% of pigs with long (non-docked) tails had tail lesions (Abriel and Jais, 2013; Lahrmann et al., 2017; Telkänranta et al., 2014a; Veit et al., 2016). As a result, tail docking is still common practice in the EU as a preventive measure for tail biting, even though routine tail docking has been prohibited by EU law since 1994 (Algers et al., 2007). Tail docking can be painful and stressful (Noonan et al., 1994), and while it reduces the occurrence of tail biting 2-4 fold, it does not fully eliminate tail biting (Larsen et al., 2018; Valros and Heinonen, 2015). Furthermore, it is likely that tail docking will be more strictly regulated in the future due to societal pressure, therefore alternatives to reduce tail biting are required.

Providing environmental enrichment could facilitate exploratory behaviour and reduce harmful behaviour, thereby improving pig welfare. However, the effectiveness of different enrichment materials varies greatly (reviewed by Buijs and Muns, 2019; van de Weerd and Day, 2009). It is generally accepted that point-source objects, which are the predominant type of commercially applied enrichment, do not maintain pig's interest for long periods of time (van de Weerd and Ison, 2019) and they do not reduce harmful behaviour such as tail biting (Buijs and Muns, 2019). In contrast, rooting substrates such as straw and peat, provided that they are regularly replenished, are very interesting for pigs, and they effectively redirect the exploratory behaviour of pigs from pen mates towards the substrate, thereby reducing harmful behaviour (Bulens et al., 2015; Schrøder-

Petersen and Simonsen, 2001; van de Weerd and Day, 2009). However, slatted flooring prohibits the wide-spread use of these substrates commercially. Finally, several studies showed that combinations of enrichment materials have many benefits for pigs (e.g., Oostindjer et al., 2011b; Telkänranta et al., 2014b), though these require higher costs and more labour, making them less economically feasible for a farmer to use.

To conclude, by diverging from the natural weaning and environmental conditions of pigs, commercial rearing diminishes pig welfare with regards to natural behaviour, health and functioning, and affective states. Providing effective environmental enrichment mitigates part of these welfare problems, and BSFL might potentially be effective environmental enrichment for pigs.

Providing BSFL to improve pig welfare

BSFL could benefit pig welfare by stimulating solid feed intake around weaning, and by facilitating exploratory and foraging behaviours in otherwise barren pens. The effects of providing whole insect larvae to pigs have, to the author's knowledge, never been studied. However, whole BSFL do possess many characteristics that were found to be attractive to pigs, such as being odorous, manipulable, destructible, chewable, palatable, and edible (Studnitz et al., 2007; van de Weerd et al., 2003). Enrichment materials with these characteristics (e.g., rooting substrate, fresh wood) were reported to be most interesting for pigs (Telkänranta et al., 2014a; van de Weerd and Day, 2009). Pigs tend to spend a lot of time interacting with chewable items, especially if they are relatively large (Clark et al., 2016; van den Brand et al., 2014) and can be chewed while laying down (Feddes and Fraser, 1994), which are both applicable for BSFL. Short behavioural tests indicated that environmental enrichment that provides edible rewards can extend the attention and motivation of pigs to interact with the enrichment (Holm et al., 2008; Machado et al., 2017; van de Weerd et al., 2003), likely because consuming edible items is rewarding and positively reinforces foraging behaviours (Tarou and Bashaw, 2007). Therefore, the palatable BSFL are expected to be desirable for pigs and to stimulate exploratory and foraging behaviour for extended periods of time. This, in turn, might reduce the occurrence of harmful social behaviour and improve overall pig welfare.

Previous studies indicated that interacting with and consuming solid feed items before weaning can ease the weaning transition, as this allows piglets to gain behavioural and physiological experience with feed. For example, extensive creep feed exploration and consumption before weaning has been linked to increased feed intake and body weight gain after weaning, improved maturation of and nutrient absorption in the GIT, and reduced post-weaning diarrhoea prevalence (Choudhury et al., 2021; Collins et al., 2013; Kuller et al., 2007; Middelkoop et al., 2019a; Sulabo et al., 2010a; Yan et al., 2011). However, simply providing creep feed is often not attractive enough to facilitate exploration and consumption (Hedemann et al., 2007; Middelkoop et al., 2019a). Many strategies did not substantially increase creep feed intake, including adding flavour to the creep feed, increasing creep feed composition complexity, and providing creep feed in a play feeder with various enrichment materials (Collins et al., 2013; Middelkoop et al., 2019a; Sulabo et al., 2010b). Furthermore, several strategies that did promote overall creep feed intake, such as providing larger creep feed pellets or providing creep feed in a play feeder with three small protrusions that facilitated exploration, did not increase the number of piglets that sampled creep feed before weaning (Clark et al., 2016; Kuller et al., 2010). In contrast to most strategies, provision of BSFL might play a multi-factorial role in increasing solid feed exploration and intake before weaning. First, if the larvae are highly palatable and attractive, piglets are expected to spend much time exploring and consuming them, already causing habituation to solid feed. The high protein, fat, and moisture content of live BSFL might make them palatable and easily accepted by young piglets that are accustomed to a liquid milk diet. Furthermore, the low fibre content of BSFL may allow for easy digestion. Second, when larvae are provided alongside creep feed, the stimulated exploratory and foraging behaviour might be redirected towards creep feed. Finally, providing larvae alongside creep feed creates a diverse diet in terms of flavour, texture, and nutrients, which was previously reported to increase overall feed intake before weaning, possibly by limiting sensory-specific satiety (Middelkoop et al., 2019b, 2018).

Providing BSFL might not only improve a piglet’s ability to cope with weaning by easing the feed transition, but it may also cause long-term welfare benefits by creating a more complex and stimulating environment. Previously, providing extensive enrichment (e.g., a combination of straw, wood shavings, peat, and branches, or sisal ropes, plastic balls, paper, and wood shavings) has been shown to be beneficial for piglets during the weaning transition. Extensive pre-weaning enrichment improved feed intake and growth directly after weaning (Brajon et al., 2017; O'Connell et al., 2005) and was sometimes found to decrease post-weaning harmful social behaviour (Telkänranta et al., 2014b), though this was not the case in all studies (Oostindjer et al., 2011b). Providing extensive enrichment directly after weaning also benefited piglets, with enriched piglets showing less harmful social behaviour, higher growth rates, greater feed efficiencies, and reduced diarrhoea (Ledergerber et al., 2015; Oostindjer et al., 2011b, 2010; Schaefer et al., 1990). Providing only chewable toys during the pre- or post-weaning period did not improve pig welfare, likely due to the limited interest of pigs in these materials (Bench and Gonyou, 2006; Fu et al., 2018).

When extensive environmental enrichment was available for a longer time (i.e., multiple weeks), this caused pigs to perform more play behaviour and more exploratory behaviour towards the enrichment, while they performed less harmful social behaviour and manipulation of pen fixtures (Beattie et al., 2000b; Bolhuis et al., 2006; Petersen et al., 1995; Rodarte et al., 2004), which sometimes resulted in fewer tail, ear, and body lesions (Fu et al., 2018; Zonderland et al., 2008). Long-term extensive enrichment combined with increased space was found to benefit pigs' affective states, as it improved the cognitive abilities of pigs (Bolhuis et al., 2013), created a more optimistic state of mind (Douglas et al., 2012), and reduced fearful responses towards novelty (Beattie et al., 2000a).

As mentioned before, BSFL are expected to be highly attractive to pigs and facilitate exploratory behaviour such as sniffing, rooting, and chewing, and they are expected to stay interesting for long periods of time as consuming the palatable larvae will likely reinforce exploratory behaviours (Tarou and Bashaw, 2007). As such, BSFL provisioning might be more efficient and effective in benefitting pig welfare during the weaning transition and subsequent production stages than previously tried (combinations of) enrichment materials, though this remains to be studied.

Conclusion and knowledge gaps

It has become clear that broilers and pigs suffer from diminished welfare because of the challenges imposed on them by their rearing environment and management practices. BSFL possess many traits that likely make them highly attractive to broilers and pigs, therefore BSFL could be provided to them as environmental enrichment that alleviates these welfare problems. While it is known that broilers show high interest in insect larvae, this must still be determined for pigs. Furthermore, it is unknown whether and how BSFL provisioning affects the welfare of broilers and pigs regarding their natural behaviour, health and functioning, and affective states, and which methods of BSFL provisioning yield the most welfare benefits. For pigs, the periods before, during, and after the weaning transition pose different welfare challenges, and the potential of BSFL to improve pig welfare in these distinct periods is unknown. Finally, if BSFL can benefit broiler and pig welfare this may change the prospects for integrating insects in sustainable livestock production systems, which requires reflection.

Aim and scope of the thesis

The current thesis aimed to investigate the impact of providing broilers and pigs with BSFL on the three pillars of animal welfare, namely natural behaviour, health and functioning, and affective state, paying particular attention to common welfare problems in broiler and pig production systems. This included determining the BSFL provisioning methods with the highest welfare benefits. As live BSFL are presumably most attractive for broilers and pigs (i.e., due to their high moisture content and movement), they were the primary focus of this thesis. Additionally, the use of BSFL as livestock feed and enrichment was placed in the broader context of sustainable livestock production. See **Figure 1** for a schematic overview of the parts and chapters of this thesis.

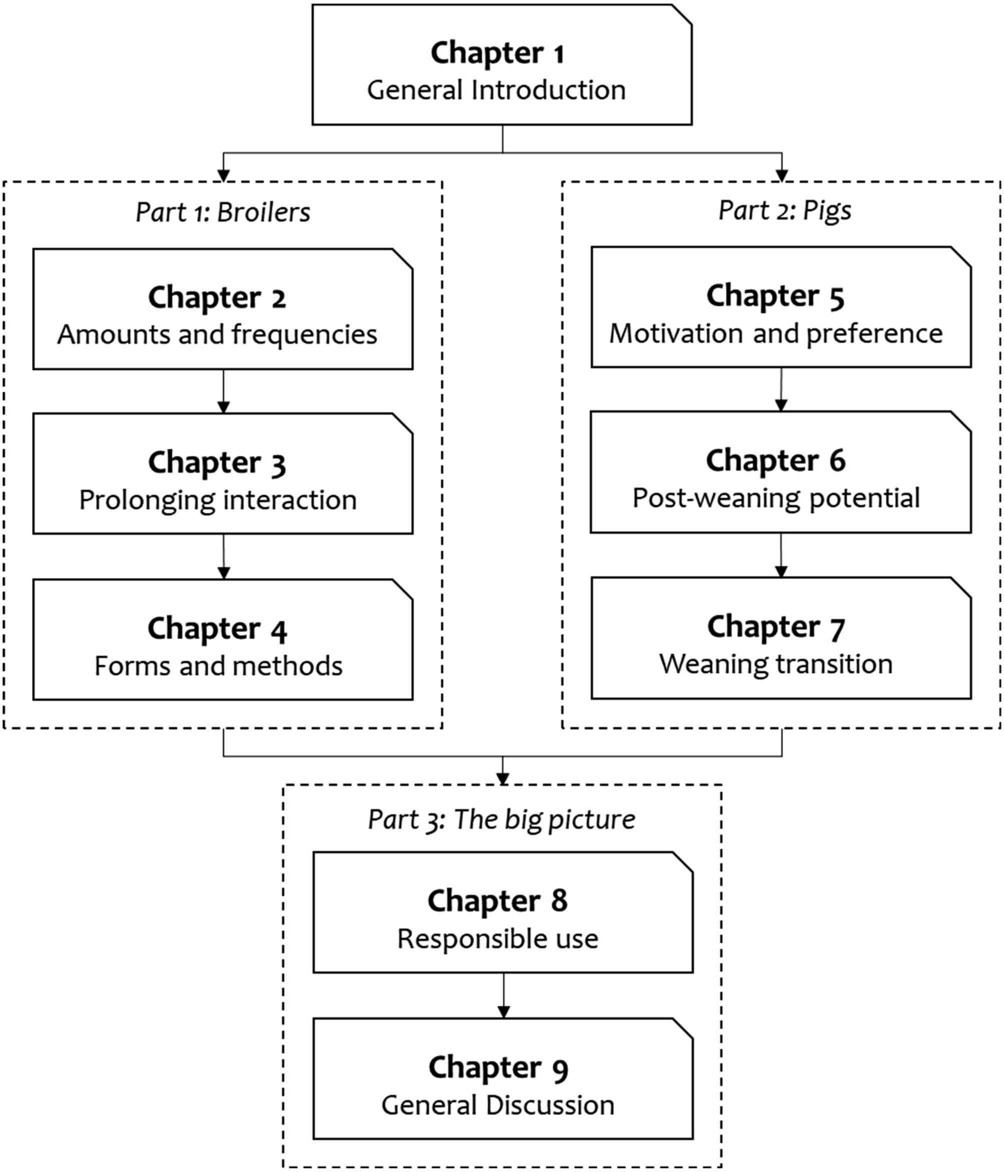

Figure 1. Schematic overview of parts and chapters in this thesis.

Part 1: Broilers

In *Part 1*, the effects of BSFL provisioning on broiler welfare were studied, and the most effective provisioning strategies were determined (**Figure 1**). The first step was to assess the impact of providing different amounts of live BSFL at different provisioning frequencies on broiler welfare, where higher amounts and frequencies were expected to increase the time broilers would interact with the larvae. Therefore, in **Chapter 2** broilers received either 5% or 10% of their expected dietary DM intake as live BSFL, and these larvae were provided by scattering them through the pen either two or four times a day. The effect of live BSFL provisioning on several welfare parameters was studied. The most effective treatments identified in **Chapter 2** were applied at commercial stocking densities in **Chapter 3**, together with treatments aimed to further benefit broiler welfare by prolonging the interaction time with larvae. In these new treatments, larvae were provided at 5% of the expected DM intake in small portions seven times a day or at 10% of the expected DM intake in transparent tubes with holes that needed to be moved by the broilers to get the larvae out. Finally, **Chapter 4** aimed to disentangle the nutritional effects on broiler welfare from the effects of the provisioning method on broiler welfare. To achieve this, broilers received larvae in different forms (i.e., live, dried, or as meal and oil), which were provided using different methods (i.e., in the feeder or scattered through the pen), and their effects on broiler welfare were observed.

Part 2: Pigs

In *Part* 2, the effects of live BSFL provisioning on pig welfare were investigated (**Figure 1**). As the motivation of pigs to access and consume live BSFL and the pigs' preference for live BSFL over other feed items and enrichment materials was unknown, this was studied in **Chapter 5**. In **Chapter 6** the welfare consequences of receiving small amounts of live BSFL scattered through the pen twice a day for 11 days after weaning were determined. Finally, **Chapter 7** aimed to gain insight into the effects of BSFL on piglet welfare during the weaning transition. In the four weeks before weaning, piglets received live BSFL *ad libitum* in a feeder next to creep feed, as opposed to receiving only creep feed in the control treatment. During three weeks after weaning piglets had access to up to 20% of their expected DM intake as live BSFL, provided in transparent tubes with holes that required rooting and pushing to get the larvae out. The effects of receiving live BSFL before and/or after weaning on piglet behaviour, health, intestinal development, affective state, and production performance during and after the weaning transition were determined.

Part 3: The big picture

In *Part 3*, the big picture of using BSFL as edible enrichment in food production systems was considered (**Figure 1**). In **Chapter 8**, the obtained knowledge from the previous chapters was integrated with information on the potential of insects as a circular feed source with a low environmental impact. This cumulated into a list of principles for the responsible use of insects in sustainable livestock production systems. Finally, **Chapter 9** provides a synthesis and discussion of the results obtained in all foregoing chapters, including recommendations for future research and reflections on the role of insects in sustainable food systems.

Supplementary data

Table S1A. Effects of black soldier fly inclusion in fast-growing broiler diets on production performance, the gastro-intestinal tract (GIT), blood composition, and health. Replaced ingredients of diets can be found in **Table S2**.

Age (d)	**Fat (FF/DF), % in diet as fed**	**Performance**				**GIT, blood, and health parameters**	**Reference**
		ADG	**FW**	**ADFI**	**FCR**		
Larvae meal							
1-10	FF, 6.6	=	=	=	=		(Elwert et al., 2010)
	DF, 5.4	=	=	=	=		
	DF, 4.7	↓	↓	↓	=		
	FF, 6.6	=	=	=	=		
	DF, 5.4	=	=	=	=		
	DF, 4.7	=	=	↓	=		
10-24	DF, 5	=	=	=	=		
1-35	FF, 0.2	=		↑		≠ crop, ileal & caecal microbiota composition	(Józefiak et al., 2018)
1-20	?, 1					Blood: = lysozyme Spleen: = cell proliferation ↑ $CD3^+$, $CD4^+$	(J. Lee et al., 2018)
	?, 2					Blood: ↑ lysozyme Spleen: ↑ cell proliferation, $CD3^+$, $CD4^+$	
	?, 3					Blood: ↑ lysozyme Spleen: ↑ cell proliferation, $CD3^+$, $CD4^+$	

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
1-35	?, 1					<u>After S. *Gallinarum* challenge</u>: = survival, counts of viable S. *Gallinarum* cells	(J. Lee et al., 2018)
	?, 2					<u>After S. *Gallinarum* challenge</u>: = survival ↓ counts of viable S. *Gallinarum* cells	
	?, 3					<u>After S. *Gallinarum* challenge</u>: ↑ survival ↓ counts of viable S. *Gallinarum* cells	
1-32	?, 1	↑		=	=		
	?, 2	↑		=	=		
	?, 3	↑		=	=		
1-10	DF, 5, 10, 15	$\uparrow^{L,Q}$	$\uparrow^{L,Q}$	$\uparrow^{L,Q}$	=		(Dabbou et al., 2018)*
10-24		$\uparrow^{L,Q}$	$\uparrow^{L,Q}$	=	$\uparrow^{L,Q}$		
24-35		$\downarrow^{L}$	$\uparrow^{L,Q}$	=	$\uparrow^{L,Q}$		
1-35						<u>Blood:</u> = erythrocytes, leukocytes, H/L ratio, urea, uric acid, AST, creatinine, triglycerides, cholesterol, Ca, Mg, Fe, TAS ↑ $P^{L,Q}$, GPx^{L} <u>Histopathological scores:</u> = spleen, thymus, bursa of Fabricius, liver	
1-35	DF, 5, 10, 15		$\uparrow^{L,Q}$		$\uparrow^{L,Q}$	<u>Caecal microbiota:</u> 5%: = α-diversity ↑ ß-diversity 10%: = α-diversity ↑ ß-diversity 15%: ↓ α-diversity ↑ ß-diversity <u>SI:</u> 5%: = VH, CD, VH/CD, villi mucin, crypt mucin 10%: = VH, CD, VH/CD, crypt mucin ↓ villi mucin 15%: = crypt mucin ↓ VH, VH/CD, villi mucin ↑ CD	(Biasato et al., 2020b; Dabbou et al., 2018; Schiavone et al., 2019)*

Table S1A continued.

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
14-56	?, 2	↓		=	=	= gizzard weight (%FW), intestine length Blood: = total protein, triglycerides ↓ ALB, uric acid	(Attivi et al., 2020)
	?, 4	↓		=	=	↓ gizzard weight (%FW), intestine length Blood: = total protein, triglycerides ↓ ALB, uric acid	
	?, 6	↓		↓	↓	↓ gizzard weight (%FW), intestine length Blood: = total protein ↑ triglycerides ↓ ALB, uric acid	
	?, 8	↑		↓	↓	↓ gizzard weight (%FW), intestine length Blood: = total protein ↑ triglycerides, ALB ↓ uric acid	
14-35	FF, 5		↑				(Popova and Petkov, 2020)
	DF, 5		↑				
1-35	DF, 21.7 (St), 12.3 (Gr)		↑				(Altmann et al., 2020)
14-35	FF, 5	↑ W1 ↓ W2 ↑ W3	↑	=	↓ W1 = W2 = W3	↓ gizzard weight (%FW)	(Popova et al., 2021, 2020)

Table S1A continued.

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
14-35	DF, 5	↑ ^{W1} ↓ ^{W2} ↑ ^{W3}	↑	=	↓ ^{W1} = ^{W2} = ^{W3}	= gizzard weight (%FW)	(Popova et al., 2021, 2020)
1-42	FF, 2.5, 5, 7.5, 10 (St), 5, 10, 15, 20 (Gr/Fi)	↑ ^{Q}	↑ ^{Q}	=	↑ ^{C,Q}	Blood: ↑ leukocytes^{L} Intestinal: = $CD3^+CD4^+$ ↓ $CD3^+$, $CD3^+CD8^+$, $CD3^+CD4^+CD8^+$	(de Souza Vilela et al., 2021a)*
1-35	DF, 7.5 (St), 7 (Gr), 6.5 (Fi)		=				(B. Kim et al., 2021)
	DF, 15 (St), 14 (Gr), 13 (Fi)		↓				
1-42	FF, 20 (St), 17 (Gr), 13 (Fi)	↓	↓	↓	=	= gizzard weight (%FW), breast & leg muscle loss of cross-striations, (multi-) focal defragmentation/necrosis of muscle fibres, infiltration of lymphoid cells & heterophils, fibromuscular dysplasia, leg muscle fibre hyalinization ↑ breast muscle fibre hyalinization ↓ breast muscle fibre diameter	(Murawska et al., 2021)
	FF, 30 (St), 25 (Gr), 20 (Fi)	↓	↓	↓	=	↑ gizzard weight (%FW) = breast & leg muscle loss of cross-striations, (multi-) focal defragmentation/necrosis of muscle fibres, infiltration of lymphoid cells & heterophils, fibromuscular dysplasia, leg muscle fibre hyalinization ↑ breast muscle fibre hyalinization ↓ breast muscle fibre diameter	

Table S1A continued.

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
1-42	FF, 40 (St), 34 (Gr), 27 (Fi)	↓	↓	↓	↑	↑ gizzard weight (%FW) = breast & leg muscle loss of cross-striations, (multi-) focal defragmentation/necrosis of muscle fibres, infiltration of lymphoid cells & heterophils, fibromuscular dysplasia, leg muscle fibre hyalinization ↑ breast muscle fibre hyalinization ↓ breast muscle fibre diameter	(Murawska et al., 2021)
1-21	FF, 10					After infectious bronchitis virus challenge: = blood CD4$^+$ ↑ blood CD8$^+$, mRNA expression of IFN-γ, MHC-I, LITAF, FAS, IL-2, protein levels of IFN-γ ↓ viral load & damage in trachea/kidney	(Zhang et al., 2021)
1-36	DF, 5 (St), 4.4 (Gr), 4.1 (Fi)	=	=	=	=	↓ gizzard weight (g) Jejunum: = VH, VA, CD, VH/CD, goblet cells villus count, mucosa layer, submucosa layer, tunica muscularis layer ↑ VW Ileum: = VH, VW, VA, CD, VH/CD, goblet cells villus count, mucosa layer, submucosa layer, tunica muscularis layer	(Hartinger et al., 2021)
	DF, 10 (St), 8.7 (Gr), 8.1 (Fi)	↓	↓	=	=	= gizzard weight (g) Jejunum: = VH, VW, VA, CD, VH/CD, goblet cells villus count, mucosa layer, submucosa layer, tunica muscularis layer Ileum: = VH, VW, VA, CD, VH/CD, goblet cells villus count, mucosa layer, submucosa layer, tunica muscularis layer	

Table S1A continued.

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
1-36	DF, 5.2 (St), 4.6 (Gr), 4.0 (Fi)	=	=	=	=	Caecal digesta: = biogenic amines, ammonia, SCFA, BCFA Colonic digesta: = biogenic amines Jejunum: = VH, VW, VA, CD, VH/CD, goblet cells villus count, mucosa layer, submucosa layer, tunica muscularis layer Ileum: = VH, VW, VA, CD, VH/CD, goblet cells villus count, mucosa layer, submucosa layer, tunica muscularis layer	(Hartinger et al., 2022)
Larvae meal & oil							
1-36	DF, 5.8 (St), 5.6 (Gr), 6.5 (Fi)	=	=	=	=	Caecal digesta: = biogenic amines, ammonia, SCFA, BCFA Colonic digesta: = biogenic amines Jejunum: = VH, VW, VA, CD, VH/CD, goblet cells villus count, mucosa layer, submucosa layer, tunica muscularis layer Ileum: = VH, VW, VA, CD, VH/CD, goblet cells villus count, mucosa layer, submucosa layer, tunica muscularis layer	(Hartinger et al., 2022)
	DF, 6,5 (St), 6.6 (Gr), 9.0 (Fi)	=	=	=	=	Caecal digesta: = biogenic amines, ammonia, SCFA, BCFA Colonic digesta: = biogenic amines Jejunum: = VH, VW, VA, CD, VH/CD, goblet cells villus count, mucosa layer, submucosa layer, tunica muscularis layer Ileum: = VH, VW, VA, CD, VH/CD, goblet cells villus count, mucosa layer, submucosa layer, tunica muscularis layer	
Larvae oil							
1-35	2.9 (St/Gr), 3.5 (Fi)	=	=	=	=	Blood: = erythrocytes, leukocytes, H/L ratio, total proteins, uric acid, creatinine, AST, ALT, GGT, triglycerides, cholesterol, P, Mg, Fe	(Schiavone et al., 2017a)

Table S1A continued.

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
1-35	5.9 (St/Gr), 6.9 (Fi)	=	=	=	=	Blood: = erythrocytes, leukocytes, H/L ratio, total proteins, uric acid, creatinine, AST, ALT, GGT, triglycerides, cholesterol, P, Mg, Fe	(Schiavone et al., 2017a)
21-48	3.4	=	=	=	=	SI: = VH, CD, VH/CD Blood: = erythrocytes, leukocytes, H/L ratio, total proteins, uric acid, creatinine, AST, ALT, GGT, triglycerides, cholesterol, P, Mg, Fe Histopathological scores: = spleen, thymus, bursa of Fabricius, liver	(Cullere et al., 2019; Schiavone et al., 2018)
	6.9	=	=	=	=	SI: = VH, CD, VH/CD Blood: = erythrocytes, leukocytes, H/L ratio, total proteins, uric acid, creatinine, AST, ALT, GGT, triglycerides, cholesterol, P, Mg, Fe Histopathological scores: = spleen, thymus, bursa of Fabricius, liver	
1-35	1.5	=	=	=	=	= duodenum, jejunum & ileum weight (g/kg FW) & length (cm/kg FW) ↓ gizzard weight (g/kg FW)	(B. Kim et al., 2020)
	3	=	=	=	=	= duodenum, jejunum & ileum weight (g/kg FW) & length (cm/kg FW) ↓ gizzard weight (g/kg FW)	
1-30	5	=	=	=	↓	= duodenum, jejunum & ileum weight (g/100 g FW) & length (cm/100 g FW), ileal & caecal SCFA = GPT, GOT, total/HDL cholesterol, triglyceride, uric acid, nitric oxide, MDA ↑ TAC	(Y. B. Kim et al., 2020)

Table S1A continued.

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
1-35	0.8 (St), 1.2 (Gr/Fi)	=		=	=	= duodenum, jejunum, cecum, gizzard & proventriculus weight (%FW), intestinal length (cm/kg FW) ↓ ileum weight (%FW) = foot pad dermatitis score	(Kierończyk et al., 2022, 2020)
	1.7 (St), 2.5 (Gr/Fi)	=		=	=	= duodenum, cecum, gizzard & proventriculus weight (%FW), intestinal length (cm/kg FW), crop, jejunum, & cecum digesta pH, jejunal microbiota composition ↓ ileum & jejunum weight (%FW) ≠ crop & caecal microbiota Blood: = glucose, triglycerides, NEFA, total protein, ALB, AST, cholesterol, IgM, IgA, IgY, IL-2, IL-6 ↓ ALT = foot pad dermatitis score	
	2.5 (St), 3.7 (Gr/Fi)	=		=	=	= duodenum, cecum, gizzard & proventriculus weight (%FW), intestinal length (cm/kg FW), caecal pH, jejunal & caecal microbiota composition ↓ ileum & jejunum weight (%FW), crop & caecal digesta pH ≠ crop microbiota = foot pad dermatitis score	

Table S1A continued.

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
1-35	3.3 (St), 4.9 (Gr/Fi)	=	=	=	=	= duodenum, cecum, gizzard & proventriculus weight (%FW), intestinal length (cm/kg FW) ↓ ileum & jejunum weight (%FW) Blood: = glucose, triglycerides, NEFA, total protein, ALB, AST, IgM, IgA, IgY, IL-2, IL-6 ↓ ALT, cholesterol = foot pad dermatitis score	(Kierończyk et al., 2022, 2020)
1-33	2.9 (St), 1.6 (Gr/Fi)	=	=	=	=	= α-diversity of faecal microbiota = gizzard, proventriculus & intestine weight (%FW) SI: = VH, CD, VH/CD Blood: = ALB, ALT, AST, ALP, GGT, total/HDL/LDL cholesterol, triglycerides, uric acid, creatinine, total protein, P, CL, K, Mg, Fe, Na, Ca Histopathological score: = spleen, liver, thymus, bursa of Fabricius, glandular stomach, intestine	(Dabbou et al., 2021)
1-36	0.8 (St), 1.6 (Gr), 2.6 (Fi)	=	=	=	=	Caecal digesta: = biogenic amines, ammonia, SCFA, BCFA Colonic digesta: = biogenic amines Jejunum: = VH, VW, VA, CD, VH/CD, goblet cells villus count, mucosa layer, submucosa layer, tunica muscularis layer Ileum: = VH, VW, VA, CD, VH/CD, goblet cells villus count, mucosa layer, submucosa layer, tunica muscularis layer	(Hartinger et al., 2022)

Table S1A continued.

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
1-36	1.5 (St), 3.1 (Gr), 5.1 (Fi)	=	=	=	=	Caecal digesta: = biogenic amines, ammonia, SCFA, BCFA Colonic digesta: = biogenic amines Jejunum: = VH, VW, VA, CD, VH/CD, goblet cells villus count, mucosa layer, submucosa layer, tunica muscularis layer Ileum: = VH, VW, VA, CD, VH/CD, goblet cells villus count, mucosa layer, submucosa layer, tunica muscularis layer	(Hartinger et al., 2022)
Pre-pupae meal							
7-48	FF, 5	=	=	=	=	= gizzard weight (%CW)	(Onsongo et al., 2018)
	FF, 10	=	=	=	=	= gizzard weight (%CW)	
	FF, 15	=	=	=	=	= gizzard weight (%CW)	
1-35	?, 5		=				(Pieterse et al., 2019)
	?, 10		=				
	?, 15		=				
1-21	FF, 5	=		=	=	= duodenum, jejunum, ileum & caecum length (cm/kg FW) & weight (g/kg FW), gizzard weight (g/kg FW) After cutaneous basophilic hypersensitivity test: = foot pad index	(Elangovan et al., 2021)
Other							
1-13	De-frosted, 8	=	=		=	≠ caecal microbiota = tibia ash % SI: = villus lysis score	(Moula et al., 2018)

Table S1A continued.

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
1-39	Live larvae, 5**	=	=	=	=	= intestine & gizzard weight (%FW), intestinal mucins, caecal length, caecal microbiota α & β-diversity SI: = VH, CD, VH/CD Jejunum: IL-2, IL-4, IL-6, INF-γ, TNF-α, MUC-2 mRNA expression Blood: = erythrocytes, leucocytes, H/L, total proteins, uric acid, ALB, AST, ALT, total/HDL/LDL cholesterol Histopathological scores: = spleen, bursa of Fabricius, liver, gut	(Bellezza-Oddon et al., 2021; Colombino et al., 2021)

Table S1A continued.

Abbreviations: d = days, FF = full fat meal, DF = (partially) defatted meal, GIT = gastro-intestinal tract, ADG = average daily gain, FW = final weight, ADFI = average daily feed intake, FCR = feed conversion ratio, St = starter diet, Gr = grower diet, Fi = finisher diet, W1 = week 1, W2 = week 2, W3 = week 3, SI = small intestine, VH = villus height, VW = villus width, VA = villus area, CD = crypt depth, SCFA = short-chain fatty acids, BCFA = branch chain fatty acids, CW = carcass weight.

= indicates no difference, ↑ indicates significant increase, ↓ indicates significant decrease, ≠ indicates significant change, ? indicates the parameter is unknown. Empty cells indicate the parameter was not measured.

*Includes statistical analysis on all inclusion levels combined for one or more parameters, for statistical differences it is indicated in superscript which models were used: C = cubic model, L = linear model, Q = quadratic model.
**Included on dry matter basis.

Table S1B. Effects of black soldier fly inclusion in pig diets on production performance, the gastro-intestinal tract (GIT), blood composition, and health. Replaced ingredients of diets can be found in **Table S2**.

Age (d)	**Fat (FF/DF), % in diet as fed**	**Performance**				**GIT, blood, and health parameters**	**Reference**
		ADG	**FW**	**ADFI**	**FCR**		
Larvae meal							
10-28	FF, 3.4	=	=	=		Blood: leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, erythrocytes, haemoglobin, haematocrit, MCHC, RDW, platelets, MPV, ALB, Ca, P, Fe, IgG, IgA, IgM ↑ MCV, MCH	(Driemeyer, 2016)
43d (Fp)	FF, 4	↑	=	=	↓	Colon: = α-diversity of microbiota ↑ SCFA ≠ microbiota composition Blood: ↑ total protein, ALB, glucose ↓ urea Colonic mucosa mRNA expression: = TLR-2, TLR-5, IL-8, TNF-α, MUC-2 ↑ IL-10, ZO-1, occludin, MUC-1 ↓ TLR-4, IFN-γ	(Yu et al., 2019b, 2019c, 2019a)
	FF, 8	=	=	=	=	Colon: = α-diversity of microbiota, SCFA ≠ microbiota composition Blood: = ALB, total protein, glucose, urea Colonic mucosa mRNA expression: = TLR-2, TLR-4, TLR-5, IL-10, IL-8, TNF-α, occludin, mucin-1, mucin-2 ↑ ZO-1 ↓ IFN-γ	

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
98d (Fp)	DF, 8.1, 12.3 (P1), 6.5, 9.7 (P2)		↑				(Altmann et al., 2019)
63d (Fp)	FF, 9	=	=	=	=	Blood: = erythrocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, haemoglobin, haematocrit, MCV, MCH, MCHC, RDW, platelets, total/HDL/LDL cholesterol, triglycerides	(Chia et al., 2019)
	FF, 12	=	=	=	=	Blood: = erythrocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, haemoglobin, haematocrit, MCV, MCH, MCHC, RDW, platelets, total/HDL/LDL cholesterol, triglycerides	
	FF, 14.5	=	=	=	=	Blood: = erythrocytes, lymphocytes, monocytes, eosinophils, basophils, haemoglobin, haematocrit, MCV, MCH, MCHC, RDW, platelets, total/HDL/LDL cholesterol, triglycerides ↑ neutrophils	
	FF, 18.5	=	=	=	=	Blood: = erythrocytes, lymphocytes, monocytes, eosinophils, basophils, haemoglobin, haematocrit, MCV, MCH, MCHC, RDW, platelets, total/HDL/LDL cholesterol, triglycerides ↑ neutrophils	

Table S1B continued.

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
20-61	DF, 5, 10	=	=	=	=	SI: = VH, CD, VH/CD, mucin composition Caecal microbiota: = α-diversity ↑ ß-diversity Blood: = erythrocytes, leukocytes, lymphocytes, eosinophils, basophils, uric acid, cholesterol, triglycerides, creatinine, total protein, P, Fe, Mg, MCV, haematocrit, MCH, MCHC, RDW, haemoglobin, platelets, MPV, procalcitonin, PDW, GOT, GPT, ALP, GGT, ALB, α-globulin, ß -globulin, γ-globulin ↑ monocytes^{L}, neutrophils^{Q} Histopathological scores: = stomach, gut, liver, kidney	(Biasato et al., 2020a, 2019)*
28-56	FF, 1	=			=	= LI weight (%FW), jejunal VH, CD, VH/CD ↑ SI weight (%FW) = diarrhoea rate Ileum: = VH, CD, VH/CD, microbiota composition, pH, lactate, SCFA Caecum: = pH, lactate, SCFA ≠ microbiota composition Blood: = total protein, ALB, globulin, creatinine, glucose, triglyceride, total/HDL/LDL cholesterol, ALT, AST, ALP, IL-8, IL-10, IFN-γ, TNF-α, IgG, IgA ↓ urea Ileal mucosa mRNA expression: = TLR-2, TLR-4, TLR-5, NF-kB, MyD88, IL-8, IL-10, TNF-α, MUC-1, MUC-2, ZO-1, occludin, claudin-2, IGF-1, IGF-1R, GLP, EGF Ileal mucosa concentrations: = IL-8, IL-10, SIgA, IgG, IFN-γ, TNF-α	(Yu et al., 2020b, 2020a)

Table S1B continued.

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
28-56	FF, 2	↑			↓	= LI weight (%FW), jejunal CD, VH/CD ↑ SI weight (%FW), jejunal VH = diarrhoea rate Ileum: = VH, CD, VH/CD, pH, SCFA ↑ lactate ≠ microbiota composition Caecum: ↑ lactate, SCFA ↓ pH ≠ microbiota composition Blood: = ALB, creatinine, glucose, total/HDL/LDL cholesterol, ALT, AST, ALP, IL-8, TNF-α, IgG ↑ total protein, globulin, IL-10, IgA ↓ urea, triglyceride, IFN-γ Ileal mucosa mRNA expression: = TLR-2, TLR-5, MyD88, IL-8, MUC-2, IGF-1R ↑ IL-10, MUC-1, ZO-1, occludin, claudin-2, IGF-1, GLP, EGF ↓ TLR-4, NF-kB, MyD88, TNF-α Ileal mucosa concentrations: = IL-8, IL-10, IgG, IFN-γ, TNF-α ↑ SIgA	(Yu et al., 2020b, 2020a)

Table S1B continued.

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
28-56	FF, 4	=			=	= LI & SI weight (%FW), jejunal VH, CD, VH/CD = diarrhoea rate Ileum: = VH, CD, VH/CD, microbiota composition, pH, SCFA ↑ lactate Caecum: = pH, lactate, SCFA ≠ microbiota composition Blood: = total protein, ALB, creatinine, glucose, triglyceride, total/HDL/LDL cholesterol, ALT, AST, ALP, IL-8, IL-10, TNF-α, IgG ↑ globulin, IgA ↓ urea, IFN-γ Ileal mucosa mRNA expression: = TLR-2, TLR-4, TLR-5, NF-kB, IL-8, TNF-α, MUC-2, claudin-2, IGF-1R, GLP ↑ IL-10, MUC-1, ZO-1, occludin, IGF-1, EGF ↓ MyD88 Ileal mucosa concentrations: = IL-8, IL-10, IgG, IFN-γ, TNF-α ↑ SIgA	(Yu et al., 2020b, 2020a)
20-63	FF, 7.4 (P1), 3.8 (P2), 0.5 (P3)	=	=	=	=	= stomach, SI & LI weight (g/kg FW), SI VH, CD, VH/CD After ovALB vaccination: = plasma haptoglobin, anti-OVA IgG, change in skinfold thickness	(Crosbie et al., 2021)
	FF, 14.8 (P1), 7.5 (P2), 1 (P3)	=	=	=	=	= stomach, SI & LI weight (g/kg FW), SI VH, CD, VH/CD After ovALB vaccination: = plasma haptoglobin, anti-OVA IgG, change in skinfold thickness	

Table S1B continued.

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
98d (Gp)	FF, 6	=	=	=	=		(Chia et al., 2021)
	FF, 9	↑	↑	=	↓		
	FF, 12	↑	=	=	↓		
	FF, 14	↑	↑	=	↓		
23d (Gp)	DF, 30.5					Microbiota: = ileal α-diversity ↑ jejunal α-diversity ↑ ileal & jejunal ß-diversity Serum: = INF-α, IL-12p40	(Kar et al., 2021)
28-56	FF, 3	=		=	=	After *Escherichia coli* K88 infection: ↓ diarrhoea incidence, diarrhoea index Jejunum: = MUC-2, occludin, ZO-1, IL-6, IL-8, IL-10, TNF-αβ, pBD2, PR39 mRNA expression; sIgA concentration ↑ VH, VH/CD; MUC-1, claudin-1, pBD1, PG1-5 mRNA expression ↓ CD; IL-1β mRNA expression Ileum: = VH, CD, VH/CD; claudin-1, ZO-1, IL-6, TNF-α, PR39, NOD2, TLR2, TLR4 mRNA expression ↑ MUC-1, MUC-2, occludin, IL-10, TGF-β, pBD1, pBD2, PG1-5, NOD1 mRNA expression; sIgA concentration ↓ IL-1β, IL-8 mRNA expression Colon: ≠ microbiota composition ↑ SCFA	(Tang et al., 2022)
28-141	DF, 4	=	=				(Zhu et al., 2022)
	DF, 8	↑	=				

Table S1B continued.

Age (d)	Fat (FF/DF), % in diet as fed	Performance				GIT, blood, and health parameters	Reference
		ADG	FW	ADFI	FCR		
Larvae oil							
21-61	2, 4, 6	↑^{L}	↑^{L}	=	=	Blood: = total protein, ALB, globulin, AST, ALT, alkaline phosphatase, γ-glutamyl transpeptidase, urea, creatinine, glucose, Ca, P, Mg, K, Na, Cl, triglycerides, amylase, lipase, creatine phosphokinase, leukocytes, erythrocytes, haematocrit, neutrophils, lymphocytes, monocytes, eosinophils, basophils ↑^{L} cholesterol, platelets	(van Heugten et al., 2019)*
Pre-pupae meal							
21-36	FF, 4	=		=	=	= stomach & SI digesta pH & intestinal microbiota, jejunal VH, CD, VH/CD	(Spranghers et al., 2018)
	FF, 8	=		=	=	= stomach & SI digesta pH & intestinal microbiota, jejunal VH, CD, VH/CD	
	DF, 5.4	=		=	=	= stomach & SI digesta pH & intestinal microbiota, jejunal VH, CD, VH/CD	
33-60	FF, 4.8, 9.5, 19.1	↓^{C}	=	=	=	= faecal DM, faecal score, reddening of jejunal/ileal/colonic mucosa, jejunum with low muscle tone and no muscle folding Stomach: = pH Jejunum: = pH, VH, CD, VH/CD Ileum: = VH, CD, VH/CD Colon: = CD, SCFA, ammonia, α- & ß -diversity of microbiota ≠ microbiota composition	(Håkenåsen et al., 2021)*

Table S1B continued.

Table S1B continued.
Abbreviations: d = days, FF = full fat meal, DF = (partially) defatted meal, GIT = gastro-intestinal tract, ADG = average daily gain, FW = final weight, ADFI = average daily feed intake, FCR = feed conversion ratio, SI = small intestine, LI = large intestine, VH = villus height, CD = crypt depth, Gp = grower pigs, Fp = finisher pigs, P1 = phase one diet, P2 = phase two diet, P3 = phase three diet, SCFA = short-chain fatty acids.

= indicates no difference, ↑ indicates significant increase, ↓ indicates significant decrease, ≠ indicates significant change. Empty cells indicate the parameter was not measured.

*Includes statistical analysis on all inclusion levels combined for one or more parameters, for significant differences it is indicated in superscript which models were used: C = cubic model, L = linear model, Q = quadratic model.

Table S2. Replaced ingredients of studies included in **Table S1A,B.**

Reference	**Replaced ingredient(s)**
Broilers	
(Elwert et al., 2010)	FM, SBO, corn/ SBM, SBO, corn
(Józefiak et al., 2018)	On top
(J. Lee et al., 2018)	On top
(Dabbou et al., 2018)	SBM, SBO, CGM
(Biasato et al., 2020b; Dabbou et al., 2018; Schiavone et al., 2019)	SBM, SBO, CGM
(Attivi et al., 2020)	Roasted SBM, FM, wheat bran
(Popova and Petkov, 2020)	SBM, sunflower oil, corn
(Altmann et al., 2020)	SBM, corn, wheat
(Popova et al., 2021, 2020)	SBM, sunflower oil, corn
(de Souza Vilela et al., 2021a)	SBM, MBM, canola oil, cotton-seed oil
(B. Kim et al., 2021)	SBM, SBO, CGM, corn
(Murawska et al., 2021)	SBM, SBO, wheat
(Zhang et al., 2021)	On top
(Hartinger et al., 2021)	SBM, corn, SBO, grass meal
(Hartinger et al., 2022)	SBM and/or SBO
(Schiavone et al., 2017a)	SBO
(Cullere et al., 2019; Schiavone et al., 2018)	SBO
(B. Kim et al., 2020)	SBO
(Y. B. Kim et al., 2020)	Corn oil
(Kierończyk et al., 2022, 2020)	SBO
(Dabbou et al., 2021)	SBO
(Onsongo et al., 2018)	SBM, FM, corn oil, corn grain, wheat pollard
(Pieterse et al., 2019)	SB, corn
(Elangovan et al., 2021)	SBM, corn
(Moula et al., 2018)	Corn meal
(Bellezza-Oddon et al., 2021; Colombino et al., 2021)	On top
Pigs	
(Driemeyer, 2016)	SBM, FM, whey, corn
(Yu et al., 2019b, 2019c, 2019a)	SBM, SBO
(Altmann et al., 2019)	SBM, SBO
(Chia et al., 2019)	FM, wheat pollard
(Biasato et al., 2020a, 2019)	SBM, SBO, corn meal
(Yu et al., 2020b, 2020a)	SBM, FM, SBO, corn
(Crosbie et al., 2021)	SBM, FM, corn, whey, blood meal, plasma, animal vegetable fat
(Chia et al., 2021)	FM, wheat pollard
(Kar et al., 2021)	SBM, SBO, corn starch

Reference	**Replaced ingredient(s)**
(Tang et al., 2022)	Corn, FM
(Zhu et al., 2022)	Corn, SBM, puffed soybeans, FM, SBO
(van Heugten et al., 2019)	Corn oil
(Spranghers et al., 2018)	SBM, SBO, corn
(Håkenåsen et al., 2021)	SBM, FM, SB protein, wheat, saturated vegetable fat

Table S2 continued.

Abbreviations: SB = soybean, SBM = soybean meal, SBO = soybean oil, FM = fishmeal, CGM = corn gluten meal, MBM = meat-and-bone meal.

Part 1: Broilers

Chapter 2

Provisioning of live black soldier fly larvae (*Hermetia illucens*) benefits broiler activity and leg health in a frequency- and dose-dependent manner

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Abstract

Fast-growing broilers spend most their time inactive and are therefore prone to experience leg problems. Environmental enrichment that facilitates intrinsically motivated behaviours can potentially promote activity and reduce leg problems, thereby improving broiler welfare. A promising environmental enrichment method is the scattering of desired feed items, such as insects, which are highly attractive to broilers. We studied the effect of providing live black soldier fly larvae (BSFL) scattered on the litter on broiler behaviour, leg health, and performance. One-day-old male broilers were assigned to one of five treatments (eight pens/treatment, nine broilers/pen): a control without BSFL and four treatments with BSFL provided from day 1 onwards in different amounts (5% or 10% of estimated dietary dry matter intake; A5 and A10 respectively) and frequencies (two or four times a day; F2 and F4 respectively). All broilers were fed diets formulated to ensure a similar energy and nutrient intake. Broiler weight and leg health were determined on day 42. The behavioural time budget was determined weekly by observations for 7 h per day using 12-min scan sampling, and activity around larval provisioning was determined by 3-min scan sampling from 9 min before, until 27 min after larval provisioning on day 15/16, 29/30 and 40/41. Broilers in all larval provisioning treatments had a different behavioural time budget than controls, with significantly higher levels of foraging behaviour, walking, standing idle, and general activity during at least three of the five observation days ($p < 0.05$ compared to controls). The increase in activity was numerically highest and most long-term in A10-F4 broilers. Time spent active and in standing posture declined from week 4 onwards in A10-F4, whereas for all other treatments this decline occurred already in week 2. Activity during 30 minutes after larval provisioning was higher for A10 than A5 treatments ($p < 0.05$ for all days) but overall not affected by frequency of larval provisioning. Hock burn occurred less in A10 birds than in controls ($p < 0.05$), and lameness occurred less in A10 and A5-F4 birds than in controls ($p < 0.01$). Only A10-F2 birds had a lower final weight than controls ($p < 0.05$). In conclusion, the largest amount combined with the highest frequency of larval provisioning applied in this study resulted in the most prominent increase in activity and better leg health, without significantly affecting broiler performance. Further investigation into BSFL provisioning

methods is required to determine the optimal method for achieving improved broiler welfare.

Introduction

Industrial experts estimate that in 2017 approximately 90-95% of the European fast-growing broilers obtained a weight of 2-2.5 kg within 6 weeks (van Horne, 2018). This rapid growth rate attributes to the development of broiler lameness, directly by impairing broiler leg bone development (Kestin et al., 1992; Olkowski et al., 2011) and indirectly through limiting broiler activity (Reiter and Bessei, 2009; Sherlock et al., 2010). Previous reports indicate that the majority of fast-growing broilers exhibit some degree of lameness (Muri et al., 2019), and that between 30% and 50% of broilers clearly show a reduced ability to walk (indicated by a gait score of 3 or higher, de Jong et al., 2019; Vasdal et al., 2018b). Lameness can be painful and limit the expression of active behaviours (Danbury et al., 2000). Furthermore, moisture and ammonia aggregate in the litter over time, and broilers that spend a lot of time resting in this litter are more prone to develop contact dermatitis (de Jong et al., 2014). The negative effects of a rapid growth rate on leg health are exacerbated under commercial conditions where broilers have limited space and environmental stimulation. The commercial environment can impair broiler welfare by limiting the possibility to perform intrinsically motivated behaviours, diminishing activity levels and thereby increasing the occurrence of associated leg problems (Vasdal et al., 2018b). The effects of fast growth and leg problems on activity and behaviour are prominent, as fast-growing broilers spend between 60-80% of their time sitting (see for example Baxter et al., 2018a; de Jong and Gunnink, 2018).

Several studies have indicated that promoting activity from a young age onwards can advance leg bone development, and even increase activity levels later in life (Reiter and Bessei, 2009; Ruiz-Feria et al., 2014; Vasdal et al., 2018b). An additional benefit of increased activity can be that the litter tends to dry easier when periodically mixed by, for example, scratching. This mixing can thus improve the litter quality and reduce the risk of contact dermatitis (de Jong and van Harn, 2012). Therefore, (early) facilitation of activity in broilers may benefit broiler welfare. Various measures, for example increasing the distance between the food and water supply, have been tested with variable levels of success (for review see

Riber et al., 2018). Facilitating foraging behaviour is one promising way to promote broiler activity. While providing whole wheat on the litter did not promote activity (Jordan et al., 2011), after scattering mealworms the activity of broilers increased substantially, at least on the short term (Pichova et al., 2016). This could be explained by the observation that broilers are highly motivated to gain access to and consume larvae (Bokkers and Koene, 2002; Clara et al., 2009). In addition, live insect larvae are potentially more attractive to broilers than dead insects, as chickens prefer to interact with moving rather than stationary objects (Jones et al., 1998).

Black soldier fly larvae (BSFL) could potentially be used as effective broiler enrichment. Broilers actively consume these larvae when provided processed (Leiber et al., 2017) or live (Oonincx, personal communication). In addition, although Leiber et al. (2017) found no effect of including BSFL meal on broiler performance, other studies have found that BSFL inclusion in the diet can increase broiler growth and feed intake (Dabbou et al., 2018), and increase the broiler's T-helper cell frequency and serum lysozyme activity, improving their nonspecific immune responses (J. Lee et al., 2018). With the aim of improving the effectiveness of providing insects as enrichment, Pichova et al. (2016) suggested that prolonged access to insects could further increase broiler activity. However, the effect of providing broilers with long-term access to BSFL on the broiler's behavioural time budget has currently not been studied. Also, the effect of BSFL provisioning on broiler leg health and performance remains to be investigated.

The objective of the current study was to investigate the effect of scattering BSFL in the litter on broiler behaviour, contact dermatitis, lameness, and performance. Specifically, the consequences of providing larvae in combinations of two amounts (5 or 10% of estimated dietary dry matter intake) and two frequencies (provided two or four times a day) were studied. We hypothesized that scattering BSFL would promote activity and reduce health problems in broilers, where the largest amount and highest frequency were expected to have the strongest effect as they allow for prolonged interaction with the larvae. An additional aim was to keep broiler performance similar between treatments, as to avoid potential confounding effects of body weight on broiler welfare.

Materials and methods

This experiment was conducted between January and March 2019 at the animal experiment facilities of Wageningen University & Research, and the protocol was approved by the Animal Care and Use committee of Wageningen University & Research (Wageningen, The Netherlands).

Animals and management

Three hundred and sixty one-day old male Ross 308 broiler chicks were obtained from a commercial hatchery. At arrival at the experimental facilities chicks were tagged with a leg ring and randomly distributed over forty pens, resulting in nine chicks per pen. Each pen of 1 × 2 m constituted of one feeder, one drinking line containing five nipples with cups, one 1 m long perch (rectangular bar of 2 × 2 cm, 10 cm high) and a 5 cm deep layer of wood shavings. Visual contact between pens was obstructed by solid panels. Feed and water were provided *ad libitum*. The IB vaccination (spray) was given at hatch and the NCD vaccination (spray) at eight days of age. Temperature at the start of the experiment was 33 °C and this was gradually decreased to 22 °C at 27 days of age, after which it was kept constant. Humidity was adjusted based on recommendations of the Aviagen Ross broiler handbook. The lighting schedule (20 lx at chick level throughout the rearing period, artificial light) was 23 L:1 D from day 1-3, and from day 4 onwards a schedule of 18 L:6 D was maintained, with the dark period lasting from 00:00-06:00.

Experimental design

Five treatments were included in this study. Broilers in the control treatment did not receive BSFL throughout the experiment. In the other treatments the amount and frequency of larval provisioning were varied. Broilers received either 5% or 10% of the estimated daily dietary DM intake as BSFL (hereafter referred to as A5 and A10, respectively), provided either two or four times a day (F2 and F4, respectively) resulting in, apart from the control treatment, treatments A5-F2, A5-F4, A10-F2 and A10-F4. Each treatment was applied to eight randomly selected pens which were distributed (balanced for treatment) over three experimental rooms. A commercial BSFL producer supplied fresh, 14-day-old BSFL weekly. Larvae were stored at 12 °C near the experimental rooms for up to one week. One day before the larvae were provided to the broilers, the larvae were stored at room temperature, to increase their activity at provisioning. Broilers received BSFL on set moments each day (08:00 and 14:00 for the F2 treatments and 08:00, 11:00, 14:00 and 17:00 for the F4 treatments). Provisioning occurred by scattering larvae across the litter throughout the pen.

Diet composition

All diets were designed to meet or exceed the broilers' nutrient requirements (CVB, 2016). All chicks received a starter feed from day 1-7 (12.46 ME/kg DM metabolizable energy, 22% crude protein). This starter feed was the same for all treatments; it was not adjusted for larval amount as digestible nutrient intake from larvae was expected to be small during day 1-7. The grower feed provided to the A5 and A10 broilers from day 8-42 was adjusted to account for the estimated nutrient intake from these larvae and ensure a similar nutrient and energy intake for all broilers. Briefly, a mix was designed mimicking the nutritional composition of BSFL, based on analysed values of three samples of BSFL for dry matter, crude protein, crude fat, calcium, and phosphorus content. In this mix the fat source was BSFL oil and the protein source was potato protein. This protein source was chosen as protein from dead BSFL is currently not allowed to be used in commercial broiler feed, and not in the feed in this experiment. The total dietary DM intake of all broilers consisted for 90% of a core feed, and for the control broilers the remaining 10% consisted of the above-mentioned mix. The A5 broilers' diet contained 5% of the mix and 5% live BSFL, and the diet of A10 broilers contained 10% live BSFL and no mix. Detailed ingredient and nutrient composition of all diets is provided in **Supplementary Tables S1,S2**. The results of the analyses on nutrient composition of BSFL were also used to determine the exact amount (g) of fresh larvae to be provided to each pen daily.

Measurements

Behavioural time budget over the day

Prior to observations all broilers were individually marked with one coloured dot, to allow for individual identification. Behaviour and posture (standing/sitting) were observed at 12-min intervals by instantaneous scan sampling on one day at the end of week 1-5 (day 7, 14, 21, 28, and 36). This involved seven one-hour periods starting at 08:00, 09:15, 10:45, 12:00, 13:45, 15:00, and 16:30, resulting in 35 scans/broiler/day. Three observers simultaneously observed all pens in the three experimental rooms, and every hour observers switched between rooms. Observers had previously been trained and inter-observer reliability was determined to be sufficient (Fleiss kappa > 0.8) before observations commenced. The ethogram is given in **Table 1.** At BSFL provisioning times observations started immediately after larvae provisioning.

Behaviour at larval provisioning

At three ages broiler behaviour around all four larval provisioning times was observed in more detail in six pens per treatment for all treatments, including the control group, also if no larvae were provided. For each age, observations occurred during two consecutive days (day 15-16, day 29-30 and day 40-41), and each day three pens per treatment were observed and foraging- and activity-related behaviour was noted according to the ethogram (**Table 2**). Three-min instantaneous scan sampling started 9 min before and ended 27 min after each larval provisioning time (08:00, 11:00, 14:00 and 17:00), when generally all larvae had been consumed.

Visual health scores

All broilers were visually scored on day 42 on various health parameters. Foot pad dermatitis was scored for both feet on a 5-point scale, with 0 = no lesions and 4 = marked swelling and enlargement of the entire foot pad, necrotic cells covering more than one-half of the total foot pad area (full descriptions by Sami Yamak et al., 2016). Hock burn, breast blisters, cleanliness, and gait score were determined according to the Welfare Quality® assessment protocol for poultry (Butterworth, 2009). Hock burn was scored for both hocks on a 5-point scale, with 0 = no lesions and 4 = severe lesions. Breast blisters were scored as being present or absent. Cleanliness was scored on a 3-point scale, with 0 = clean plumage, 1 = slightly dirty plumage, and 2 = large patches of dirty plumage on breast or breast completely covered with dirty plumage. The walking ability (indicative of lameness) of broilers was assessed by encouraging broilers to walk approximately 1 m in the pen and assigning a gait score between 0 (normal, dextrous and agile walk) and 5 (incapable of walking, Butterworth, 2009).

Table 1. Ethogram for the behavioural time budget observations.

Item	Description
Behaviour	
Eating	Standing/sitting with head above or in the feeder and/or pecking at feed in the feeder or on the floor.
Drinking	Drinking from nipple or cup beneath nipple.
Defecation	Excretes faeces.
Walking	Locomoting in an upright position with a normal speed or quick steps without performing any other behaviour.
Shuffling	Half standing/half sitting (hocks might touch the ground or be slightly above the ground) and is moving a few steps after which it sits down.
Standing idle	Standing on the ground without performing any other activity.
Perching	Perching without any other activity.
Resting	Sitting with hocks resting on ground without any other activity, possibly with head on the ground or under wing.
Scratching	Scraping of the litter with the claws.
Ground pecking	Performing pecking movements directed at the ground.
Food running	Running with food in beak while pen mates follow and attempt to grab the food item.
Dust bathing	Performed with fluffed feathers while lying, head rubbed on floor, wings opened, scratching at ground, distributing substrate over body.
Stretching	Stretching of wing and/or leg.
Preening	Grooming of own feathers with the beak.
Wing flapping	Bilateral up-and-down wing flapping.
Pecking pen mate head	Pecking movements directed at the head of a pen mate.
Pecking pen mate other	Pecking movements directed at the body or beak of a pen mate.
Interaction other	Jumping at pen mate, chasing pen mate, threatening pen mate.
Other	Any behaviour not mentioned above.
Posture	
Standing	On floor: hocks not in contact with the litter. On perch: knees not bent.
Sitting	On floor: hocks in contact with the litter. On perch: knees bent.

Table 2. Ethogram for the behavioural observations at larval provisioning.

Behaviour	**Description**
Foraging behaviour	Ground pecking and/or scratching.
Food running	Running with food in beak while pen mates follow and attempt to grab the food item.
Walking	Locomoting in an upright position with a normal speed or quick steps.
Standing idle	Standing without any other activity, on perch or on ground.
Resting	Sitting with hocks resting on ground without any other activity, possibly with head on the ground or under wing.
Agonistic behaviour	Pecking pen mate, jumping, chasing, threatening etc.
Other	Any behaviour not mentioned above.

Performance

All broilers were individually weighed at placement and on day 6, 13, 20, 27, 34 and 42. Feed consumption on pen level was determined weekly by weighing feed remains in the feeder on day 8, 15, 22, 29, 35 and 42. Morbidity and mortality were recorded daily.

Statistical analysis

Data processing

Based on the intake of feed and larvae, the percentage of the total DM intake consisting of BSFL was determined per pen. All larvae were assumed to be consumed as determined by regular observations and further supported by absence of adult flies in the rooms. The average daily gain (ADG) and average daily DM intake (with and without including larvae) in g/day/chick were determined and based on this the average daily metabolizable energy (ME) intake and the feed conversion ratio (FCR), based on dry matter, were calculated. Behaviours observed during the behavioural time budget observations were combined per chick per day and expressed as the percentage of time spent performing that behaviour. Besides being analysed independently, ground pecking, scratching and food running were combined into "foraging behaviour", preening and dust bathing were pooled into "comfort behaviour", and all behaviours except resting and perching while sitting were pooled into "activity", and these pooled behaviours were analysed in addition to the separate behaviours. Behaviours that occurred in fewer than 0.5% of the observations (interaction other, other, defecation, food-running, dustbathing, pecking pen mate head/other, and wing flapping) were not analysed independently. Time spent in standing posture was analysed separately by pooling all behaviours displayed whilst standing. Concerning the detailed observations at larval provisioning times, preliminary analysis showed similar patterns in several foraging-related behaviours. Therefore, these behaviours could be well exemplified by patterns in "active behaviour" (all behaviours except resting), and only "active behaviour" around larval feeding was analysed. For this analysis observations were grouped per pen and expressed as percentage of active chicks. A curve representing activity over time was plotted, and the areas under the curve (AUC) before (3 scans) and after (10 scans) larval provisioning were analysed separately. Breast blisters and cleanliness scores above 0 were never observed. Hock burn and foot pad dermatitis scores were combined into absence (score = 0) or presence (score > 0) of the affliction as no scores above 1 were observed, and per chick only the leg with the highest score was included in the analysis. Gait scores were pooled into "absence of lameness" (score = 0), "slight walking abnormality" (score = 1) and "obvious walking abnormality" (score > 1) as scores higher than 2 were uncommon (< 2% of all broilers).

Data analysis

Data were analysed with SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Performance parameters (ADG, final weight, average daily DM intake, average daily ME intake and FCR based on DM) were analysed using a linear mixed model (MIXED in SAS). The ADG was analysed per week. The average daily DM and energy intake, and the FCR were analysed for the total grower period (day 8-42) only as partial floor feeding applied during the starter period prevented accurate measurements of feed intake. Models included treatment and experimental room as fixed effects, and the random effects of pen nested within room and treatment and (for ADG and final weight) chick nested within pen, room, and treatment. To analyse the relationship between activity (percentage of time spent active on the observation day closest to weighing) and ADG a similar model was used with activity as covariate. Behaviours observed in the behavioural time budget observations were analysed with a generalised linear mixed model (GLIMMIX in SAS) using a binomial distribution, logit link function, and additional multiplicative over-dispersion parameter. The model included a fixed effect of room, treatment, week (1-5) and the treatment by week interaction, a random effect of pen nested in room and treatment, and a random effect of week with chick as subject nested within pen, room, and treatment, with a heterogeneous first-order autoregressive covariance structure. The AUC of activity before and after larval provisioning was analysed for each of the three ages in a mixed model with room, treatment, larval provisioning time (08:00, 11:00, 14:00 and 17:00) and the treatment by provisioning time interaction as fixed effects, and larval provisioning time as repeated effect with pen nested in room and treatment as subject, with a homogenous first-order autoregressive covariance structure. The model also included two contrast statements to test overall effects of amount (5% vs 10%) and frequency (2× vs 4×) of larval provisioning. Concerning visual health parameters, the distribution of foot pad dermatitis, hock burn, and gait scores were analysed in a GLIMMIX with a binary distribution and logit link function for foot pad dermatitis and hock burn scores, and a multinomial distribution and cumulative logit link for gait score. The models included treatment and room as fixed effects and as random effects pen nested within room and treatment and chick nested within pen, room, and treatment. Significant fixed effects were analysed further using differences in least square means with a Tukey's HSD correction for pairwise comparisons, except for gait score for which pairwise differences were analysed with a Kruskal-Wallis test. Data are presented as means ± SEM based on pen averages, unless indicated otherwise. P-values below 0.05 were considered statistically significant and p-values between 0.05 and 0.1 indicate a trend. Pairwise differences with $p < 0.05$ are presented in the results.

Results

Behavioural time budget over the day

Treatment had no effect on shuffling (0.5 ± 0.3 %), perching (2.6 ± 0.1 %), stretching (1.4 ± 0.1 %), and comfort behaviour (4.5 ± 0.1 %, $p > 0.1$ for all). For all other analysed behaviours, effects of treatment, week and/or their interaction were found (see below).

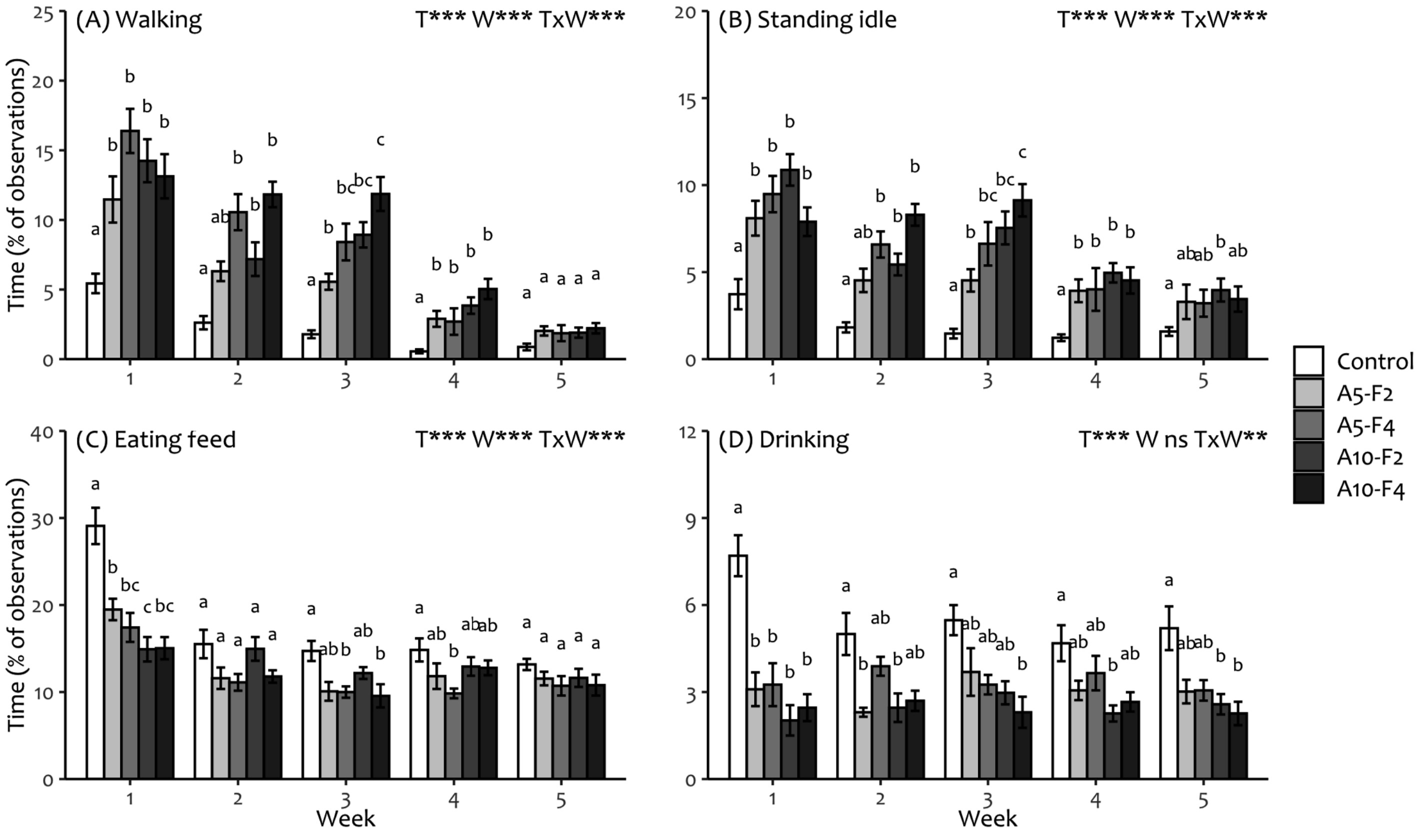
Walking

Walking was influenced by treatment, week, and their interaction ($p < 0.001$, **Figure 1A**). During the first four weeks, broilers in all larval provisioning treatments spent more time walking than controls without larvae, except for A5-F2 in week 2. In week 3, A10-F4 broilers also spent more time walking than A5-F2 broilers. Compared to week 1, a decline in time spent walking from week 2 onwards was observed in the control, F2 and A5-F4 broilers, whereas for the A10-F4 broilers this decline started in week 4.

Standing Idle

Standing idle was affected by treatment, week, and their interaction ($p < 0.001$, **Figure 1B**). Controls spent less time standing idle than broilers in the other treatments in several weeks (A5-F2: week 1, 3 & 4, A5-F4: week 1-4, A10-F2: week 1-5, A10-F4: week 1-4). In week 3, A10-F4 broilers also spent more time standing idle than A5-F2 broilers. A decline in time spent standing idle occurred from week 2 onwards for the controls and A5-F2 broilers, whereas for the other treatments time spent standing idle remained relatively constant during the first three weeks, after which a decline occurred.

Figure 1 (next page). Behavioural activities (% of observations) of broilers receiving no larvae (Control) or provided with larvae in different amounts (5% or 10% of the total dietary DM replaced with larvae, A5 and A10 respectively) and frequencies (two or four times a day, F2 and F4 respectively) scored at the end of week 1-5. Activity encompasses all behaviours except resting and perching while sitting. Foraging behaviour encompasses ground pecking, scratching and food running. Data are presented as means ± SEM. Effects of Treatment (T), Week (W) and their interaction (TxW) are indicated as ns (not significant), ** ($p < 0.01$) or *** ($p < 0.001$). Different letters within one week indicate significant ($p < 0.05$) differences between treatments.


(A) Walking
T*** W*** TxW***
(B) Standing idle
T*** W*** TxW***
(C) Eating feed
T*** W*** TxW***
(D) Drinking
T*** W ns TxW**
Time (% of observations)
Time (% of observations)
Week
Week
1
2
3
4
5
Control
A5-F2
A5-F4
A10-F2
A10-F4
0
5
10
15
20
25
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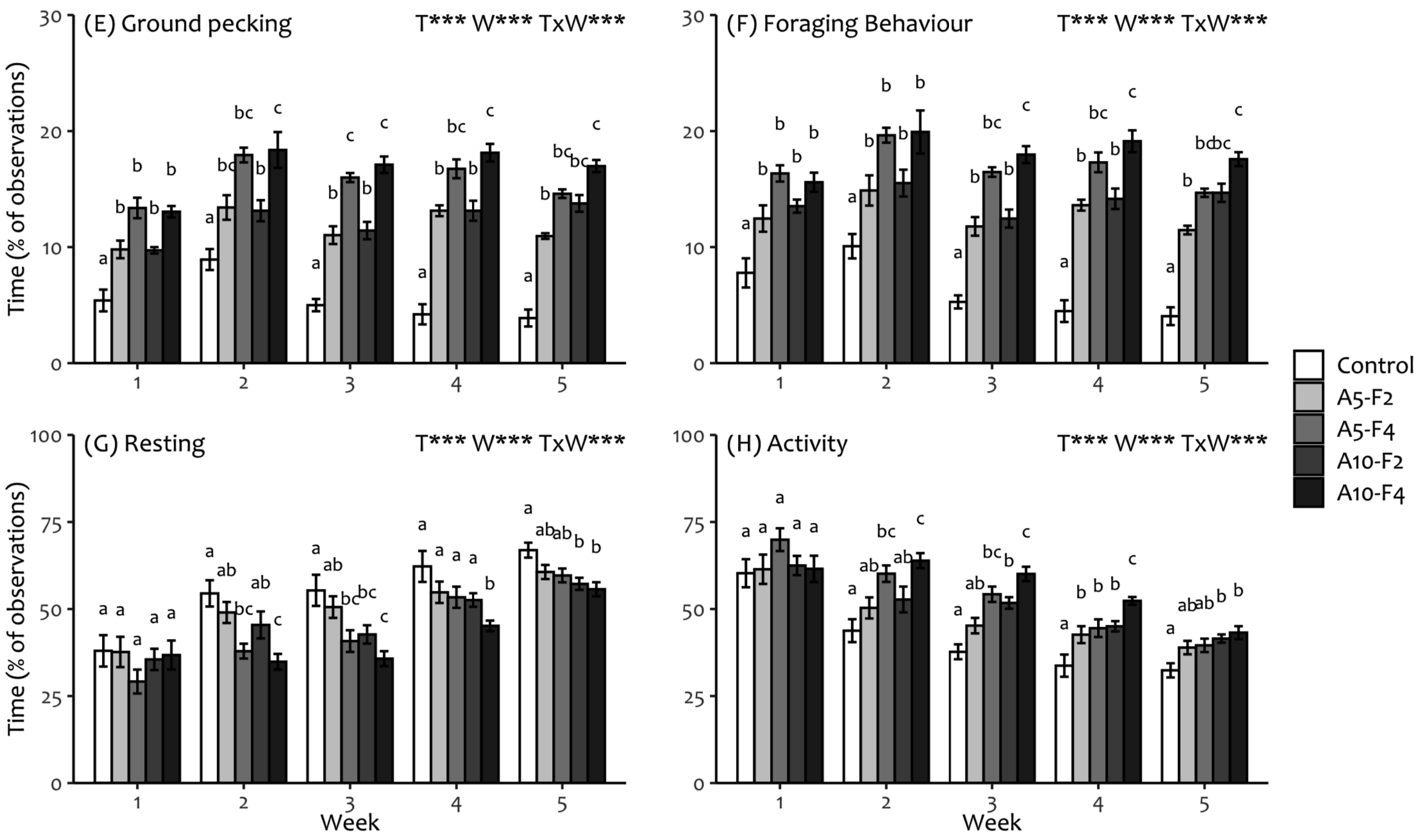


Figure 1 continued.

Eating feed and Drinking

The time spent eating feed was influenced by treatment, week, and their interaction ($p < 0.001$, **Figure 1C**), and time spent drinking was influenced by treatment and the treatment by week interaction ($p < 0.01$, **Figure 1D**). In week 1, controls spent more time eating feed than broilers in all other treatments and A5-F2 broilers spent more time eating feed than A10-F2 broilers. In week 3, controls spent more time eating feed than F4 broilers and this difference remained in week 4 for A5-F4 broilers. Controls also spent more time drinking for one or more weeks than broilers in all other treatments (A5-F2: week 1-2, A5-F4: week 1, A10-F2: week 1-3 & 5, A10-F4: week 1, 3 & 5).

Foraging behaviour

The time spent scratching was affected by treatment ($p = 0.001$) and was overall higher in A10-F2 broilers (1.7 ± 0.2%) than in controls (0.8 ± 0.1%) and A5-F4 broilers (0.7 ± 0.1%). Ground pecking and total foraging behaviour were influenced by treatment, week, and their interaction ($p < 0.001$, **Figure 1E**,**F**), and these behaviours were performed more in all larval provisioning treatments compared to controls in all weeks. In addition, A10-F4 broilers spent more time on these behaviours during several weeks than A5-F2 broilers (week 3-5) and A10-F2 broilers (week 3-4). Time spent on ground pecking (but not total foraging behaviour) increased between week 1 and 2 for all broilers. From week 2 onwards, controls showed a decline in ground pecking and total foraging behaviour, while the occurrence of these behaviours remained relatively constant in all BSFL treatments.

Resting

Resting behaviour was influenced by treatment, week, and their interaction ($p < 0.001$, **Figure 1G**). Controls spent more time resting in several weeks than A5-F4 (week 2-3), A10-F2 (week 3 & 5) and A10-F4 broilers (week 2-5). In one or more weeks A10-F4 broilers also spent less time resting than A5-F2 (week 2-4), A5-F4 (week 4) and A10-F2 broilers (week 2 & 4). Time spent resting increased from week 2 onwards for the control, F2 and A5-F4 broilers, and from week 4 onwards in A10-F4 broilers.

Overall activity

Overall activity, i.e., any behaviour except resting and perching while sitting, was affected by treatment, week, and their interaction ($p < 0.001$, **Figure 1H**), and was lower in control broilers in several weeks compared to A5-F2 (week 4), A5-F4 (week 2-4), A10-F2 (week 3-5) and A10-F4 broilers (week 2-5). Additionally, A10-F4 broilers were more active than F2 broilers in week 2-4 and A10-F2 broilers in week 4. Time spent active decreased from week 2 onwards for the control, F2 and A5-F4 treatments, and from week 4 onwards in A10-F4.

Posture

Posture was affected by treatment, week, and their interaction ($p < 0.001$ for all, **Figure 2**). Time spent in standing posture was lower in controls than in A10-F2 and F4 broilers during week 2-5. In several weeks A5-F2 broilers also showed less time standing than A5-F4 (week 2-3) and A10-F4 broilers (week 2-4), and in week 2 and 4 A10-F2 broilers spent less time standing than A10-F4 broilers. No other within-week differences between treatments were observed. Time spent in standing posture decreased from week 2 onwards in controls and F2 broilers, from week 3 onwards in A5-F4 broilers and from week 4 onwards in A10-F4 broilers.

Behaviour at larval provisioning

The percentage of active chicks per treatment around each larval provisioning time at three different ages (15/16, 29/30 and 40/41 days of age) is illustrated in **Figure 3**. Broilers in F4 treatments received larvae at all four larval delivery moments (08:00, 11:00, 14:00 and 17:00), while F2 broilers received larvae only at 08:00 and 14:00. Significant differences between the area under the curve of the activity plots before (3 scans) and after (10 scans) larval provisioning are presented below.

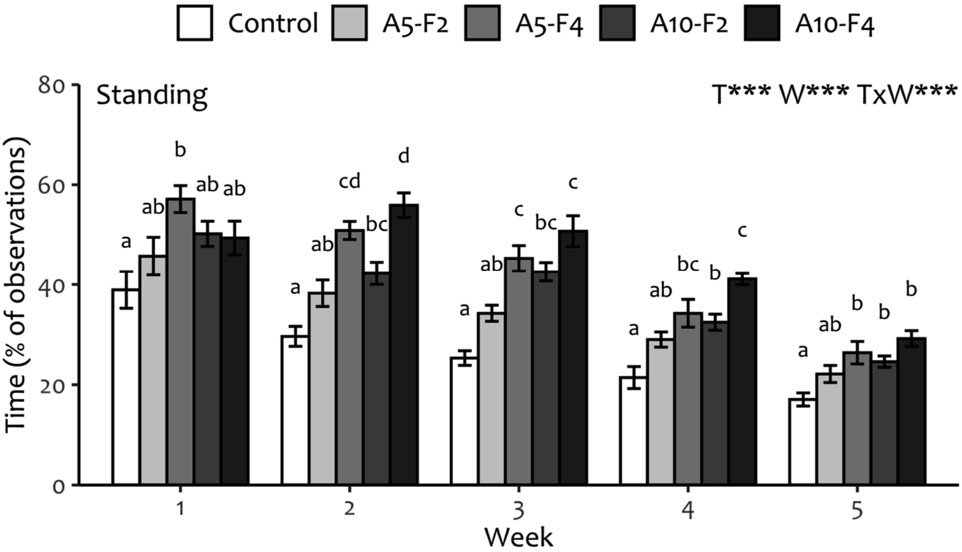
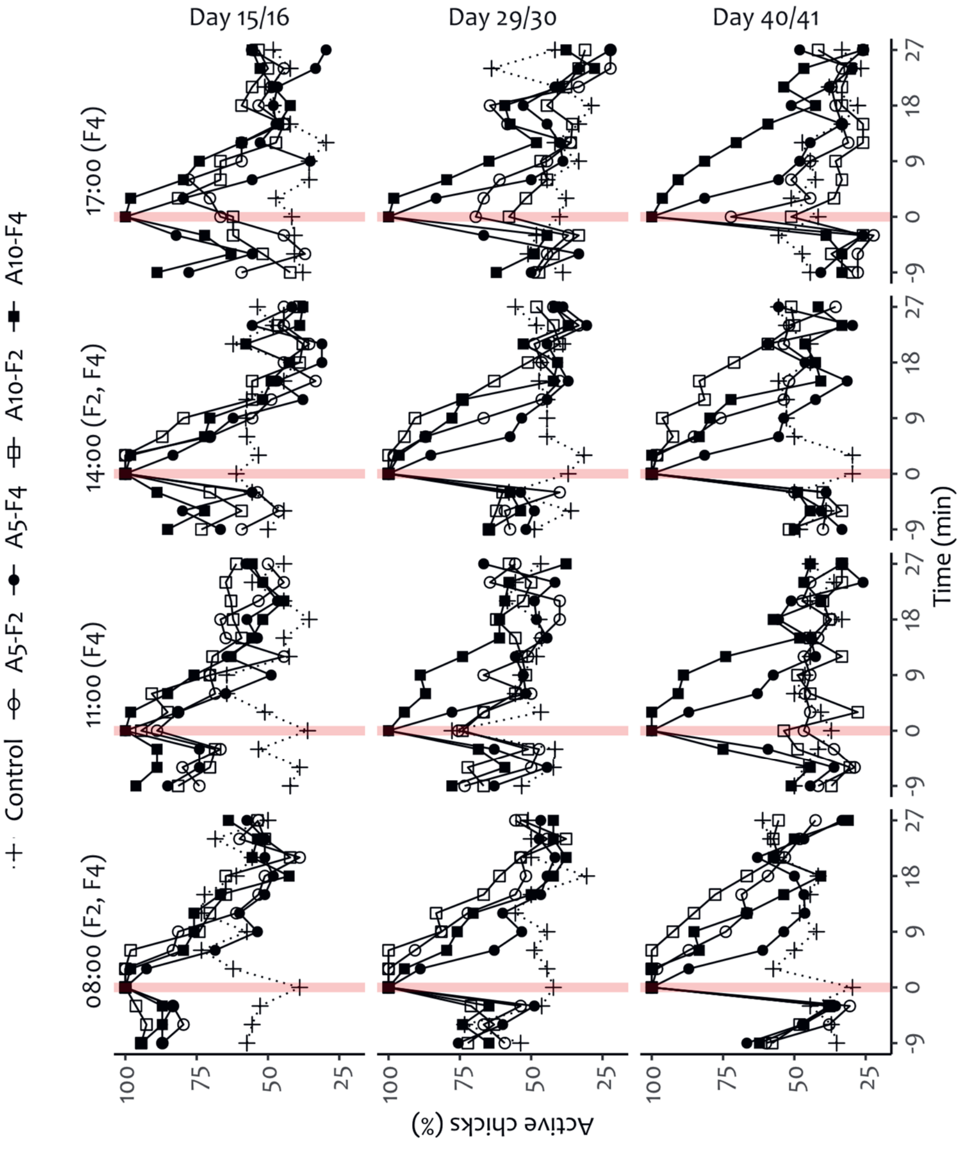


Figure 2. Posture (% of observations spent standing) of broilers receiving no larvae (Control) or provided with larvae in different amounts (5% or 10% of the total dietary DM replaced with larvae, A5 and A10 respectively) and frequencies (two or four times a day, F2 and F4 respectively) scored at the end of week 1-5. Data are presented as means ± SEM. Effects of Treatment (T), Week (W) and their interaction (TxW) are indicated as *** ($p < 0.001$). Different letters within one week indicate significant ($p < 0.05$) differences between treatments.

Figure 3 (next page). Percentage of broilers active around larval provisioning moments. Broilers received no larvae (Control) or were provided with larvae in different amounts (5% or 10% of the total dietary DM replaced with larvae, A5 and A10 respectively) and frequencies (two or four times a day, F2 (received larvae at 08:00 and 14:00) and F4 (received larvae at 08:00, 11:00, 14:00 and 17:00) respectively). Each row represents one observation period (day 15/16, 29/30 and 40/41), each column represents one moment of larval provisioning (08:00, 11:00, 14:00 and 17:00) with the treatments receiving larvae at that moment between brackets. Instantaneous 3-min scan sampling was done from 9 minutes before until 27 minutes after larvae delivery. Larval delivery (at t = 0) is indicated by the vertical line.



Activity before larval provisioning

On day 15/16 activity during the 9 min before larval provisioning, as calculated by the area under the curve, was influenced by treatment ($p < 0.001$) and the treatment by provisioning time interaction ($p = 0.038$). During several provisioning times some BSFL treatments were more active than controls (A5-F4: 08:00 & 11:00; A10-F2: 08:00; A10-F4: 08:00, 11:00, 14:00 & 17:00). On day 29/30 activity before larval provisioning was influenced by treatment ($p = 0.014$), where only A10-F4 broilers were more active than controls. On day 40/41 activity before larval provisioning was similar for all treatments (**Figure 3**).

Activity after larval provisioning

On day 15/16 activity during the 27 min following larval provisioning, as calculated by the area under the curve, was influenced by treatment ($p < 0.001$), provisioning time ($p < 0.001$), and their interaction ($p = 0.038$). During several provisioning times broilers in some BSFL treatments were more active than controls (A5-F2: 17:00; A10-F2: 11:00; A10-F4: 11:00 & 17:00). The A5-F4 broilers never differed in activity from controls, and no differences between BSFL treatments occurred. On day 29/30 and 40/41 activity following larval provisioning was affected by treatment, provisioning time and treatment by provisioning time interaction ($p < 0.001$ for all). Day 29/30 showed a similar trend as day 15/16, with larval provisioning treatments at some time points being more active than controls (A5-F2: 08:00; A10-F2: 08:00 and 14:00; A10-F4: 11:00 & 17:00). On day 40/41 all larval provisioning treatments except A5-F4 showed higher activity compared to controls after receiving larvae (F2: 08:00 & 14:00; A10-F4: 08:00, 11:00, 14:00 & 17:00). In line with this, controls (which never received larvae) and F4 broilers (which received larvae at all four time points) exhibited consistent activity levels across the four provisioning times, whereas F2 treatments showed higher activity following larvae provisioning at 08:00 and 14:00 than following 11:00 and 17:00 (when no larvae were provided to them). Also, A10-F2 broilers were more active after receiving larvae than A5-F4 broilers (08:00 & 14:00) and this was reversed when no larvae were provided to A10-F2 broilers at 17:00. Similarly, during moments when no larvae were provided to the F2 broilers (11:00 & 17:00) they were less active than A10-F4 broilers. When testing for an effect of amount and frequency by using contrast statements, it was determined that treatments receiving a higher amount of larvae were overall more active after larval delivery on all days whereas frequency of provisioning did not significantly affect activity after larval provisioning (**Figure 3**).

Visual health scores

Percentages of chicks with distinct visual health scores are depicted in **Figure 4**. The occurrence of foot pad dermatitis was low (< 4%) and not affected by treatment (p = 0.99). Treatment did influence the incidence of hock burn (p = 0.007), and post-hoc analysis indicated that the percentage of chicks with hock burn was higher in controls than in A10-F2 (p = 0.033) and A10-F4 (p = 0.017). An effect of treatment (p = 0.005) was also found on gait score (score of 0, 1, and > 1). Elevated gait scores occurred more in controls compared to A5-F4 (p = 0.003), A10-F2 (p < 0.001) and A10-F4 (p = 0.001) broilers, and they occurred more in A5-F2 broilers compared to A10-F2 (p = 0.001) and A10-F4 (p = 0.014) broilers.

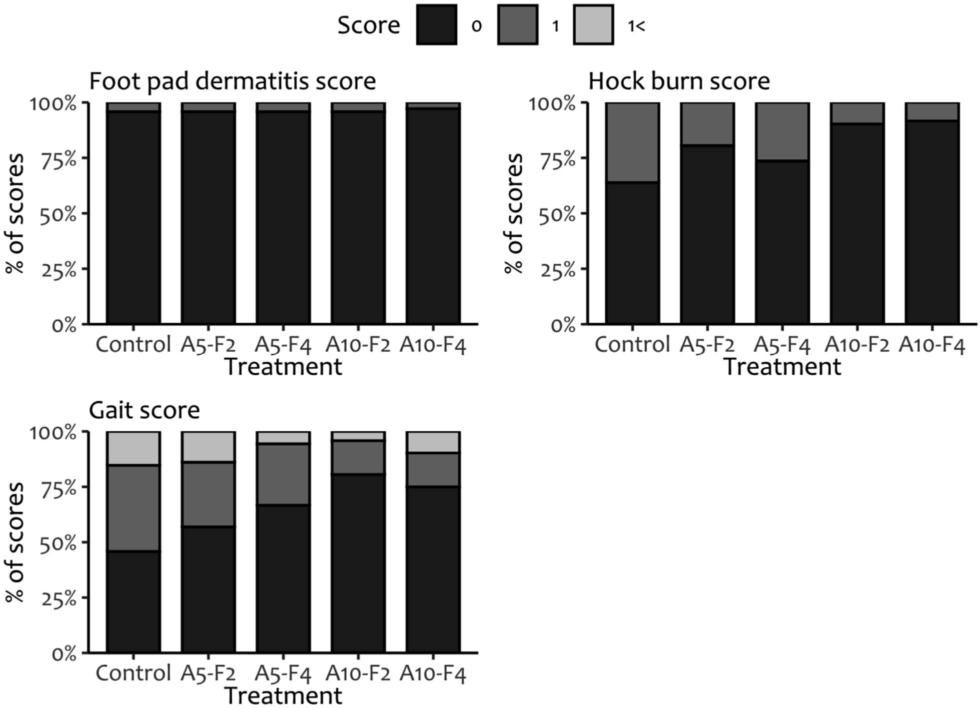


Figure 4. Percentage of broilers with distinct foot pad dermatitis scores, hock burn scores and gait scores, per treatment. Treatments include broilers receiving no larvae (Control) or provided with larvae in different amounts (5% or 10% of the total dietary DM replaced with larvae, A5 and A10 respectively) and frequencies (two or four times a day, F2 and F4 respectively).

Performance

The percentage of the total DM intake consisting of BSFL was 6.3 ± 0.1% for A5-F2, 6.4 ± 0.1% for A5-F4, 12.7 ± 0.1% for A10-F2 and 12.3 ± 0.3% for A10-F4 broilers. Performance parameters are shown in **Table 3**. Controls had a higher ADG than A10 broilers during days 13-20 and 21-27 ($p < 0.020$ for all) and a higher final weight than A10-F2 broilers ($p = 0.049$). Activity added as a covariate in the model for ADG influenced ADG ($p < 0.001$ for all weeks), in addition to removing the significant treatment effect during day 13-27. The average daily DM intake excluding BSFL was higher in control broilers compared to all others, in A5-F2 compared to A10 broilers and in A5-F4 compared to A10-F2 broilers. The average daily DM intake including BSFL and the average daily ME intake did not differ between treatments. The FCR based on DM was higher in the A10 treatments than in the A5 treatments, with the FCR of the control treatment being in between. No morbidity (besides the visually scored health problems) or mortality was observed.

Table 3. Performance parameters of broilers receiving no larvae (Control) or provided with larvae in different amounts (either 5% or 10% of the total dietary dry matter replaced with larvae, A5 and A10 respectively) and frequencies (two or four times a day, F2 and F4 respectively).

Measure	**Control**	**A5-F2**	**A5-F4**	**A10-F2**	**A10-F4**	**P-value**
ADG (g)						
d1-d6	9.2 ± 0.3	9.3 ± 0.2	8.5 ± 0.3	9.2 ± 0.3	9.7 ± 0.2	0.52
d7-d13	29.5 ± 0.6	29.7 ± 0.6	27.9 ± 0.7	27.6 ± 0.7	29.5 ± 0.6	0.27
d14-d20	55.8 ± 1.0^{a}	53.2 ± 1.0^{ab}	52.2 ± 1.0^{ab}	50.4 ± 1.1^{b}	49.9 ± 1.0^{b}	**0.01**
d21-d27	82.1 ± 1.6^{a}	79.2 ± 1.5^{ab}	79.6 ± 1.6^{ab}	75.8 ± 1.3^{b}	75.4 ± 1.6^{b}	**0.01**
d28-d34	102.2 ± 2.1	105.8 ± 2.2	108.2 ± 1.8	103.4 ± 1.6	107.8 ± 2.2	0.26
d35-d42	111.5 ± 2.7	114.6 ± 2.2	112.2 ± 1.9	103.4 ± 2.2	109.2 ± 2.6	0.18
FW (g)	2890 ± 37.9^{a}	2902 ± 39.4^{a}	2866 ± 38.5^{ab}	2726 ± 36.9^{b}	2808 ± 42.6^{ab}	**0.02**
ADMI[1] (g)						
-BSFL[2]	97.03 ± 1.84^{a}	90.67 ± 1.13^{b}	89.83 ± 1.12^{bc}	82.83 ± 0.61^{d}	85.28 ± 2.32^{cd}	**<0.001**
+BSFL[3]	97.03 ± 1.84	96.54 ± 1.13	95.7 ± 1.12	95.06 ± 0.61	97.52 ± 2.33	0.86
ADMEI[1,3] (MJ)	1.20 ± 0.02	1.20 ± 0.01	1.19 ± 0.01	1.19 ± 0.01	1.22 ± 0.03	0.85
DMCR[1,3] (g/g)	1.20 ± 0.02^{ab}	1.20 ± 0.01^{a}	1.20 ± 0.01^{a}	1.26 ± 0.01^{b}	1.25 ± 0.01^{b}	**0.001**

ADG: average daily gain. FW: final weight. ADMI: average daily dry matter intake. ADMEI: average daily metabolizable energy intake. DMCR: dry matter conversion ratio. Data are reported as means ± SEM. Within each row different letters indicate significant ($p < 0.05$) differences between treatments.

[1] Based on the grower period, day 8-42.

[2] Based on intake of feed (meal).

[3] Based on intake of feed (meal) and BSFL.

Discussion

The present study investigated the effect of providing live black soldier fly larvae (BSFL) in different amounts and frequencies on broiler behaviour, leg health and performance. Broilers receiving larvae showed a profound increase in active behaviours compared to controls. In line with our hypothesis, the largest amount of larvae provided at the highest frequency, i.e., 10% of the dietary DM as larvae spread over four provisioning times a day, seemed most effective in promoting activity and lowering the occurrence of hock burn and lameness, while the final weight of these broilers was not significantly reduced compared to controls.

All broilers receiving BSFL increased their time spent walking, standing idle, ground pecking and foraging, whilst their time spent resting was decreased compared to controls. Behavioural observations of the daily time budget and around larval provisioning showed that the activity of broilers receiving 5% of their dietary DM as BSFL provided two times a day was only occasionally elevated. Providing this amount four times a day resulted in a strong but short-term peak in activity around larval provisioning, explaining why the area under the curve of activity around larval provisioning was not affected. However, in most weeks the activity in the daily time budget observations was increased in these A5-F4 broilers compared to controls. A comparable increase in activity was observed in broilers receiving 10% of their dietary DM as BSFL twice a day. Finally, the highest activity level was achieved by broilers receiving 10% of their dietary DM as BSFL four times a day. In several weeks this group showed more active behaviours than other groups, apart from the controls, particularly the A5-F2 group. Furthermore, for all broilers the time spent walking and being active decreased over time and the time spent resting increased over time, which is in line with other studies (Bailie et al., 2013; Baxter et al., 2018c). However, broilers receiving 10% of their dietary DM as BSFL provided four times a day started showing this decline in activity later in the production period than all other treatments. Increasing and maintaining activity levels from an early age onwards was found to promote good leg bone development in broilers (Reiter and Bessei, 2009), and the observed prolonged elevated activity levels could therefore indicate improved leg health in these broilers.

Our results suggest that providing BSFL shifts the behavioural repertoire of broilers towards more active, natural behaviours such as foraging, and that this shift is strongest when a large amount of larvae is provided in a high frequency. This is in line with previous studies showing modest increases in activity around insect provisioning in small amounts (Oonincx, personal communication; Pichova et al., 2016), where authors suggested that prolonged access to larvae would further increase broiler activity. Other previously tested enrichment materials such as straw bales or strings caused a temporary elevation of broiler activity only (Bailie et al., 2013; Bailie and O'Connell, 2015), and providing wood shavings, perches, and metal chains even reduced activity compared to controls (de Jong and Gunnink, 2018). The main difference between these materials and BSFL is likely the strong appetitive value for broilers and the fact that BSFL are alive and moving, which could make them highly interesting for broilers (Bokkers and Koene, 2002; Jones et al., 1998) and therefore more effective in increasing foraging behaviour and thus activity. This is supported by the observation that the increase in foraging-related behaviours was more prominent and long term than that of other behaviours in all BSFL treatments. As being active becomes more energy-expensive with increased broiler weight (Tickle et al., 2018), it is likely that the presence of consumable larvae is required to increase activity levels later in the production period, and that this activity primarily consists of foraging behaviour. This premise is further supported by the observation that the delivery of desirable mealworms also caused a stark increase in foraging behaviours in a previous study (Pichova et al., 2016). Our results suggest that BSFL in contrast to many other enrichment materials strongly motivate broilers to show active behaviour, particularly foraging, until the end of the rearing period, despite the energetic costs.

Apart from affecting broiler activity, the consumption of BSFL influenced broiler performance, even though by mimicking the nutritional value of BSFL in the feed a similar ME intake was achieved for all treatment groups. The relative consumption of BSFL was slightly higher than anticipated (approximately 6% and 12% instead of the expected 5% and 10% of dietary DM), which could have caused a slight imbalance in amino acid uptake, affecting broiler growth. Broilers receiving 10% of their dietary DM as larvae showed a reduced growth during day 13-27, and A10 broilers receiving those larvae two times a day had a lower final weight compared to controls. Previous studies found that BSFL consumption in low levels (up to 5% of the dietary DM) had a neutral (J. Lee et al., 2018) or positive (Dabbou et al., 2018) effect on broiler growth, whereas diets containing 10-15% live BSFL resulted in diminished broiler growth and final weight (Oonincx, personal communication). Chitin could play a role in the observed reduction in broiler growth, as suggested by Dabbou et al. (2018). Chitin can be only partially digested by broilers (Hossain and Blair, 2007; Khempaka et al., 2006), and it can hinder digestibility of crude protein in the broiler digestive tract (Khempaka et al., 2011). In our study, providing 10% of the dietary DM as BSFL twice a day resulted in relatively large portions of larvae, therefore the digestion-inhibiting effect of chitin could have been strongest for these broilers, resulting in the observed diminished performance. This is also in line with the absence of an effect on final weight of broilers receiving this amount provided four times a day, and thus in smaller portions.

In our experiment broilers were kept at a relatively low stocking density, with good litter quality, which could explain the low occurrence of leg problems compared to commercial conditions (Bessei, 2006; de Jong et al., 2019). Even so, the occurrence and severity of hock burn and lameness was significantly lower in broilers receiving 10% of their dietary DM as larvae compared to controls, and the severity of lameness was lower in broilers receiving 5% of the dietary DM as larvae four times a day compared to controls. Leg problems can be painful and inhibit natural behaviour (Danbury et al., 2000), suggesting these broilers experienced improved welfare compared to others. We expect that the beneficial effect of larvae provisioning on leg health will be more prominent under commercial conditions where broilers can benefit more from enrichment, however the applicability of providing BSFL in commercial settings remains to be investigated. For instance, research on the effect of lower light intensities and higher stocking density on BSFL detection and potential negative effects of anticipation of BSFL delivery on bird-directed pecking behaviour deserve attention. In addition, the costs, supply, and distribution methods of the larvae must be considered.

Providing BSFL affected broiler activity, weight and leg health, and these variables may be interconnected. A positive association between leg health and activity was found in our study and in previous studies (Bassler et al., 2013; de Jong et al., 2014). Causality in this relationship is unclear, as activity could promote better leg development and increase leg strength (Reiter and Bessei, 2009), but painful leg problems could also inhibit broiler activity (Danbury et al., 2000). Besides, a link between activity and leg health may also arise from the bi-directional relationship between broiler activity and growth. On the one hand, increased activity levels could result in less energy being available for broiler growth (Tickle et al., 2018). On the other hand, an inherently lower growth rate facilitates higher activity levels as observed in slow-growing broilers (Bokkers and Koene, 2003a). In the current study providing 10% of the dietary DM as larvae provided two times a day improved broiler leg health, however as this group displayed a lower ADG and final weight, the effect might be confounded with body weight. Overall, the causality of the relationship between growth, activity and leg health in the current experiment is unclear and requires further examination. However, the improved leg health and activity levels without significantly reduced growth observed in broilers receiving 5% or 10% of their dietary DM as BSFL provided four times a day indicates that improved activity and welfare by larval provisioning can be obtained without impairing performance.

Not only leg health but also specific broiler behaviours are linked to broiler welfare. In the current study, comfort behaviour was not affected by larval provisioning. Similarly, providing mealworms did not promote nor diminish comfort behaviour in broilers (Pichova et al., 2016). In laying hens, comfort behaviour was exhibited in anticipation of a reward, namely access to mealworms (Zimmerman et al., 2011), and it has been suggested that comfort behaviour is an indicator of good welfare (Nicol et al., 2009). However, Moe et al. (2014) found that anticipatory comfort behaviour was not affected by a dopamine blockade in layers, indicating a potential disengagement between comfort behaviour and the reward system in chickens. Other natural behaviours such as foraging behaviour were increased in the current study. Performing such intrinsically motivated behaviours can be rewarding (Moe et al., 2014, 2012), and therefore improve broiler welfare (Bracke and Hopster, 2006), although this was not directly investigated in the current experiment. Further research including other welfare indicators, preferably also assessing affective state, is needed to determine the effect of larval provisioning on broiler welfare, as well as studies on the impact of BSFL provisioning on welfare of broilers kept under commercial conditions.

Conclusion

In conclusion, this study showed long-term elevated levels of foraging behaviour and general activity in broilers receiving BSFL, and this effect was largest and most consistent for broilers receiving BSFL in the highest amount and frequency tested, i.e., 10% of their dietary DM as larvae provided four times a day. Broilers receiving 5% or 10% of their dietary DM as BSFL four times a day also experienced improved leg health. These broilers had a similar final weight as controls, despite a temporary period of reduced growth for broilers receiving 10% of their dietary DM as BSFL four times a day. Thus, by facilitating natural behaviour and activity, and by reducing leg health problems, larval provisioning can benefit broiler welfare. Further studies will focus on strategies facilitating prolonged access of BSFL for broilers.

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Supplementary data

Table S1. Ingredients and (analysed) chemical composition of dietary components.

	Starter feed[1,5]	Core feed[2,3,5]	BSFL-replacer mix[2,4,5]	BSFL[2,6]
Ingredients (g/kg)				
Corn	368.1	225.1	-	-
Wheat	250.0	409.6	-	-
Soybean meal	290.0	201.3	-	-
Fishmeal	-	69.9	160	-
Rapeseed meal	40.0	33.2	-	-
Potato protein	-	-	350	-
Sunflower oil	16	33.6	-	-
BSFL oil		-	350	-
Premix	5	5.6	-	-
Lime fine	14	-	-	-
Monocalcium phosphate	8.3	4.5	11.0	-
Salt	2.1	2.3	-	-
$CaCO_3$	-	10.6	-	-
$NaHCO_3$	1.8	1.2	-	-
Phytase	0.2	0.2	-	-
DL-methionine	2.1	2.1	-	-
L-threonine	0.45	0.6	-	-
L-valine	0.1	0.1	-	-
L-lysine HCl	1.85	-	-	-
Diamol	-	-	129	-
Chemical composition				
Dry matter	876.8	883	900	350.7
ME	12.46	11.53	19.5	19.5
Crude protein	220	223	419	419
Crude fat	36.5	80	371	371
Calcium	9	7.6	6.3	6.3
Phosphorus	7.1	6.0	6.8	6.8

Nutrients are presented in g/kg dry matter, except ME (MJ/kg of dry matter) and dry matter (g/kg).

[1] Provided to all broilers during the starter period (day 1-7).

[2] Provided in different combinations during the grower period (day 8-42).

[3] Core diet component of the grower feed supplied to all broilers.

[4] Diet component with similar chemical composition as BSFL.

[5] Chemical composition according to CVB (2006).
[6] Chemical composition based on analysis via standardized protocols (DM 10032, Protein 10005, Fat 10112, Calcium and Phosphorus 10040).

Table S2. (Chemical) composition of grower feed.

	Control	**A5**	**A10**
Dietary component[1], % of total DM intake			
Core feed	90	90	90
BSFL-replacer mix	10	5	0
BSFL	0	5	10
Chemical composition			
ME (MJ/kg of DM)	12.4	12.3	12.3
Crude protein (g/kg of DM)	244	243	243
Crude fat (g/kg of DM)	111	111	110
Calcium (g/kg of DM)	7.5	7.5	7.5
Phosphorus (g/kg of DM)	6.0	6.0	6.1

Control, A5 and A10: 0%, 5% and 10%, respectively, of the total dry matter intake replaced with live larvae.

[1] Distributions of diet components are based on larval supply, to provide all broilers with iso-energetic diets with a similar chemical composition.

Chapter 3

Long-term access to live black soldier fly larvae (*Hermetia illucens*) stimulates activity and reduces fearfulness of broilers, without affecting health

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Abstract

Commercially housed broilers frequently experience limited environmental stimulation and various health issues, compromising their welfare. Providing environmental enrichment can alleviate these problems by facilitating natural behaviour and activity. We investigated the effect of providing live black soldier fly larvae (BSFL) to broilers housed at commercial densities (33 kg/m^2) on behaviour, fearfulness, health, and performance. One-day-old broilers were distributed over five treatments with eight pens/treatment: a control treatment without BSFL; two treatments where 5% of the daily nutrient intake was replaced with live BSFL, provided four or seven times a day; and two treatments where 10% of the daily dietary intake was replaced with live BSFL provided four times a day or in transparent, movable tubes with holes. In all BSFL treatments foraging behaviour, and thereby broiler activity, was increased. Prolonged access to live BSFL, either by providing larvae seven times a day or in tubes, caused the largest increase in activity while also decreasing the time spend in tonic immobility, indicating reduced fearfulness. Broiler final weight and health were not affected. Overall, long-term access to live BSFL seems most effective in improving broiler welfare by facilitating natural behaviour and reducing fearfulness, without hindering broiler performance and health.

Introduction

Decades-long genetic selection on increased growth rate and feed efficiency in broilers has caused broiler meat to become one of the most consumed animal meats worldwide (OECD/FAO, 2019). Fast growth combined with a commercial rearing environment can compromise broiler welfare and cause low levels of activity. Broilers housed in large commercial systems spend around 70% of their time sitting (Baxter et al., 2018a; de Jong and Gunnink, 2018), with the highest levels of sitting occurring at the end of the production period. The legs of the inactive broilers are in long-term contact with moist and ammonia-rich litter, which can result in contact dermatitis (Bassler et al., 2013; de Jong et al., 2014), including foot pad dermatitis and hock burn. Furthermore, inactivity has been linked to a range of developmental leg problems such as tibial dyschondroplasia, resulting in lameness (Bessei, 2006; Bradshaw et al., 2002). Reports indicate that 20-30% of broilers suffer from moderate to severe lameness (de Jong et al., 2019;

Kittelsen et al., 2017; Vasdal et al., 2018a), and as lameness can be painful (Danbury et al., 2000), limit the expression of active behaviours (Weeks et al., 2000), and reduce access to feed and water (Weeks et al., 2000), it impairs broiler welfare. Welfare problems tend to be worsened under commercial conditions with high stocking densities, restricted space, and minimal environmental stimulation, as these conditions limit the broiler's range of behavioural choices and can cause elevated levels of fearfulness (Altan et al., 2013; ESFA, 2010).

Providing environmental enrichment that facilitates a broader range of behaviours might increase broiler activity, resulting in improved leg health and broiler welfare. However, in previous studies enrichment materials such as straw bales, perches or strings, had varying effects on broiler activity and welfare (for review see Pedersen and Forkman, 2019; Riber et al., 2018). In a recent study, foraging behaviour and activity of broilers were successfully increased by means of lasers which moved around the pen four times a day in 4-min periods (Meyer et al., 2019). While this type of enrichment increases broiler activity, facilitating foraging behaviour without including a rewarding aspect (e.g., consumption of a feed item) could cause frustration. Providing a preferred feed item as enrichment could increase foraging behaviour without causing frustration. Also, foraging for and consuming a preferred feed item can be rewarding and positively influence dopaminergic systems in the broiler's brain (Moe et al., 2014), and through this promote a more positive affective state (Spruijt et al., 2001). Laying hens with outdoor access showed both a more positive affective state and lower levels of fearfulness than layers kept indoors, and if a similar link can be found in broilers, providing preferred feed items could reduce broiler fearfulness through improving their affective state (Campbell et al., 2019).

Feed items that could be used as effective enrichment are insects, which broilers are highly motivated to interact with and consume (Bokkers and Koene, 2002). Insects are part of the natural diet of chickens, and several insect meals have been included in broiler feed without negatively affecting broiler health and/or performance (Dabbou et al., 2018; Khan et al., 2018; Leiber et al., 2017). Providing a small amount of mealworms increased broiler activity for several minutes (Pichova et al., 2016). In our previous study, replacing 5 or 10% of the dietary dry matter intake of broilers with live black soldier fly larvae (BSFL) and scattering them across the pen twice or four times a day promoted active behaviours. Furthermore, providing live BSFL four times a day reduced the occurrence of lameness and hock burn without affecting performance (Ipema et al., 2020b). While BSFL provisioning successfully promoted activity, the broilers in this study were kept at low stocking densities (4.5 broiler/m^2) (Ipema et al., 2020b). Stocking density can affect broiler behaviour and welfare (Sanotra et al., 2001). Therefore, broilers might respond differently to insect provisioning when housed at commercial densities. Furthermore, several authors have suggested prolonging the period during which broilers have access to insects, as this can amplify the increase in broiler activity, as well as the resulting welfare benefits (Pichova et al., 2016). Prolonging the access duration could have the additional benefit of allowing broilers to habituate more to the presence of novel items that do not require a fear response, potentially reducing broiler fearfulness. Currently, it is unclear whether prolonging access to live BSFL expands the welfare benefits for broilers, and which methods for prolonging access to insects are most effective in improving broiler welfare.

The aim of this study was to determine the effect of prolonged access to live BSFL on the behaviour, fearfulness, health, and performance of broilers kept under commercial densities (33 kg/m^2), while maintaining a similar nutrient intake between treatments. We hypothesized that prolonged access to live BSFL would cause the largest activity increase, fearfulness reduction, and health improvement compared to controls.

Methods

The Animal Care and Use committee of Wageningen University & Research approved the experimental protocol under project licence number 2018.W-0036, and the protocols were in accordance with the Dutch law on animal experimentation, which complies with European Directive 2010/63/EU. The experiment was executed at the animal experiment facilities of Wageningen University & Research (Wageningen, The Netherlands).

Animals and management

At arrival, 880 one-day old male Ross 308 broilers received a neck tag after which they were randomly distributed over forty pens, resulting in 22 broilers/pen. Ten randomly selected focal broilers per pen were marked with a coloured dot (stock marker) for individual identification. Each pen was 1 × 2 m in size and contained two feeders, five drinking nipples with cups, one 1 m long perch (rectangular bar of 2 × 2 cm, 10 cm high) and a 5 cm deep layer of wood shavings. Feed and water were provided *ad libitum*. The lighting schedule (20 lux at chick level) was 1 D:23 L on day 1-3, after which it was gradually decreased to 6 D:18 L and kept constant throughout the remainder of the experiment. Temperature and humidity were based on the Aviagen Ross broiler handbook. At hatch, all chicks received an IB vaccination (spray) and on day 16 they received NCD vaccination (spray).

Experimental design

The study included one control treatment without BSFL provisioning and four treatments with live BSFL provisioning. All treatments were designed to maintain identical nutrient composition of the total feed plus live BSFL offered, as outlined below. The two most effective treatments from our previous study (Ipema et al., 2020b), i.e., replacing 5 or 10% of the dietary dry matter (DM) intake with live BSFL scattered throughout the pen in four equal portions a day, were included in the current study to determine their effect under commercial stocking densities. Two additional treatments were included; one in which 5% of the dietary DM intake was replaced with live BSFL, scattered throughout the pen in seven equal portions a day, and one in which 10% of the dietary intake was replaced with live BSFL, provided in transparent tubes with holes. Scattering a small amount seven times a day reduces portion size, making the larvae harder to locate, and providing larvae in tubes requires broilers to actively manipulate the tubes to acquire the live larvae. These treatments were chosen with the aim of prolonging access to the live larvae. Tubes were filled every morning with the total daily amount of live BSFL, and broilers had continuous access to the tubes. Based on a pilot study, tube size and the number of holes in the tubes were chosen as they resulted in limited larvae release solely by larvae activity, meaning that broilers had to actively get the live larvae out of the tubes. Tube dimensions and holes were adjusted throughout the experimental period to complement the increasing broiler size and the increasing BSFL amount (**Supplementary Table S1**). In the remainder of the text, all BSFL treatments will be referred to by the BSFL amount (5 or 10%, called A5 and A10) and BSFL delivery method (scattered four or seven times a day, called S4 and S7; and in tubes, called TB, **Supplementary Table S2**). Each treatment was applied to eight pens, which were equally distributed over eight blocks, with four blocks per experimental room (**Supplementary Table S3**). A commercial BSFL producer supplied live 14-day-old BSFL each week, which were stored at 12 °C near the experimental rooms until use.

Diet composition

All diets met or exceeded the broilers' nutrient requirements (CVB, 2016). From day 1-7, broilers received a starter feed (12.46 ME/kg DM, 22% crude protein) that was not adjusted for larval intake as the digestible nutrient intake from BSFL was expected to be low for young broilers. From day 8-42, broilers received a grower feed which was adjusted to the estimated larvae intake to ensure a similar nutrient and energy intake between treatments. The grower feed was designed as follows. First, three samples of BSFL were analysed for dry matter, crude protein, crude fat, calcium, and phosphorus content. Based on this analysis, a feed mix was designed mimicking the nutritional composition of BSFL. A basal grower feed containing 10% of this mix was provided to control broilers. The grower feeds for the A5 and A10 broilers were designed by omitting half or all this mix from the basal feed, respectively (nutrient composition in **Supplementary Table S4**). Results from the BSFL analysis were also used to determine the daily portions of live larvae.

Measurements

Behavioural observations

The behaviour and posture of focal broilers were observed in 8-min intervals by instantaneous scan sampling one day in week 1-5 (day 5, 12, 19, 26, and 33) according to the ethogram in **Table 1**. Each day had seven 64-min observation periods, and each period consisted of one scan before and seven scans after the time of larval provisioning (of the A5-S7 group, as not all treatments received larvae in each observation period). Four observers simultaneously observed 10 pens each, and every observation period the observers switched between pens. The observers were trained, and inter-observer reliability was deemed to be sufficient (Fleiss kappa > 0.8) before observations took place.

Table 1. Ethogram of behavioural observations.

Item	Description
Behaviour	
Eating	Having head above or in the feeder and/or pecking at feed in the feeder or on the floor.
Drinking	Drinking from nipple or cup beneath nipple.
Locomotion	Walking (locomoting in upright position with a normal speed or quick steps) or shuffling (half standing/half sitting and moving a few steps before sitting down), without performing any other behaviour.
Defecation	Excreting faeces.
Standing idle	Standing on the ground without performing any other behaviour.
Perching	Perching without performing any other behaviour.
Resting	Sitting with hocks resting on ground without performing any other behaviour, possibly with head on the ground or under wing.
Scratching	Scraping of the litter with the claws.
Ground pecking	Performing pecking movements directed at the ground.
Food running	Running with food in beak while pen mates follow and attempt to grab the food item.
Dust bathing	Performed with fluffed feathers while lying, head rubbed on floor, wings opened, scratching at ground, distributing substrate over body.
Stretching	Stretching of wing and/or leg.
Preening	Grooming of own feathers with beak.
Wing flapping	Bilateral up-and-down wing flapping.
Agonistic	Jumping at pen mate, threatening pen mate, pecking movements directed at head of pen mate.
Pecking pen mate	Pecking movements directed at the body or beak of pen mate.
Interaction tube	Pecking movement directed at tube or moving tube.
Other	Any behaviour not mentioned above.
Posture	
Standing	On floor: hocks not in contact with the litter. On perch: knees not bent.
Sitting	On floor: hocks in contact with the litter. On perch: knees bent.

Tonic immobility test

On day 15 and 16 between 08:30-12:30, four focal birds per pen were subjected to a tonic immobility (TI) test, a well-validated test used to assess chicken fearfulness (Forkman et al., 2007). Broilers were tested in a randomized order balanced for treatment, in a separate room close to the experimental rooms. Each broiler was placed on its back on a table with its head hanging over the edge of the table. To induce TI, the experimenter restrained the bird for 15 seconds by placing one hand on its sternum and one on its head covering the eyes. After induction, the experimenter slowly stepped back and the latency to vocalize, move their head and stand up were recorded. If the bird stood up within 10 seconds, the induction attempt was immediately repeated. If the bird stood up within 10 seconds on three attempts, this was noted, and the test was terminated. If a bird was still in TI after ten minutes, it received the maximum score of 600 s.

Health measurements

On day 40, all 10 focal broilers per pen were visually scored on their degree of hock burn, foot pad dermatitis, lameness, cleanliness, and thigh scratches. On day 42, six focal broilers per pen were sacrificed and analysed post-mortem. Due to mechanical malfunction, half of the birds per pen were euthanised by electrocution, and half were euthanised by lethal injection with Euthasol, balanced per treatment. Broiler body and heart weight were measured, and the degree of white striping and wooden breast of the pectoral muscles, and the presence of abdominal fluid were scored. The legs of the sacrificed broilers were removed and stored at -20 °C for several days. After thawing, the tibias were removed, and tibia proximal length and lateral cortex width were measured with a digital calliper. Fluctuating asymmetry was calculated by dividing the absolute length difference between the left and right tibia by the average length of the left and right tibia. A veterinarian scored tibial dyschondroplasia on the left tibia by making a diagonal cut in the femoral proximal head and scoring lesions (for full descriptions of scores, see **Supplementary Table S5**) (Butterworth, 2009; Karaarslan and Nazlıgül, 2018; Kuttappan et al., 2016). The breaking strength (maximum load to break the tibia in Newton) of the right tibia was determined using an Instron Electromechanical Universal Testing Machine (Instron, Norwood, MA, USA).

Litter quality

On day 41, the level of litter friability and wetness were scored visually in each pen based on the protocol described by van Harn et al. (2019). Additionally, litter samples from under the drinking line, next to the feeder and in the centre of each pen were taken and mixed, and the moisture percentage was determined by weighing the litter sample before and after drying at 70 °C for 24 hours.

Performance

All broilers were weighed before placement and on day 7, 14, 21, 28, 35 and 41, and based on this the average daily gain (ADG) per broiler was calculated. Feed consumption on pen level was determined over the entire growing period (day 8–42). Regular observations indicated that most of the live larvae were consumed each day and based on this the percentage of the total DM intake consisting of BSFL was determined per pen. Morbidity and mortality were recorded daily, and the amount of live larvae provided was adjusted if chick mortality occurred.

Statistical analysis

Data processing

For the grower period the average daily dietary DM (with and without BSFL) in g/day/chick was determined and based on this the average daily energy intake per chick was calculated. Observed behaviours and postures were grouped per chick per day and expressed as the percentage of observations in which the behaviour occurred. All behaviours occurring in more than 0.5% of the observations (eating, drinking, locomotion, standing idle, perching, resting, scratching, ground pecking, stretching, and preening) were analysed independently. Additionally, the grouped items “foraging behaviour” (ground pecking, scratching, food running and interaction tube), “comfort behaviour” (preening and dust bathing) and “activity” (all behaviours except resting and sitting while perching) were analysed. Foot pad dermatitis, wooden breast and abdominal fluid were uncommon (< 2%) and the effect of treatment on these parameters was therefore not analysed. Per chick only the leg with the highest hock burn score was included in the analysis. Hock burn scores of 3 and 4 and gait scores of 4 and 5 were rare (< 1%) and therefore these scores were grouped with a score of 2 for hock burn and a score of 3 for gait score. All broilers had a white striping score of 1 or 2, therefore this was analysed as a binary variable.

Data analysis

Data were analysed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). All mixed models included block and pen (nested in block and treatment) as random effects. Behaviours were analysed with a generalized linear mixed model (GLIMMIX in SAS) with a binomial distribution, logit link function and additional multiplicative over-dispersion parameter. Here, the fixed effects were treatment, week, and the treatment by week interaction. For the behaviour "interaction tube" the model included only week as fixed effect as only A10-TB birds could perform this behaviour. A random effect of week with chick as subject (nested within pen, block, and treatment) was added, with a heterogeneous first-order autoregressive covariance structure. Tonic immobility latencies, health scores and performance parameters were analysed with treatment as fixed effect and density as covariate. Ordinal scores were analysed in a GLIMMIX with either a binary or multinomial distribution, and continuous scores were analysed in a MIXED procedure. Tonic immobility latencies and fluctuating asymmetry values were square root transformed for normalization. Visual litter friability and wetness scores were analysed with a Kruskal-Wallis test due to the occurrence of empty subclasses. The proportion of moisture was analysed with a GLIMMIX with binomial distribution and additional over-dispersion parameter. Significant treatment effects were further analysed using differences in least square means with a Tukey's HSD correction or, for visual litter quality scores, a Dwass- Steel-Critchlow-Fligner multiple comparisons test. Data are presented as means ± SEM based on pen averages. P-values below 0.05 were considered statistically significant.

Results

Behavioural observations

Treatment did not influence the percentage of time spent perching (1.21 ± 0.06%, $p > 0.05$) and stretching (0.89 ± 0.03%, $p > 0.05$). The behaviours locomotion, standing idle, eating, scratching, ground pecking, foraging behaviour, resting and total activity were influenced by treatment, week, and the treatment by week interaction ($p < 0.01$ for all, **Figure 1**). Treatment and week, but not the treatment by week interaction, affected drinking, preening and comfort behaviour, and week affected the time spent on interacting with the tubes ($p < 0.001$). Pairwise significant ($p < 0.05$) differences are discussed below.

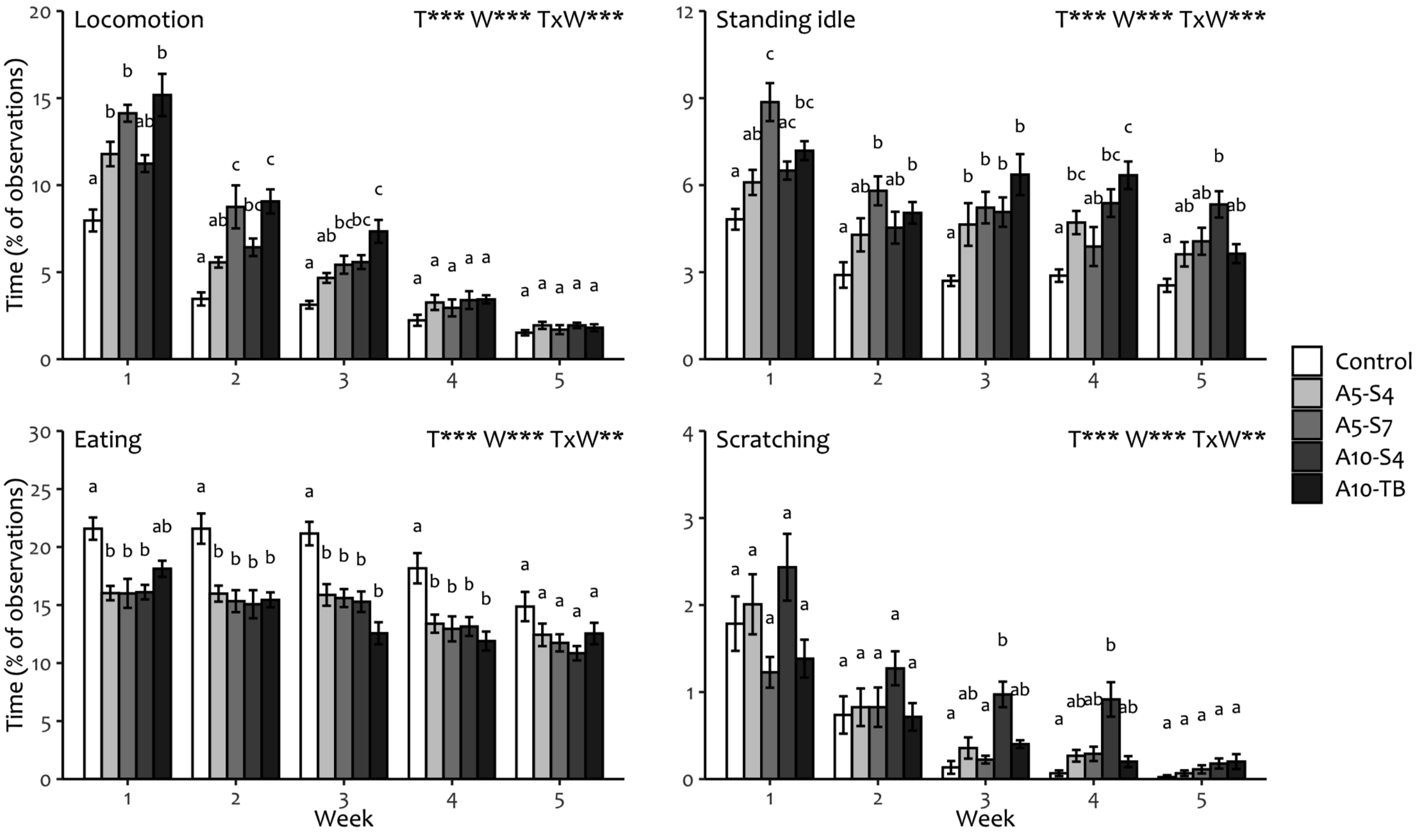
Locomotion

In week 1, broilers in treatments A10-TB, A5-S7, and A5-S4 walked more than control broilers. During week 2 and 3 A10-TB, A5-S7 and A10-S4 broilers walked more than controls, and in these two weeks A10-TB broilers also walked more than A5-S4 broilers. Over time, the stimulation of locomotion by live BSFL provisioning decreased, until no treatment effects were observed in week 4 and 5.

Standing Idle

During one or several weeks broilers in all BSFL treatments spent more time standing idle than control broilers (A5-S4: week 3 & 4, A10-S4: week 3-5, A5-S7: week 1-3, A10-TB: week 1-4). In week 1, A5-S7 broilers spent more time standing idle than A5-S4 broilers, and in week 4 A10-TB broilers stood idle more than A5-S7 broilers. The time spent standing idle declined in week 5 for A5-S4 broilers, while for the other treatments this decline occurred first in week 2. After the initial decline the time spent standing idle remained relatively constant for broilers in the control, A5-S7 and A10-S4 treatments. In contrast, for A10-TB broilers the initial decline was followed by an incline in time spent standing idle in week 3, and this was again followed by a decline in time spent standing idle in week 5.

Figure 1 (next page). Behavioural activities (% of observations) of broilers receiving no larvae (Control), or provided with live larvae in different amounts (5 or 10% of the total dietary DM replaced with larvae, A5 and A10 respectively) and provisioning methods (scattered four or seven times a day, S4 and S7 respectively, or in tubes, called TB) scored in week 1-5. Foraging behaviour encompasses ground pecking, scratching, food running and interaction with tube. Activity encompasses all behaviours except resting and sitting while perching. Data presented as means ± SEM. Effects of Treatment (T), Week (W) and their interaction (TxW) are indicated as **$p < 0.01$ or ***$p < 0.001$. Different letters within one week indicate significant ($p < 0.05$, Tukey's HSD correction) differences between treatments.


Locomotion
T*** W*** TxW***
Standing idle
T*** W*** TxW***
Eating
T*** W*** TxW**
Scratching
T*** W*** TxW**
Time (% of observations)
Week
1
2
3
4
5
0
5
10
15
20
25
30
0
3
6
9
12
0
1
2
3
4
Control
A5-S4
A5-S7
A10-S4
A10-TB

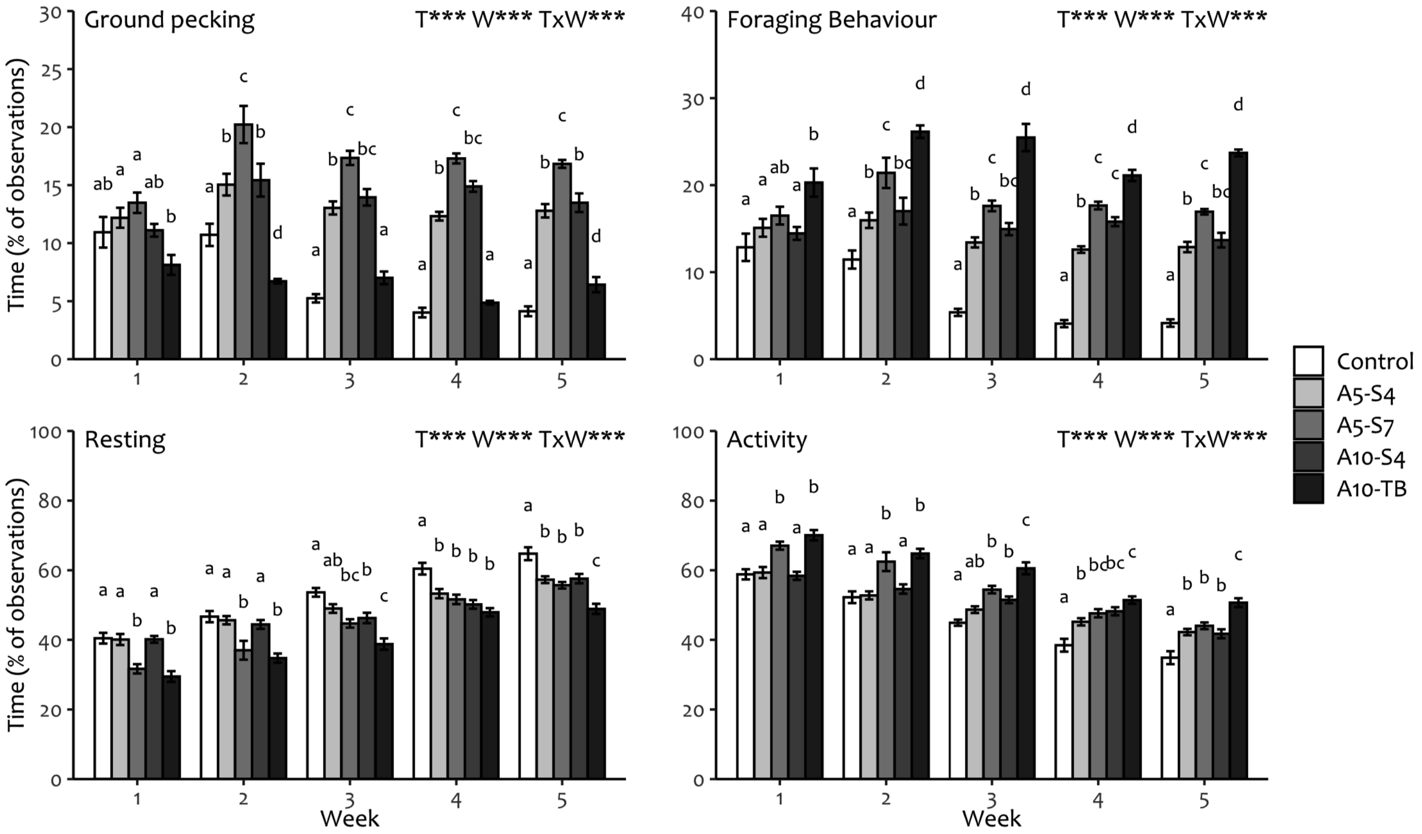


Figure 1 continued.

Eating and Drinking

In the first four weeks, control broilers spent more time eating feed than those in all BSFL treatments, except for A10-TB broilers in week 1. In week 5, treatment did not affect time spent eating. The time spent eating feed of all broilers decreased over time, independent of the live BSFL amount. Controls spent more time drinking than broilers in the BSFL treatments (C = 5.5 ± 0.3%, A5-S4 = 4.0 ± 0.2%, A5-S7 = 4.2 ± 0.2%, A10-S4 = 3.8 ± 0.2%, A10-TB = 3.8 ± 0.2%, $p < 0.001$) and for all treatments the time spent drinking decreased steadily during week 1 until and including week 3, after which it increased again in week 5.

Foraging behaviour

Time spent interacting with the tubes (which could only be performed by A10-TB broilers) was lower in week 1 compared to all other weeks, and in week 4 compared to week 2 and 3. The A10-S4 broilers spent more time scratching than A5-S7 broilers in week 3 and than controls in week 3 and 4, and all treatments showed variations in time spent scratching over time. In week 2-5, A5-S4, A5-S7 and A10-S4 broilers spent more time on ground pecking than controls and A10-TB broilers. Additionally, A5-S7 broilers spent more time on ground pecking than A5-S4 broilers in week 2-5 and A10-S4 broilers in week 2 and 5. In week 2, controls also spent more time on ground pecking than A10-TB broilers, and in week 5 this was reversed. Overall, all methods of live BSFL provisioning persistently increased the time spent on foraging behaviour. The effects on foraging behaviour were similar as for ground pecking, except that A10-TB broilers foraged more than controls, A5-S4 and A10-S4 broilers in all weeks, and they foraged more than A5-S7 broilers in week 2–5. The control broilers' time spent ground pecking and total foraging behaviours decreased after two weeks after which it remained constant. In contrast, A5-S4, A5-S7 and A10-S4 broilers showed a relatively constant high level of ground pecking and foraging behaviour throughout all weeks. While A10-TB broilers spent a consistently low amount of time on ground pecking, their level of total foraging behaviour started high and increased further in week 2, 3 and 5 compared to week 1.

Resting and Activity

The patterns for resting and activity were almost complementary as activity consists of all behaviours except resting and sitting while perching, therefore only activity is discussed. During several weeks control broilers were less active than broilers in the BSFL treatments (A5-S4: week 4 & 5, A10-S4: week 3-5, A5-S7 & A10-TB: week 1-5). In addition, A10-TB broilers were more active than A5-S4 broilers during all weeks, and A5-S7 broilers were more active than A5-S4 broilers in week 1 and 2. Birds from all treatments showed a decline in activity over time, and this decline started in week 2 for control, A5-S4 and A10-TB broilers, and in week 3 (compared to week 1) for A5-S7 and A10-S4 broilers.

Comfort behaviour

A10-TB broilers spent less time on preening (C = 4.0 ± 0.2%, A5-S4 = 4.2 ± 0.2%, A5-S7 = 3.9 ± 0.2%, A10-S4 = 4.1 ± 0.2%, A10-TB = 3.2 ± 0.2%, $p < 0.001$) and total comfort behaviour (C = 4.3 ± 0.3%, A5-S4 = 4.5 ± 0.2%, A5-S7 = 4.2 ± 0.2%, A10-S4 = 4.4 ± 0.2%, A10-TB = 3.4 ± 0.2%, $p < 0.001$) than broilers in all other treatments. The time spent on comfort behaviours decreased in week 2 compared to week 1, and in week 3 compared to week 2, after which it remained constant.

Posture

Treatment, week, and the treatment by week interaction affected posture ($p < 0.001$ for all, **Figure 2**). During several weeks, controls stood less than broilers in the BSFL treatments (A5-S4: week 5, A10-S4: week 3-5, A5-S7 & A10-TB: week 1-5). A10-TB broilers spent more time standing than A5-S4 broilers during all weeks except week 4, and A5-S7 spent more time standing than A5-S4 broilers in week 1 and 2. Birds in all treatments showed a decline in time spent standing over time. For the controls this decline started in week 2 (compared to week 1), for A5 broilers the decline started in week 3, and for A10 broilers the decline started in week 4.

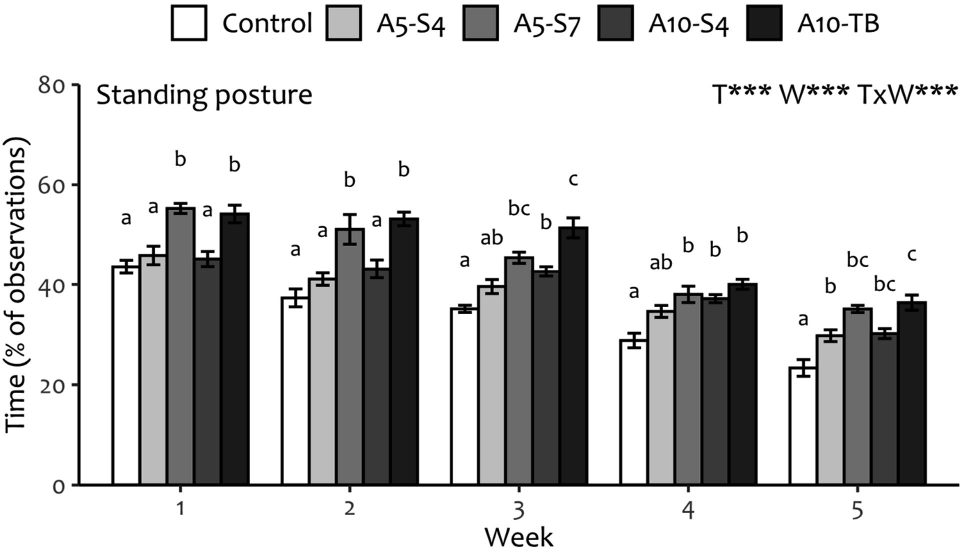


Figure 2. Time spent in standing posture (% of observations) of broilers receiving no larvae (Control) or provided with live larvae in different amounts (5 or 10% of the total dietary DM replaced with larvae, A5 and A10 respectively) and provisioning methods (scattered four or seven times a day, S4 and S7 respectively, or in tubes, called TB) scored in week 1-5. Data are presented as means ± SEM. Effects of Treatment (T), Week (W) and their interaction (TxW) are indicated as ***$p < 0.001$. Different letters within one week indicate significant ($p < 0.05$, Tukey's HSD correction) differences between treatments.

Tonic immobility

Treatment influenced the time spent in tonic immobility, where controls stayed in tonic immobility longer than A5-S7 and A10-TB broilers, with the A5-S4 and A10-S4 in between. Treatment did not affect latency to vocalize and latency to first head movement (**Table 2**).

Health

Neither hock burns, lameness, cleanliness, thigh scratches and white striping (**Table 3**), nor the broilers' heart weight, tibia length, tibia fluctuating asymmetry and tibia breaking strength (**Table 4**) were influenced by treatment. Treatment did influence broiler tibia width ($p = 0.024$), where the tibias of controls were wider than those of A10-S4 broilers.

Table 2. Tonic immobility (TI) responses of broilers receiving no larvae (Control) or provided with live larvae in different amounts (5 or 10% of the total dietary DM replaced with larvae, A5 and A10 respectively) and provisioning methods (scattered four or seven times a day, S4 and S7 respectively, or in tubes, called TB). Broilers that were not induced in tonic immobility after three attempts, and broilers that did not vocalize or move their heads were excluded from the respective analysis. Data are presented as means. Different letters indicate significant ($p < 0.05$, Tukey's HSD correction) differences between treatments. *$p < 0.05$.

Measure (s)	Control	A5-S4	A5-S7	A10-S4	A10-TB	SEM	F-statistic and df	P
Time in TI	183.8^{a}	119.8^{ab}	95.6^{b}	168.2^{ab}	103.0^{b}	12.0	$F_{(4,35)} = 3.87$	0.010*
Latency to vocalize	33.6	25.0	36.2	47.7	14.9	5.6	$F_{(4,33)} = 1.04$	0.400
Latency to move head	54.6	24.9	17.4	20.4	21.0	4.2	$F_{(4,28)} = 1.15$	0.356

Table 3. Frequencies of health scores of broilers receiving no larvae (Control) or provided with live larvae in different amounts (5 or 10% of the total dietary DM replaced with larvae, A5 and A10 respectively) and provisioning methods (scattered four or seven times a day, S4 and S7 respectively, or in tubes, called TB). Health parameters were analysed with linear mixed models. Foot pad dermatitis, wooden breast and abdominal fluid levels were not analysed due to low variance.

Score	Control	A5-S4	A5-S7	A10-S4	A10-TB	F-statistic and df	P
Gait							
0	8	5	5	8	5	$F_{(4,34)} = 0.48$	0.750
1	22	24	26	27	33		
2	42	42	44	40	35		
3	8	8	5	5	5		
Hock burn							
0	35	49	38	40	41	$F_{(4,34)} = 0.85$	0.506
1	38	23	33	27	33		
> 1	7	8	9	13	6		
Foot pad dermatitis							
0	79	80	78	75	80	-	-
1	1	0	2	5	0		
Cleanliness							
1	6	3	3	9	2	$F_{(4,35)} = 0.21$	0.933
2	35	47	49	29	40		
3	39	30	28	42	37		

Score	Control	A5-S4	A5-S7	A10-S4	A10-TB	F-statistic and df	P
Thigh scratches							
Absent	61	66	68	63	57	$F_{(4,35)} = 1.13$	0.357
Present	19	14	12	17	22		
Wooden breast							
Absent	43	45	48	48	48	-	-
Mild	4	3	0	0	0		
Severe	1	0	0	0	0		
White striping							
Mild	22	24	32	26	27	$F_{(4,35)} = 1.08$	0.382
Severe	26	24	16	22	21		
Abdominal fluid							
Absent	47	48	48	47	48	-	-
Present	1	0	0	1	0		

Table 3 continued .

Table 4. Heart and tibia measurements of broilers receiving no larvae (Control) or provided with live larvae in different amounts (5 or 10% of the total dietary DM replaced with larvae, A5 and A10 respectively) and provisioning methods (scattered four or seven times a day, S4 and S7 respectively, or in tubes, called TB). All parameters analysed by linear mixed models (fluctuating asymmetry (FA) was first square root transformed). Within each row different letters indicate significant ($p < 0.05$, Tukey's HSD correction) differences. *$p < 0.05$.

Measure	Control	A5-S4	A5-S7	A10-S4	A10-TB	SEM	F-statistic and df	P
Heart weight (g)	11.6	11.6	11.9	11.6	11.8	0.1	$F_{(4,35)} = 0.19$	0.943
Tibia measures								
Length (mm)	102.2	101.8	102.3	101.3	101.3	0.2	$F_{(4,35)} = 0.93$	0.460
Width (mm)	8.7^{a}	8.6^{ab}	8.5^{ab}	8.2^{b}	8.4^{ab}	0.05	$F_{(4,35)} = 3.23$	0.024*
Length FA (mm)	0.6	0.5	0.5	0.5	0.5	0.03	$F_{(4,35)} = 0.31$	0.869
Width FA (mm)	0.3	0.3	0.2	0.2	0.3	0.02	$F_{(4,35)} = 0.11$	0.980
Breaking strength (N)	445.2	471.2	449.9	448.8	463.4	5.1	$F_{(4,35)} = 0.87$	0.492

Litter quality

Treatment influenced the friability of the litter ($p = 0.008$). The A10-S4 pens had less friable litter than control and A10-TB pens. The visual wetness score and the proportion of moisture in the litter were not affected by treatment (**Table 5**).

Table 5. Litter quality scores of pens with broilers receiving no larvae (Control) or provided with live larvae in different amounts (5 or 10% of the total dietary DM replaced with larvae, A5 and A10 respectively) and provisioning methods (scattered four or seven times a day, S4 and S7 respectively, or in tubes, called TB). Visual friability and wetness scores were analysed with a Kruskal-Wallis test and a post-hoc DSCF test and moisture percentage was analysed with a generalized linear mixed model. $^{**}p < 0.01$.

Measure	Control	A5-S4	A5-S7	A10-S4	A10-TB	SEM	Test-statistic and df	P
Visual friability score (frequency of scores)								
2	0	1	3	5	0	-		
3	4	3	4	3	4			
4	3	4	0	0	4		$H_{(4)} = 13.72$	0.008**
5	1	0	1	0	0			
Sum of scores[1]	213^{a}	193^{ab}	129^{ab}	81^{b}	206^{a}			
Wetness score (frequency of scores)								
2	0	0	0	1	1	-		
3	6	4	3	5	4		$H_{(4)} = 3.88$	0.423
4	2	4	5	2	3			
Sum of scores[1]	146	184	203	134	153			
Moisture (%)	56.3	57.8	58.4	60.4	55.9	0.7	$F_{(4,35)} = 1.25$	0.320

[1] Sum of scores calculated by a Kruskal-Wallis test.

Performance

Over the course of the experiment, several broilers were removed due to health problems: 3 broilers from the control, A5-S4, A5-S7 and A10-S4 treatments, and 4 broilers from the A10-TB treatment. In the third week, broilers in both A10 treatments had a lower ADG than controls, and in the fourth week broilers in the A10-S4 and A5-S7 treatments had a lower ADG than controls. In week 1, 2, 5 and 6, broilers showed no differences in ADG, and their final weight was also not affected by treatment. Considering feed intake, the percentage of the total dry matter (DM) intake consisting of BSFL was 6.3 ± 0.1% for A5-S4 broilers, 6.4 ± 0.1% for A5-S7 broilers, 13.8 ± 0.2% for A10-S4 broilers and 13.8 ± 0.1% for A10-TB broilers. The daily dietary DM intake excluding BSFL dropped proportionally with the amount of live BSFL offered, where it was higher in control broilers than in all other broilers and in A5 broilers than in A10 broilers. The average daily DM and energy intake including BSFL did not differ between treatments (**Table 6**).

Table 6. Performance parameters of broilers receiving no larvae (Control) or provided with live larvae in different amounts (5 or 10% of the total dietary DM replaced with larvae, A5 and A10 respectively) and provisioning methods (scattered four or seven times a day, S4 and S7 respectively, or in tubes, called TB). All parameters were analysed with linear mixed models. Within each row different letters indicate significant ($p < 0.05$, Tukey's HSD correction) differences. $^{**}p < 0.01$, $^{***}p < 0.001$.

Measure	Control	A5-S4	A5-S7	A10-S4	A10-TB	SEM	F-statistic and df	P
Average daily gain (g/d)								
Week 1	12.1	12.4	11.7	12.8	12.2	0.15	$F_{(4,35)} = 1.50$	0.225
Week 2	41.0	41.0	38.4	39.7	38.6	0.44	$F_{(4,34)} = 1.68$	0.176
Week 3	63.5^{a}	61.2^{ab}	60.1^{ab}	58.4^{b}	57.6^{b}	0.50	$F_{(4,34)} = 5.89$	0.001**
Week 4	88.9^{a}	87.0^{ab}	82.8^{b}	82.1^{b}	83.8^{ab}	0.75	$F_{(4,35)} = 4.06$	0.009**
Week 5	104.2	107.5	103.3	102.8	103.3	0.92	$F_{(4,34)} = 1.85$	0.143
Week 6	125.5	128.5	125.6	125.7	130.5	1.25	$F_{(4,34)} = 0.59$	0.673
Final weight (g)								
d42	2,963.5	2,970.6	2,874.4	2,873.9	2,918.0	17.0	$F_{(4,34)} = 1.58$	0.201
Feed intake (g/d)								
Excl. BSFL	96.5^{a}	92.2^{b}	91.2^{b}	85.2^{c}	84.8^{c}	0.83	$F_{(4,34)} = 23.36$	<0.001***
Incl. BSFL	96.5	98.1	97.0	96.9	96.6	0.44	$F_{(4,34)} = 0.43$	0.789
Energy intake incl. BSFL (Mj/d)	1.19	1.21	1.21	1.21	1.21	0.005	$F_{(4,34)} = 0.67$	0.616

Discussion

The current study showed that providing broilers with 5 or 10% of their dietary dry matter (DM) intake as live BSFL four times a day increased activity during the second half of the production period, mainly by facilitating foraging behaviour. Moreover, increasing the frequency of live BSFL access by providing larvae seven times a day or prolonging live BSFL access by offering them in tubes with holes increased broiler activity throughout the entire production period, while it reduced broiler fearfulness. A temporarily reduced growth rate was observed in A5-S7, A10-S4 and A10-TB broilers, but this did not influence the final weight of broilers. Broiler health was not affected by live BSFL provisioning.

The observed increase in foraging behaviour, activity, and time spent standing in broilers receiving live larvae compared to controls was in line with previous studies showing short-term effects of scattering mealworms (Pichova et al., 2016) and long-term effects of scattering BSFL (Ipema et al., 2020b). Our previous study already indicated positive effects of live BSFL provisioning on activity of broilers housed at low densities, and the current study demonstrates that under commercial densities this effect is still present. As foraging is considered a behavioural need (Lindqvist et al., 2002), being rewarding (Bracke and Hopster, 2006) for chickens, the welfare of these broilers is improved. Previous studies in which maize roughage or sand and strings were provided to broilers also found an increase in foraging behaviour (Arnould et al., 2004; Bach et al., 2019), however this increase was numerically lower and occurred for a shorter time compared to the current study. The difference in foraging behaviour between studies could be attributed to the high motivation of broilers to gain access to high-value feed items such as insects (Bokkers and Koene, 2002; Pichova et al., 2016) compared to items with a low nutritional value, making them relatively more willing to work for access to larvae. This is supported by the observation that overall locomotion was increased by live larvae provisioning but not by providing maize roughage or sand and strings (Arnould et al., 2004; Bach et al., 2019). In addition, the provisioning methods used in our study, i.e., scattering live larvae throughout the pen or providing live larvae in tubes, required more foraging and/or locomotion to obtain the resource compared to providing stationary, easily accessible items, independent of the type of resource.

Overall activity of A5-S4 and A10-S4 broilers was only increased during the second half of the production period. This is in line with our previous study in which these treatments were applied to broilers kept at lower (14 versus 33 kg/m^2) densities (Ipema et al., 2020b). Furthermore, in the current experiment these broilers had almost identical behavioural time budgets, suggesting that frequency is more important than amount when it comes to promoting broiler activity. In line with this, increasing the frequency to seven times a day or prolonging access to live BSFL by providing them in tubes not only caused a higher increase in foraging behaviour and activity, but also increased activity during more weeks. This is in accordance with previous studies that suggested (Pichova et al., 2016) or showed (Ipema et al., 2020b) that prolonging access to insects would increase broiler activity even more. Prolonged access can increase activity in multiple ways. In our study, a higher frequency of live larvae provisioning meant that broilers received smaller portions and were stimulated to be active multiple times a day. Scattering fewer live larvae throughout the pen makes larvae harder to locate, which promotes foraging behaviour. Furthermore, BSFL have a high water content of approximately 65%, and consuming high amounts of water in a short amount of time could increase satiety in broilers (Hocking et al., 2004), reducing their motivation to forage. When providing smaller portions this effect is less likely to occur. Also, at a higher frequency of live larvae provisioning the disturbance caused by providing the enrichment could also have increased broiler activity. Though, this is unlikely as broiler activity was not increased by providing wheat or wood shavings (Pichova et al., 2016), showing that disturbance alone does not promote broiler activity. For live larvae provisioning in tubes, obtaining BSFL requires substantial effort of manipulating the tube, therefore more active behaviour is required to consume the same amount of larvae. As frequent or prolonged access appears key in increasing broiler activity, further research should focus on alternative, cost- and labour-efficient methods to achieve this. For example, a recent study tested a device that allowed for gradual live BSFL provisioning to layer hens (Star et al., 2020), and such a device could also be applied in the broiler industry. Also, further research is required to determine the effect of BSFL amount on the success of these methods. The observation that performance was not hindered by live larvae provisioning up to 10% despite the increased activity and decreased pellet intake indicates that BSFL are a good source of nutrients and could even be provided in higher amounts, potentially increasing the welfare benefits.

The investigated live larvae provisioning methods had differential effects on individual behaviours. Scattering live BSFL resulted mainly in increases in ground pecking. Providing live BSFL in tubes resulted in a large amount of time spent interacting with these tubes, and we observed that the interaction consisted mainly of pecking and scratching at the tubes. Pecking at the ground or at objects is a natural behaviour of chickens (Murphy et al., 2014), therefore performing this behaviour could satisfy a motivational need of broilers. Interacting with a plastic tube might be less natural for broilers, though broilers were clearly motivated to interact with the tubes as reflected by the large amount of time they spent performing foraging-related behaviour directed towards the tubes. Therefore, broilers seem to adjust their behaviours to the environment, and both methods of provisioning can satisfy their behavioural needs. Despite differences in the target of the foraging behaviour, both more frequent and prolonged access treatments caused a similar higher increase in time spent in standing posture occurring during more weeks compared to the lower frequency treatments and controls. This is in line with a study showing that increasing standing activity from a young age onwards caused long-term increases in activity (Reiter and Bessei, 2009). Finally, comfort behaviour occurred significantly less in broilers receiving live BSFL in tubes compared to controls. Comfort behaviour is often considered as a sign of good welfare (Nicol et al., 2009), therefore this could indicate that these birds experience diminished welfare. However, as these broilers spent between 20-25% of their time on foraging behaviour, which is considerably more than broilers kept under regular commercial conditions (Baxter et al., 2018a; de Jong and Gunnink, 2018), foraging behaviour might occur at the expense of comfort behaviour. It is therefore unlikely that the reduced time spent on comfort behaviour is an indicator of diminished welfare in broilers receiving live BSFL, but rather a consequence of the increased foraging possibilities.

Providing 5% of the diet as live BSFL given seven times a day or providing 10% of the diet as live BSFL in tubes both reduced the time broilers spent in tonic immobility compared to controls, which indicates reduced fearfulness (Forkman et al., 2007). While previously tested enrichment methods such as providing mealworms once a day or adding strings and perches did not affect fearfulness (Bailie and O'Connell, 2015; Pichova et al., 2016), adding perches in combination with peat dust baths did reduce fearfulness in broilers compared to barren-housed controls (Baxter et al., 2018a). Prolonged access to preferred enrichment such as peat dust baths or larvae could have extended the interaction with non-threatening stimuli and adapted the birds to potentially frightening situations (Jones, 1996), reducing their tonic immobility response. However, providing live BSFL four times a day did not influence broiler fearfulness, indicating that four provisioning moments are not enough to familiarize the birds to novelty. In addition, the increased interaction with live larvae could have promoted a more positive affective state in broilers through positive influences on the dopaminergic systems (Moe et al., 2014). A more positive affective state could be linked to reduced fearfulness, as shown in layer hens (Campbell et al., 2019), though the cause and effect relationship is not completely clear.

Considering the observed health parameters, only tibia width was influenced by live BSFL provisioning, where control broilers had wider tibia than broilers receiving 10% of their diet as live BSFL four times a day, which could indicate better leg health of the controls. The A10-S4 broilers grew slower than controls during week 3 and 4 of the experiment, suggesting broiler growth rate could be linked to tibia development. In line with this, feed restriction during the second week of the production period also slowed broiler growth and reduced tibia length and width (Bruno et al., 2000). However, other studies found no relation between growth rate and tibia development (Leterrier and Constantin, 1999; Rutten et al., 2002). Alternatively, the increased activity of the A10-S4 broilers compared to controls could have reduced tibia width, as Foutz et al. (2007) found that increasing broiler activity through treadmill training reduced tibia length and width. However, as broilers in the other BSFL treatments also had increased activity without showing differences in tibia measures compared to controls, this cannot be the sole explanation.

The absence of a treatment effect on the other examined health parameters was unexpected, as activity was increased by live BSFL provisioning, and increased activity has positively affected walking ability and bone development in previous studies (Bradshaw et al., 2002; Reiter and Bessei, 2009; Shipov et al., 2010). The occurrence and severity of lameness and contact dermatitis in the control and BSFL broilers was moderately lower compared to previous large-scale studies on broiler leg health (Dinev et al., 2019; Kittelsen et al., 2017), and one possible explanation for this could be the stocking density applied in our study. While stocking densities of 33 kg/m^2 are applied commercially, it is the lower margin, and stocking densities up to 42 kg/m^2 are allowed in the EU (Council Directive 2007/43/EC). Stocking densities below 35 kg/m^2 have been linked to reduced incidence of foot pad dermatitis and hock burn (Buijs et al., 2009), and walking ability can be limited by higher stocking densities (Knowles et al., 2008). This is in line with the even lower incidence of these health issues in our previous study that applied a lower stocking density (14 kg/m^2) (Ipema et al., 2020b). However, in that study live BSFL provisioning did improve leg health. The absence of a treatment effect on leg health in the current study could be due to the higher stocking density combined with the relatively small pen size used. It is probable that, even though broilers performed more active behaviours, the large walking distances previously shown to improve broiler leg health (e.g., Foutz et al., 2007; Reiter and Bessei, 2009) cannot be achieved in these conditions. This is in line with several previous studies that also applied small pens, where enrichment increased activity temporarily without affecting leg health (Bizeray et al., 2002b; Meyer et al., 2019). In commercial-sized pens the activity increase caused by live BSFL provisioning could promote longer walking distances, potentially improving leg health. However, at higher commercial stocking densities the potential benefits of having larger pens might be negated by the limited space. Additional research in large-scale broiler housing systems is necessary to determine if and how live BSFL provisioning can benefit broiler welfare under these conditions.

The prevalence of tibial dyschondroplasia (TD) and white striping was higher compared to other studies (Kaukonen et al., 2017a; Kuttappan et al., 2016; Pedersen et al., 2020). For TD, the results are in line with a previous study that found a numerically higher occurrence in birds housed at 10 birds/m^2, a similar stocking density as in the current study, compared to densities of 15 or 20 birds/m^2, though the cause for this was unclear (Tablante et al., 2003). The relatively high occurrence of white striping could be a result of including only male broilers in the study, as white striping tends to be more common in male than female broilers (Kuttappan et al., 2013). The lack of effect of live BSFL provisioning on these health parameters could be due to the large influence of growth rate and weight on them (Kuttappan et al., 2013; Shim et al., 2012). The final weight was similar for broilers from all treatments, and it seems that the effect of growth could not be compensated by the increased activity caused by live larvae provisioning.

A strong risk factor for contact dermatitis is poor litter quality (Bradshaw et al., 2002; de Jong et al., 2014), therefore the comparable moisture levels in the pens of all treatments could explain the absence of differences in foot pad dermatitis and hock burn. However, we expected that the increase in activity caused by live BSFL provisioning would improve the litter quality. Especially scratching behaviour, which was temporarily increased in broilers receiving 10% of their diet as live BSFL four times a day, can have this effect (de Jong et al., 2012), yet litter friability was lowest for this treatment. It is possible that the consumption of whole BSFL negatively affected litter quality. Whole BSFL contain high levels of fat, and animal fat cannot be digested well by young poultry (Mossab et al., 2000), meaning it might be excreted on the litter. This in turn can reduce the water uptake capabilities of the litter (Collett, 2012). The negative effects of BSFL consumption might have outweighed the putative beneficial effects of activity on litter quality. If this is the case, then providing a lower amount of BSFL could be more beneficial for broiler litter quality, and in turn, broiler health.

Conclusion

Our results indicate that live BSFL provisioning can reduce fearfulness and amplify broiler activity, especially foraging behaviour, and that this effect is most prominent in broilers with frequent or prolonged access to live BSFL. Feed intake decreased proportionally to the amount of BSFL, yet the final weight of broilers was not influenced by live BSFL provisioning. No positive or negative effects on broiler health were observed. While a reduction in fearfulness and facilitation of natural behaviour can already improve broiler welfare, live BSFL provisioning does have the potential to further benefit welfare by reducing health problems under intense commercial conditions in which health problems are more common

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Supplementary data

Table S1. Tube details.

Week	Tube diameter (mm)	Tube length (mm)	Hole diameter (mm)	Number of holes
1	40	100	9	2
2	40	150	9	3
3	75	100	9	6
4-6	75	150	9	8

Table S2. Amounts and methods of live black soldier fly larvae (BSFL) provisioning in different treatments.

Treatment	% Inclusion of BSFL in complete ration on dry matter basis	BSFL provisioning method per pen	Provisioning time (number of provisioning moments)
Control	0	-	-
A5-S4	5	Scattering	08:00, 11:00, 14:00, 17:00 (4)
A5-S7	5	Scattering	08:00, 09:30, 11:00, 12:30, 13:00, 14:30, 17:00 (7)
A10-S4	10	Scattering	08:00, 11:00, 14:00, 17:00 (4)
A10-TB	10	Five tubes with holes	08:00 (1, continuous access to tubes)

Table S3. Visual representation of treatment and block distribution across experimental rooms. Treatments include broilers receiving no larvae (Control) or provided with live larvae in different amounts (5 or 10% of the total dietary DM replaced with larvae, A5 and A10 respectively) and provisioning methods (scattered four or seven times a day, S4 and S7 respectively, or in tubes, called TB). Blocks are bordered by solid lines. Each block contains five pens with five different treatments in a randomized order.

Room 1		Room 2	
Pen 1: Control	Pen 11: A5-S4	Pen 21: A5-S4	Pen 31: A10-S4
Pen 2: A5-S7	Pen 12: A10-S4	Pen 22: A10-S4	Pen 32: A5-S7
Pen 3: A10-S4	Pen 13: A5-S7	Pen 23: A5-S7	Pen 33: Control
Pen 4: A10-TB	Pen 14: A10-TB	Pen 24: A10-TB	Pen 34: A5-S4
Pen 5: A5-S4	Pen 15: Control	Pen 25: Control	Pen 35: A10-TB
Pen 6: A5-S7	Pen 16: A5-S4	Pen 26: A10-S4	Pen 36: A10-S4
Pen 7: Control	Pen 17: A10-S4	Pen 27: A5-S7	Pen 37: A5-S4
Pen 8: A5-S4	Pen 18: A10-TB	Pen 28: A5-S4	Pen 38: A5-S7
Pen 9: A10-TB	Pen 19: Control	Pen 29: Control	Pen 39: A10-TB
Pen 10: A10-S4	Pen 20: A5-S7	Pen 30: A10-TB	Pen 40: Control

Table S4A. Ingredients and (analysed) chemical composition of dietary components.

	Starter feed[1,5]	**Core feed**[2,3,5]	**BSFL-replacer mix**[2,4,5]	**BSFL**[2,6]
Ingredients (g/kg)				
Corn	368.1	225.1	-	-
Wheat	250.0	409.6	-	-
Soybean meal	290.0	201.3	-	-
Fishmeal	-	69.9	160	-
Rapeseed meal	40.0	33.2	-	-
Potato protein	-	-	350	-
Sunflower oil	16	33.6	-	-
BSFL oil		-	350	-
Premix	5	5.6	-	-
Lime fine	14	-	-	-
Monocalcium phosphate	8.3	4.5	11.0	-
Salt	2.1	2.3	-	-
$CaCO_3$	-	10.6	-	-
$NaHCO_3$	1.8	1.2	-	-
Phytase	0.2	0.2	-	-
DL-methionine	2.1	2.1	-	-
L-threonine	0.45	0.6	-	-
L-valine	0.1	0.1	-	-
L-lysine HCl	1.85	-	-	-
Diamol	-	-	129	-
Chemical composition				
Dry matter	876.8	883	900	350.7
ME	12.46	11.53	19.5	19.5
Crude protein	220	223	419	419
Crude fat	36.5	80	371	371
Calcium	9	7.6	6.3	6.3
Phosphorus	7.1	6.0	6.8	6.8

Nutrients are presented in g/kg dry matter, except ME (MJ/kg of dry matter) and dry matter (g/kg).

[1] Provided to all broilers during the starter period (day 1-7).

[2] Provided in different combinations during the grower period (day 8-42).

[3] Core diet component of the grower feed supplied to all broilers.

[4] Diet component with similar chemical composition as BSFL.

[5] Chemical composition according to CVB (2006).

[6] Chemical composition based on analysis via standardized protocols (DM 10032, Protein 10005, Fat 10112, Calcium and Phosphorus 10040).

Table S4B. (Chemical) composition of grower feed. Control, A5 and A10: 0%, 5% and 10%, respectively, of the total dietary dry matter replaced with live larvae. Distributions of diet components are based on larval supply, to provide all broilers with iso-energetic (based on ME) diets with a similar chemical composition.

	Control	**A5**	**A10**
Dietary component, % of total DM intake			
Core feed	90	90	90
BSFL-replacer mix	10	5	0
BSFL	0	5	10
Chemical composition			
ME (MJ/kg of DM)	12.4	12.3	12.3
Crude protein (g/kg of DM)	244	243	243
Crude fat (g/kg of DM)	111	111	110
Calcium (g/kg of DM)	7.5	7.5	7.5
Phosphorus (g/kg of DM)	6.0	6.0	6.1

Table S5. Health scores protocol.

Score	Description	Reference
Gait score		
0	Normal, dextrous, and agile.	(Butterworth, 2009)
1	Slight abnormality, but difficult to define.	
2	Definite and identifiable abnormality.	
3	Obvious abnormality, affects ability to move.	
4	Severe abnormality, only takes a few steps.	
5	Incapable of walking.	
Hock burn score[1]		
0	No evidence of hock burn.	(Butterworth, 2009)
1	Minimal evidence of hock burn.	
2	Minimal evidence of hock burn.	
3	Evidence of hock burn.	
4	Evidence of hock burn.	
Foot pad dermatitis[1]		
0	No lesions.	(Butterworth, 2009)
1	Raised central pad, reticulate scales are separated, with or without small, black necrotic area(s).	
2	Marked swelling of the foot pad, black reticulate scales forming scale-shaped necrotic areas, with necrosis evident on less than one-quarter of the total foot pad area.	
3	Marked swelling and enlargement of the entire foot pad, necrosis extending up to one-half of the total foot pad area.	
4	Marked swelling and enlargement of the entire foot pad, necrotic cells covering more than one-half of the total foot pad area.	
Cleanliness[1]		
0	The feathers and skin are completely clean.	(Butterworth, 2009)
1	The feathers and skin on the belly of the bird are slightly dirty.	
2	The feathers and skin on the belly of the bird are quite dirty. Dirt is caked on the feathers. Other parts of the plumage may be slightly dirty.	
3	The feathers and skin on the belly of the bird are quite dirty. Dirt is caked on the feathers and also the rest of the plumage (back, wings is visibly dirty).	

Score	Description	Reference
Thigh scratches		
Absent	No thigh scratches present on both thighs.	
Present	One or more thigh scratches present on one or both thighs.	
Wooden breast		
Absent	Soft breast muscle.	
Mild	Part of the breast muscle is hardened.	
Severe	Almost the whole area of the breast muscle is hardened.	
White striping[1]		
0	No distinct white lines.	(Kuttappan et al., 2016)
1	Small white lines, generally < 1 mm thick, but apparently visible on the filet surface.	
2	Large white lines (1-2 mm thick) very visible on the filet surface.	
3	Thick white bands (> 2 mm thickness) covering almost entire filet surface.	
Abdominal fluid		
Absent	No abdominal fluid present.	
Present	Abdominal fluid present.	
Tibial dyschondroplasia[1]		
0	No lesion.	(Karaarslan and Nazlıgül, 2018)
1	Lesion less than 0.5 cm in size.	
2	Lesion between 0.5-1 cm in size.	
3	Lesion more than 1 cm in size.	

Table S5 continued.

[1] See reference for detailed illustrations of individual scores.

Chapter 4

Provision of black soldier fly larvae (*Hermetia illucens*) in different ways benefits broiler welfare and performance, with largest effects of scattering live larvae

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Abstract

Including black soldier fly larvae (BSFL) in broiler diets has the potential to benefit broiler welfare and increase production performance, but the effects of dietary BSFL likely depend on the way BSFL are provided. In this study we aimed to discern the effects of different BSFL forms and provisioning methods by providing male broilers with no BSFL (CON), processed BSFL meal and oil incorporated in the feed pellets (INC-F), dried BSFL in the feeder on top of the feed (D-F), or dried or live BSFL scattered through the pen (D-S and L-S, respectively), and evaluating various indicators of broiler welfare and production performance. In all dietary BSFL treatments 8% of the total dietary dry matter content was replaced with BSFL. Dried and live larvae were provided in four equal daily portions at 08:00, 11:00, 14:00, and 17:00. Compared to a diet without BSFL, scattering dried or live larvae through the pen increased active behaviours, though only live larvae increased the time broilers spent standing. Broilers in the D-F, D-S and L-S treatments had higher average daily body weight gain during some periods, and they had higher final weights, despite L-S broilers having a lower total dry matter intake than CON broilers. Furthermore, the dry matter conversion ratio of INC-F, D-S and L-S broilers was reduced. At the end of the rearing period, pens in all dietary BSFL treatments had better litter quality than CON pens. Furthermore, food pad dermatitis was less severe for INC-F and D-S broilers than for CON broilers, and for L-S broilers than for broilers in all other treatments, and hock burn severity was less for L-S than for CON broilers. Broiler lameness, cleanliness, plasma natural antibody titers, and whole blood serotonin were not influenced by dietary BSFL treatment. Feather corticosterone concentrations were affected by treatment, though without any significant post-hoc differences. Our results indicate that BSFL meal and oil, and dried and live BSFL are all promising feed ingredients for broilers as they all benefit some aspects of broiler welfare and production performance. Scattering BSFL through the pen results in more welfare benefits than providing BSFL in the feeder, with live BSFL having the most beneficial effects on broiler welfare.

Introduction

Black soldier fly larvae (BSFL) are considered a suitable feed ingredient for broilers as they contain sufficient quantities of micro- and macronutrients (e.g., protein, fat, minerals, vitamins and fibres, Barragan-Fonseca et al., 2017; Makkar et al., 2014). They can be reared on a wide range of biological waste streams (Lalander et al., 2013; St-Hilaire et al., 2007) and compared to fishmeal and soybean meal BSFL rearing is expected to use less land and water and to produce less greenhouse gas emissions, thereby contributing less to global warming (van Huis and Oonincx, 2017; Vauterin et al., 2021). Additionally, BSFL contain compounds with prebiotic and/or antibiotic functions, such as chitin and antimicrobial peptides, that could benefit broiler immunity and intestinal functioning (Khempaka et al., 2011; Makkar et al., 2014; Xia et al., 2021).

Before August 2021 only whole live insects and insect fat could be included in livestock feed in the EU, but commission regulation 2021/1372 changed this to also allow processed insect protein to be included in livestock feed. The range of insect forms allowed in livestock feeds is thus expanding, and in the future, it may include other forms such as whole dried larvae. Different insect forms will have varying effects on broiler physiology, behaviour, and welfare. For example, dietary full-fat or defatted BSFL meal has been reported to increase broiler body weight gain and feed intake, enhance innate immune activity, and stimulate a more diverse caecal microbiota composition (Biasato et al., 2020b; Dabbou et al., 2018; de Souza Vilela et al., 2021b; J. Lee et al., 2018). Conversely, dietary BSFL oil did not affect broiler body weight gain and feed intake, and it had minimal effects on gastro-intestinal tract development (Dabbou et al., 2021; Kierończyk et al., 2020; B. Kim et al., 2020). Provision of live BSFL in feeding trays also did not affect body weight gain, but it did cause a more diverse caecal microbiota composition (Bellezza-Oddon et al., 2021; Colombino et al., 2021). Variability in the observed effects of different BSFL forms will partly be due to different inclusion levels across studies (ranging from 1-20%). For example, a meta-analysis on dietary insects for poultry indicated that replacing more than 10% of the diet with insects often reduces poultry body weight gain (Moula and Detilleux, 2019). Additionally, the different processing methods required for the different BSFL forms play a role. For example, heat treatment reduces moisture content, changes product texture, and can cause lipid oxidation and protein denaturization, which in turn can change the nutrient availability and palatability of BSFL, affecting broiler functioning (Kröncke et al., 2019; Melgar-Lalanne et al., 2019). The level of defatting also plays a role, as partially defatted BSFL meal was found to be more digestible than highly defatted BSFL meal for broilers (Schiavone et al., 2017b).

Not only the BSFL form but also the provisioning method influences how broilers respond to dietary BSFL, especially considering broiler behaviour. The fast growth rate of broilers can hinder leg development and reduce their ability to be active (Bessei, 2006), and consequently broilers spend up to 70% of their time sitting near the end of the rearing period (Baxter et al., 2018a; de Jong and Gunnink, 2018). Many common welfare issues of broilers, such as contact dermatitis and lameness, are partly a result of their low activity levels and are exacerbated by their barren, unstimulating housing environments (de Jong et al., 2014; Vasdal et al., 2018b). Incorporating BSFL meal or oil in feed pellets is unlikely to stimulate activity and thereby improve leg health, which is supported by the observation that neither dietary BSFL meal nor BSFL oil influenced the occurrence of foot pad dermatitis (Kierończyk et al., 2020; van der Heide et al., 2021). In contrast, two recent studies showed that regularly scattering small amounts of live BSFL through the pen or providing live BSFL in tubes that had to be manipulated to access the larvae stimulated foraging behaviour and activity and, in some cases, reduced the occurrence of activity-related leg problems (Ipema et al., 2020a, 2020b). Moreover, one of these studies demonstrated that broilers with frequent or prolonged access to live BSFL were less fearful (Ipema et al., 2020a), suggesting that providing live BSFL also benefits the affective state of broilers and thereby promotes welfare in a broad sense. Dried larvae are more suitable for commercial use than fresh live larvae, as the latter cannot be stored for long periods of time and their high moisture content may cause feed safety risks related to degradation and microbial spoilage (Kröncke et al., 2019). It is, however, unknown whether offering dried BSFL will have welfare benefits similar to those of live BSFL, as chickens seem particularly attracted to moving prey (Clara et al., 2009)

Distinct combinations of BSFL forms and provision methods are thus expected to differentially affect broilers. Therefore, the aim of the current study was to determine the effects of different BSFL forms (i.e., BSFL meal and oil incorporated in the feed, dried BSFL, and live BSFL) and different provisioning methods (i.e., in the feeder or scattered across the pen) on various indicators of broiler welfare (e.g., behaviour, health) and production performance (e.g., body weight gain, feed intake). We hypothesized that incorporating BSFL meal and oil in the diet and providing dried BSFL in the feeder would be less effective in stimulating broiler activity and therefore less beneficial for welfare than scattering dried or live BSFL through the pen. In addition, we expected that scattering live BSFL would be more attractive to broilers and thereby more proficient in improving broiler welfare than scattering dried BSFL. Furthermore, based on the expected effect on activity, it is possible that the different BSFL supplementations strategies will have differential effects on broiler production performance.

Methods

This experiment was carried out at the research facility of ForFarmers (Bathmen, The Netherlands). The experimental protocol was approved by the Animal care and Use committee of Wageningen University & Research, under project license number AVD1040020187184. The protocol was in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific research. The ARRIVE guidelines for reporting animal experiments were accounted for in this study (du Sert et al., 2020).

Animals, housing, and management

At the start of the experiment 1680 one-day-old male Ross 308 broilers were obtained from a commercial hatchery and randomly distributed across 60 pens at the experimental facility, resulting in 28 broilers per pen. Each pen of 1.45 × 1.45 m contained one feeder (1.42 × 0.2 m), one drinking line containing 6 nipples with cups, and a 1-cm layer of wood shavings. After placement, per pen ten randomly selected broilers without signs of health problems were given a neck tag for individual identification. These broilers were the focal broilers for individual measurements throughout the experiment. Feed and water were available *ad libitum* throughout the 35-day experiment, and all broilers received routine vaccinations. The lighting schedule was 23 L:1 D on day 1-2, 20 L:4 D on day 3-7, 18 L:6 D on day 8-33 and 20 L:4 D on day 34-35. The temperature was 34 °C during the first two days, after which it was gradually decreased to 20 °C on day 35.

Experimental design

The experimental room was divided in 12 blocks of five adjacent pens, and within each block pens were randomly assigned to one of five treatments. Broilers in the control (CON) treatment did not receive any black soldier fly larvae (BSFL). In the four BSFL treatments, 8% of the ingredients from the CON pellets was replaced by BSFL as follows: BSFL meal and oil incorporated in the pellets and thus provided in the feeder (INC-F), dried whole BSFL provided in the feeder (D-F), dried whole BSFL scattered through the pen (D-S), or live BSFL (on dry matter (DM) basis) scattered through the pen (L-S). For the INC-F treatment, the ratio between BSFL meal and oil was chosen to have a similar protein to fat ratio as whole BSFL. The dried and live larvae were provided in equal portions four times a day (08:00, 11:00, 14:00 and 17:00). Protix B.V. (Dongen, The Netherlands) supplied the BSFL meal (ProteinX) and oil (LipidX), and Bestico B.V. (Berkel en Rodenrijs, The Netherlands) supplied the dried and live BSFL. Live larvae were supplied weekly and stored at 10 °C near the pens until provisioning.

All broilers were fed a three-phase diet, with starter feed provided on day 1-9, grower feed provided on day 9-27, and finisher feed provided on day 27-35. The composition of all pellets was adjusted to similar protein, fat, and energy intakes among the dietary treatments based on preliminary analyses of the composition of the applied BSFL forms, assuming DM intake was unaffected by the BSFL supplementation strategy (**Supplementary Table S1-S3**). All dietary treatments were designed to meet or exceed broiler dietary requirements (CVB, 2016).

Measurements

Home-pen behaviour and posture

Eight pens/treatment were included in the home-pen behavioural observations. Before observations, the ten focal broilers per pen were marked with a coloured dot (stock marker) for individual identification. Both behaviour and posture of all focal broilers were scored by means of 6-min instantaneous scan sampling on day 12, 23 and 33 (ethogram in **Table 1**), using a tablet with the program Observer 3.3 (Noldus Information Technology B.V., Wageningen, The Netherlands). Behaviour was observed for seven 1-hour periods, starting at 08:00, 09:30, 11:00, 12:15, 14:00, 15:30 and 17:00. If an observation period included the provisioning of dried or live

BSFL, the larvae were provided immediately before the first sampling point of each pen. Four observers simultaneously observed ten pens each, switching pens every observation period. Before observations, all observers had been trained and inter-observer reliability was deemed sufficient (Fleiss kappa > 0.8, Landis and Koch, 1977).

Table 1. Ethogram for home-pen behavioural observations. Both behaviour and posture were scored at each scan sampling point.

Item	Description
Behaviour class	
Eating from feeder	Having head above or in the feeder and/or pecking at feed or larvae in the feeder.
Drinking	Drinking from nipple or cup beneath nipple.
Standing idle and Walking	Standing, walking (locomoting in upright position with a normal speed or quick steps) or shuffling (half standing/half sitting and moving a few steps before sitting down), without performing any other behaviour.
Defecating	Excreting faeces.
Resting	Sitting with hocks resting on ground without performing any other behaviour, possibly with head on the ground or under wing.
Foraging	Performing pecking movements directed at the ground, or scraping the litter with claws, or food running (running with food in beak while pen mates follow and attempt to grab the food item).
Comfort behaviour	Grooming of own feathers with beak, or dust bathing (performed with fluffed feathers while lying, head rubbed on floor, wings opened, scratching at ground, distributing substrate over body).
Stretching	Stretching of wing and/or leg.
Wing flapping	Bilateral up-and-down wing flapping.
Agonistic behaviour	Jumping at pen mate, chasing pen mate, threatening pen mate, pecking movements directed at head of pen mate.
Pecking pen mate	Pecking movements directed at the body or beak of pen mate.
Other	Any behaviour not mentioned above.
Posture class	
Standing	Hocks not in contact with the litter.
Sitting	Hocks in contact with the litter.

Litter quality

On day 34 the litter quality was scored according to a protocol adjusted from Van Harn et al. (2019) (**Table 2**). The level of friability was scored on a scale from 1 (completely friable litter) to 5 (completely clumped litter), and the level of wetness was scored on a scale from 1 (completely dry litter) to 5 (very wet litter).

Visual leg health and cleanliness scores

On day 34 the lameness of four randomly selected focal broilers per pen was assessed by prompting the broilers to walk at least 1 m in the pen and assigning a gait score between 0 (normal, dexterous, and agile walk) and 5 (incapable of walking, Butterworth, 2009). On day 35 all focal broilers were visually scored on several leg health parameters and cleanliness. Foot pad dermatitis on both feet was scored on a scale of 0 (no lesions) to 4 (marked swelling and enlargement of the entire foot pad, necrotic cells covering more than half of the total foot pad area, Butterworth, 2009; Yamak et al., 2016). Hock burn on both hocks was scored on a scale of 0 (no hock burn) to 4 (severe lesions, Butterworth, 2009). Cleanliness of the belly was scored on a scale of 0 (feathers and/or skin completely clean) to 2 (feathers and/or skin have severe discoloration and mattered, clumped feathers of > 10 cm, Taylor et al., 2018). Full descriptions of scores are present in **Table 2**.

Table 2. Litter quality, visual leg health, and cleanliness scores.

Score	Description	Reference
Litter friability		
1	Completely friable litter.	(van Harn et al., 2019)
2	25% of litter is clumped.	
3	50% of litter is clumped.	
4	75% of litter is clumped.	
5	Completely clumped litter.	
Litter wetness		
1	Completely dry litter.	(van Harn et al., 2019)
2	Mildly moist litter.	
3	Moist litter.	
4	Wet litter.	
5	Very wet litter.	

Score	Description	Reference
Gait		
0	Normal, dexterous, and agile.	(Butterworth, 2009)
1	Slight abnormality, but difficult to define.	
2	Definite and identifiable abnormality.	
3	Obvious abnormality, affects ability to move.	
4	Severe abnormality, only takes a few steps.	
5	Incapable of walking.	
Foot pad dermatitis[1]		
0	No lesions.	(Butterworth, 2009)
1	Raised central pad, reticulate scales are separated, with or without small, black necrotic area(s).	
2	Marked swelling of the foot pad, black reticulate scales forming scale-shaped necrotic areas, with necrosis evident on less than one-quarter of the total foot pad area.	
3	Marked swelling and enlargement of the entire foot pad, necrosis extending up to one-half of the total foot pad area.	
4	Marked swelling and enlargement of the entire foot pad, necrotic cells covering more than one-half of the total foot pad area.	
Hock burn[1]		
0	No evidence of hock burn.	(Butterworth, 2009)
1	Minimal evidence of hock burn.	
2	Minimal evidence of hock burn.	
3	Evidence of hock burn.	
4	Evidence of hock burn.	
Cleanliness[1]		
0	Clean feathers.	(Butterworth, 2009; Taylor et al., 2018)
1	Discoloured feathers.	
2	Severe discoloration and mattered, clumped feathers > 10 cm.	

Table 2 continued.

[1] See reference for detailed illustrations of individual scores.

Immunological and hormonal measures

On day 35 the four focal broilers per pen of which the gait was previously assessed also had blood and feather samples taken for immunological and hormonal measures. Per broiler, 2 ml blood was collected in EDTA-containing tubes. Half of the blood was stored as whole blood at -80 °C until analysis. The other half was centrifuged at 5000 x g for 10 min at room temperature (RT), after which plasma was collected and stored at -20 °C until analysis. Additionally, the second and eighth primary feathers of each wing were clipped, and the four feathers from each broiler were collected in a bag and stored in the dark until analysis. All laboratory analyses were performed blind to treatment.

Plasma natural antibodies

Plasma was used to determine IgM and IgG natural antibody (NAb) titers against keyhole limpet hemocyanin (KLH) by ELISA. Natural antibodies are antigen-binding antibodies without known exposure to the antigen, and they play a role in innate immunity (Coutinho et al., 1995). After thawing, plasma was pre-diluted 1/10 for IgM and IgG binding KLH in dilution buffer (PBS containing 0.5% horse serum and 0.05% Tween®20), based on a pilot. Briefly, 96-wells plates were coated with a coating buffer (5.3 g/L Na_2CO_3 + 4.2 g/L $NaHCO_3$, pH 9.6) containing 2 µg/ml KLH. All washing steps were done with tap water containing 0.05% Tween. After washing, plates were incubated for 90 min at RT with a serial 4-step dilution in dilution buffer, resulting in 1:40, 1:160, 1:640 and 1:2560 test dilutions. Duplicate standard positive plasma samples (a pool of male plasmas) were stepwise 1:1 diluted with dilution buffer. After washing again, plates were incubated for 90 min at RT with goat-anti-chicken IgM labelled with horse radish peroxidase (PO) (1:10,000, GASwIgM/PO, Bethyl Laboratories Inc., Montgomery, USA) or goat-anti-chicken IgG labelled with PO (1:10,000, GASwIgGFC/PO, Bethyl Laboratories Inc., Montgomery, USA) in dilution buffer. After washing again, plates were incubated with tetramethylbenzidine for approximately 15 min at RT, after which the reaction was stopped with 1.25 M H_2SO_4. Absorbance was measured with a Multiskan Go (Thermo scientific, Breda, The Netherlands) at 450 nm and expressed relative to that of the standard positive control sample. Antibody titers are $\log_2$ values of dilutions that gave an extinction closest to 50% of E_{max}, with E_{max}

representing the highest mean extinction of the standard positive sample present on all plates.

Whole blood 5-HT

Whole blood serotonin (5-Hydroxytryptamine, or 5-HT) was measured according to Bolhuis et al. (2009). In short, after thawing 1 ml whole blood was pipetted into 50 ml tubes and 2 ml NaCl solution (9 g/L), 1 ml ascorbic acid solution (3%), 5 ml phosphate buffer (2 M K_2HPO_4, pH 10.0) and 20 ml n-butanol were added. Tubes were shaken for 5 min and centrifuged at 1000 x g for 15 min. Fifteen ml of the butanol layer was pipetted into new tubes after which 2 ml 0.1 M HCl and 25 ml cyclohexane were added. These tubes were shaken for 20 sec and centrifuged at 1000 x g for 4 min. The cyclohexane/butanol layer was removed, and 1 ml of the acidic phase was pipetted into a new tube containing 0.3 ml 12 M HCl. Tubes were vortexed shortly and samples were measured at 295/540 nm on the Aminco-Bowman fluorescence spectrofluorophotometer (PerkinElmer Inc., Waltham, USA). Absorbance was compared to a standard curve, and 5-HT levels were expressed as nmol/ml.

Feather corticosterone

To extract feather corticosterone (CORT), all feathers were cleaned by placing them in demi-water for 10 sec and gently rubbing them with a tissue, after which they were air dried overnight. The calamus, downy bars and tip of the feathers were removed and after this the length of the feathers was determined. Then, the vanes were collected by cutting next to the rachis, and vanes from the four feathers of each broiler were combined and weighed to the nearest 0.1 mg. The vanes were cut in flakes of < 3 mm^2 and thoroughly mixed. A sub-sample of approximately 35 mg (weighed to the nearest 0.1 mg) was placed in an Eppendorf tube with 3 metal beads (3.2 mm stainless steel balls, Cat. No. 11079132ss, Biospec Products, Bartlesville, USA). These tubes were dropped in liquid nitrogen for 1-2 minutes, and immediately thereafter they were placed in a Tissuelyser (Qiagen, Hilden, Germany) at 30 Hz for 5 min. This step was repeated three times. Then, 0.5 ml PBS was added to each sample and the samples were placed in a rotator (IKA Loopster, Staufen, Germany) at 300 rpm for 24 at RT, after which they were frozen at -20 C° until analysis.

After thawing, the PBS extract was centrifuged at 1000 g x for 5 min and the supernatant was pipetted in Eppendorf tubes for analysis. CORT concentrations were determined in duplicate by using a commercial CORT ELISA kit (Enzo Life Sciences, NY, USA) following a standard protocol (see online manual http://www.enzolifesciences.com/ADI-900-097/corticosterone-eia-kit). The CORT concentrations were expressed as a function of the feather length (pg/mm).

Production performance

At placement and on day 9, 19, 27 and 35 the average weight and feed intake of all broilers were determined on pen level. Additionally, on these days all focal broilers were weighed individually to determine if the focal broilers were representative of the whole pen. Morbidity and mortality were recorded daily.

Statistical analysis

Data processing

During the experiment, 48 broilers (of which 19 focal broilers) died from health issues (CON n = 3, INC-F n = 8, D-F n = 12, D-S n = 15, L-S n = 10) and they were excluded from analysis. If a focal broiler died, it was immediately replaced by a randomly selected pen mate, and this was accounted for in the analyses. The behaviours and postures observed in the home-pen were averaged per broiler per day and expresses as a percentage of the total observations. Behaviours that occurred in more than 5% of the observations (comfort behaviour, drinking, standing idle and walking, foraging, and resting) were analysed. Additionally, the behaviour "eating from feeder" was analysed as it is an indicator for pellet intake, and in case of the D-F treatment also for dried larvae intake. Based on the pellet intake measures the average daily dry matter intake in g/broiler/day with and without larvae was calculated, as well as the dry matter conversion into body weight gain. Per broiler only the leg with the most severe foot pad dermatitis or hock burn score was included in the analysis. The score "4" for both foot pad dermatitis and hock burn was present in less than 1% of the broilers, therefore this score was combined with score "3" for both parameters. To assure normality of residuals from general linear mixed models, a Grubbs test was applied to all continuous data, and the indicated outliers (mostly deemed a result of sampling errors or health problems) were omitted from analysis of the average daily gain (d1-9 five outliers, d9-19 four outliers, d19-27 five outliers, d27-35 five outliers), the final weight (three outliers), and the feather corticosterone concentration (three outliers) of focal broilers. The focal broilers were deemed a reliable representation of the total pen as treatment effects on the average daily gains and final weights of the focal broilers were similar to that of the total pen weights (**Supplementary Table S4**).

Data analysis

The statistical software SAS 9.4 (SAS Institute Inc., Cary, NC, USA) was used to analyse the data. All general linear mixed model residuals showed normality, except for the feather CORT concentration, which was ln transformed to achieve normality. All general and generalized linear mixed models included a fixed effect of dietary treatment and a random effect of block, and all models with individual broilers as experimental units additionally included a random effect of pen nested in treatment and block.

The proportion of observations in which the different behaviours and postures were shown were analysed with generalized linear mixed models (GLIMMIX in SAS) using a binomial distribution, logit link function, and an additional multiplicative over-dispersion parameter. Besides the aforementioned fixed effect of dietary treatment, these models included a fixed effect of day and the treatment by day interaction. Additionally, these models included a repeated effect of day with broiler as subject, using a heterogenous first-order autoregressive covariance structure. The average daily gain and final weight (measured at pen and individual level) and the average daily feed intake, dry matter conversion ratio, plasma antibody titers, whole blood 5-HT concentration, and feather CORT concentration (measured at individual level) were analysed with general linear mixed models (MIXED in SAS). The model for final weight included d1 weight as covariate. Significant fixed effects were further analysed using differences in least square means with a Tukey HSD correction

As litter quality scores contained empty subclasses, these scores were analysed with a Kruskal-Wallis test, and in case of significant treatment effects a Dwass-Steel-Critchlow-Fligner multiple comparisons test was used for pair-wise comparisons. Leg health scores were analysed with the GLIMMIX procedure using multinomial distribution and cumlogit link, and cleanliness scores were analysed with the GLIMMIX procedure using a binary distribution and logit link. Significant fixed effects on health and cleanliness scores were further analysed using estimate comparisons with Bonferroni correction.

Data are presented as pen means ± SEM unless indicated otherwise. Effects were considered significant at $p < 0.05$ and a tendency at $0.05 < p < 0.1$.

Results

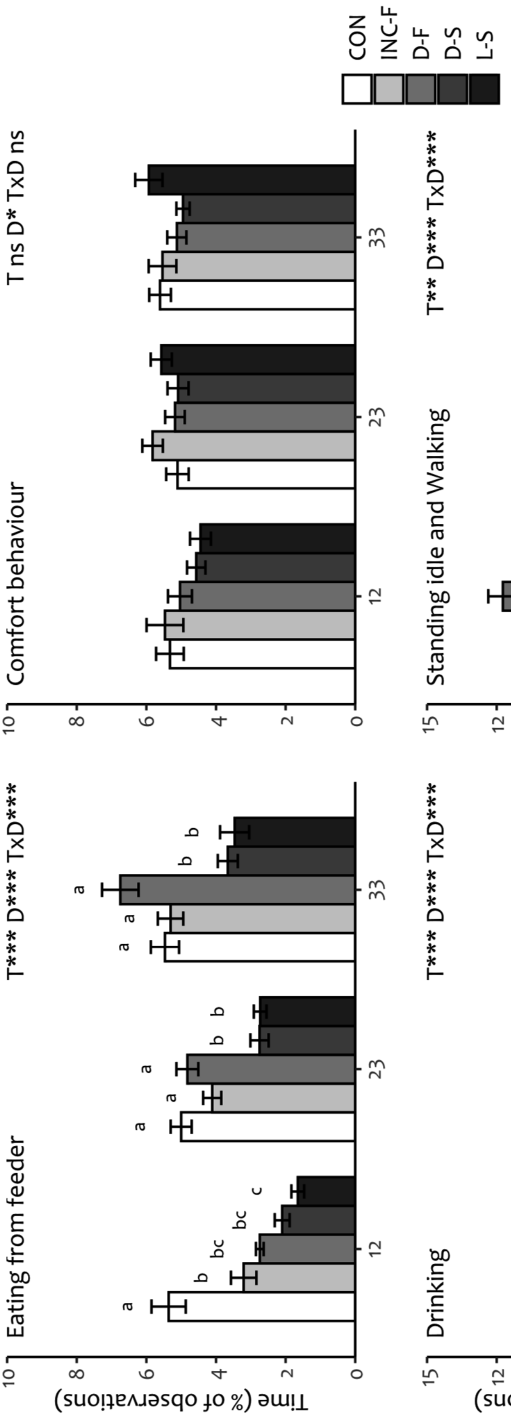
Home-pen behaviour and posture

The time spent on comfort behaviour was only influenced by day ($F_{(2,1150)} = 3.03$, $p = 0.049$, **Figure 1**), with no significant post-hoc differences. The time spent on the behaviours eating from feeder, drinking, standing idle and walking, foraging, resting (**Figure 1**), and the time spent in standing posture (**Figure 2**) were influenced by treatment, day, and the treatment by day interaction, and pairwise significant ($p < 0.05$) differences are described below.

Eating from feeder

The time spent on eating from the feeder was influenced by treatment ($F_{(4,35)} = 28.93$, $p < 0.001$), day ($F_{(2,1150)} = 44.01$, $p < 0.001$, **Figure 1**), and the treatment by day interaction ($F_{(8,1150)} = 4.43$, $p < 0.001$). On d12 CON broilers spent more time eating from the feeder than broilers in all other treatments, and broilers in the INC-F treatment spent more time eating from the feeder than broilers in the L-S treatment. On d23 and 33 broilers in the CON, INC-F and D-F treatments spent more time eating from the feeder than broilers in the D-S and L-S treatments. The time CON broilers spent eating from the feeder did not change over time, while it increased from d12 to 23 and d23 to 33 for D-F broilers and it increased from d12 to 33 for INC-F, D-S and L-S broilers.

Figure 1 (next page). Home-pen behaviour (% of observations) of broilers receiving no larvae (CON), larvae meal and oil incorporated in the feed (INC-F), dried larvae in the feeder (D-F), dried larvae scattered through the pen (D-S), or live larvae scattered through the pen (L-S). Data presented as means ± SEM. Effects of Treatment (T), Day (D), and their interaction (TxD) are indicated as ns (not significant), * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$). Different letters within one day indicate significant ($p < 0.05$, Tukey's HSD correction) differences between treatments.


Eating from feeder
T*** D*** TxD***
Comfort behaviour
T ns D* TxD ns
Drinking
T*** D*** TxD***
Standing idle and Walking
T** D*** TxD***
Time (% of observations)
Day
12
23
33
a
b
bc
bc
c
a
a
a
b
b
a
a
a
b
b
a
a
a
a
b
a
a
ab
a
b
a
ab
bc
abc
c
0
2
4
6
8
10
0
3
6
9
12
15
CON
INC-F
D-F
D-S
L-S

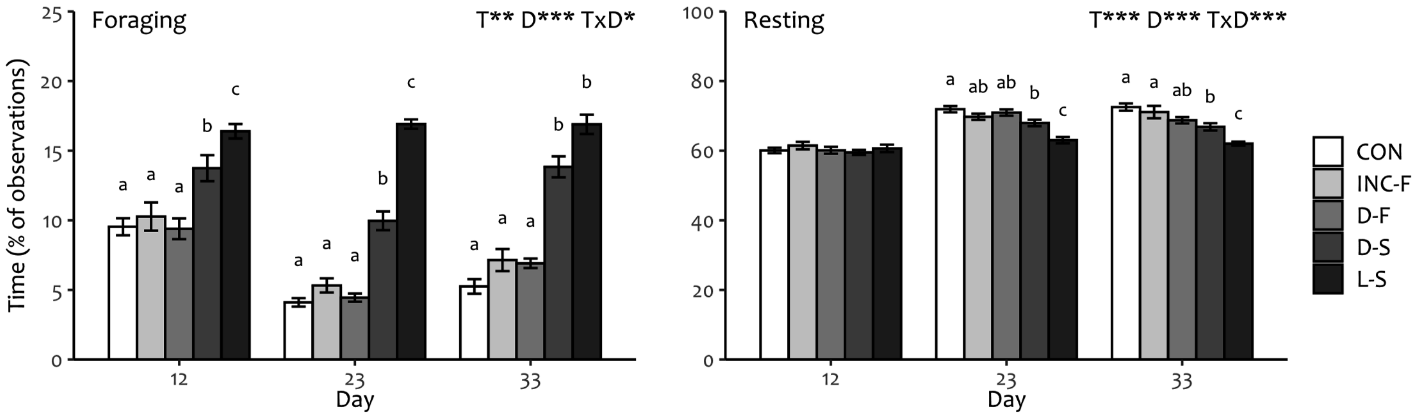


Figure 1 continued.

Drinking

The time spent drinking was influenced by treatment ($F_{(4,35)} = 6.85$, $p < 0.001$), day ($F_{(2,1150)} = 18.23$, $p < 0.001$), and the treatment by day interaction ($F_{(8,1150)} = 3.73$, $p < 0.001$, **Figure 1**). On d12 L-S broilers spent less time drinking than all other broilers, and on d23 they spent less time drinking than CON, INC-F and D-S broilers. The time spent drinking did not differ between treatments on d33. CON and L-S broilers did not change in their time spent drinking over time, while INC-F and D-S broilers spent less time drinking on d33 than on d23, and D-F broilers spent less time drinking on d33 than on d12.

Standing idle and Walking

The time spent standing idle and walking was influenced by treatment ($F_{(4,35)} = 5.54$, $p = 0.002$), day ($F_{(2,1150)} = 467.47$, $p < 0.001$), and the treatment by day interaction ($F_{(8,1150)} = 3.36$, $p < 0.001$, **Figure 1**). On d12 and 23 the time spent standing idle and walking did not differ between treatments. On d33 CON broilers spent less time standing idle and walking than D-F and L-S broilers, and INC-F broilers spent less time standing idle and walking than L-S broilers. The time spent standing idle and walking decreased from d12 to 23 and d23 to 33 for CON, INC-F and D-S broilers, while it decreased only from d12 to 23 for D-F and L-S broilers after which it stayed constant.

Foraging

The time spent foraging was influenced by treatment ($F_{(4,35)} = 98.81$, $p < 0.001$), day ($F_{(2,1150)} = 129.84$, $p < 0.001$), and the treatment by day interaction ($F_{(8,1150)} = 18.59$, $p < 0.001$, **Figure 1**). On all days D-S and L-S broilers foraged more than CON, INC-F and D-F broilers. Additionally, on d12 and 23 L-S broilers foraged more than D-S broilers. L-S broilers did not differ in their time spend foraging on different days. INC-F and D-F broilers spent less time foraging on d23 than d12, and on d33 than d23. CON broilers spent less time foraging on d23 and 33 than d12, and D-S broilers spent less time foraging on d23 than on d12 and 33.

Resting

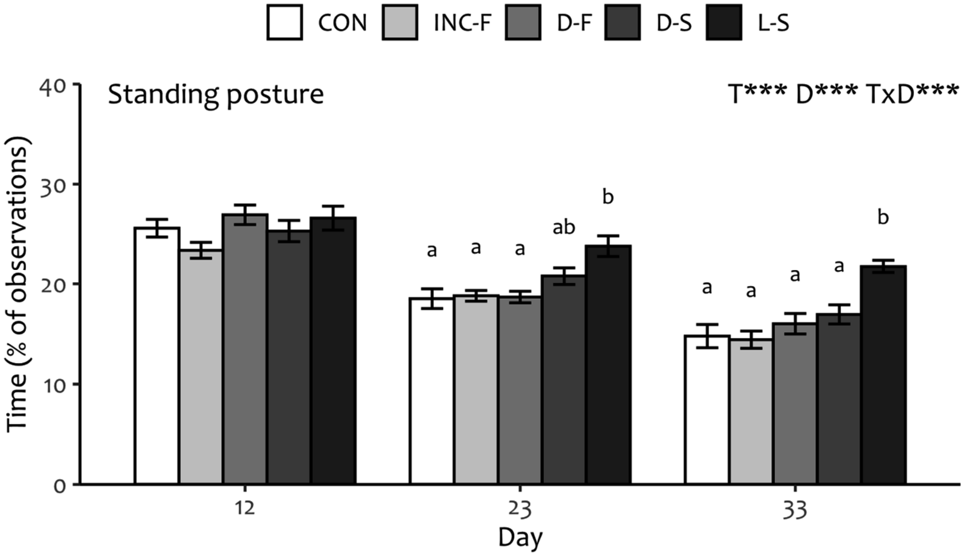
The time spent resting was influenced by treatment ($F_{(4,35)} = 19.14$, $p < 0.001$), day ($F_{(2,1150)} = 205.04$, $p < 0.001$), and the treatment by day interaction ($F_{(8,1150)} = 10.31$, p

< 0.001, **Figure 1**). On d12 there was no difference in time spent resting between treatments. L-S broilers spent less time resting than broilers in all other treatments on d23 and 33. D-S broilers spent less time resting than CON broilers on d23 and 33 and than INC-F broilers on d33. The time spent resting of L-S broilers did not change over time, whereas in the other treatments it increased from d12 to 23 after which it remained constant.

Standing posture

The time spent in standing posture was influenced by treatment ($F_{(4,35)} = 12.81$, $p < 0.001$), day ($F_{(2,1150)} = 219.61$, $p < 0.001$), and the treatment by day interaction ($F_{(8,1150)} = 6.08$, $p < 0.001$, **Figure 2**). Treatment did not influence the time spend in standing posture on d12. On d23 and 33 L-S broilers spent more time standing than CON, INC-F and D-F broilers, while on d33 L-S broilers also spent more time standing than D-S broilers.

Figure 2. Time spent in standing posture (% of observations) of broilers receiving no larvae (CON), larvae meal and oil incorporated in the feed (INC-F), dried larvae in the feeder (D-F), dried larvae scattered through the pen (D-S), or live larvae scattered through the pen (L-S). Data presented as means ± SEM. Effects of Treatment (T), Day (D), and their interaction (TxD) are indicated as *** ($p < 0.001$). Different letters within one day indicate significant ($p < 0.05$, Tukey's HSD correction) differences between treatments.



Litter quality

Treatment influenced both litter friability and wetness ($p < 0.001$, **Table 3**). CON pens had less friable litter than D-F, D-S and L-S pens, and CON pens had wetter litter than pens in all other treatments.

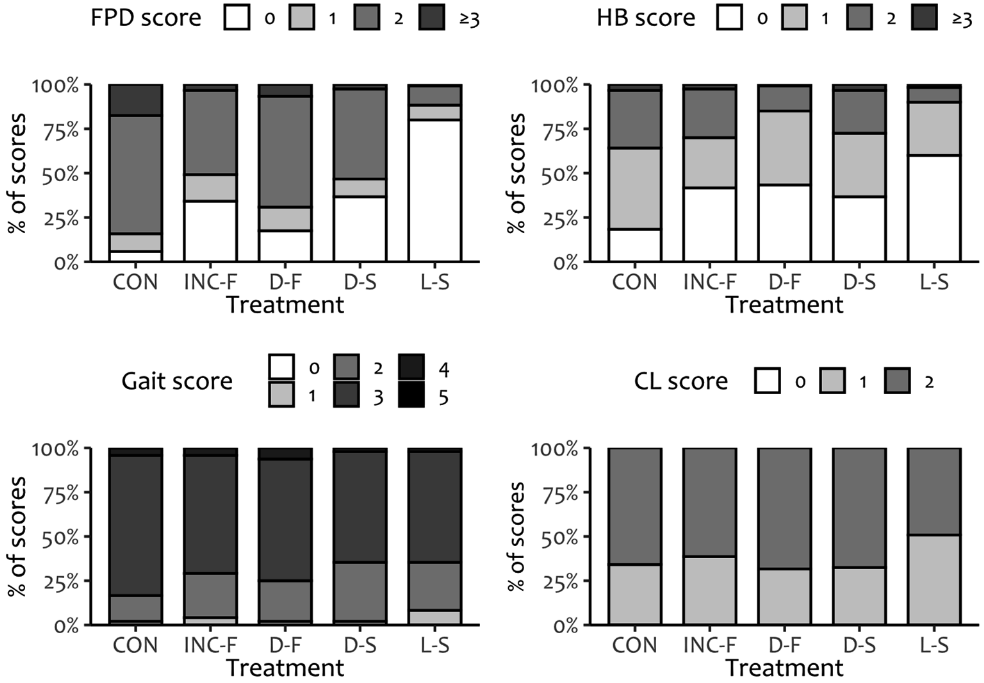
Table 3. Frequencies of visual litter quality scores of pens with broilers receiving no larvae (CON), larvae meal and oil incorporated in the feed (INC-F), dried larvae in the feeder (D-F), dried larvae scattered through the pen (D-S), or live larvae scattered through the pen (L-S). Significant treatment effects ($p < 0.05$) are indicated in bold, and within the "sum of scores" rows different superscript letters indicate significant ($p < 0.05$, DSCF correction) differences between treatments.

Measure	CON	INC-F	D-F	D-S	L-S	Test-statistic and df	P-value
Friability score							
1	0	0	0	0	0		
2	1	3	7	3	7		
3	1	4	5	8	4		
4	4	4	0	1	1	$H_{(4)} = 23.24$	**<0.001**
5	6	1	0	0	0		
Sum of scores[1]	579.5^{a}	414^{ab}	239.5^{b}	341.5^{b}	255.5^{b}		
Wetness score							
1	0	0	0	0	0		
2	0	4	8	8	2		
3	3	6	4	3	10		
4	9	2	0	1	0	$H_{(4)} = 26.54$	**<0.001**
5	0	0	0	0	0		
Sum of scores[1]	597^{a}	368^{b}	234^{b}	253^{b}	378^{b}		

Visual health and welfare scores

There was a tendency for treatment to affect gait score ($p = 0.098$) and cleanliness ($p = 0.052$, **Figure 3**). Foot pad dermatitis was influenced by treatment ($p < 0.001$), with the L-S broilers having less severe foot pad dermatitis scores than broilers in all other treatments, and the INC-F and D-S broilers having less severe foot pad dermatitis scores than CON broilers. Hock burn was also affected by treatment (p = 0.002), where L-S broilers had less severe hock burn scores than CON broilers (**Figure 3**).

Figure 3. Percentage of broilers with each foot pad dermatitis (FPD), hock burn (HB), gait, and cleanliness (CL) score of broilers receiving no larvae (CON), larvae meal and oil incorporated in the feed (INC-F), dried larvae in the feeder (D-F), dried larvae scattered through the pen (D-S), or live larvae scattered through the pen (L-S). Please note, no birds received a score of 0 for gait and CL, and no birds received a score of 5 for gait.



Immunological and hormonal measures

Feather CORT concentration was affected by treatment (p = 0.037), but there were no significant post-hoc differences between treatments. Plasma IgM natural antibody titers against KLH tended to be influenced by treatment (p = 0.059). Plasma IgG natural antibody titers against KLH and whole blood 5-HT were not influenced by treatment (**Table 4**).

Table 4. Blood plasma KLH-IgG and KLH-IgM antibody titers, whole blood 5-HT concentrations, and feather corticosterone (CORT) concentrations of broilers receiving no larvae (CON), larvae meal and oil incorporated in the feed (INC-F), dried larvae in the feeder (D-F), dried larvae scattered through the pen (D-S), or live larvae scattered through the pen (L-S). Data presented as means ± SEM. Significant treatment effects ($p < 0.05$) are indicated in bold.

Measure	**CON**	**INC-F**	**D-F**	**D-S**	**L-S**	**Test-statistic and df**	**P-value**
Plasma KLH-IgG titers	2.4 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.5 ± 0.1	$F_{(4,55)} = 0.67$	0.618
Plasma KLH-IgM titers	3.1 ± 0.1	3.3 ± 0.1	2.9 ± 0.1	3.2 ± 0.1	3.4 ± 0.1	$F_{(4,55)} = 2.42$	0.059
Whole blood 5-HT (nmol/ml)	44.4 ± 1.3	46.0 ± 1.9	44.2 ± 2.4	46.0 ± 1.8	43.2 ± 2.1	$F_{(4,55)} = 0.58$	0.680
Feather CORT (pg/mm)	0.44 ± 0.13	0.24 ± 0.06	0.30 ± 0.11	0.41 ± 0.14	0.38 ± 0.12	$F_{(4,55)} = 2.76$	**0.037**

Production performance

The production performance parameters of the entire pens are shown in **Table 5**. During several days the broilers' average daily gain was influenced by treatment (all $p < 0.01$). During d1-9 D-F and L-S broilers grew faster than CON broilers, with INC-F and D-S broilers in between. During d9-19 D-F, D-S and L-S broilers grew faster than CON and INC-F broilers. During d19-27 the D-F and D-S broilers grew faster than CON broilers, and during d27-35 treatment did not influence broiler average daily gain. The final weight was also influenced by treatment ($p < 0.001$). The final weight of D-F and L-S broilers was higher than that of CON broilers, and the final weight of D-S broilers was higher than that of CON and INC-F broilers.

When calculating the average daily dry matter intake, we assumed that all larvae provided were indeed consumed (**Table 5, Supplementary Table S5**), though this may not have been the case for all pens (see Discussion). Based on this calculation, the BSFL percentage of the total dry matter consumption was estimated to be 8.5 ± 0.1% for D-F broilers, 8.7 ± 0.1% for D-S broilers, and 9.1 ± 0.1 % for L-S broilers

Treatment influenced the average daily dry matter intake of pellets ($p < 0.001$). L-S broilers had a lower intake than broilers in all other treatments, and D-F and D-S broilers had a lower pellet intake than CON and INC-F broilers. Treatment also influenced the estimated average daily dry matter intake of pellets and dried or live BSFL combined ($p < 0.001$). Here, L-S broilers had a lower intake than broilers in all other treatments, and D-F broilers had a higher intake than INC-F broilers. Periodic differences in average daily dry matter intake are shown in **Supplementary Table S5**.

The dry matter conversion ratio (DMCR) was influenced by treatment ($p < 0.001$). The DMCR of L-S broilers was lower than that of broilers in all other treatments, and the DMCR of INC-F and D-S broilers was lower than that of CON broilers.

Table 5. Production performance on pen level of broilers receiving no larvae (CON), larvae meal and oil incorporated in the feed (INC-F), dried larvae in the feeder (D-F), dried larvae scattered through the pen (D-S), or live larvae scattered through the pen (L-S). Data are presented as means ± SEM. Significant treatment effects ($p < 0.05$) are indicated in bold, and within each row different superscript letters indicate significant ($p < 0.05$, Tukey’ s HSD correction) differences between treatments.

Measure	**CON**	**INC-F**	**D-F**	**D-S**	**L-S**	**Test-statistic and df**	**P-value**
Average daily gain (g/d)							
d1-9	22.6 ± 0.3^{a}	23.0 ± 0.1^{ab}	23.7 ± 0.2^{b}	23.4 ± 0.3^{ab}	23.8 ± 0.3^{b}	$F_{(4,44)} = 4.50$	0.004
d9-19	61.3 ± 0.6^{a}	61.6 ± 0.3^{a}	64.8 ± 0.4^{b}	64.2 ± 0.4^{b}	65.8 ± 0.7^{b}	$F_{(4,44)} = 17.15$	<0.001
d19-27	102.4 ± 1.3^{a}	103.1 ± 0.9^{ab}	107.0 ± 1.3^{b}	107.6 ± 1.3^{b}	104.0 ± 1.4^{ab}	$F_{(4,44)} = 4.16$	0.004
d27-35	122.4 ± 1.2	124.4 ± 1.2	123.6 ± 1.5	125.5 ± 1.5	124.6 ± 2.5	$F_{(4,44)} = 0.57$	0.688
FW (g)	2660 ± 19.7^{a}	2694 ± 11.4^{ab}	2758 ± 9.8^{bc}	2772 ± 18.9^{c}	2747 ± 16.1^{bc}	$F_{(4,44)} = 9.88$	<0.001
Average daily dry matter intake (g/d)							
Pellets	93.4 ± 0.7^{a}	92.4 ± 0.3^{a}	86.9 ± 0.6^{b}	85.8 ± 0.6^{b}	81.3 ± 0.6^{c}	$F_{(4,44)} = 73.48$	<0.001
Pellets & larvae*	93.4 ± 0.7^{ab}	92.4 ± 0.3^{b}	95.0 ± 0.6^{a}	94.0 ± 0.6^{ab}	89.4 ± 0.5^{c}	$F_{(4,44)} = 13.73$	<0.001
DMCR (g/g)	1.25 ± 0.002^{a}	1.23 ± 0.003^{b}	1.24 ± 0.008^{ab}	1.22 ± 0.004^{b}	1.16 ± 0.004^{c}	$F_{(4,44)} = 49.63$	<0.001

FW = Final weight, DMCR = Dry matter conversion ratio.

* Estimated based on the assumption that all larvae are consumed.

Discussion

In this study we investigated the effects of replacing 8% of the dietary dry matter intake of broilers with black soldier fly larvae (BSFL) as meal and oil incorporated in the pellets (INC-F), as dried larvae provided in the feeder (D-F) or scattered through the pen four times a day (D-S), and as live larvae scattered through the pen four times a day (L-S) on various indicators of broiler welfare and production performance. The four different BSFL inclusion methods all did not affect or improved the investigated parameters compared to a diet similar in protein, fat, and energy content but without BSFL (CON treatment), though the specific responses varied between treatments. Generally, broiler welfare benefitted most from scattering live BSFL, followed by scattering dried BSFL and then BSFL meal and oil incorporated in the pellets. Also, broiler production performance was increased most by providing dried or live BSFL.

Home-pen behaviour and posture

Compared to the controls, D-S and L-S broilers performed more foraging behaviour throughout the whole rearing period (on average 12.5% and 16.7% of the observed time, respectively, vs. 6.3% of time of controls). Furthermore, they performed more standing idle and walking and less resting near the end of the rearing period. The L-S broilers typically spent more time on active behaviours than D-S broilers, and only the L-S broilers showed more standing postures than controls on d23 and 33. Contrarily, INC-F and D-F broilers did not show more active behaviours than controls. The increased activity as a result of scattering larvae was also observed in previous studies using live BSFL (Ipema et al., 2020a, 2020b) or mealworms (Pichova et al., 2016), and our study shows that scattering larvae throughout the pen promotes activity in contrast to providing dried larvae localized in a feeder. Performing natural behaviours such as foraging is considered essential for good welfare as it satisfies intrinsic motivations (Bracke and Hopster, 2006; Fraser, 2008) and the benefits can extend to a broiler's affective state (e.g., reduced fearfulness) and health (e.g., reduced leg problems) (Ipema et al., 2020a; Reiter and Bessei, 2009). Live larvae may be more interesting to broilers than dried larvae because their movement can be attractive to birds (Clara et al., 2009), and/or due to consequences of the drying process such as reduced moisture contents and changed odours that may make dried larvae less palatable (Mishyna et al., 2020), though unravelling the exact reasons will require more extensive research. Based on our observations, scattering live larvae through the pen is most advantageous for promoting broiler activity.

On d12 and 23, the time spent drinking was lower for L-S broilers compared to broilers in all other treatments. This is likely a results of the high moisture content of live BSFL, and is in line with what we found previously (Ipema et al., 2020b). As expected, the time spent eating from the feeder was lower in D-S and L-S broilers compared to CON broilers on all observation days, because 8% of their diet was provided outside of the feeder. On d12 INC-F and D-F broilers also spent less time eating from the feeder than CON broilers, although their average daily intake from the feeder was not lower during this period. Previous studies have suggested that diets including insect meals or oils are more palatable than diets without (Ballitoc and Sun, 2013; Cullere et al., 2018), which could have resulted in faster consumption of the more palatable diets by INC-F and D-F broilers. However, as the observed differences did not persist throughout the rearing period it is difficult to pinpoint the exact cause.

Litter quality

We observed that pens in all BSFL treatments had dryer litter than CON pens, and additionally pens in D-F, D-S, and L-S treatments had more friable litter than CON pens. The increased activity of D-S and L-S broilers likely regularly tousled the litter, which is known to promote drying and improve litter quality (Bailie et al., 2013; de Jong and van Harn, 2012). However, as INC-F and D-F pens also showed better litter quality without broilers being more active, it can be assumed that the consumption of larvae also improved litter quality independent from activity. Previous studies indicate that diets including BSFL meal have high fat digestibility (de Souza Vilela et al., 2021b), which can be beneficial for litter quality as faecal lipid compromises litter absorption abilities (Collett, 2012). However, in contrast to the current study, former studies on dietary BSFL found either no effects or a decrease of litter quality (Ipema et al., 2020a; van der Heide et al., 2021), and these discrepancies between studies highlight the need to further explore the mechanisms through which dietary BSFL affect litter quality.

Visual leg health and cleanliness scores

The percentage of broilers with a score above 0 for foot pad dermatitis (FPD, 63.3%), hock burn (HB, 61,3%), lameness (100%), and cleanliness (100%) are in the range of those found in previous studies (de Jong et al., 2019, 2012; Granquist et al., 2019). The improved litter quality in the BSFL treatments partially coincides with improved leg health, as INC-F and D-S broilers had less severe FPD scores, and L-S broilers had less severe FPD and HB scores than CON broilers. Both FPD and HB have been linked to poor litter quality, as high levels of moisture and ammonia cause contact dermatitis (Bassler et al., 2013; de Jong et al., 2014; Haslam et al., 2007), therefore improved litter quality can benefit broiler leg health. In contrast to our results from the INC-F treatment, previous studies found that providing dietary BSFL meal or oil did not affect the severity of FPD (Kierończyk et al., 2020; van der Heide et al., 2021), and the mechanisms of this effect require further attention.

Only L-S broilers had reduced HB severity, and that may be because they showed the highest activity and time spent standing. Increased activity reduces the time that hocks are in contact with the litter, limiting the development of hock burns (de Jong et al., 2014). This is in agreement with our previous study that found reduced HB severity after live BSFL provisioning only in treatments with the most active broilers (Ipema et al., 2020b). Also, D-F broilers did not show a reduced severity of FPD and HB despite the improved litter quality, which could be because their activity level was similar to that of the CON broilers. Furthermore, D-F broilers had a higher average daily gain and final weight than CON broilers (discussed later), and higher weights can increase the risk of contact dermatitis due to increased pressure of the skin against the litter (Kjaer et al., 2006; Mayne, 2005), possibly outweighing any benefits of improved litter quality. In previous studies several treatments applying live BSFL provisioning did not affect FPD and HB severity, however in these treatments the occurrence of leg health problems was low, presumably due to beneficial rearing conditions (e.g., lower stocking densities or litter supplementation, Ipema et al., 2020a, 2020b).

Lameness severity can also be reduced by regular activity as this stimulates leg development (Bassler et al., 2013; Vasdal et al., 2018b) and it can be increased by higher daily body weight gains because of the extensive load this places on the legs (Kestin et al., 2001; Knowles et al., 2008). The D-S and L-S broilers had both increased activity and higher body weight gains than controls, and these effects may have cancelled each other out, explaining why gait score was not influenced by dietary BSFL. This is consistent with a previous study in which regular provisioning of live larvae reduced broiler lameness but also reduced their average daily gain (Ipema et al., 2020b). Cleanliness of the broilers was also not influenced by dietary BSFL in the current and a previous study (Ipema et al., 2020a). Some studies indicate a link between cleanliness and litter quality (de Jong et al., 2014) though others find no such connection (Kaukonen et al., 2017b), and cleanliness has also been linked to other parameters such as leg health and performance (Bassler et al., 2013; de Jong et al., 2014). While there were no significant differences in lameness and cleanliness scores, there was a tendency for dietary BSFL to benefit these parameters, which warrants further investigation.

Immunological and hormonal measures

Both IgG and IgM titers against KLH were not affected by dietary BSFL. It has been suggested that the prebiotic compounds in BSFL (e.g., chitin, antimicrobial peptides) and their derivatives can modulate humoral and cell-mediated immunological responses of broilers (Deng et al., 2008; Xia et al., 2021), though evidence of immuno-enhancing effects of dietary BSFL is scarce and contradictory. For example, blood leukocyte concentrations were increased by including up to 20% of BSFL meal in broiler diets (de Souza Vilela et al., 2021b), but in another study this concentration was unchanged by up to 15% inclusion of BSFL meal (Dabbou et al., 2018). One study found that replacing 6.5% of the diet of layer hens with BSF pupa for 15 weeks increased serum IgG concentrations (Park et al., 2017), and another study found that mealworms fermented with probiotics increased broiler IgG levels while not affecting IgM levels after a *Salmonella enteritidis* challenge compared to controls (Islam and Yang, 2017), but the current study could not corroborate these results. It is possible that an immunological challenge is required to observe any immunomodulatory effects of dietary BSFL, which warrants experimental investigation.

Whole blood serotonin (5-HT) concentrations were also not influenced by dietary BSFL. Whole blood 5-HT reflects storage of 5-HT and thus long-term 5-HT system functioning (Shajib and Khan, 2015). Relatively higher whole blood 5-HT levels have been linked to reduced fear-related behaviour in layer hens (Bolhuis et al., 2009) and pigs (Ursinus et al., 2013), and 5-HT depletion has been associated with pessimistic affective states in pigs (Stracke et al., 2017). Providing broilers with environmental enrichment such as perches and dust baths (Baxter et al., 2018a), or live BSFL in tubes (Ipema et al., 2020a), was found to reduce fearfulness, though in these studies, 5-HT levels were not investigated. Our results do not suggest an effect of dietary BSFL on 5-HT concentrations, though the relationship between dietary BSFL, 5-HT system functioning, and broiler affective states remains to be studied.

Feather CORT concentrations are considered a novel indicator of long-term stress in broilers (D. Y. Kim et al., 2021; Weimer et al., 2018). Compared to previous reports, the observed feather CORT concentrations were similar (Carbajal et al., 2014) or lower (D. Y. Kim et al., 2021) and differences between studies are expected to be a result of different feathers used or alternative processing methods (Ataallahi et al., 2021). Despite a main effect of treatment, there were no significant differences between individual treatments in the feather CORT concentration. Numerically, the feather CORT concentration was highest in the control treatment and lowest in the INC-F treatment, which might suggest that consuming BSFL can lower chronic stress in broilers. INC-F broilers had less severe leg problems than controls, and better leg health has previously been linked to reduced stress in broilers (Weimer et al., 2021). However, as broilers in several other BSFL treatments also showed improved leg health, even to a larger extent, but had similar feather CORT concentrations as controls, it cannot be excluded that other factors affected feather CORT accumulation in our study. A recent paper demonstrated that contamination with faeces can increase feather CORT concentrations, even when feathers were washed prior to analysis (Bartels et al., 2021). As the friability and wetness of the litter as well as time spent standing, which may influence exposure to faeces, were all influenced by treatment, it is possible that potential effects of stress on feather CORT concentrations were confounded with effects of contact with faecal matter. Future studies are needed to affirm whether and how different BSFL provisioning methods affect broiler stress.

Production performance

Broilers in the INC-F treatment did not differ in average daily feed intake from CON broilers. This is in line with preceding studies that observed no difference in feed intake when BSFL meal (Elwert et al., 2010; Popova et al., 2021) or oil (Kierończyk et al., 2020; B. Kim et al., 2020; Schiavone et al., 2017b) was incorporated in broiler diets at similar inclusion percentages, and confirms that including 8% processed BSFL in broiler diets does not negatively influence feed intake. As anticipated, D-F and D-S broilers consumed less pellets but had a similar daily total dry matter intake as CON broilers. It must be noted that personal observations indicate that several broilers from the D-F and D-S treatments did not consume the dried larvae during approximately the first two weeks of the trial, and this could have resulted in an over-estimation of the daily total dry matter intake. Therefore, additional studies that record dried larvae consumption are required to obtain certainty about dry matter intake levels.

In contrast to broilers that received dried larvae, L-S broilers had a lower daily pellet intake than all other treatments, and their daily total dry matter intake was also lower than that of CON broilers. This contradicts previous studies that did not observe reduced total dry matter intake when up to 10% of the dry matter intake consisted of live BSFL (Bellezza-Oddon et al., 2021; Ipema et al., 2020a, 2020b). However, pigs that received up to 20% of their diet as live larvae did show a reduced total dry matter intake (Ipema et al., 2022). Differences between studies in the effect of feeding live BSFL on daily dry matter intake could be caused by differences in dietary composition (e.g., different protein and energy levels) or experimental set-up (e.g., stocking density), as these parameters affect feed intake (Feddes et al., 2002; Namroud et al., 2010). Live BSFL have a higher moisture content and consequently a higher volume at similar dry matter weights than dried larvae, and it is plausible that this increased the stomach fill and thereby satiety in broilers, as broilers are known to eat to their maximum physical ability

(Bokkers and Koene, 2003b). This could have resulted in a lower motivation to consume pellets and thus an overall lower dry matter intake.

Despite having a similar or lower daily dry matter intake, the average daily gain of broilers in the D-S and L-S treatments was higher during several days than that of controls, resulting in a higher final weight and lower dry matter conversion ratio (DMCR). These results are not in line with previous studies that found either similar or temporarily lower average daily gains in broilers that received live larvae, which was mainly attributed to their increased activity (Bellezza-Oddon et al., 2021; Ipema et al., 2020a, 2020b). However, in these studies live BSFL provisioning had no or minimal effects on broiler leg health (Ipema et al., 2020a, 2020b). While higher broiler body weight gains can impair leg health (discussed before), alternatively leg health can influence broiler body weight gain. For example, body weight gain was reduced by inducing FPD through wet litter (de Jong et al., 2014) and by inducing lameness through bacterial chondronecrosis with osteomyelitis (Weimer et al., 2021). As such, any benefits of BSFL provisioning on activity and leg health may have improved the body weight gain of D-S and L-S broilers.

However, D-F broilers also had increased average daily gains and a higher final weight on d35 without higher activity levels or improved leg health, and INC-F broilers had a lower DMCR than controls, even though their feed intake and average daily gain only differed numerically. These results suggest that not only activity and leg health play a role in broiler production performance. Several studies on dietary BSFL meal did indicate higher average daily gains at similar feed intake levels (Attivi et al., 2020; de Souza Vilela et al., 2021b), sometimes resulting in a lower feed conversion ratio (Attivi et al., 2020). In these studies, the beneficial effects of dietary BSFL on broiler production performance were attributed to the nutrition composition of BSFL, including their prebiotic and antibiotic compounds (e.g., chitin and antimicrobial peptides (AMPs)), however the exact cause remains to be studied. Furthermore, in the current study diets were adjusted based on estimated digestibility levels from studies including BSFL meal, as to the authors' knowledge the digestibility of diets containing dried and live larvae has not been investigated. Underestimation of the digestibility of diets including BSFL may have led to higher metabolizable energy and/or protein contents in these diets, which in turn could have increased production performance. Research into the effect of dietary dried and live BSFL on nutrient digestibility is required to unravel their role in broiler performance, and to understand why in-feed BSFL meal and oil, dried BSFL, and live BSFL affect the studied parameters of broiler production performance differently.

Conclusion

Replacing 8% of the diet of broilers with BSFL meal and oil incorporated in feed pellets, dried larvae provided in the feeder or scattered through the pen four times a day, or live larvae scattered through the pen four times a day all increased broiler average daily gains and/or dry matter conversion ratio, and improved litter quality. Incorporating BSFL meal and oil in the diet and scattering dried or live larvae through the pen improved broiler leg health, and scattering larvae also increased broiler activity. Plasma natural antibodies and whole blood serotonin concentrations were not influenced by dietary BSFL. Feather CORT concentrations were affected by BSFL provisioning, though post-hoc differences between treatments were absent. Overall, we confirmed that processed and live BSFL can benefit broiler welfare and increase broiler production performance. Scattering BSFL through the pen results in more welfare benefits than providing BSFL in the feeder, with live BSFL having the strongest effects on broiler behaviour and leg health, and therefore being most beneficial for broiler welfare.

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Supplementary data

Table S1. Analysed composition of black soldier fly larvae (BSFL) forms.

	BSFL meal	**BSFL oil**	**Dried BSFL**	**Live BSFL**
Dry matter (dm, g/kg)	950	1000	1000	279
Protein (g/kg of dm)	560	0	413	428
Fat (g/kg of dm)	150	990	411	375

Table S2. Ingredients (g/kg) of starter, grower, and finisher pellets of broilers receiving no larvae (CON), larvae meal and oil incorporated in the feed (INC-F), dried larvae in the feeder (D-F), dried larvae scattered through the pen (D-S), or live larvae scattered through the pen (L-S). BSFL = black soldier fly larvae.

	Starter (d1-9)			**Grower (d9-27)**		
	CON	**INC-F**	**D-F, D-S, L-S***	**CON**	**INC-F**	**D-F, D-S, L-S***
Corn	272.15	253.6	231.78	250	250	272
Wheat	300	300.01	300	349.36	368.49	352.69
Soybean meal	304.95	270.38	357.35	267.75	200.96	259.16
Sunflower meal	10	10	10.9	10	10	10.9
Rapeseed meal	10	10	10.9	20	20	21.8
Oat hulls	20	20	21.74	20	20	21.74
Soy oil	28.33	5.16	15	38.41	7.35	15
Limestone	11.73	12.08	11.9	8.53	8.69	8.2
Monocalcium phosphate	7.4	4.8	6.89	2.88	0.56	3.72
Sodium bicarbonate	1.4	1.38	0.75	2.9	3.35	3.05
Salt	2.28	2.13	2.84	1.23	0.76	1.25
VM VLK 0-20 d 8754	20	20	21.73	20	20	21.74
VM Maxiban 0.3%	3	3	3.26	3	3	3.26
L-Lysine HCl	2.82	2.72	1.08	1.98	2.83	1.9
DL-methionine	3	2.9	2.68	2.15	2.28	2.22
L-threonine	1.22	0.74	0	0.71	0.63	0.2
Valine 98	0.62	0	0	0	0	0
Xylanase 6.25%	1	1	1.09	1	1	1.09
Fytase Axtra Phy 5000L	0.1	0.1	0.11	0.1	0.14	0.11
BSFL oil	0	22.4	0	0	22.4	0
BSFL meal	0	57.6	0	0	57.6	0

	Finisher (d27-35)		
	CON	**INC-F**	**D-F, D-S, L-S***
Corn	200	200	217.4
Wheat	440.45	450.41	494.57
Soybean meal	222.49	163.96	178.02
Sunflower meal	10	10	10.9
Rapeseed meal	20	20	21.74
Oat hulls	20	20	21.74
Soy oil	45.89	15.85	16.91
Limestone	10.3	10.09	6.05
Monocalcium phosphate	1.62	0	0
Sodium bicarbonate	2.34	2.67	3.3
Salt	1.13	0.74	0.81
VM VLK 0-20 d 8754	20	20	21.74
VM Maxiban 0.3%	0	0	0
L-Lysine HCl	2.17	2.78	3.03
DL-methionine	1.8	1.87	2.02
L-threonine	0.71	0.53	0.57
Valine 98	0	0	0
Xylanase 6.25%	1	1	1.09
Fytase Axtra Phy 5000L	0.1	0.1	0.11
BSFL oil	0	22.4	0
BSFL meal	0	57.6	0

Table S2 continued.

* Dried and live larvae not included as ingredients.

Table S3. Nutrient composition of starter, grower, and finisher pellets of broilers receiving no larvae (CON), larvae meal and oil incorporated in the feed (INC-F), dried larvae in the feeder (D-F), dried larvae scattered through the pen (D-S), or live larvae scattered through the pen (L-S). AMEn = nitrogen-corrected apparent metabolizable energy.

	Starter (d1-9)			**Grower (d9-27)**		
	CON	**INC-F**	**D-F, D-S, L-S***	**CON**	**INC-F**	**D-F, D-S, L-S***
AMEn (kcal/kg)	2850	2850	2698	2937.5	2937.5	2794
Dry matter (g/kg)	877	879	875	872	871	868
Crude protein (g/kg)	219.2	231	240	206	207	205
Crude fat (g/kg)	55.6	61	42	65	64	43
Crude fiber (g/kg)	33.7	37	36	34	38	36
Crude ash (g/kg)	65	64	69	57	54	58
Starch brunt (g/kg)	355	344	330	369	380	385
Calcium (g/kg)	8.8	8.8	9.0	6.8	6.8	6.9
Phosphorus (g/kg)	5.5	5.2	5.6	4.4	4.1	4.6
Sodium (g/kg)	1.4	1.4	1.45	1.4	1.4	1.44
6-Phytase E4a1640 (ftu)	500	500	545	500	500	545
Digestible lysine (g/kg)	12.0	12.0	12.0	10.6	10.6	10.4
Digestible methionine (g/kg)	5.9	5.8	5.8	4.9	5.0	5.0
Digestible methionine +cysteine (g/kg)	8.8	8.8	8.9	7.7	7.7	7.8
Digestible threonine (g/kg)	7.7	7.7	7.2	6.8	6.8	6.2
Digestible tryptophan (g/kg)	2.35	2.44	2.64	2.23	2.17	2.21
Retainable P broiler (g/kg)	4.0	4.0	4.1	3.1	3.1	3.4

	Finisher (d27-35)		
	CON	**INC-F**	**D-F, D-S, L-S***
AMEn (kcal/kg)	3000	3000	2861
Dry matter (g/kg)	869	870	864
Crude protein (g/kg)	190	194	178
Crude fat (g/kg)	72	71	44
Crude fiber (g/kg)	34	37	35
Crude ash (g/kg)	53	51	47
Starch brunt (g/kg)	389	395	431
Calcium (g/kg)	6.2	6.2	4.3
Phosphorus (g/kg)	4.0	3.9	3.6
Sodium (g/kg)	1.2	1.2	1.3
6-Phytase E4a1640 (ftu)	500	500	545
Digestible lysine (g/kg)	9.7	9.7	9.5
Digestible methionine (g/kg)	4.4	4.4	4.5
Digestible methionine +cysteine (g/kg)	7.1	7.1	7.1
Digestible threonine (g/kg)	6.2	6.2	5.6
Digestible tryptophan (g/kg)	2.06	2.03	1.89
Retainable P broiler (g/kg)	2.8	3.0	2.6

Table S3 continued.

* Dried and live larvae not included in nutrient composition.

Supplementary Table S4. Average daily gain and final weight of focal broilers (n = 10/pen) receiving no larvae (CON), larvae meal and oil incorporated in the feed (INC-F), dried larvae in the feeder (D-F), dried larvae scattered through the pen (D-S), or live larvae scattered through the pen (L-S). Data are presented as pen means ± SEM. Significant treatment effects ($p < 0.05$) are indicated in bold, and within each row different superscript letters indicate significant ($p < 0.05$, Tukey’s HSD correction) differences between treatments.

Measure	CON	INC-F	D-F	D-S	L-S	Test-statistic and df	P-value
Average daily gain (g/d)							
d1-9	22.1 ± 0.3^{a}	22.7 ± 0.2^{ab}	23.5 ± 0.2^{b}	22.8 ± 0.4^{ab}	23.4 ± 0.2^{b}	$F_{(4,55)} = 6.21$	**<0.001**
d9-19	62.5 ± 0.6^{a}	62.5 ± 0.6^{ab}	66.9 ± 0.6^{d}	64.6 ± 0.5^{bc}	66.5 ± 0.7^{cd}	$F_{(4,55)} = 15.88$	**<0.001**
d19-27	100.7 ± 1.6^{a}	103.0 ± 1.3^{ab}	107.1 ± 1.9^{b}	106.7 ± 2.2^{b}	104.0 ± 1.4^{ab}	$F_{(4,55)} = 3.98$	**0.007**
d27-35	119.3 ± 1.5	120.9 ± 1.2	120.3 ± 1.7	121.2 ± 1.7	119.1 ± 2.6	$F_{(4,55)} = 0.31$	0.868
FW (g)	2628 ± 20^{a}	2670 ± 17^{ab}	2732 ± 27^{b}	2730 ± 20^{b}	2713 ± 22^{ab}	$F_{(4,55)} = 3.52$	**0.013**

FW = Final weight.

Supplementary Table S5. Periodic average daily dry matter intake of pellets or pellets and larvae combined, of broilers receiving no larvae (CON), larvae meal and oil incorporated in the feed (INC-F), dried larvae in the feeder (D-F), dried larvae scattered through the pen (D-S), or live larvae scattered through the pen (L-S). Data are on pen level and presented as means ± SEM. Significant treatment effects ($p < 0.05$) are indicated in bold, and within each row different letters indicate significant ($p < 0.05$, Tukey' s HSD correction) differences between treatments.

Measure	**CON**	**INC-F**	**D-F**	**D-S**	**L-S**	**Test-statistic and df**	**P-value**
Average daily dry matter intake of pellets (g/d)							
d1-9	20.8 ± 0.2^{ab}	20.8 ± 0.2^{ab}	20.4 ± 0.2^{b}	21.2 ± 0.3^{a}	18.2 ± 0.2^{c}	$F_{(4,44)} = 31.95$	**<0.001**
d9-19	69.8 ± 0.7^{a}	69.0 ± 0.3^{a}	67.5 ± 1.0^{a}	63.3 ± 0.6^{b}	63.5 ± 0.6^{b}	$F_{(4,44)} = 19.27$	**<0.001**
d19-27	127.3 ± 1.5^{a}	126.2 ± 0.9^{ad}	118.9 ± 1.8^{b}	118.6 ± 1.3^{b}	110.0 ± 1.5^{c}	$F_{(4,44)} = 29.79$	**<0.001**
d27-35	170.7 ± 1.2^{a}	168.4 ± 1.3^{a}	154.1 ± 1.0^{b}	153.9 ± 1.6^{b}	145.7 ± 2.4^{c}	$F_{(4,44)} = 61.51$	**<0.001**
Average daily dry matter intake of pellets & larvae (g/d)*							
d1-9	20.8 ± 0.2^{a}	20.8 ± 0.2^{a}	22.6 ± 0.2^{b}	23.5 ± 0.3^{b}	20.5 ± 0.2^{a}	$F_{(4,44)} = 41.77$	**<0.001**
d9-19	69.8 ± 0.7^{a}	69.0 ± 0.3^{a}	73.6 ± 1.0^{b}	69.4 ± 0.6^{a}	69.6 ± 0.6^{a}	$F_{(4,44)} = 7.29$	**<0.001**
d19-27	127.3 ± 1.5^{a}	126.2 ± 0.9^{a}	129.5 ± 1.8^{a}	129.3 ± 1.3^{a}	120.7 ± 1.5^{b}	$F_{(4,44)} = 7.87$	**<0.001**
d27-35	170.7 ± 1.2^{a}	168.4 ± 1.3^{a}	168.8 ± 1.0^{a}	168.8 ± 1.6^{a}	160.5 ± 2.3^{b}	$F_{(4,44)} = 8.58$	**<0.001**

* Estimated based on the assumption that all larvae are consumed.

Part 2: Pigs

Chapter 5

Live black soldier fly larvae (*Hermetia illucens*) provisioning is a promising environmental enrichment for pigs as indicated by feed- and enrichment-preference tests

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Abstract

Live black soldier fly larvae (BSFL) could function as environmental enrichment for pigs as they are edible, odorous, manipulable, and destructible, though the interest of pigs in live BSFL is unestablished. In this study, three trials were performed to assess this interest in order to verify the suitability of live BSFL as enrichment. Eight (Trial 1 & 2) and 16 (Trial 3) pairs of weaned pigs were included. In Trial 1, the relative motivation of pigs to consume live BSFL compared to corn, raisins and feed pellets was evaluated in no-choice and subsequent two-choice preference tests including all (combinations of) feed items. In Trial 2, the feed items in the no-choice and two-choice tests were provided in tubes that had to be rooted or pushed to access the feed items. In this trial BSFL, corn, feed pellets, and empty tubes were compared. In Trial 1 & 2 each day had two 15-min tests occurring six hours apart. In Trial 3 the long-term interest in live BSFL provided in the tubes for a 5-day period was compared to feed pellets provided in tubes, to jute sacks, and to rubber balls attached to chains. Tubes with live BSFL or pellets were refilled every morning. The relative interest was determined through intake of feed items and/or time spent interacting with the items, as measured by 1.5- or 1-min scan sampling. In Trial 3, the time spent on oral manipulation of pen mates was also scored through scan sampling. In all trials the interaction with and consumption of live BSFL was significantly higher compared to the other items, indicating a clear preference for live BSFL, and pigs remained engaged with BSFL as long as they were accessible. In Trial 3, interest in live BSFL decreased on day 5 compared to day 1, interest in jute sacks and feed pellets decreased on day 3 compared to day 1, and interest in rubber balls attached to chains was continuously low. Pigs also spent less time on oral manipulation of pen mates when having access to live BSFL, pellets, or jute sacks, compared to rubber balls attached to chains. Overall, pigs showed high and extended interest in live BSFL, confirming their suitability as edible environmental enrichment. Pigs were willing to work for access to live BSFL by rooting and pushing tubes, which can be employed when providing live BSFL as enrichment commercially.

Introduction

The consequences of incorporating insects into the diet of pigs have been of recent interest, yet their potential effectiveness as environmental enrichment has received little attention. Insects generally have a low feed conversion ratio and can turn biological waste into a high-quality feedstuff that can act as a substitute for increasingly limited feedstuffs such as soybean meal and fishmeal (Makkar et al., 2014; Veldkamp and Bosch, 2015). Black soldier fly larvae (*Hermetia illucens*, BSFL) are suitable to be included in pig diets due to their richness in fat, and in protein with an appropriate amino acid profile for pigs (Barragan-Fonseca et al., 2017; Biasato et al., 2019; Müller et al., 2017). BSFL meal (full-fat or partially or fully defatted) has been included in pig diets without interfering with normal growth, feed intake, nutrient digestibility or intestinal morphology (Biasato et al., 2019; Chia et al., 2019; Crosbie et al., 2020; Spranghers et al., 2018; Yu et al., 2020a), or in some cases with beneficial effects on growth and feed/gain ratio (Yu et al., 2020a, 2019a) and on caecal microbiota composition (Biasato et al., 2020a).

BSFL meal has a predominantly nutritional function. Meanwhile, providing BSFL as whole, live larvae could serve an additional function as environmental enrichment and, as such, benefit pig welfare, as was found in broilers (Ipema et al., 2020a, 2020b). Whole BSFL could be applied as environmental enrichment as pigs can not only consume, but also smell, manipulate, and destroy the larvae, aspects that have all been attributed to effective enrichment (Studnitz et al., 2007; Tarou and Bashaw, 2007; van de Weerd and Day, 2009). In barren environments without enrichment, pigs have limited possibilities to perform exploratory behaviour, which often causes them to redirect these behaviours towards pen fixtures and pen mates (Oostindjer et al., 2011b; van de Weerd et al., 2005b), resulting in stress and injuries (Beattie et al., 2000a; Zonderland et al., 2008). Providing environmental enrichment allows pigs to exhibit a broader range of species-specific behaviour in their home-pen (van de Weerd and Day, 2009). For example, enrichment materials such as straw and peat facilitate exploratory behaviours, while they in turn reduce the occurrence of maladaptive behaviours such as oral manipulation of pen mates (Beattie et al., 2000b; Oostindjer et al., 2011b; Vanheukelom et al., 2011). Previous studies have indicated that pigs are especially interested in edible enrichment objects (Durán et al., 2019; Machado et al., 2017; Nannoni et al., 2019) and that they prefer items with food feedback over items without (Holm et al., 2008). In our preceding study we found that scattering small amounts of whole live BSFL on the pen floor during 8 days after weaning increased the piglets' exploratory behaviour towards the floor from approximately 20% to 30% of the observation time, compared to piglets that did not receive larvae. In this study larvae provisioning also decreased pig- and pen-directed oral manipulative behaviours (Ipema et al., 2021a).

These results are promising, but the motivation of pigs to interact with and consume live BSFL has not yet been studied. Determining pigs' interest in live BSFL as compared to other feed and enrichment materials will verify potential advantages of using live BSFL as edible enrichment. As effective enrichment often stimulates extended interaction (Studnitz et al., 2007; van de Weerd et al., 2003), it is also relevant to determine if pigs are willing to work for access to live BSFL, as a way to prolong the interaction with BSFL. Finally, pigs provided with common commercial enrichment items such as a rubber ball attached to a chain often quickly loose interest in them (van de Weerd and Day, 2009), therefore determining the pigs' long-term interest in live BSFL compared to commercially applied items is relevant. To evaluate these aspects, the current study consisted of three separate trials aimed at investigating the motivation of pigs: to consume live BSFL (Trial 1), to work for access to live BSFL provided in an enrichment device consisting of tubes with holes (Trial 2), and to interact with live BSFL provided in the tubes over a 5-day period (Trial 3). In all trials the voluntary interaction with and consumption of live BSFL, as well as the preference for live BSFL over other items was determined. It was expected that pigs would be highly motivated to access and consume live BSFL, and that they would maintain interest in live BSFL longer than in other (commercially applied) enrichment materials.

Methods

Three trials were carried out at the animal experiment facilities of Wageningen University & Research (Wageningen, The Netherlands). The experimental protocols were approved by the Animal Care and Use committee of Wageningen University & Research under project license number AVD1040020187184, and they were in accordance with the Dutch animal experimentation law which complies with European Directive 2010/63/EU.

Animals, Housing and Management

Thirty-two female, tail docked pigs (Pietrain × TN70, age at start: 38.7 ± 0.8 days, weight at start: 9.7 ± 0.2 kg) were housed in pairs at the experimental facility. They were housed in pens of 2.85 × 1.20 m equipped with a feed trough (12 × 50 cm, with three feeding places) placed in a corner of the pen, a drinking nipple and rubber flooring covered by 5 cm of wood shavings. One chew object (a plastic cross or cylinder attached to a chain) was always present in the pens, except during the experimental days of Trial 3 (see below). Pigs had *ad libitum* access to water and to a commercial pelleted feed (Optima 3, AgruniekRijnvallei). In the experimental rooms the lights and a radio were on from 07:00 to 19:00 h, and between 19:00 and 07:00 h the radio was off, and the lights were dimmed. Room temperature was 23 °C at the start of the experiment, and gradually decreased to 21 °C at day 10, after which it remained constant. During all tests, both pigs were present in the pen, and pigs were marked weekly on their back with stock marker spray for individual identification during the behavioural observations.

Experimental design

Three trials were conducted to determine the relative interest of pigs in live BSFL provided in a feeder (Trial 1, or T1) or in an enrichment device, i.e., tubes with holes (Trial 2, or T2, and Trial 3, or T3) compared to other feed and enrichment items (**Table 1**). The enrichment device used in T2 and T3 consisted of two horizontally suspended, transparent tubes (32 cm long, 7.5 cm Ø) with four 1 cm Ø holes at the top, that had to be rotated at least 90° for the feed items to fall in the empty feeder below the tubes (**Figure 1A**). Pigs could rotate the tubes by pushing and rooting them. T1 and T2 were performed in parallel, with each trial including 8 pens, followed by T3 which included all 16 pens. The provisioning order of the tested materials was based on a Latin square design (**Supplementary Table S1**).

Table 1. Overview of included feed and enrichment items, types of tests, and measures taken during the tests of Trial 1, 2 & 3.

Trial	Feed/enrichment items	Tests	Measures
1	• BSFL • Corn • Raisins • Pellets	• No-choice tests (15 min/test) • Two-choice tests (15 min/test)	• Intake of feed items (% of item consumed) • Interaction with feed items (% of scans)
2	• Hanging tubes containing BSFL • Hanging tubes containing corn • Hanging tubes containing pellets • Empty hanging tubes	• No-choice tests (15 min/test) • Two-choice tests (15 min/test)	• Intake of feed items (% of item consumed) • Interaction with tubes and feed items (% of scans)
3	• Hanging tubes containing BSFL • Hanging tubes containing pellets • Hanging jute sacks • Hanging rubber balls on chains	• Long-term enrichment interaction test (5 days/test)	• Interaction with enrichment items and feed items (% of scans) • Oral manipulation of pen mates (% of scans)

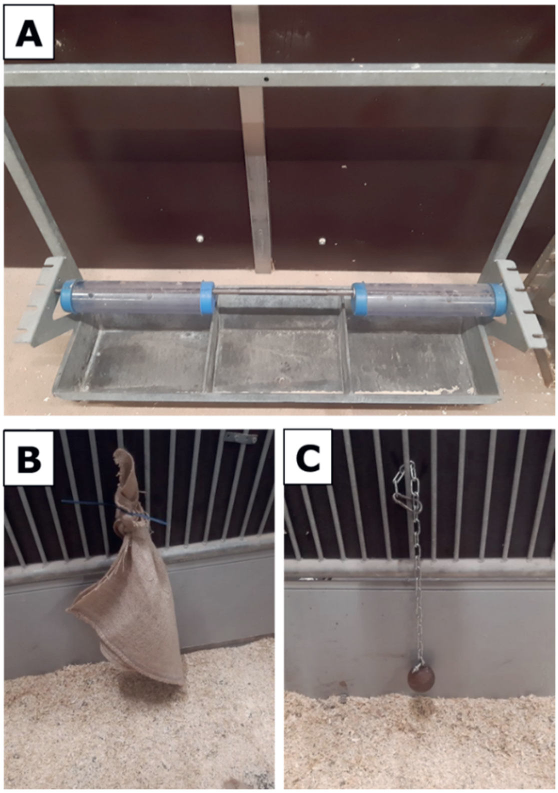

Figure 1. A) Enrichment device applied in Trial 2 & Trial 3, tubes can be filled with feed items and require turning to get the feed items out; B) jute sack as provided in Trial 3; C) rubber ball attached to a chain as provided in Trial 3.

In T1, the pigs' interest in live BSFL was compared to interest in their regular pelleted feed, canned corn, and raisins. In T2, the pigs' interest in live BSFL was compared to interest in their regular pelleted feed and canned corn, all provided in tubes, and to empty tubes. The comparison with their pelleted feed was included to determine the overall acceptance of live BSFL, while canned corn and raisins were included as these are known palatable feed items for pigs. Corn is attractive for pigs as demonstrated by its common use in feral pig traps (Karlin and Khan, 2020), and raisins are assumed to be attractive for pigs as they have previously been used as edible rewards in several studies (de Jonge et al., 2008; Reimert et al., 2014a). The nutritional composition of the feed items can be found in **Supplementary Table S2**, and the approximate dry matter levels are 36% for BSFL (based on chemical analysis), 88% for pellets (according to manufacturer), 32% for corn and 76% for raisins (de Jong, 2010). A pilot trial indicated that the tubes had a similar release rate in g of fresh material per rotation for live BSFL, corn and pellets. In T3, the pigs' long-term interest in live BSFL provided in tubes was compared to interest in pellets provided in tubes, two jute sacks hanging from the pen wall, and two rubber balls attached to chains hanging from the pen wall (**Figure 1**). Rubber balls attached to chains were included as these are commonly used commercially (Bracke et al., 2013), while jute sacks were included as they are highly interesting for pigs (Ursinus et al., 2014).

Live, 14-day old larvae were provided weekly (by Bestico B.V., Berkel en Rodenrijs, The Netherlands) and stored at 12 °C near the experimental rooms, together with the other feed items. From approximately 30 minutes before provisioning, the feed items were stored at room temperature and the canned corn was dried with paper towels. Behavioural observations were done by four observers using the program Observer 14.2 (Noldus Information Technology B.V., Wageningen, The Netherlands). Prior to the experiment the observers were trained, and inter-observer reliability was regarded to be "almost perfect" (Fleiss kappa > 0.8, Landis and Koch, 1977). After each test, the left-over feed items, including any feed that was spilled, were weighed to determine the feed intake.

Trial 1 (T1)

T1 consisted of a habituation period (week 1 & 2, 4 days/week), no-choice voluntary feed intake tests (week 3, 4 days) and two-choice feed preference tests (week 4 & 5, 6 days/week), in which the relative interest in live BSFL compared to feed pellets, corn, and raisins was determined (**Table 1**). The feed items were presented in a feeder (12 × 50 cm with three feeding places) placed near the pen wall during the test. During habituation, pigs had access to one feed item each day. In the first week of habituation, each day a feeder with 300 g of the feed item was placed in the pen between 09:30-15:30 h, and in the second week of habituation the feeder was placed in the pen for 15 min twice a day, at 10:00 and 16:00 h. Weighing back of left-over feed confirmed that all feed items were sampled in all pens during habituation. During all test days, pigs had access to one (no-choice test) or two (two-choice preference test) feed items for 15 min twice a day, starting at 10:00 and 16:00 h. In each test 300 g of each feed item was provided. In the two-choice preference tests two adjacent feeders each contained one feed item, and each combination of feed items was tested on one day in both weeks, with the location of the feed items balanced between weeks. During the tests, the behaviour of all piglets was scored by instantaneous scan sampling every 1.5 min, resulting in 10 scans/15-min test. For each scan, the pig was scored as either "Interacting", which included eating or manipulating the feed item and/or exploring the experimental feeder by sniffing, nosing, rooting, or chewing, or "Not interacting".

Trial 2 (T2)

T2 had a similar set-up as T1, except the feed items were provided in the enrichment device with tubes, requiring pigs to work for access to the feed items (**Figure 1A**). Instead of raisins, empty tubes were included in T2 (**Table 1**). For habituation and for the no-choice tests, two tubes, each containing 150 g of the feed item, hanging above a feeder were placed near the pen wall. For the two-choice preference tests, two such devices were placed adjacent to each other, and each device contained two tubes with the same feed item, or with no feed item in case of comparison to the empty tubes. During the first week of habituation, a handful of the feed item was always placed in the feeder beneath the tubes. All pens had sampled all feed items during habituation. Testing started daily at 09:00 and 15:00 h and lasted 15 min. Behaviour was observed as in T1, except that 1 min instantaneous scan sampling was applied in the two-choice preference test, resulting in 15 scans/15-min test. The behaviours scored were

"Interacting" which included eating or manipulating the feed, exploring the tubes or the feeder and turning the tubes, and "Not Interacting".

Trial 3 (T3)

In T3, pens were provided with the different enrichment items for five days a week during four consecutive weeks (**Table 1**). For the live BSFL and the feed pellets, every morning at 08:30 h 300 g was added to each tube, and any feed still in the tubes was taken out and weighed back to determine the daily intake. On day 1, 3 and 5, individual pig behaviours were scored through 1-min instantaneous scan sampling for 5 hours a day, starting at 08:30, 10:00, 11:30, 13:00 and 14:30 h. Two observers each observed eight pens in one room, switching rooms every hour. The scored behaviours were "Interacting with enrichment" which included exploring the enrichment, playing with the enrichment, and eating edible enrichment, "Manipulating pen mate" which included (belly) nosing and biting of the other pig, and "Other".

Statistical analysis

Intake of feed items and behavioural data were averaged per pen prior to analysis. For each feed item in each test the proportion of the provided feed that was consumed was used in the analysis. Behavioural data were grouped per 15-min test (T1 & T2) or per day (T3) and expressed as the proportion of scans in which the behaviour was registered. Data from the two-choice tests were analysed separately for each unique combination of feed or enrichment items. All data were analysed with the statistical software SAS 9.4 (SAS Institute Inc., Cary, NC, USA) with generalized linear mixed models (GLIMMIX in SAS) with a binomial distribution, logit link, and additional multiplicative over-dispersion parameter. Models included 15-min test (T1 & T2) or day (T3) as repeated effect with pen as subject using a compound symmetry covariance structure. Models for T1 and T2 included feed type and test order as fixed effects, though as the effect of test order was never significant this was left out of the final models. Models for T3 included enrichment item, day, and their interaction as fixed effects. P-values below 0.05 were considered statistically significant, and in case of the two-choice tests, this indicated a preference for one item over the other. Significant fixed effects identified by the models on data from the no-choice tests and the data from T3 were further analysed with post-hoc tests on pairwise differences in least square means with a Tukey's HSD correction. Data are presented as pen means ± SEM.

Results

Trial 1

Intake of feed items in the no-choice tests was influenced by feed type ($F_{(3,21)} = 29.3$, $p < 0.001$, **Figure 2**). Post-hoc tests indicated differences between the intake of all feed items, in the order of live BSFL > corn > raisins > pellets. The behavioural interaction with the feed items was also influenced by feed type ($F_{(3,21)} = 23.5$, $p < 0.001$), where pigs interacted more with live BSFL than with all other feed items, and no other differences occurred.

The intake of feed items during the two-choice tests differed for all feed type comparisons (BSFL vs. corn: $F_{(1,7)} = 37.4$, $p = 0.001$, BSFL vs. raisins: $F_{(1,7)} = 80.2$, $p < 0.001$, BSFL vs. pellets: $F_{(1,7)} = 59.4$, $p < 0.001$, corn vs. raisins: $F_{(1,7)} = 7.7$, $p = 0.028$, corn vs. pellets: $F_{(1,7)} = 12.1$, $p = 0.010$, raisins vs. pellets: $F_{(1,7)} = 5.7$, $p = 0.048$), with the order of preference being BSFL > corn > raisins > pellets (**Figure 2**). Considering behaviour, the interaction with live BSFL was higher than the interaction with all other items (BSFL vs. corn: $F_{(1,7)} = 103.3$, $p < 0.001$, BSFL vs. raisins: $F_{(1,7)} = 147.5$, $p < 0.001$, BSFL vs. pellets: $F_{(1,7)} = 190.6$, $p < 0.001$). Pigs also interacted more with raisins than with pellets when both were present ($F_{(1,7)} = 8.6$, $p = 0.022$), and there was a trend for pigs interacting more with corn than pellets when both were present ($F_{(1,7)} = 5.4$, $p = 0.053$). There was no difference in interaction when corn and raisins were present simultaneously ($F_{(1,7)} = 0.7$, $p = 0.446$).

Trial 2

In the no-choice tests, the feed item in the tubes influenced the intake of feed items ($F_{(2,14)} = 21.9$, $p < 0.001$), in the order of live BSFL > corn > pellets as indicated by post-hoc tests (**Figure 3**). The behavioural interaction with the tubes also depended on feed type ($F_{(3,21)} = 31.4$, $p < 0.001$). Post-hoc tests identified a difference between all items except between pellets and empty tubes, resulting in the preference order of live BSFL > corn > pellets and empty tubes.

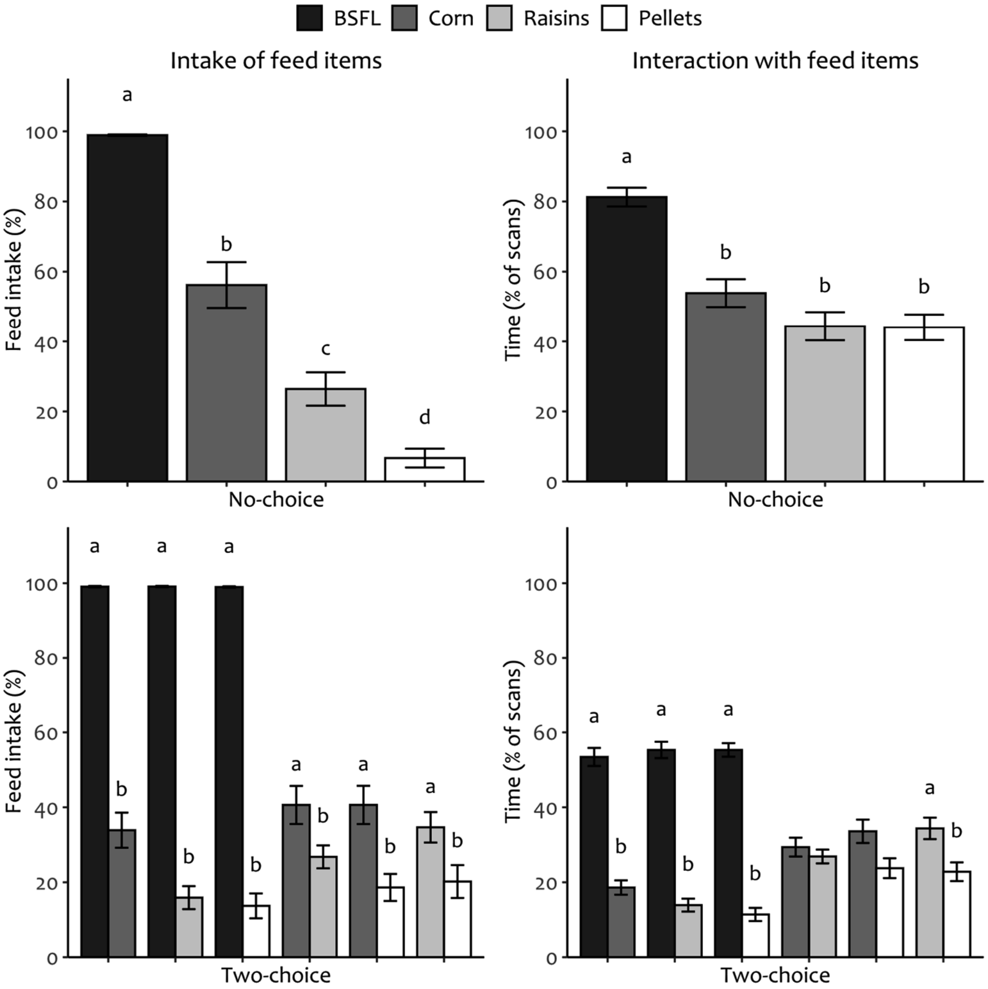

Figure 2. The percentual intake of each feed item and the time spent on interacting with each feed item provided in no-choice and two-choice tests in Trial 1. BSFL = Black soldier fly larvae, Pellets = the pigs' pelleted feed. For the no-choice tests all bars with different letters differ significantly ($p < 0.05$), for the two-choice tests bars within one feed combination with different letters differ significantly ($p < 0.05$). Data are presented as pen means ± SEM.

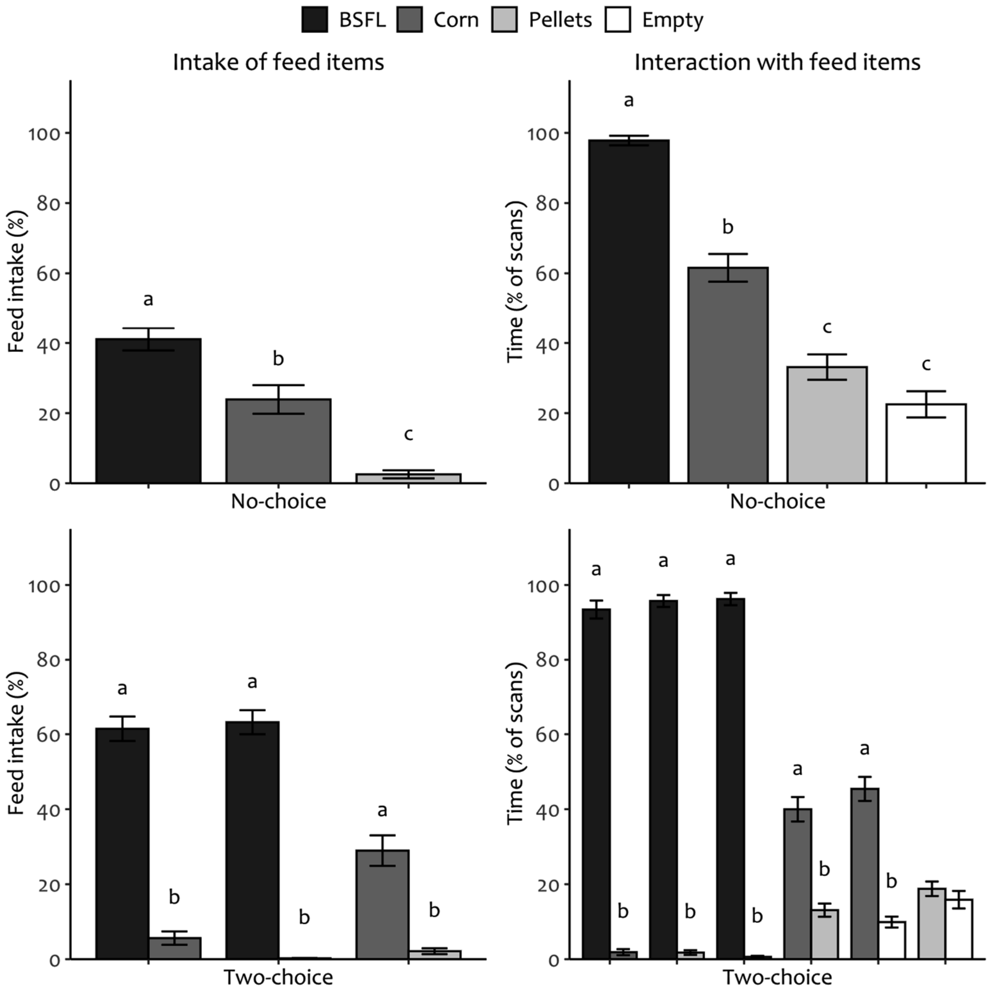

Figure 3. The percentual intake of each feed item and time spent on interacting with each feed item provided in the enrichment device in no-choice and two-choice tests in Trial 2. BSFL = Black soldier fly larvae, Pellets = the pigs' pelleted feed, Empty = empty tubes in the enrichment device. For the no-choice tests all bars with different letters differ significantly ($p < 0.05$), for the two-choice tests bars within one feed combination with different letters differ significantly ($p < 0.05$). Data are presented as pen means ± SEM.

For the two-choice preference tests, a feed item intake preference occurred for all combinations (BSFL vs. corn: $F_{(1,7)} = 89.4$, $p < 0.001$, BSFL vs. pellets: $F_{(1,7)} = 42.7$, $p < 0.001$, corn vs. pellets: $F_{(1,7)} = 27.3$, $p = 0.001$), with the preference order BSFL > corn > pellets (**Figure 3**). Pigs interacted more with tubes containing live BSFL than with tubes containing corn ($F_{(1,7)} = 95.3$, $p < 0.001$), pellets ($F_{(1,7)} = 139.4$, $p < 0.001$), or nothing ($F_{(1,7)} = 82.8$, $p < 0.001$). Furthermore, pigs interacted more with tubes containing corn than with tubes containing pellets ($F_{(1,7)} = 50.9$, $p < 0.001$) or nothing ($F_{(1,7)} = 84.0$, $p < 0.001$). The time spent on interacting did not differ when tubes with pellets and empty tubes were present simultaneously ($F_{(1,7)} = 0.9$, $p = 0.372$).

Trial 3

In T3, pigs consumed more live BSFL than feed pellets provided in tubes (BSFL: 97.3 ± 1.3%, pellets: 22.5 ± 3.4%, $F_{(1,15)} = 68.4$, $p < 0.001$), and consumption was not influenced by day ($F_{(4,55)} = 0.6$, $p = 0.643$) or the feed type by day interaction ($F_{(4,54)} = 1.9$, $p = 0.119$). A main effect of enrichment type ($F_{(3,45)} = 146.0$, $p < 0.001$), day ($F_{(2,30)} = 59.1$, $p < 0.001$), and the enrichment type by day interaction ($F_{(6,90)} = 10.9$, $p < 0.001$) was found on the time spent interacting with the enrichment. According to post-hoc tests on the feed type by day interaction, the daily preference based on the level of interaction was as follows: on day 1 BSFL & jute sacks > pellets > balls, on day 3 BSFL > jute sacks & pellets > balls, and on day 5 BSFL > jute sacks > pellets & balls (**Figure 4**). These post-hoc tests also indicated that the time spent interacting with rubber balls attached to chains did not differ throughout the week, while the time spent interacting with jute sacks and tubes containing pellets dropped on day 3 compared to day 1, after which it did not change. The time spent interacting with tubes containing live BSFL was lower on day 5 compared to day 1.

The time spent on oral manipulation of pen mates was influenced by enrichment type ($F_{(3,45)} = 13.5$, $p < 0.001$) and by day ($F_{(2,30)} = 12.7$, $p < 0.001$), but not by their interaction ($F_{(6,90)} = 1.48$, $p = 0.193$, **Figure 4**). Post-hoc tests indicated that oral manipulation of pen mates occurred more when the balls attached to chains were present (2.6 ± 0.2%) compared to when any of the other items were present (BSFL: 1.3 ± 0.1%, jute sacks: 1.6 ± 0. 2%, pellets: 1.8 ± 0.2%). On day 1 the time spent on manipulating pen mates (1.3 ± 0.1%) was lower than on day 3 (1.8 ± 0.2%) and day 5 (2.3 ± 0.2%).

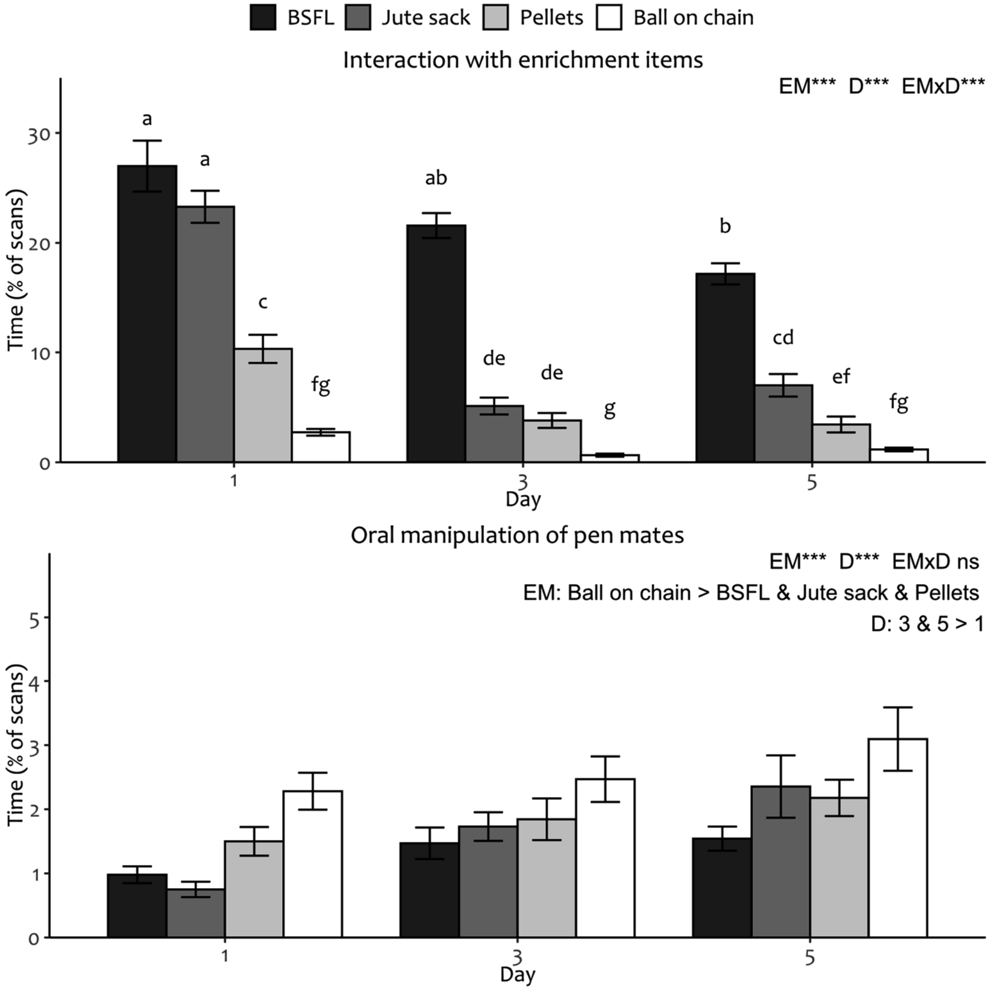

Figure 4. The time spent on interacting with the enrichment items and on oral manipulation of pen mates in the home pen in Trial 3. BSFL = Black soldier fly larvae, Pellets = the pigs' pelleted feed. Effects of enrichment material (EM), day (D) and their interaction (EMxD) are indicated as ns (not significant), or *** ($p < 0.001$). If EMxD is significant, different letters above bars indicate significant ($p < 0.05$) differences; if EMxD is not significant, the independent differences between enrichment materials and days are indicated. Data are presented as pen means ± SEM.

Discussion

All three trials demonstrate that pigs are highly motivated to interact with and consume live BSFL. Pigs showed relatively more interest in live BSFL compared to their regular feed pellets, canned corn, and raisins, either provided in a feeder (T1), or provided in an enrichment device that required pigs to push and root tubes to access the feed items (T2, only pellets and corn). In T3, pigs also interacted more with live BSFL provided in tubes compared to pellets provided in tubes, jute sacks, and balls attached to chains. The interaction of pigs with live BSFL only went down on day 5, as opposed to day 3 for jute sacks and pellets, while the interest in balls attached to chains was continuously low. Pigs spent more time on manipulating pen mates when they had access to balls attached to chains compared to all other enrichment materials.

In T1 and T2 the results of the no-choice and subsequent two-choice preference tests were in high agreement, all indicating a clear preference for live BSFL over all other feed items. In T1, the intake of live BSFL was always close to 100%, while the time spent interacting with BSFL was around 50-80%. This may be explained by our observation that the last larvae in the feeder were near impossible to reach, which could result in the pigs losing interest over time. Machado et al. (2017) similarly observed that enrichment that required high effort to obtain rewards stimulated exploration less than enrichment that required moderate effort to obtain rewards. The results from T2 also support this notion. Moderate effort was required to obtain the larvae provided in tubes, and here up to 40% of the provided BSFL were still available at the end of the 15-min tests, while pigs spent close to 100% of the scans interacting with the larvae. Both trials therefore confirm that pigs will keep interacting with live BSFL if the larvae can be obtained with moderate effort.

The reason that live BSFL are preferred over corn, raisins, and pellets is likely multi-factorial. It is possible that long-term nutritional consequences for the pigs may have played a role, although with the current set-up this is unlikely. Kyriazakis et al. (1991) claimed that pigs adapted their feed intake to the nutritional composition of the diet only when they had sufficient time to experience the nutritional consequences. Pigs may have experienced the nutritional consequences of consuming the feed items during the first week of habituation in T1 and T2, when each item was available throughout one day. However, during the tests the pigs only had access to individual feed items one day a week for two 15-minute periods, separated by six hours without access, limiting the opportunity to adjust intake based on nutritional consequences.

Alternatively, immediate signals such as taste, odour, and other sensory characteristics influence pigs' intake and interest in feed (Aubé et al., 2019a; Solà-Oriol et al., 2009; van de Weerd et al., 2003). In T1, pigs rarely differed in the time they spent interacting with corn, raisins, and pellets, yet their consumption, and consequently also rate of consumption, differed in the order corn > raisins > pellets. In previous studies, pigs did not prefer hard and fragile feed items (Solà-Oriol et al., 2007), and did prefer items with a higher moisture content that are easier to consume (Aubé et al., 2019a; Olsen et al., 2000). These studies support a faster consumption rate of moisture-laden corn as opposed to raisins and pellets, which are dryer, harder, and therefore more difficult to chew. However, pigs preferred consuming and interacting with live BSFL over corn while they have a similar moisture content, indicating that moisture content cannot solely explain this preference. Besides the high moisture content, live BSFL contain at least 3× more fat than the other feed items, and fat is known to improve palatability (Mizushige et al., 2007). BSFL are also richer in protein than corn and raisins, and in line with our results sows were previously found to prefer forages with a high protein level in short-term preference tests (Aubé et al., 2019a). While feed pellets contained marginally more protein than BSFL, these are plant-based proteins that can be less preferred by pigs than animal proteins (Solà-Oriol et al., 2011). Like animal protein, BSFL protein is high in glutamic acid (Makkar et al., 2014) which can produce umami flavours that pigs show a preference for (Figueroa et al., 2019).

Finally, items that are novel and/or only periodically available facilitate exploration more than familiar items (Docking et al., 2008; van de Weerd et al., 2003), explaining the overall preference for live BSFL, corn and raisins over the pellets that were available *ad libitum*. This is consistent with the observations that pigs in T2 were equally interested in tubes containing pellets and in empty tubes. Overall, while the preference for live BSFL over other feed items is obvious, further research is needed to determine the exact properties that prelude this preference.

In T1 and T2 pigs actively consumed live BSFL. Likewise, in T3 where enrichment materials were provided over a 5-day period pigs consumed almost all live BSFL daily, while the consumption of pellets provided in the enrichment device was much lower. The time spent on interacting with live BSFL throughout the day started off at the same level as for jute sacks, however the interaction with jute sacks decreased already on day 3 while the interest in live BSFL only decreased on day 5, and on this day, it was still higher than the interest in all other items. The prolonged high interest in live BSFL coincides with our previous study where newly weaned piglets remained highly interested in exploring live BSFL for the observed 8 days (Ipema et al., 2021a). The reduction in interaction on day 5 may be due to increased familiarity with the larvae, as was previously also observed for other enrichment items over a 5-day period (Docking et al., 2008; van de Weerd et al., 2003). An alternative explanation is that pigs became more efficient over time in retrieving the larvae from the enrichment device. This is supported by the observation that larvae intake did not differ over days, despite the lower time spent interacting with the tubes containing larvae on day 5. In support of this, in T2 the interaction with live BSFL provided in the enrichment device was similar for the no-choice and the subsequent two-choice preference tests, yet the intake of live BSFL in the latter test was substantially higher (60% vs. 40%), likely because pigs learned how to better retrieve the larvae out of the tubes. Increasing the difficulty of obtaining larvae and/or changing the manner of larvae provisioning over time may counteract this increased efficiency of pigs in procuring the larvae, and thereby prolong the time they spend interacting with the device that delivers larvae.

In T3, the occurrence of oral manipulation of pen mates was higher in pigs that had access to balls attached to chains than pigs that had access to any other enrichment material. Previous studies found that enrichment material that facilitates rooting, such as straw or peat, is of high interest and reduces manipulation behaviour directed towards other pigs (Oostindjer et al., 2011b; Telkänranta and Valros, 2020; Vanheukelom et al., 2011). Balls on chains provide relatively little opportunity for rooting behaviour (Day et al., 2002), which may explain the higher level of oral manipulation of pen mates when pigs only had access to this enrichment. Besides, from all studied items, the balls on chains were most similar to the chew items provided in the pigs' home pen during T1 and T2, and familiarity with such items likely decreased interest in them, reducing their effectiveness as enrichment (van de Weerd et al., 2003). In the current study the level of pig-directed oral manipulation was likely already low because pigs were housed in pairs with much space and bedding (Beattie et al., 2000b; Chaloupková et al., 2007; Fu et al., 2016), and because regular testing may have functioned as a form of environmental enrichment. Pigs housed under commercial conditions often exhibit more damaging behaviours (Oostindjer et al., 2011b; van de Weerd et al., 2005b), therefore live BSFL provisioning could have more pronounced effects under such conditions.

Overall, the observed reduction in maladaptive behaviour compared to balls attached to chains, combined with the greater interest in live BSFL compared to other feed and enrichment items observed in all trials highlights the potential advantage of using live BSFL as environmental enrichment. Accordingly, research into BSFL provisioning under commercial conditions is required, with focus on the consequences for pig behaviour, performance, and health. Commercially, providing other varieties of BSFL such as dried or popped larvae may be more practical and should thus be studied. Also, commercial BSFL provisioning methods should allow all pigs to access the larvae simultaneously, as pigs show synchronized behaviour (Docking et al., 2008) and limited access could result in aggression (Zwicker et al., 2013). Finally, regulated access is likely required to ensure prolonged interest and thereby increased beneficial effects of live BSFL provisioning (van de Weerd et al., 2003).

Conclusion

In conclusion, this study demonstrated that pigs are highly motivated to consume and interact with live BSFL, and they prefer live BSFL over a range of other feed and enrichment items. Over a 5-day period, the occurrence of pig-directed oral manipulation behaviour was reduced in pigs having access to live BSFL in an enrichment device compared to rubber balls attached to chains. These results emphasize the suitability of live BSFL to be used as edible environmental enrichment for pigs. Further research on live BSFL provisioning under commercial conditions is required to establish the total consequences for pigs.

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Supplementary data

Table S1. Experimental set-up of Trial 1-3. Test order was based on a Latin square design with tested items as treatment, test days as row and pen as column. BSFL = Black soldier fly larvae, Pellets = the pigs' pelleted feed, Empty = empty tubes.

Week	Day	**Trial 1**				**Trial 2[t]**			
		Pen 1-2	**Pen 3-4**	**Pen 5-6**	**Pen 7-8**	**Pen 9-10**	**Pen 11-12**	**Pen 13-14**	**Pen 15-16**
Habituation									
1-2	1	BSFL	Raisins	Pellets	Corn	BSFL	Empty	Pellets	Corn
	2	Corn	BSFL	Raisins	Pellets	Corn	BSFL	Empty	Pellets
	3	Pellets	Corn	BSFL	Raisins	Pellets	Corn	BSFL	Empty
	4	Raisins	Pellets	Corn	BSFL	Empty	Pellets	Corn	BSFL
No-choice tests									
3	1	BSFL	Raisins	Pellets	Corn	BSFL	Empty	Pellets	Corn
	2	Corn	BSFL	Raisins	Pellets	Corn	BSFL	Empty	Pellets
	3	Pellets	Corn	BSFL	Raisins	Pellets	Corn	BSFL	Empty
	4	Raisins	Pellets	Corn	BSFL	Empty	Pellets	Corn	BSFL
Two-choice tests									
4-5	1	BSFL vs. Corn	Raisins vs. Pellets	BSFL vs. Raisins	Pellets vs. Corn	BSFL vs. Corn	Empty vs. Pellets	BSFL vs. Empty	Pellets vs. Corn
	2	Raisins vs. Pellets	BSFL vs. Corn	Pellets vs. Corn	BSFL vs. Raisins	Empty vs. Pellets	BSFL vs. Corn	Pellets vs. Corn	BSFL vs. Empty
	3	BSFL vs. Pellets	Corn vs. Raisins	Corn vs. BSFL	Raisins vs. Pellets	BSFL vs. Pellets	Corn vs. Empty	Corn vs. BSFL	Empty vs. Pellets
	4	Corn vs. Raisins	BSFL vs. Pellets	Raisins vs. Pellets	Corn vs. BSFL	Corn vs. Empty	BSFL vs. Pellets	Empty vs. Pellets	Corn vs. BSFL
	5	BSFL vs. Raisins	Pellets vs. Corn	Pellets vs. BSFL	Corn vs. Raisins	BSFL vs. Empty	Pellets vs. Corn	Pellets vs. BSFL	Corn vs. Empty
	6	Pellets vs. Corn	BSFL vs. Raisins	Corn vs. Raisins	Pellets vs. BSFL	Pellets vs. Corn	BSFL vs. Empty	Corn vs. Empty	Pellets vs. BSFL

Week	Day	Trial 3[t]							
		Pen 1-2	**Pen 3-4**	**Pen 5-6**	**Pen 7-8**	**Pen 9-10**	**Pen 11-12**	**Pen 13-14**	**Pen 15-16**
Long-term enrichment interaction tests									
6	1-5	Ball on chain	Pellets	Jute sack	BSFL	Ball on chain	Pellets	Jute sack	BSFL
7	1-5	BSFL	Ball on chain	Pellets	Jute sack	BSFL	Ball on chain	Pellets	Jute sack
8	1-5	Jute sack	BSFL	Ball on chain	Pellets	Jute sack	BSFL	Ball on chain	Pellets
9	1-5	Pellets	Jute sack	BSFL	Ball on chain	Pellets	Jute sack	BSFL	Ball on chain

Table S1 continued.

[t] All feed items in the Trial are provided in tubes.

Table S2. Nutritional composition of feed items. BSFL = Black soldier fly larvae, Pellets = the pigs' pelleted feed, ME = metabolizable energy, NE = net energy.

	BSFL[1]	Canned corn[2]	Raisins[2]	Pellets[2]
ME (MJ/kg of Fresh)	6.8	4.6	13.8	
NE (MJ/kg of Fresh)				9.9
Crude protein (g/kg of Fresh)	147	29	31	166
Crude fat (g/kg of Fresh)	130	15	5	40
Sugar (g/kg of Fresh)	-	20	592	44
Starch (g/kg of Fresh)	-	170	-	407
Fibres (g/kg of Fresh)	-	30	37	115

[1] Based on analysis of chemical composition via standardized protocols (Protein 10005, Fat 10112).

[2] Provided by manufacturer.

Chapter 6

Providing live black soldier fly larvae (*Hermetia illucens*) improves welfare while maintaining performance of piglets post-weaning

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Abstract

During weaning, piglets experience concurrent social, physical, and nutritional stressors. Consequently, piglets often have poor feed intake and display increased oral manipulative behaviours post-weaning, indicative of compromised welfare. Black soldier fly larvae (BSFL) possess many attractive properties for pigs and could therefore function as effective edible enrichment, potentially alleviating weaning stress by facilitating exploration and promoting feed intake. In this study, pairs of piglets received a small amount of either live BSFL or wood shavings (8 pens/treatment) scattered throughout the pen twice a day for 11 days after weaning. Home-pen behaviour was scored by instantaneous scan sampling on day 2, 5 and 8, and behavioural responses to a novel environment and novel object were scored on day 10/11. Performance-related parameters were observed regularly. Larvae provisioning increased floor-directed exploration and decreased object-directed exploration, pig-directed oral manipulation, fighting and eating of pellets, and reduced neophobia towards a novel object. Pellet intake was significantly decreased by BSFL provisioning during day 4-11 post-weaning, although feed and net energy intake including BSFL never differed between treatments. BSFL provisioning did not influence piglet growth, feed efficiency, energy efficiency, and faecal consistency. To conclude, live BSFL provisioning positively affected post-weaning piglet behaviour while maintaining performance.

Introduction

On commercial pig farms, piglets are often weaned abruptly around 3-4 weeks of age. This process involves several stressors, including separation from the sow, transitioning from a mainly liquid (milk) diet to a completely solid diet, and usually relocation to a new environment and mixing with unfamiliar piglets. Piglets are generally neophobic towards unfamiliar feed items (Rozin and Vollmecke, 1986), and early and abrupt weaning often results in decreased feed consumption post-weaning. Decreased feed consumption can reduce growth and intestinal integrity, and increase disease susceptibility, reflected by the high incidence of post-weaning diarrhoea (as reviewed by Campbell et al., 2013; Dong and Pluske, 2007). Furthermore, abruptly weaned piglets frequently display increased levels of aggression and pig- and pen-directed oral manipulation behaviour, especially when housed in barren environments (Colson et al., 2006; Oostindjer et al., 2011b). Early and abrupt weaning can severely impact the welfare of piglets, and may have lifelong detrimental effects on pig performance and intestinal health (Cabrera et al., 2010; Moeser et al., 2017).

One approach to ease the weaning transition would be to implement systems that allow for more gradual weaning, such as group farrowing systems with a prolonged lactation period (van Nieuwamerongen et al., 2017), though this requires large-scale adjustments to current husbandry management. Alternatively, providing environmental enrichment post-weaning can benefit piglet welfare, as its presence may facilitate enrichment oriented exploration, reducing the motivation to explore and manipulate pen mates (Oostindjer et al., 2011b). Furthermore, previous studies have indicated that enriched-housed piglets often respond less neophobic towards novel objects (Beattie et al., 2000a) and novel feed items (Oostindjer et al., 2011a) than barren-housed piglets. Hemsworth et al. (1996) attributed reduced neophobia to regular confrontation with novel items which likely habituates pigs to novelty. Additionally, stressful situations were found to increase food neophobia in mammals such as sheep and humans (Burritt and Provenza, 1997; Pliner et al., 1995). For pigs, it was found that environmental enrichment can reduce stress (Munsterhjelm et al., 2010), and as such enrichment could lessen feed neophobia. Reduced feed neophobia may result in increased feed intake (Oostindjer et al., 2011a) and consequently better performance post-weaning. It has been shown, for example, that providing hanging ropes and tyres to piglets during several weeks after weaning improved piglet growth and reduced fearful responses towards a person (Rodarte et al., 2004), and providing hanging ropes and wood shavings also improved piglet growth and increased exploration behaviour (Oliveira et al., 2016). Other studies demonstrated that providing peat (for 5 weeks post-weaning) or providing increased space combined with straw, peat and branches (for 2 weeks post-weaning) reduced pig-directed oral manipulation behaviour, and increased exploration behaviour and growth (Oostindjer et al., 2011b, 2010; Vanheukelom et al., 2011).

Edible items (e.g., straw) are very effective as enrichment, as they provide a consumable reward and thereby reinforce exploration behaviour (van de Weerd et al., 2005a). Exploring and eating edible enrichment allows piglets to develop feeding-related behaviours such as chewing, adapting them to consuming solid feeds (Cox and Cooper, 2001), while simultaneously benefitting gut functioning (Pluske et al., 2007a). Furthermore, providing edible enrichment creates dietary diversity, which has been shown to increase feed intake before weaning and may have a similar effect after weaning (Middelkoop et al., 2019b, 2018). Durán et al. (2019) found that newly weaned piglets actively interact with and consume whey-based edible enrichment. Also, providing straw post-weaning increased exploration behaviour and reduced pig- and pen-directed oral manipulation (Bolhuis et al., 2005; Kelly et al., 2000). These studies highlight the potential of using edible enrichment post-weaning to ease the weaning transition, though this has not been widely studied.

Living insect larvae, which are a natural feed source of wild boar (Ballari and Barrios-García, 2014), have the potential to function as effective edible enrichment. One species suitable for consumption by commercial pigs is the black soldier fly. Black soldier fly larvae (BSFL) are rich in fat, calcium, and protein with an appropriate amino acid profile for pigs (Barragan-Fonseca et al., 2017; Makkar et al., 2014; Spranghers et al., 2017). Their palatability and high moisture content (55-65%, Makkar et al., 2014; Spranghers et al., 2017; Veldkamp and Bosch, 2015) might stimulate intake by newly weaned piglets that are accustomed to a liquid milk diet, and their consistency could allow larvae to function as a transitory feed between milk and concentrate. BSFL are essentially free of carbohydrates (Makkar et al., 2014), therefore digestion is not limited by low secretion of starch-degrading enzymes in recently weaned piglets. In addition, BSFL possess many characteristics of effective enrichments. The larvae are not only edible, but also odorous, destructible, and manipulable, and these properties are highly interesting for pigs and facilitate exploration behaviour (Studnitz et al., 2007; van de Weerd et al., 2003). The ability of live larvae to crawl to different places could decrease the predictability of interacting with the larvae, and decreased predictability is known to prolong interest in enrichment (van de Weerd and Ison, 2019). Moreover, piglets seem to prefer interacting with larger sized feed items. Previous studies found that piglets prefer interacting with 5-mm diameter feed pellets over smaller 1.8-mm diameter pellets (Edge et al., 2005), and they prefer interacting with 12-mm diameter pellets over 2-mm diameter pellets (van den

Brand et al., 2014). BSFL can grow up to 6 mm wide and up to 15-27 mm long (Jucker et al., 2017; Makkar et al., 2014; Tinder et al., 2017), and are thus substantially larger than most commonly provided pelleted feeds. Like other enrichment items, the presence of larvae might habituate pigs to novelty and/or have stress-reducing effects on pigs, potentially reducing neophobia.

Altogether, the properties of live BSFL suggest that they could function as effective edible enrichment post-weaning. This study aims to explore this potential by determining the effect of live BSFL provisioning post-weaning on piglet behaviour, fearfulness and neophobia, growth, feed and energy intake, feed and energy efficiency, and faecal consistency. It is expected that live BSFL provisioning will promote behaviours that are indicative of good welfare, e.g., exploration and play behaviours, and reduce aggression and pig- and pen-directed oral manipulation behaviour. Furthermore, providing live BSFL is expected to reduce neophobia and improve the performance and faecal consistency of post-weaning piglets.

Methods

The experimental protocol was approved by the Animal Care and Use committee of Wageningen University & Research under project licence number AVD1040020187184. The protocol was in accordance with the Dutch animal experimentation law and complies with European Directive 2010/63/EU. The experiment was carried out at the animal experiment facilities of Wageningen University & Research (Wageningen, The Netherlands). The ARRIVE guidelines for reporting animal experiments were taken into account in this study (du Sert et al., 2020).

Animals, Housing and Management

A total of 32 female, tail docked piglets (Pietrain × TN70) from 15 litters (range parity sows: 1-5) were weaned at 24.8 ± 0.8 days of age (day 0) and transported to the experimental facility. Pairs of unfamiliar piglets were selected based on body weight, to achieve a similar average weight (7.4 ± 0.2 kg) per pen. Pens were distributed over two rooms, balanced for treatment. Pairs of pigs were housed in pens of 2.85 × 1.20 m equipped with a feed trough (12 × 50 cm with three feeding places), one drinking nipple and one rubber ball on a chain. Feed and water were provided *ad libitum*. Flooring of all pens was a rubber mat covered by a 5 cm layer of wood shavings. Pigs were fed the creep feed they had also received during the suckling period (*Speenkruimel Fit*, ABZ Diervoeding) until day 2 after weaning. This feed was then mixed with the weaner feed (*Speen Havic Top*, AgruniekRijnvallei) for one day, after which only the weaner feed was provided. As feed consumption was initially very low, in all pens a spoonful of sugar was added when the weaner diet was first provided to ease the feed transition. In the experimental rooms a radio and lights were on from 07:00 to 19:00 h, and between 19:00 and 07:00 h the lights were dimmed, and the radio was off. Room temperature was 27 °C on arrival and was gradually decreased to 24 °C on day 11 post-weaning (the end of the experiment).

Experimental design

Pens were assigned to either the control (CON) or the black soldier fly larvae (LAR) treatment (8 pens/treatment). Piglets were provided with wood shavings (CON) or live BSFL (LAR) twice a day, at 09:00 and 13:00 h, from day 1 after weaning, by scattering the item in the pen on the wood shavings bedding. Wood shavings were provided to the CON treatment to create a similar disturbance in all pens. LAR pens received either 75 grams (day 1-4) or 150 grams (day 5-11) of live larvae twice a day, and CON pens always received a similar volume of wood shavings (approximately 100 and 200 ml, respectively). Live, 14-day-old BSFL were supplied weekly (by Bestico B.V., Berkel en Rodenrijs, The Netherlands) and stored at 12 °C. From approximately 30 minutes before provisioning, larvae were stored at room temperature to increase larvae activity.

Measurements

Home-pen behaviour

At arrival and on day 7 of the experiment, each piglet was marked with a colour stripe (stock marker spray) on their back for individual identification. The behaviour of all pigs was observed by 1-min interval instantaneous scan sampling on day 2, 5 and 8 post-weaning according to the ethogram in **Table 1**. Each of these three days had seven observation periods, starting at 07:15, 09:00, 10:15, 11:30, 13:00, 14:15 and 17:00 h. The periods starting at 09:00, 10:15, 13:00 and 14:15 h were observed live, where two observers observed 8 experimental pens (4 CON and 4

LAR) in one room each, switching pens every hour. Before observing, the observers were trained, and inter-observer reliability was regarded "almost perfect" (Cohen's kappa > 0.8, Landis and Koch, 1977). The remaining observation periods were observed afterwards from video by one person (one of the two observers). All observations were done using the program Observer 14.2 (Noldus Information Technology B.V., Wageningen, The Netherlands).

Table 1. Ethogram of behaviours observed in the home pen.

Behaviour	Description
Pig-directed oral manipulation	Nosing, rooting, sucking, nibbling, or chewing body of pen mate, or belly nosing (rubbing belly of a pen mate with up and down snout movements).
Fighting	Pushing, pressing, ramming, head knocking, nudging, or lifting pen mate, can include aggressive biting.
Social play	Group wise running, jumping, rolling, and/or turning in the pen.
Non-social play	Running, jumping, or turning in the pen individually, and/or shaking head while holding toy/bedding, throwing bedding in the air.
Exploring floor	Sniffing, touching, rooting, or chewing the floor or bedding material, potentially including larvae.
Exploring object	Sniffing, touching, rooting, or chewing walls, feeders, or toys (above floor level).
Eating feed	Having head in feeder while eating or manipulating feed.
Inactive	Sitting or lying, without performing any other behaviour.
Other	Performing any other behaviour.

Novel Environment and Novel Object Test

To determine pig responses to novelty, all experimental pigs, except one pig from the LAR treatment which was lame on the day of testing, were subjected to a Novel Environment Test and subsequent Novel Object Test. Tests were performed on day 10 and 11 post-weaning between 09:00 and 15:00 h. Half of the pigs were tested on one day and the other half on the other day, with the order being balanced for treatment and room. Prior to testing, pigs were individually caught, placed in a closed cart, and transported to an experimental room nearby. Here, the pig was placed in a start box alongside the experimental area. The door of the start box was opened within 10 seconds, giving the pig access to an unfamiliar area of 5.3 × 5.3 m with a rubber floor, surrounded by 1 m high hardwood walls. The Novel Environment Test started when the pig had entered the area and the door was closed behind it. After 5 minutes, the Novel Object Test was started by lowering an unfamiliar object (a metal bucket) from the ceiling in the centre of the area. The bucket was placed on the floor, which resulted in a noise. The rope attached to the bucket was held loosely. After five more minutes the test ended, and the pig was transported back to the home pen. During the tests, two observers were in the same room but out of sight of the pig, viewing the pig on a screen. One observer scored the behavioural and postural states, and the other observer scored behavioural events, according to the ethogram in **Table 2**. Scoring was done on a tablet with the program Observer 14.2 (Noldus Information Technology B.V., Wageningen, The Netherlands). Between tested pigs, faeces and urine were removed and the experimental area was cleaned with water and soap and dried with towels.

Performance

Piglets were individually weighed directly after arrival at the experimental facilities, and on day 1, 4, 7 and 11 post-weaning. Pellet intake per pen was determined by weighing leftovers on day 1, 2, 4, 7 and 11. Regular observations of the bedding indicated that live larvae were almost absent within a few hours after provisioning, therefore complete consumption of the larvae was assumed. Prior to the experiment, three samples of BSFL were analysed for their dry matter (DM), crude protein, crude fat, calcium, and phosphorus content, and based on this the net energy content (MJ NE/kg DM) of the larvae was calculated (**Supplementary Table S1**). The net energy content of the pelleted feed (MJ NE/kg DM) was calculated using the energy values provided by the feed manufacturers. The DM intake per pen excluding and including larvae were calculated and analysed separately and based on the total DM intake including larvae the net energy intake (MJ NE), feed efficiency (weight gain (g)/dry matter intake (g)), and energy efficiency (weight gain (g)/ net energy intake (MJ NE)) were calculated to account for the large difference in the net energy content between pellets and BSFL.

Table 2. Ethogram of behaviours observed in the Novel Environment Test and Novel Object Test.

Item	Description
Exclusive behavioural states	
Exploring environment	Sniffing, nosing, rooting, chewing, or licking the floor or wall.
Exploring object	Sniffing, nosing, rooting, chewing, or licking the object.
Drawing back	Quickly moving a few steps away from the object after being oriented towards the object.
Other	Performing any other behaviour.
Exclusive postural and locomotive states	
Moving	Walking or running.
Freezing	Standing motionless on four legs with head fixed (up or down) and ears upright.
Standing	Standing motionless on four legs, not alert.
Sitting	Sitting or kneeling on the floor.
Lying	Lying with side or belly touching the floor.
Behavioural events	
Defecating	Excreting faeces.
Urinating	Excreting urine.
High-pitched vocalizations	Screams, squeals, or grunt-squeals.
Low-pitched vocalizations	Short or long grunts.

Faecal consistency and dry matter content

Every day at 08:30 h the faecal consistency of individual pigs was scored by two observers. Faecal consistency scores as visible around the anus of the pig at that time were based on the definition by Pedersen and Toft (2011). Score 1 represents firm faeces, score 2 soft but shaped faeces, score 3 loose faeces and score 4 water thin faeces. On day 4 and 7 faecal samples were collected during the weighing procedure, either by allowing the piglet to defecate by itself, or by stimulating defecation through gentle anal manipulation with a cotton swab for at most 10 seconds. The faecal samples were weighed before and after drying at 70 C ° for 24 hours and based on this the dry matter percentage was calculated.

Statistical analyses

Data processing

The feeder in one CON pen had fallen over on the last experimental day and this data was excluded from analysis. Home-pen behaviours were summed per piglet per day and expressed as the percentage of observed scans. To limit the number of variables, a factor analysis was conducted on data from the Novel Environment Test and the Novel Object Test (e.g., Reimert et al., 2014). Behavioural states and postures were expressed as a percentage of time, and behavioural events were expressed as absolute frequencies. During the Novel Environment and Novel Object Test the behaviours “drawing back”, “defecating”, and “urinating”, and the postures “sitting” and “lying” were very rare, and they were not included in the factor analysis. The behaviours “high-pitched vocalizations” and “low-pitched vocalizations” were combined into “vocalizations” for the factor analyses. The latency (s) to explore the novel object was calculated and included in the factor analysis. Prior to analysis, a Pearson’s correlation test performed on all variables showed that the variable “standing” was strongly negatively correlated to “moving” in the Novel Object Test ($r = -0.80$), therefore “standing” was excluded from the factor analysis. For the Novel Environment test, the variables “freezing” and “moving” each contained one outlier (based on the Grubb’s test), and these outliers were excluded from analysis. The variables “freezing” (for the Novel Environment Test and the Novel Object Test) and “exploring object” (for the Novel Object Test) were arcsine square root transformed, and “latency to explore object” was logarithmically transformed for normalization.

Data analysis

All data were analysed using the statistical software SAS 9.4 (SAS Institute Inc., Cary, NC, USA). General linear (mixed) model residuals were checked for normality.

The proportions of time spent on each observed behaviour in the home pen were analysed in generalised linear mixed models (GLIMMIX in SAS) with a binomial distribution, logit link function, and additional multiplicative over-dispersion parameter. Treatment, room, and day were included as fixed effects. The interaction between day and treatment was removed from the final models as this effect was never significant. Furthermore, the models included a random effect of pen nested within treatment and room, and a repeated effect of test day with pig as subject, using a homogenous first-order autoregressive covariance structure.

The variables obtained from the Novel Environment Test and the Novel Object Test were put in a factor analysis with orthogonal varimax rotation, and factors with an eigenvalue above one were retained. This resulted in one factor for the Novel Environment Test and one factor for the Novel Object Test (**Table 3**), explaining 63% and 72% of the variation, respectively. The scores of each pig for each factor were analysed with a linear mixed model (MIXED in SAS) including a fixed effect of treatment, room and test day and a random effect of pen nested in treatment and room.

Table 3. Loadings of the factors with an eigenvalue above one that were extracted by factor analysis with orthogonal varimax rotation on the behaviours and postures scored during the Novel Environment Test (NET) and the Novel Object Test (NOT). High loadings (loadings ≤ −0.5 or ≥ 0.5) are indicated in bold.

Variable	**NET factor 1 “Fearful”**	**NOT factor 1 “Neophobic”**
Exploring environment (% of time)	**-0.57**	-0.34
Moving (% of time)	**-0.93**	**-0.67**
Standing (% of time)	**0.76**	
Freezing (% of time)	**0.60**	**0.81**
Vocalizations (frequency)	**0.56**	-0.26
Exploring object (% of time)		**-0.69**
Latency to explore object (s)		**0.76**
Eigenvalues	2.45	2.35
% of variance explained	63%	72%

Body weight and average daily gain were analysed in a linear mixed model including a fixed effect of treatment and room, a random effect of pen nested in treatment and room, and weight directly after weaning as a covariate. Dry matter intake excluding and including BSFL, net energy intake, feed efficiency, and energy efficiency were analysed for treatment effects on pen level in general linear models. The proportion of faecal dry matter was analysed per day in a similar model as the home-pen behaviours but without a repeated effect.

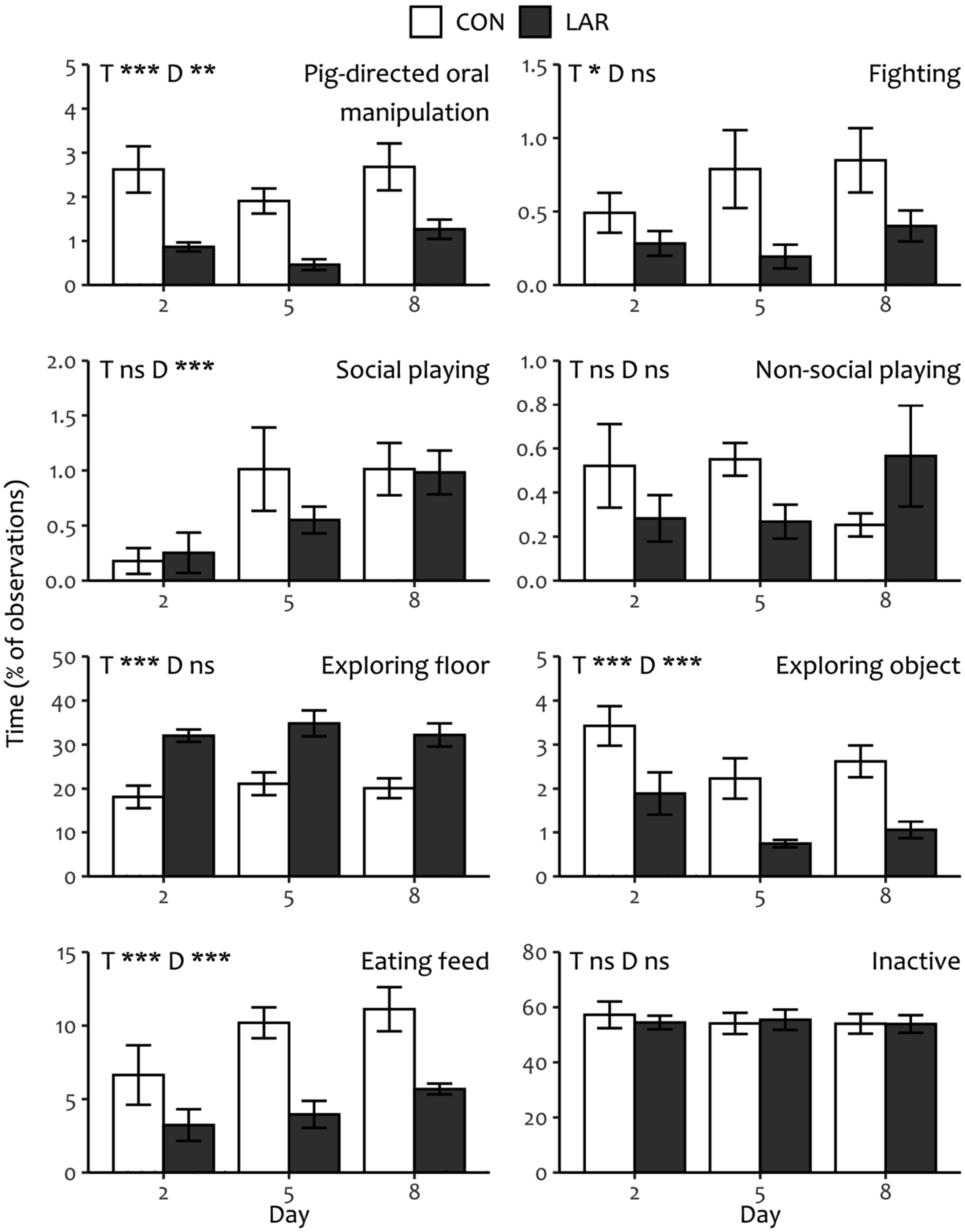
P-values below 0.05 were considered statistically significant. Significant fixed effects were further analysed using differences in least square means with a Tukey's HSD correction. Data are presented as pen means ± SEM unless stated otherwise.

Results

Home-pen behaviour

Averaged over the three time points, LAR piglets spent less time on pig-directed oral manipulation ($F_{(1,13)} = 24.27$, $p < 0.001$), fighting ($F_{(1,13)} = 6.92$, $p = 0.02$), exploring objects ($F_{(1,13)} = 17.89$, $p < 0.001$) and eating feed ($F_{(1,13)} = 23.89$, $p < 0.001$), and they spent more time on exploring the floor ($F_{(1,13)} = 29.14$, $p < 0.001$) than the CON piglets. The time spent inactive ($F_{(1,13)} = 0.02$, $p = 0.89$), social playing ($F_{(1,13)} = 0.39$, $p = 0.54$), and non-social playing ($F_{(1,13)} = 0.09$, $p = 0.77$) did not differ between treatments. Considering the effect of day, the time spent on pig-direct oral manipulation varied over time ($F_{(1,78)} = 5.13$, $p = 0.01$), with the lowest levels occurring on day 5 as compared to day 2 and 8 after weaning. The time spent on exploring objects decreased over time ($F_{(1,78)} = 9.33$, $p < 0.001$) and the time spent on social play ($F_{(1,78)} = 18.18$, $p < 0.001$) and eating feed ($F_{(1,78)} = 7.93$, $p < 0.001$) increased over time. The time spent fighting ($F_{(1,78)} = 2.43$, $p = 0.09$), non-social playing ($F_{(1,78)} = 0.00$, $p = 1.00$), exploring the floor ($F_{(1,78)} = 1.90$, $p = 0.16$) and being inactive ($F_{(1,78)} = 0.45$, $p = 0.64$) were not influenced by day (**Figure 1**).

Figure 1 (next page). Behaviours observed in the home pen. Behavioural time budgets expressed as pen means ± SEM of pigs receiving wood shavings (CON) or black soldier fly larvae (LAR) twice a day. Effects of Treatment (T) and Day (D) are indicated as ***p < 0.001, **p < 0.01, *p < 0.05 and ns if p > 0.05. The Treatment x Day interaction was never significant and is therefore not shown.

CON
LAR
Time (% of observations)
T *** D **
Pig-directed oral manipulation
T * D ns
Fighting
T ns D ***
Social playing
T ns D ns
Non-social playing
T *** D ns
Exploring floor
T *** D ***
Exploring object
T *** D ***
Eating feed
T ns D ns
Inactive
2
5
8
Day

Novel Environment and Novel Object Test

The one retained factor of the Novel Environment Test had high positive loadings for standing, freezing and vocalizations, and high negative loadings for exploring the environment and moving, therefore this factor will be designated as "fearful" (**Table 3**). Treatment did not influence the pigs' scores for this factor (CON: 0.02 ± 0.20, LAR: 0.03 ± 0.28, $F_{(1,13)} = 0.02$, $p = 0.90$).

The one retained factor of the Novel Object Test had high positive loadings for freezing and the latency to explore the object, and high negative loadings on time spent moving and exploring the object, and this factor will be designated as "neophobic" (**Table 3**). Here, treatment influenced piglet scores, where CON piglets scored higher on the "neophobic" factor than LAR piglets (CON: 0.43 ± 0.27, LAR: -0.39 ± 0.25, $F_{(1,13)} = 5.30$, $p = 0.04$).

Performance

Performance parameters measured over the experimental period are shown in **Table 4**. Treatment did not affect average daily gain ($F_{(1,13)} = 0.07$, $p = 0.79$) and final weight ($F_{(1,13)} = 0.07$, $p = 0.79$) of the piglets, and it also did not influence their feed efficiency (based on dry matter intake including BSFL, $F_{(1,12)} = 2.64$, $p = 0.13$) and energy efficiency (based on dry matter intake including BSFL, $F_{(1,12)} = 0.71$, $p = 0.42$). Dry matter intake excluding BSFL over the whole experimental period was lower in LAR piglets than in CON piglets ($F_{(1,12)} = 6.35$, $p = 0.03$). Detailed analysis of the separate periods for which feed intake was determined showed that dry matter intake excluding BSFL was lower in LAR piglets than in CON piglets during day 4-7 ($F_{(1,13)} = 5.57$, $p = 0.03$) and during day 7-11 ($F_{(1,12)} = 10.18$, $p = 0.01$, **Supplementary Table S2**). Treatment did not influence the dry matter intake including BSFL ($F_{(1,12)} = 0.06$, $p = 0.82$) or the total energy intake including BSFL ($F_{(1,12)} = 2.31$, $p = 0.15$).

Table 4. Average daily gain, body weight, dry matter intake (excluding and including black soldier fly larvae (BSFL)), energy intake (including BSFL), feed efficiency, and energy efficiency of pigs receiving wood shavings (CON) or black soldier fly larvae (LAR) twice a day. Data are expressed as means ± SEM. Significant p-values are presented in bold.

Measure	**CON**	**LAR**	**P**
Average daily gain (g/pig/day)	208 ± 17	214 ± 14	0.79
Body weight (g)			
Day 0	7325 ± 47	7390 ± 64	0.79
Day 11	9611 ± 173	9748 ± 189	0.79
Dry matter intake excl. BSFL (g/pig/day)	215 ± 14	168 ± 11	**0.03**
Dry matter intake incl. BSFL (g/pig/day)	215 ± 14	211 ± 11	0.82
Energy intake incl. BSFL (MJ NE/pig/day)	4.7 ± 0.3	5.3 ± 0.2	0.15
Feed efficiency (body weight gain (g/pig) / dry matter intake incl. BSFL (g/pig))	0.92 ± 0.04	1.01 ± 0.04	0.13
Energy efficiency (body weight gain (g/pig) / energy intake incl. BSFL (MJ NE/pig))	84.2 ± 3.5	79.8 ± 3.1	0.42

Faecal consistency and dry matter content

Faecal consistency scores of 3 and 4 were rare (< 2%), and as only these scores indicate diarrhoea, the faecal consistency scores were not further analysed. The faecal dry matter content on day 4 and 7 was not influenced by treatment (Day 4, CON: 31.6 ± 1.7%, LAR: 28.7 ± 1.4%, $F_{(1,13)} = 1.14$, $p = 0.30$, Day 7, CON: 28.6 ± 3.4%, LAR: 27.7 ± 1.7%, $F_{(1,13)} = 0.02$, $p = 0.89$).

Discussion

In this study, we investigated the potential of using live black soldier fly larvae (BSFL) as edible enrichment for piglets post-weaning. Providing a small amount of live BSFL twice a day increased floor-directed exploration, while it decreased object-directed exploration, pig-directed oral manipulation, fighting, and time spent eating pellets. In addition, a factor analysis suggests that LAR piglets were less neophobic, i.e., fearful of a novel object, than CON piglets when this object was introduced to them in an unfamiliar environment. Daily consumption of a small amount of live BSFL reduced pellet intake, but total feed and net energy intake (including BSFL), average daily gain, feed efficiency, energy efficiency, and faecal consistency were not affected by live BSFL provisioning.

In line with our expectations, live BSFL provisioning redirected exploration and manipulation behaviours away from objects and conspecifics and towards the floor where the larvae were provided, while simultaneously decreasing aggression. These effects occurred over the whole day despite the larvae being only transiently present each day and indicate that temporary access to live BSFL can benefit piglet welfare. In the ethogram applied in the current study, pig-directed oral manipulation included nosing of pen mates, which does not necessarily negatively impact piglet welfare. However, under barren (i.e., without bedding and with limited space) commercial conditions, pig-directed oral manipulation often accumulates into damaging behaviours that are harmful to pigs, such as tail biting (Oostindjer et al., 2011b; Vanheukelom et al., 2011; Zupan et al., 2012). Additionally, post-weaning manipulation behaviours and aggression tend to be more common in barren commercial conditions compared to our experimental setting (Day et al., 2008; Vanheukelom et al., 2011; Weary et al., 2008). Therefore, the observed welfare benefits associated with live BSFL provisioning are likely amplified in commercial pig husbandry conditions, and this merits investigation in future studies.

As live BSFL possess many features interesting for pigs (Studnitz et al., 2007; van de Weerd et al., 2003), they likely provided ample exploration opportunities to satisfy the piglet's exploratory needs. By promoting exploration, the motivation to perform other behaviours such as oral manipulation behaviours directed at pen mates may be decreased. Moreover, providing enrichment can have stress-reducing effects on pigs (Munsterhjelm et al., 2010), and as such, the presence of larvae may have attenuated the piglet's weaning stress. This could have shifted their behavioural repertoire towards more favourable behaviours and reduced aggressive behaviours. In line with our results, increased exploration and reduced harmful social behaviour were also found in studies using peat, straw, or a complex environment with increased space and a multitude of enrichment materials (Bolhuis et al., 2005; Oostindjer et al., 2011b; Vanheukelom et al., 2011). However, while these studies also observed increased play behaviour in enriched pigs, live BSFL provisioning did not affect social and non-social play. Possibly, the elevated level of exploratory behaviour directed towards the BSFL reduced the motivation to perform other behaviours including (social) play. Also, in the current study piglets were housed in relatively small groups with 2 piglets per pen as compared to 4-11 piglets per pen in the previous studies, and facilitation of social play was therefore relatively low. On the other hand, all CON and LAR piglets in the current study had access to bedding, which can already promote play as compared with barren environments (Tuyttens, 2005). The influence of the experimental conditions may have outweighed any additional effects of live BSFL provisioning on play behaviour, and differential effects on play behaviour may be found under commercial conditions.

Besides studying home-pen behaviour, behavioural responses to novel situations were determined and analysed with a factor analysis. It can be speculated that piglets with reduced neophobic responses can better cope with novel situations such as changes to their environment. The factor resulting from the analysis of the Novel Object Test data had high positive loadings on the time spent freezing and the latency to explore the unfamiliar object, and high negative loadings on time spent moving and exploring the object. Previous studies indicated that relatively slower and lesser exploration of a (novel) object and being less active in unfamiliar situations can be associated with increased fearfulness and/or anxiety (Brown et al., 2009; Dalmau et al., 2009; Ursinus et al., 2013), and an alert standing posture, i.e., freezing, is often exhibited in response to aversive situations (Reimert et al., 2013). Donald et al. (2011) found a factor with similar behavioural components and loadings for piglets participating in an open field test in which novel objects were present, and concluded that this factor mainly reflected the degree of neophobia, as the fear-related behaviours occurred in response to novelty. Likewise, the factor found in our study is assumed to reflect the level of neophobia, and as LAR piglets scored lower on this factor compared to CON piglets, LAR piglets are assumed to be less neophobic. The transient presence of live larvae is therefore sufficient to decrease neophobia. This may indicate that the feedback provided by tasting and eating larvae enhances the positive associations with novelty enough to compensate for the occasional absence of larvae. In line with our results, long-term access to live BSFL decreased broiler fearfulness, as shown by a decreased amount of time spent in tonic immobility (Ipema et al., 2020a). Similarly, previous studies found that housing pigs with marginally increased space and more toys elevated exploration of a novel object (Tönepöhl et al., 2012), and providing hanging ropes together with hanging rubber tyre tubes reduced the latency to approach a person (Rodarte et al., 2004).

It should be noted that reduced neophobic responses by piglets that were given larvae were only observed in the Novel Object Test, and not in the preceding Novel Environment Test. The factors retained in this test had, similarly to the Novel Object Test, a high negative loading for moving and a high positive loading on freezing. Additionally, this factor had a high negative loading for time spent on exploring the environment, and high positive loadings for standing and the frequency of vocalizations. In a previous study, pigs which had received fear-reducing drugs spent more time on exploring their environment and less time on vocalizing compared to saline-treated pigs (Donald et al., 2011), indicating that these behaviours are also related to fearfulness. Taken together, the factor retained from the Novel Environment Test is assumed to reflect general fearfulness and/or anxiety. The observation that scores for this factor were not affected by treatment likely relates to the stimulus-specific responses of piglets towards novelty. This was demonstrated by Hemsworth et al. (1996) who found that piglets that received objects in their home pen were more willing to explore a novel object compared to piglets that had received human contact in their home pen. The absence of any observed behaviour difference in the Novel Environment Test could therefore be due to the absence of novel items in this test. A Novel Object Test was previously found to cause more fear-related behaviour and higher serotonergic responses compared to a Novel Environment Test (Ursinus et al., 2013), and the lower fear response prompted by a novel environment might not be sufficient to observe any treatment effects on fearfulness. While our results demonstrate the potential of live BSFL provisioning to reduce neophobia in piglets, further research is required to assess whether general fearfulness is also affected.

Some studies have found a link between increased exploration behaviour and increased feed intake pre-weaning (Kuller et al., 2010) and post-weaning (Middelkoop et al., 2019a). As live BSFL provisioning increased exploratory behaviours, it was expected to also increase feed intake, and potentially increase the time spent eating pellets. In contrast, LAR piglets spent less time eating pellets than CON piglets, and pellet consumption during day 4-11 was reduced in LAR piglets. The main reason that pellet consumption was reduced by live BSFL provisioning appears to be that consumption of live BSFL sufficiently fulfilled the pigs' motivation to eat. In a previous study in which a range of dietary options were available pre-weaning, consumption of the more palatable feed items was preferred (Middelkoop et al., 2019b), and the high palatability of BSFL likely caused a similar preference and high consumption. Including BSFL meal in pig diets has previously had positive (Yu et al., 2020a) or neutral (Chia et al., 2019; Spranghers et al., 2018) effects on pig growth, while the impact of consuming whole, live BSFL on piglet performance has, to our knowledge, not been studied. The absence of any effect of whole live BSFL consumption on total dry matter intake, net energy intake, feed efficiency, energy efficiency and average daily gain illustrates the nutritional equivalence of BSFL to feed on a dry matter basis in the current experimental setting. This finding highlights the potential nutritional value of BSFL for pigs, as suggested previously (Makkar et al., 2014; Veldkamp and Bosch, 2015). It is important to note that availability of BSFL in the current study was restricted to a fixed daily portion, and *ad libitum* provisioning could differentially influence piglet performance. Immediately post-weaning, when solid feed intake is generally low (Dong and Pluske, 2007), *ad libitum* BSFL provisioning might boost piglet performance by increasing nutrient intake. This, in turn, likely supports good intestinal integrity and growth (Heo et al., 2013), warranting future investigation. Furthermore, when a substantial part of the diet is replaced with larvae, it will be necessary to adjust the nutrient composition of the regular feed to assure adequate pig performance. Optimizing a pig diet including larvae requires further research into the complete nutrient composition and digestibility of BSFL.

The piglets' faecal dry matter content was not affected by live BSFL consumption. The occurrence of diarrhoea, indicated by a faecal consistency score of 3 or 4 (Pedersen and Toft, 2011), was very low in both treatment groups. This could be the results of the wood shavings present in all pens, which are known to reduce the number of days pigs have diarrhoea and benefit the overall faecal consistency score (Munsterhjelm et al., 2009). Under commercial conditions, diarrhoea is more common, for example due to the reduced feed intake and increased stress around weaning (as reviewed by Heo et al., 2013). Investigating live BSFL provisioning in this setting is recommended to determine their effect on post-weaning diarrhoea.

To conclude, post-weaning live BSFL provisioning had beneficial effects on piglet behaviour, by facilitating exploration behaviours and reducing the need to orally manipulate objects and pen mates. The presence of larvae also reduced neophobic responses towards a novel object. The performance of piglets that consumed a small amount of larvae was maintained, as their total feed and net energy intake, feed efficiency, energy efficiency, and average daily gain did not differ from that of control piglets. Under barren commercial conditions, larvae provisioning has the potential to further benefit piglet welfare, though this remains to be confirmed by future studies. These studies should also consider appropriate larvae provisioning methods under commercial conditions, as scattering larvae on slatted floors is impractical.

Acknowledgements

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Supplementary data

Table S1. Analysed chemical composition of back soldier fly larvae (BSFL). Chemical composition analysis via standardized protocols (DM 10032, Protein 10005, Fat 10112, Calcium and Phosphorus 10040).

	BSFL
Dry matter (DM, g/kg)	350.7
ME (MJ/kg of DM)	19.5
Crude protein (g/kg of DM)	419
Crude fat (g/kg of DM)	371
Calcium (g/kg of DM)	6.3
Phosphorus (g/kg of DM)	6.8

Table S2. Average daily gain and dry matter intake (excluding and including black soldier fly larvae (BSFL)) per period of pigs receiving wood shavings (CON) or black soldier fly larvae (LAR) twice a day. Data are expressed as means ± SEM. Significant p-values are presented in bold.

Measure	**CON**	**LAR**	**F-statistic and df**	**P**
Average daily gain (g/pig/day)				
d0-d1	-240 ± 22	-188 ± 32	$F_{(1,13)} = 1.65$	0.22
d1-d4	144 ± 38	133 ± 23	$F_{(1,13)} = 0.06$	0.81
d4-d7	242 ± 34	204 ± 31	$F_{(1,13)} = 0.62$	0.45
d7-d11	342 ± 15	384 ± 36	$F_{(1,13)} = 1.08$	0.32
Dry matter intake excl. BSFL (g/pig/day)				
d0-d1	13 ± 3	8 ± 3	$F_{(1,13)} = 1.59$	0.23
d1-d2	58 ± 26	43 ± 21	$F_{(1,13)} = 0.20$	0.66
d2-d4	120 ± 23	101 ± 14	$F_{(1,13)} = 0.52$	0.48
d4-d7	220 ± 27	136 ± 23	$F_{(1,13)} = 5.57$	**0.03**
d7-d11	367 ± 14	297 ± 14	$F_{(1,12)} = 10.18$	**0.01**
Dry matter intake incl. BSFL (g/pig/day)				
d0-d1	13 ± 3	21 ± 3	$F_{(1,13)} = 2.87$	0.11
d1-d2	58 ± 26	69 ± 21	$F_{(1,13)} = 0.11$	0.75
d2-d4	120 ± 23	134 ± 14	$F_{(1,13)} = 0.24$	0.63
d4-d7	220 ± 27	188 ± 23	$F_{(1,13)} = 0.78$	0.39
d7-d11	367 ± 14	350 ± 14	$F_{(1,12)} = 0.62$	0.45

Chapter 7

Assessing the effectiveness of providing live black soldier fly larvae (*Hermetia illucens*) to ease the weaning transition of piglets

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Abstract

Weaning is a stressful event for piglets, involving substantial changes to their nutritional and social environment. Providing edible enrichment around weaning may ease the weaning transition by increasing pre-weaning feed intake and improving post-weaning performance, health, behaviour, and affective state. In this study, we investigated the effects of providing live black soldier fly larvae (BSFL) as edible enrichment pre- and/or post-weaning. Pre-weaning, piglets received either only creep feed (Pre-C, n = 14 litters) or creep feed and live BSFL (Pre-L, n = 15 litters) *ad libitum*, and post-weaning piglets either had no access to live BSFL (Post-C, n = 24 pens) or they could rotate tubes that released BSFL (Post-L, n = 24 pens) at levels up to 20% of their expected daily dry matter intake, resulting in treatments CC, CL, LC, and LL. No interaction between pre- and post-weaning treatment was found for any of the measured parameters. Before weaning, Pre-L piglets preferred to interact with larvae over creep feed, and Pre-C piglets interacted more with creep feed than Pre-L piglets. Total time spent on feed-directed behaviours did not differ. Continuous larvae provisioning increased caecum length and proximal stomach digesta pH, while it decreased the passage of glucose and fluorescein isothiocyanate through the colon wall on d3 post-weaning (CC vs. LL, n = 12 piglets/treatment). Post-weaning diarrhoea and final body weight were not affected by treatment. After weaning, Pre-C piglets tended to eat more and grew marginally faster than Pre-L piglets. Post-C piglets spent more time eating and had a higher feed intake post-weaning than Post-L piglets. Based on home-pen behavioural observations, Post-L piglets actively explored and ate the larvae. Post-C piglets spent more time on exploring the environment and nosing pen mates, and they spent more time on manipulating pen mates on d8 and played more on d8 & 15 compared to Post-L piglets. Piglet responses to a novel environment and an attention bias test on d4 & 5 post-weaning were not influenced by larvae provisioning. In conclusion, pre-weaning larvae provisioning did not improve pre-weaning feed intake and post-weaning performance, however post-weaning larvae provisioning did benefit piglet behaviour as less manipulation of pen mates was observed.

Introduction

Weaning is a critical period for commercially housed piglets. Under natural conditions weaning is a gradual process lasting several months (Jensen and Recén, 1989; Newberry and Wood-Gush, 1985), whereas commercial weaning often takes place abruptly when piglets are 3-4 weeks old. As a result all weaning-related stressors, including separation from the sow, switching from a milk to a concentrate diet, relocation to a new environment, and often mixing with unfamiliar conspecifics, occur acutely and simultaneously, intensifying the stress experienced by newly weaned piglets (Hötzel et al., 2011; Weary et al., 2008). At this young age, piglets tend to have little to no experience with eating solid (creep) feed, and pre-weaning feed intake varies markedly between piglets and litters (Collins et al., 2013; Hedemann et al., 2007; Middelkoop et al., 2020a; Pluske et al., 2007b). Inexperience with solid feed combined with the stress of early weaning often causes a drop in feed intake and growth directly after weaning (Bruininx et al., 2002; Colson et al., 2006). This, in turn, can compromise the development of the gastro-intestinal tract (GIT), resulting in, among others, villous atrophy and increased intestinal permeability (Hu et al., 2013; Moeser et al., 2017; Pohl et al., 2017). Suboptimal GIT functioning poses a threat to piglet performance and health, demonstrated by a high incidence of post-weaning diarrhoea (Heo et al., 2013; Pluske et al., 2018). In addition to facing nutritional challenges, newly weaned piglets are often confronted with social and environmental challenges after weaning. Mixing of unacquainted piglets at weaning causes aggressiveness and fighting (D'Eath, 2005; Hayne and Gonyou, 2006), especially when piglets are housed in barren environments (Oostindjer et al., 2011b). Furthermore, pigs are highly motivated to perform foraging behaviours such as rooting (Studnitz et al., 2007), and when barren environments do not facilitate this behaviour, it is often redirected to pen fixtures, or to other pigs in the form of damaging oral manipulations such as ear and tail biting (de Oliveira et al., 2020; Luo et al., 2020b). The combined challenges of early and abrupt weaning thus diminish the welfare of newly weaned pigs and can have long-term ramifications for pig performance and health (Cabrera et al., 2010; Moeser et al., 2017).

A potentially effective strategy for easing the weaning transition is providing edible environmental enrichment pre- and/or post-weaning. Compared to providing only creep feed, providing additional edible items pre-weaning can create a more diverse diet in terms of texture, taste, smell, and nutrients. Previous studies found that providing a diet composed of several feed types before weaning enhances pre-weaning feed exploration and feed intake (Adeleye et al., 2014; Middelkoop et al., 2019b, 2018), and it can increase the number of piglets that sample solid feed before weaning (Middelkoop et al., 2019b). The increased interest in and consumption of a diverse diet likely occurs due to a decrease in sensory-specific satiety (Rolls et al., 1981) and increased opportunities for exploration (Middelkoop et al., 2019b). Sufficient experience with eating feed before weaning can have post-weaning benefits such as improved performance (Berkeveld et al., 2007; Bruininx et al., 2004; Pluske et al., 2007b), enhanced nutrient absorption in the small intestine (Kuller et al., 2007), a more matured intestinal microbiota, and increased weight of several GIT segments (Choudhury et al., 2021). Furthermore, providing edible enrichment such as straw before weaning has been shown to attenuate stress responses towards humans and during transport at weaning (Chaloupková et al., 2007). This indicates that piglets exposed to such enrichment may be better able to cope with the weaning transition.

Post-weaning, environmental enrichment such as increased space and/or access to straw or peat has been found to increase feed intake (Oostindjer et al., 2010) and growth (Vanheukelom et al., 2011), and it can facilitate exploration and decrease damaging behaviours such as fighting and pig-directed oral manipulation (Beattie et al., 2000a; Oostindjer et al., 2011b; Vanheukelom et al., 2011). Enrichment can also benefit the affective state of pigs. Providing piglets with a combination of space, straw, and manipulatable objects caused a more positive affective state compared to barren-housed piglets (Douglas et al., 2012). Similarly, having access to a wooden box with popcorn and wood shavings and/or an object made of plastic tubing caused fewer fear-related behaviours to be exhibited during social isolation (de Oliveira et al., 2020). Edible enrichment items are expected to maintain interest longer than non-edible enrichment due to the positive reinforcement of consumption (Holm et al., 2008; Tarou and Bashaw, 2007).

A type of feed that is highly appropriate to be used as edible enrichment for pigs during the weaning transition is live black soldier fly larvae (*Hermetia illucens*, BSFL). These larvae are high in moisture, fat, and protein and low in carbohydrates (Barragan-Fonseca et al., 2017; Makkar et al., 2014; Nguyen et al., 2015). This makes them very palatable, and it makes them a suitable transition feed in the weaning period, because piglets are accustomed to liquid milk diets and digestion of larvae does not require starch-degrading enzymes that are uncommon in young piglets. Young piglets actively consumed live BSFL in previous studies (Ipema et al., 2021a, 2021b). Some observed benefits of BSFL inclusion (in different amounts of full-fat or defatted meal) in weaner pig diets are increased growth, increased beneficial bacteria in the gut, and increased villus height in the jejunum (Biasato et al., 2020a; Spranghers et al., 2018; Yu et al., 2020a). In other cases, however, BSFL meal inclusion did not affect weaner pig performance (Crosbie et al., 2021; Spranghers et al., 2018). In our previous study, piglets that were provided with small amounts of live BSFL for 11 days after weaning showed high levels of larvae-directed exploration, decreased levels of pig-directed oral manipulation and fighting, and decreased neophobic responses towards an unfamiliar object (Ipema et al., 2021a).

Taken together, we hypothesize that providing live BSFL around weaning has the potential to ease the weaning transition. In this study we therefore investigated the effects of larvae provisioning pre- and/or post-weaning on piglet performance, GIT development, health, behaviour, and affective state. We expected that larvae provisioning before weaning would increase pre-weaning feed intake and benefit GIT development, and thereby improve post-weaning performance and health. We also expected that larvae provisioning during three weeks after weaning would benefit piglet feed intake, reduce the occurrence of maladaptive behaviours, and benefit the piglets' affective state. Keeping the type of feed or the environment similar during the pre- and post-weaning period is known to benefit, respectively, piglet feed intake (Heo et al., 2018) and behaviour (Luo et al., 2020b; Munsterhjelm et al., 2009; Oostindjer et al., 2011b) after weaning, and reducing the amount of change during weaning can reduce weaning stress (Hötzel et al., 2011; Weary et al., 2008). Therefore, piglets with continuous access to live BSFL around weaning were expected to experience the greatest benefits of larvae provisioning.

Methods

The Animal Care and Use committee of Wageningen University & Research (Wageningen, The Netherlands) approved the applied protocols under project license number AVD1040020187184. The protocols were in accordance with the Dutch animal experimentation law that complies with European Directive 2010/63/EU. The inclusion of indigo carmine in creep feed was approved by the Medicines Evaluation Board (Utrecht, The Netherlands).

Animals, housing, and management

In this study a 2 × 2 factorial design was applied, where piglets either had no access to larvae (Control, C) or had access to larvae (Larvae, L) pre-weaning (Pre-C and Pre-L) and/or post-weaning (Post-C and Post-L, resulting in the four treatment combinations CC, CL, LC, and LL, explained below). Twenty-nine multiparous pregnant sows (TN70 sows inseminated by Tempo boar semen, Topigs Norsvin, Vught, The Netherlands) were divided over two farrowing rooms in two successive batches (balanced for treatment), and they were assigned to treatments based on sow parity and piglets' birthdate (Pre-C: n = 14 sows, parity 4.7 ± 0.7, Pre-L: n = 15 sows, parity 4.6 ± 0.6). Approximately two weeks before farrowing the sows were transported from a conventional farm to the research facility of Wageningen University & Research. Here, sows were housed in groups of four or five familiar sows until one week before farrowing, and subsequently they were individually housed in farrowing pens until weaning. Sows received commercial gestation and lactation feeds (ForFarmers, Lochem, The Netherlands) in weighed portions at 7:30 and 16:00 h. To prevent piglets from consuming sow feed, any leftovers were removed 30 min after feed provisioning.

The farrowing pen had a section with slatted flooring (2.85 × 1.80 m) containing the sow crate (2.85 × 0.6 m), and an adjacent piglet feeding area with concrete flooring (1.30 × 1.80 m). A rubber mat was placed under the sow and in the piglet nest for comfort. One heating lamp was placed at either side of the sow, and the height of the lamps was adjusted over the pre-weaning period. The sow crate contained a feed trough, drinking nipple and a chew object (a chain with either a rubber ball, several bolts, or a plastic ring with protrusions attached to it) that was changed every three days. Around farrowing one jute sack was available to the sow. Piglets had access to a drinking nipple and a chain with bolts attached to it in the slatted area. From d3 after birth, piglets could access the feeding area that contained two feeders with either two (Pre-C treatment) or four (Pre-L treatment) feeding bowls (17.5 × 13.5 cm per bowl, **Figure 1**). Room temperature at farrowing was 25 °C, and this was decreased gradually to 21 °C on d13, after which it remained constant.

Within 24h after birth, piglets were weighed, and they received an ear tag and a 1 cc intramuscular iron injection. No castration, tail docking or teeth clipping were done. Within two days of age litter size was standardized by cross-fostering. At weaning on d28, litter size (Pre-C: 13.7 ± 0.3, Pre-L: 13.2 ± 0.3 piglets/litter) and weaning age (Pre-C: 26.4 ± 0.4, Pre-L: 27.1 ± 0.4 days old) did not differ significantly between treatments. At weaning, a subset of 240 piglets were transported to two weaner rooms in the two successive batches. Piglets were selected on health (no leg problems), sex, and body weight on d21 (close to average weight of the treatment group and the litter). Post-weaning, piglets were initially housed in groups of five piglets from five different litters to simulate commercial mixing at weaning. Piglets were housed in either a two female/three male or three female/two male ratio, and pens were assigned to one of the four treatment combinations (n = 12 pens per treatment combination), balanced per room. Three days after weaning, one piglet/pen was removed, and the removed piglets from the CC and LL treatments were sacrificed for post-mortem gastro-intestinal tract analysis. From d3-21 post-weaning (the end of the experiment), all pens had a two female/two male ratio.

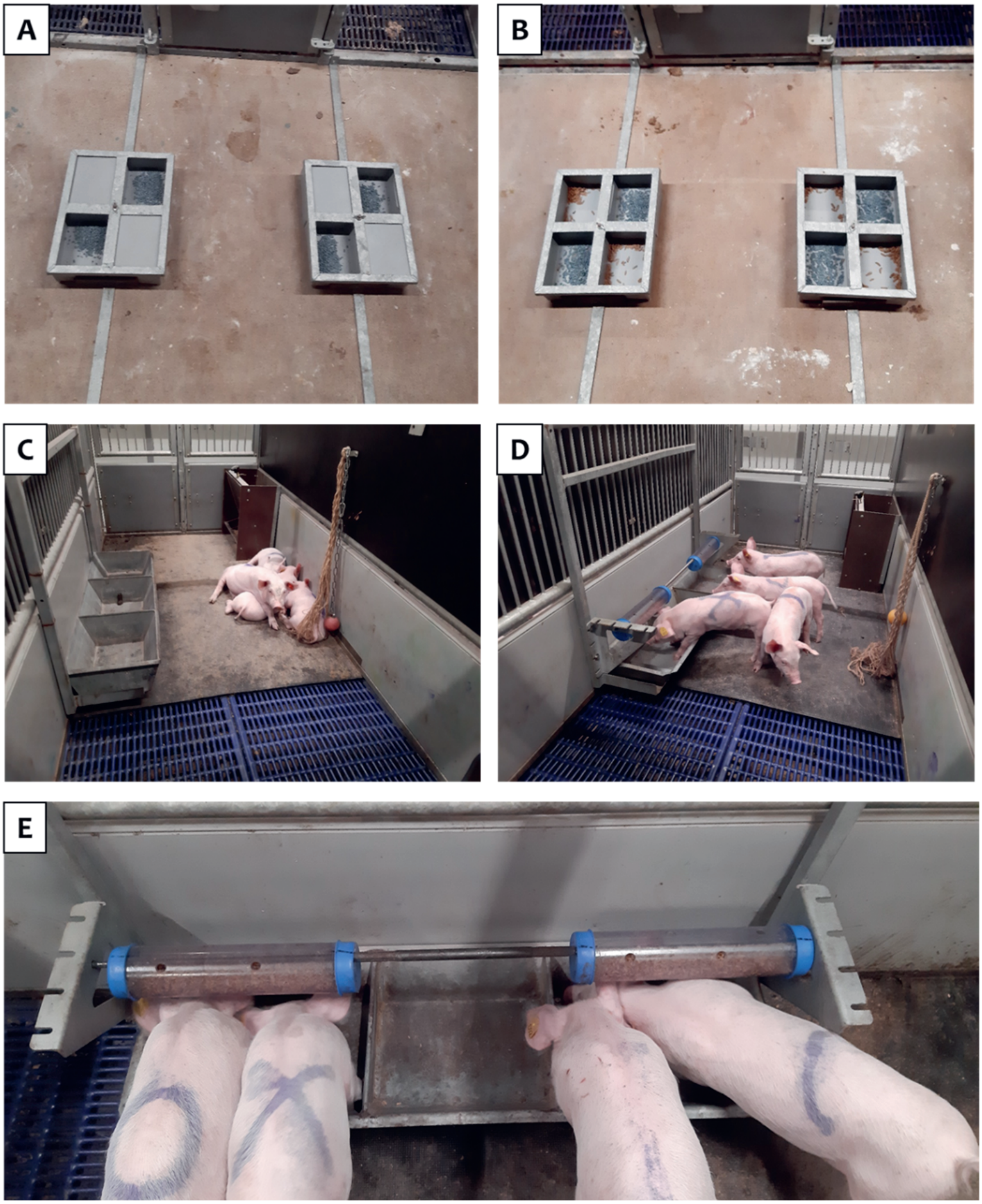


Figure 1. Set-up of the pre- and post-weaning treatments. From d3 pre-weaning, piglets could access the feeding area that contained either two feeders with two feeding spaces containing creep feed **(A)**, or two feeders with four feeding spaces, two containing creep feed and two containing larvae **(B)**. Post-weaning, piglets either had access to an empty feeder **(C)**, or access to a feeder to which two horizontally suspended tubes containing larvae were attached **(D)**. A close-up of the tubes filled with larvae can be seen in **(E)**.

Pens in the weaner rooms (2.85 × 1.20 m) had half slatted and half rubber flooring and were equipped with a feed trough (12 × 50 cm with three feeding places), drinking nipple, hanging jute rope, and a chew object (a chain with either a rubber ball or a plastic ring with protrusions attached to it) that was changed weekly. All pens also contained an experimental feeder (1.0 × 0.3 m), and in the Post-L pens two transparent tubes (32 cm long, 7.5 cm Ø) with four 1 cm Ø holes at the top were horizontally suspended above the feeder at approximately piglet shoulder height (**Figure 1**). Piglets had *ad libitum* access to water and to a commercial pelleted weaner feed (Vida Prima 3, ForFarmers, Lochem, The Netherlands). At weaning, the room temperature was 25 °C and this was gradually decreased to 23 °C at d10, after which it remained constant.

In both the farrowing and weaner rooms the light and a radio were on from 07:00 to 19:00 h, and the lights were dimmed and the radio was off from 19:00 to 07:00 h.

Experimental design

Piglets were assigned to one of four treatment combinations in a 2 × 2 factorial design, where piglets did or did not receive larvae pre-weaning and/or for three weeks post-weaning. Live, 14-day-old black soldier fly larvae (BSFL) were provided weekly (by Bestico B.V., Berkel en Rodenrijs, The Netherlands) and they were stored at 12 °C until provisioning. The creep feed (**Supplementary Tables S1, S2**) provided pre-weaning was pelleted by Research Diet Services (Wijk bij Duurstede, The Netherlands) and contained 5 g/kg Indigo carmine feed colorant (E132 Eurocert 311811, Pomona Aroma, Hedel, The Netherlands) that turned the feed blue and allowed for visual identification of creep feed consumption in the faeces.

Pre-weaning treatments

In the farrowing pens, piglets had unrestricted access to the feeding area containing two feeders from d3 after birth. In pens in the Control (Pre-C) treatment each feeder had two diagonally adjacent feed bowls containing creep feed, and in pens in the Larvae (Pre-L) treatment each feeder had four adjacent feed bowls, two containing creep feed and two containing live BSFL (**Figure 1**). Creep feed was always provided in portions of approximately 25 g, and larvae were provided in portions of 25 g until d22, and from d22-28 they were provided in portions of 150 g to accommodate the increasing demand. Every morning, one portion of the appropriate feed item was added to each feed bowl to keep the feed fresh. Feed bowls were checked at least four times a day, and a portion was added to a feed bowl if it was almost empty, to ensure near *ad libitum* access to the feed items while minimizing leftovers. Once a week the feed bowls were cleaned, and the feed items were completely refreshed.

Post-weaning treatments

After weaning, piglets were assigned either to the Control (Post-C) treatment that did not receive larvae, or to the Larvae (Post-L) treatment that received larvae in horizontally suspended tubes above a feeder (**Figure 1**), resulting in treatment combinations CC, CL, LC, and LL. Post-L piglets had to root or push the tubes to turn them at least 90° for the larvae to fall out. During the first two days after weaning, larvae were provided *ad libitum*, and on these days every morning and afternoon 420 g of larvae were placed in the feeder under the tubes to attract piglets to the tubes. After d2, piglets received up to 20% of their expected dry matter intake (based on manufacturer's recommendations) as live larvae in tubes, calculated per week. The maximum amount of live larvae provided per piglet per day was 140 g in w1, 270 g in w2, and 380 g in w3 post-weaning. Every day at 08:00, 12:00, and 16:00 h the tubes were checked, and near-empty tubes were refilled if the pen had not yet received its maximum daily amount of larvae.

Measurements

Identification of creep feed eaters based on rectal swabs pre-weaning

Rectal swabs were taken from each piglet on d7, 14, 21 and 28 pre-weaning (during the weighing procedure) to identify creep feed eaters based on the blue colour of the creep feed that was visible in the faeces. Piglets were scored as being an eater (blue coloured swab) or a non-eater (no blue coloured swab). The percentage of eaters per litter on each measuring day was calculated. The creep feed eater types of piglets for the total pre-weaning period were determined, where piglets were ranked as non, bad, moderate, or good creep feed eaters if they had respectively zero, one, two or three-four blue coloured swabs (modified from Pluske et al., 2007).

Feed-directed behaviour and identification of eaters based on behaviour pre-weaning

One day before behavioural observations pre-weaning, up to fourteen piglets per pen were marked (stock marker spray) for individual identification. Feed-directed behaviour in the home pen was scored on d8, 15, 22 and 27 (ethogram in **Table 1**). Each day, scoring was done by 3-min instantaneous scan sampling for seven one-hour periods, starting at 08:00, 09:15, 10:30, 12:15, 14:00, 15:15 and 16:30 h. Two observers scored one room each, switching rooms every hour. Observations were performed on a tablet with the software Observer 3.3 (Noldus Information Technology B.V., Wageningen, The Netherlands). Before observations, the observers were trained, and inter-observer reliability was sufficient (Fleiss kappa > 0.8, Landis and Koch, 1977). For both creep feed and total (creep feed and/or larvae) eaters the percentage of eaters per litter per day was calculated, and for the whole pre-weaning period piglets were ranked as non, bad, moderate, or good eaters if they had been observed eating at least once on respectively zero, one, two or three-four observation days.

Table 1. Ethogram of feed-directed behaviour observed in the home pen pre-weaning.

Behaviour	Description
Exploring feeder	Sniffing, touching (with snout) or rooting feeder.
Exploring or playing with feed	Sniffing, touching (with snout) or rooting feed, rolling feed item over the floor, walking around the pen with feed item in mouth, shaking head while having feed item in mouth.
Eating feed	Eating or chewing feed from the feeders or the floor.

Behaviours scored during the pre-weaning home-pen observations of pigs having access to either only creep feed (Pre-C) or creep feed and live black soldier fly larvae (Pre-L). For the Pre-L pigs, scoring included the type of feed item, either creep feed or larvae, that the behaviour was directed to. Ethogram adjusted from Middelkoop et al. (2019).

Performance

Piglets were individually weighed within 24h after birth, and on d7, 14, 21 and 28 (day of weaning) pre-weaning, and d1, 2, 7, 14 and 21 post-weaning. Feed intake pre-weaning could not be determined as piglets regularly spilled feed and larvae, which then got mixed with faeces. Post-weaning, leftover feed and larvae were weighed back on d1, 2, 7, 14 and 21 to determine feed and larvae intake at pen level.

Faecal consistency scores

Post-weaning, the faecal consistency at piglet level was scored daily at 09:00 h by two observers. Score 1 represented firm faeces, score 2 soft but shaped faeces, score 3 loose faeces and score 4 water thin faeces (Pedersen and Toft, 2011). Each piglet was given a score based on the faecal consistency visible around the anus, and the total number of days piglets had diarrhoea (score 3 or 4) or watery diarrhoea (score 4) post-weaning were determined.

Gastro-intestinal tract development

On d3 post-weaning, one piglet/pen of the CC and LL treatments (n = 12/treatment, balanced for sex and coming from different litters) was sacrificed by sedation and subsequent lethal injection with Euthasol for post-mortem gastro-intestinal tract measurements. The selected LL piglets were of similar weight and were observed to eat larvae at least three times during the pre-weaning behavioural observation on d22. The CC piglets were selected to be near the average weight of the selected LL piglets. The length, empty weight and digesta weight of the stomach, small intestine, caecum, and colon were recorded. The stomach was divided into the proximal and distal part by tying the middle off with a tie-wrap, and the proximal and distal digesta were collected separately and their pH was measured separately. Colonic digesta pH was also measured. Furthermore, a 20-cm section from the jejunum (proximal of small intestine midpoint) and from the colon (at colon midpoint) were cleaned with water, stripped of muscle, carefully everted, filled with a Ringer-HEPES solution, and tied off on both ends with rubber bands. These everted gut sacs were suspended in a closed Erlenmeyer in a solution containing Ringer, HEPES, glucose (900 μg/ml), and the fluorescent markers fluorescein isothiocyanate and tetramethyl rhodamine isothiocyanate (FITC and TRITC, respectively, 30 μg/ml, Sigma-Aldrich co. LLC., Saint Louis, USA). Before use, this solution was kept in a jerrycan containing an oxygen pump for oxygenation. The Erlenmeyers were placed in a water bath with shaker at 39 °C for one hour. Then, the sacs were removed, and their full and empty weight were determined, as well as their width and length to calculate the sac surface. The sac content was collected in black Eppendorf tubes and stored at -20 °C. Before analysis, the samples were thawed at room temperature. The glucose concentration was measured using the Glucose liquiUV mono kit (HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany). FITC and TRITC concentrations were determined by spectrophotometry (FITC measured at 485 nm excitation and 530 nm emission, TRITC measured at 528 nm excitation and 590 nm emission), and sample fluorescence was compared to a standard curve to determine the marker concentrations. From this, the transport per everted sac surface area (µg or nm/cm^2) was calculated.

Post-weaning home pen behaviour

Piglets were marked weekly (stock marker spray) for individual identification. On d8, 15 and 20 post-weaning home pen behaviour was observed through 2-min scan sampling for seven one-hours periods a day, starting at 08:00, 09:15, 10:30, 12:15, 14:00, 15:15 and 16:30 h (ethogram in **Table 2**). As for the pre-weaning observations, two observers with sufficient inter-observer reliability (Fleiss kappa > 0.8, Landis and Koch, 1977) scored one room each, switching rooms every hour, and observations were done on a tablet with the software Observer 3.3.

Affective state

As coping style can affect a piglet's behavioural response in a novel environment test (Reimert et al., 2014b) and in an attention bias test (Luo et al., 2019), piglet coping style was assessed through a back-test (based on Bolhuis et al., 2003) on d16-17 pre-weaning. In short, piglets were individually transported in a closed cart to a quiet room near the farrowing room, where they were placed on their back on a soft surface and manually restrained for 60 s during which the number of struggles and vocalizations were recorded. In accordance with Melotti et al. (2011) piglets were classified as low resisters if they struggled zero or one times, or if they struggled twice and vocalized less than 25 times. Piglets that struggled three times or more, or that struggled twice and vocalized 25 times or more were classified as high resisters.

Table 2. Ethogram of behaviour observed in the home pen post-weaning.

Behaviour	Description
Ingestive behaviour	
Eating feed	Chewing or swallowing feed pellets.
Eating larvae	Chewing or swallowing larvae.
Drinking	Drinking from water nipple.
Postures and locomotion	
Inactive	Sitting, or lying on side or belly, without performing any other behaviour.
Standing and Walking	Standing idle with four hooves on the floor or walking without performing any other behaviour.
Exploratory behaviour	
Exploring environment	Sniffing, touching (with snout), rooting, or chewing the pen floor, wall, toy/rope, feeder, or water nipple, or chewing air or faeces.
Exploring feeder (including tubes)	Sniffing, touching (with snout), rooting, or chewing the tubes containing larvae (present in Post-L treatment) or the experimental feeder to which the tubes can be attached (present in Post-C and Post-L treatment).
Pig-directed behaviour	
Nose-to-nose	Having nose to nose contact with a pen mate.
Nosing pen mate	Sniffing or touching (with snout) body of pen mate except the snout, including anal nosing.
Manipulating pen mate	Mounting pen mate or nibbling, sucking, rooting, or chewing any part of a pen mate, including belly nosing.
Fighting	Mutual pushing, pressing, ramming, head knocking, nudging, aggressively biting, or lifting pen mate.
Other behaviours	
Play	Running, jumping, or turning in the pen (either individually or with pen mates), shaking head while holding toy/rope, pulling on toy/rope.
Comfort behaviour	Rubbing body against wall/floor, scratching body with hind leg, or stretching (part of) body.
Other	Any behaviour not described.

Behaviours scored during the post-weaning home pen observations of piglets that had access to no larvae (Post-C) or had access to hanging tubes containing live black soldier fly larvae (Post-L).

To assess affective state after weaning a novel environment test (NET, d4 post-weaning) and an attention bias test (ABT, d5 post-weaning) were performed. Two piglets per pen (one female/one male) were included in both tests. For the NET, piglets were individually caught and transported in a closed cart to an experimental room near the weaner rooms. Here, they were placed in a start box alongside the test area. Within 15 s a door was opened, and the piglet entered an unfamiliar area with a 5.3 × 5.3 m rubber floor surrounded by 1 m high hardwood walls. In the centre of the floor stood a feed bowl containing 0.5 kg of their regular feed mixed with ten raisins and ten pieces of corn. After entering the area, the piglet's behaviour was recorded for 5 min by two observers, on a tablet with the Observer 3.3 software (ethogram in **Table 3**). One observer scored behavioural states and another scored behavioural events. After the test ended, the piglet was transported to the home pen, and the test area was cleared of faeces and urine and cleaned with cleaning solution and a moist mop.

For the ABT, two-thirds of the piglets (n = 16/treatment) were tested with a threat, and one-third of the piglets (n = 8/treatment) were tested without a threat to assess threat effectiveness. The experimental procedure of the ABT was similar as that of the NET, however the ABT lasted 3 min. The threat was a combination of a flashing light and a siren. Ten s after entering the test area a door in the right wall (in relation to the start box) opened, and the flashing light was shown, and the siren was turned on. After 10 s the door closed, and the flashing light and siren were turned off. In addition to scoring the behaviours that were also scored in the NET, one observer scored the attention of the piglets towards the threat (**Table 3**).

Table 3. Ethogram of behaviours observed during the Novel Environment Test and the Attention Bias Test.

Behaviour	Description
Attentive states[1]	
Attention to the threat	Having head oriented toward the location of the threat.
Attention not to the threat	Having head oriented away from the location of the threat.
Behavioural states	
Standing alert	Standing motionless with head fixed (up or down) and ears upright.
Moving	Walking or running without performing any other described behaviour. All four legs move, or the pig turns around on the same spot without moving all four legs.
Standing	Standing, not alert, with four hooves on the floor without performing any other described behaviour.
Sitting/lying	Sitting on the floor, or lying on side or belly, without performing any other described behaviour.
Exploring environment	Exploring the floor or wall by sniffing, touching (with snout), rooting, chewing, or licking it.
Exploring feed bowl	Exploring the feed bowl by sniffing, touching (with snout), rooting, chewing, or licking it. Rooting disc can be in contact with feed bowl, but pig is not eating.
Eating feed	Chewing or swallowing feed. The eating event continues while the pig is chewing, provided that the head stays close to the feed bowl and the pig remains non-vigilant. Once the pig becomes vigilant or moves away from the feed bowl, this is the end of eating, even if the pig continues chewing.
Behavioural events	
Low-pitched vocalizations	Short or long grunts.
High-pitched vocalizations	Grunt-squeals, squeals, or screams.
Eliminating	Excreting urine or faeces.
Escape attempt	Jumping in air or against the wall of the area.

[1] Only observed during the Attention Bias Test.

Statistical analysis

Data processing

Pre- and post-weaning piglet behaviours in the home pen were averaged per piglet per day and expressed as the proportion of scans. Post-weaning, the behaviours “Drinking”, “Fighting”, “Nose-to-nose” and “Comfort behaviour” were excluded from analysis due to their low occurrence (< 1.5% of observations). A factor analysis was conducted on behaviours performed in the NET, and on the behaviours performed during the 150 s following the threat in the ABT. The behaviours “Sitting/lying” and “Escape attempt” were rare and therefore excluded from analysis. The occurrence of “Eating feed” was very low and therefore it was included in “Exploring feed bowl”, and the latency of this behaviour was also incorporated in the analysis. When piglets did not interact with the feed bowl, the latency was set to the maximum possible time. The distribution of “High-pitched vocalizations” was skewed, and this behaviour was combined with “Low-pitched vocalizations” into “Vocalizing”. The behaviours “Standing” (NET and ABT after threat) and “Exploring feed bowl” (ABT after threat) were arcsine square root transformed, and “Eliminating” (NET) was squared for normalization.

Data analysis

All analyses were performed with the statistical software SAS 9.4 (SAS Institute Inc., Cary, NC, USA). General linear (mixed) model residuals were checked for normality. Except for the models on larvae consumption and larvae-directed behaviour, all models on pre-weaning data included pre-weaning treatment as fixed effect, and all models on post-weaning data included pre-weaning treatment, post-weaning treatment and their interaction as fixed effects. Additionally, all models included batch as fixed effect. If data was on piglet level and multiple piglets from the same pen were included, a random pen effected nested within batch and in pre-weaning treatment or pre- and post-weaning treatment was always included.

Piglet growth rate, body weight, feed intake and GIT measurements were analysed in general linear (mixed) models (MIXED in SAS). For analysis of piglet weaning weight and the weight on d21 post-weaning birth weight was included as covariate. For models analysing organ and digesta weights, piglet body weight on d2 post-weaning was included as covariate.

The proportion of creep feed eaters and total (creep feed and/or larvae) eaters per pen based on swabs and behaviour, and the proportion of scans spent on pre-weaning and post-weaning home-pen behaviours were analysed in generalized linear (mixed) models (GLIMMIX in SAS) with a binomial distribution, logit link function and an additional multiplicative overdispersion parameter. Pre-weaning home-pen behaviour was analysed per day, and post-weaning home-pen behaviour was analysed for the entire post-weaning period combined. As such, models on post-weaning behaviour included a fixed effect of day and its two-way interactions with pre- and post-weaning treatment, a random pen by day effect, and a repeated effect of day with piglet as subject using a heterogenous first-order autoregressive covariance structure. Initial models included the three-way interaction between pre-weaning treatment, post-weaning treatment, and day, however as this never had a significant effect it was removed from the final models.

The proportion of observations Pre-L piglets spent on interacting with larvae vs. creep feed was analysed in a GLIMMIX with binomial distribution, logit link function, and overdispersion parameter. This model included a fixed effect of feed type, and a repeated effect of feed type with piglet as subject, using a compound symmetry covariance structure. Pre-weaning creep feed and total eater types were analysed in a GLIMMIX with a multinomial distribution and cumulative logit link function. Number of days with (watery) diarrhoea was analysed in a GLIMMIX with a Poisson distribution, log link function and an additional multiplicative overdispersion parameter.

To assess the effectiveness of the threat during the ABT, the behaviour of pigs that did or did not receive a threat was assessed in models with threat (yes or no) as a fixed effect. The proportion of time spent on behaviours was analysed in a GLIMMIX with a binomial distribution, logit link function, and an additional multiplicative overdispersion parameter, the frequencies of behaviours were analysed in a GLIMMIX with a Poisson distribution, log link function, and an additional multiplicative overdispersion parameter, and the latency to explore the feed bowl was analysed in a MIXED model. For piglets receiving the threat, similar models were used to analyse behaviour during the 10 s threat, and the fixed effects in this model were pre-weaning treatment, post-weaning treatment, and their interaction. The behaviours “Standing” and “Eliminating” were rare during the 10 s threat and were therefore not analysed. The variables from the NET and ABT after the threat were put in factor analyses with orthogonal Kaiser-Varimax rotation. For the NET, the behaviour “Eliminating” had a communality estimate below 0.3, therefore this behaviour was excluded from the factor analysis. Based on Kaiser’s criterium, factors with an eigenvalue above one were retained, resulting in two factors for the NET and three factors for the ABT after the threat (**Table 4**). The scores of each piglet for each factor were analysed with MIXED models which initially included an additional fixed effect of coping style, however as this effect was never significant it was removed from the final models.

Data are presented as pen means ± SEM unless stated otherwise. P-values below 0.05 were considered significant, and p-values between 0.05 and 0.1 were considered a trend. Significant fixed effects were analysed on post-hoc differences in least square means with a Tukey’s HSD correction.

Table 4. Loadings of the factors with an eigenvalue above one that were extracted by factor analysis with orthogonal Kaiser-Varimax rotation on the behaviours and attention scored during the Novel Environment Test and the Attention Bias Test during the 150 s after the threat.

Variable	**Novel Environment Test**		
	Factor 1	***Factor 2***	
Moving (% of time)	**0.90**	-0.14	
Standing (% of time)	0.11	**0.55**	
Standing alert (% of time)	0.09	**0.86**	
Exploring environment (% of time)	**-0.82**	-0.41	
Exploring feed bowl (% of time)	0.02	**-0.61**	
Latency exploring feed bowl (s)	**-0.47**	0.39	
Vocalizing (frequency)	**0.59**	0.17	
Eigenvalues	2.07	1.79	
% of variance explained	42.7%	31.6%	
	Attention Bias Test after threat		
	Factor 1	***Factor 2***	***Factor 3***
Attention to threat (% of time)	**-0.48**	-0.40	-0.08
Moving (% of time)	**0.88**	0.19	0.17
Standing (% of time)	-0.06	0.03	**0.83**
Standing alert (% of time)	**-0.59**	**-0.79**	-0.14
Exploring environment (% of time)	-0.09	**0.98**	-0.04
Exploring feed bowl (% of time)	**0.64**	0.09	-0.29
Latency exploring feed bowl (s)	**-0.75**	-0.05	0.28
Vocalizing (frequency)	**0.72**	-0.02	-0.03
Eliminating (frequency)	-0.06	0.03	**0.67**
Eigenvalues	2.86	1.79	1.35
% of variance explained	48.7%	23.3%	16.3%

High loadings (≤ – 0.45 or ≥ 0.45) are indicated in bold.

Results

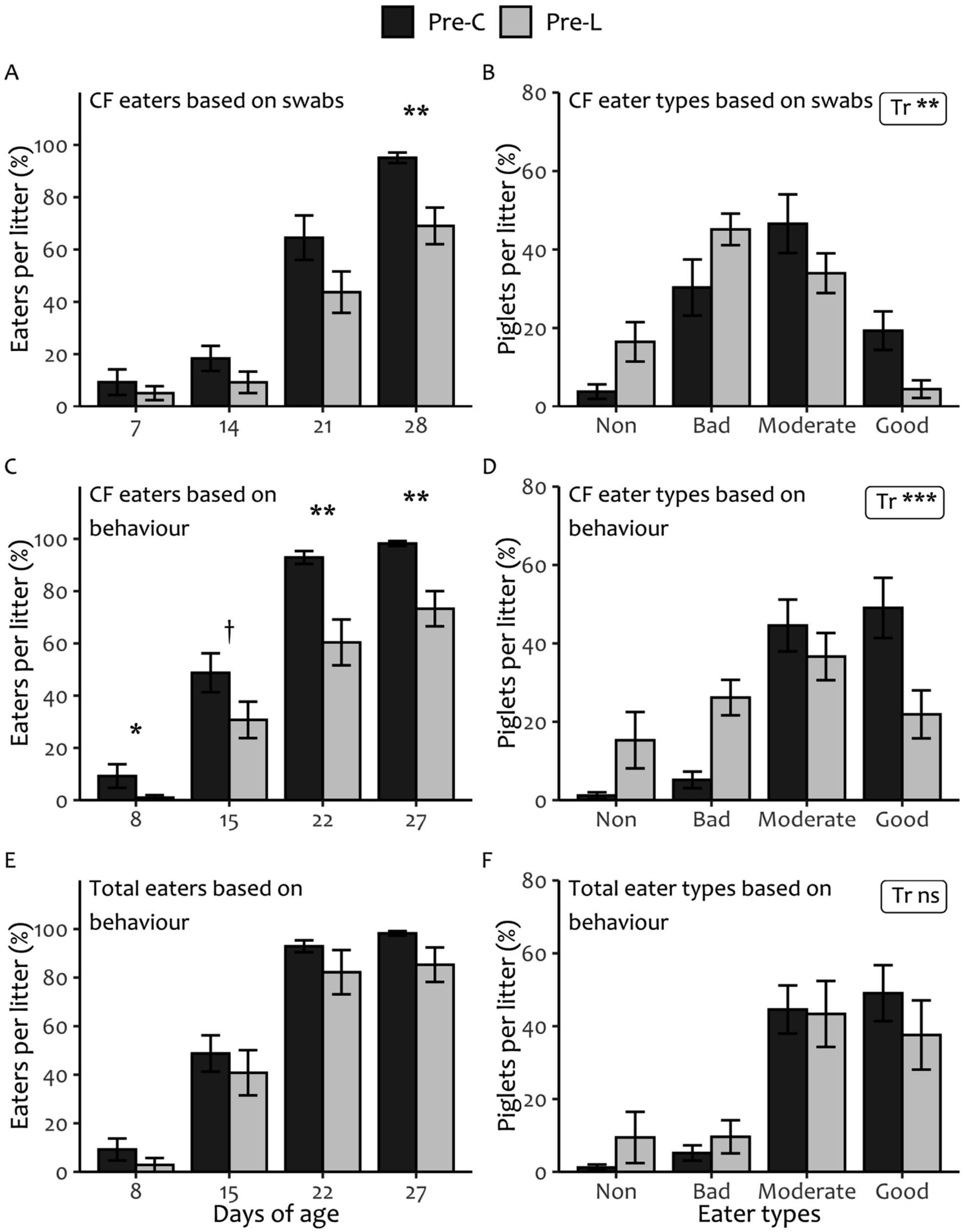
Pre-weaning eaters

The percentage of creep feed eaters per litter based on rectal swabs was significantly higher in the Pre-C treatment than in the Pre-L treatment at weaning but not on preceding days (**Figure 2A**). The distribution of creep feed eater types based on swabs was also affected by treatment, with the Pre-C treatment having more piglets in the better eater categories (**Figure 2B**). Similar results were found for the behavioural observations, where the percentage of creep feed eaters per litter was higher in the Pre-C treatment on d8, 22 and 27, and it tended to be higher on d15 (**Figure 2C**). Creep feed eater types based on behaviour were also affected by treatment, with the Pre-C treatment again having more piglets in the better eater categories (**Figure 2D**). The percentage of total (creep feed and/or larvae) eaters per litter and the total eater types based on behaviour were not influenced by treatment (**Figure 2E, 2F**).

Pre-weaning feed-directed behaviour

Piglets in the Pre-C treatment tended to spend more time eating creep feed on d8 and 15, and they spent more time eating creep feed on d22 and 27 compared to Pre-L piglets (**Figure 3A**). On d8 treatment did not influence the time spent exploring and playing with the feed items, however Pre-L piglets spent more time exploring and playing with feed on d15, 22 and 27 compared to Pre-C piglets (**Figure 3B**). Only on d27 there was a trend for Pre-C piglets to spend more time exploring the feeder (**Figure 3C**). Treatment did not influence the total time spent eating (creep feed and/or larvae, **Figure 3D**). Within the Pre-L treatment, the time spent on interacting with creep feed or with larvae did not differ on d8 and 15, but piglets interacted more with larvae than with creep feed on d22 and 27 (**Figure 4**).

Figure 2 (next page). The percentage of piglets per litter that ate creep feed (CF) or creep feed and/or larvae (Total) per day and individual eater types determined at the end of the pre-weaning period based on blue coloured rectal swabs or pre-weaning home pen behavioural observations. Piglets had access to either only creep feed (Pre-C) or creep feed and live black soldier fly larvae (Pre-L). For the percentage of eaters (per day) and for the eater types (distribution of all types) the treatment effect is indicated as † ($p < 0.1$), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or ns (not significant). Data are presented as pen means ± SEM.



Pre-C
Pre-L
A
CF eaters based on swabs
Eaters per litter (%)
0
20
40
60
80
100
7
14
21
28
**
B
CF eater types based on swabs
Tr **
Piglets per litter (%)
0
20
40
60
80
Non
Bad
Moderate
Good
C
CF eaters based on behaviour
Eaters per litter (%)
*
†
**
**
8
15
22
27
D
CF eater types based on behaviour
Tr ***
Piglets per litter (%)
Non
Bad
Moderate
Good
E
Total eaters based on behaviour
Eaters per litter (%)
8
15
22
27
Days of age
F
Total eater types based on behaviour
Tr ns
Piglets per litter (%)
Non
Bad
Moderate
Good
Eater types

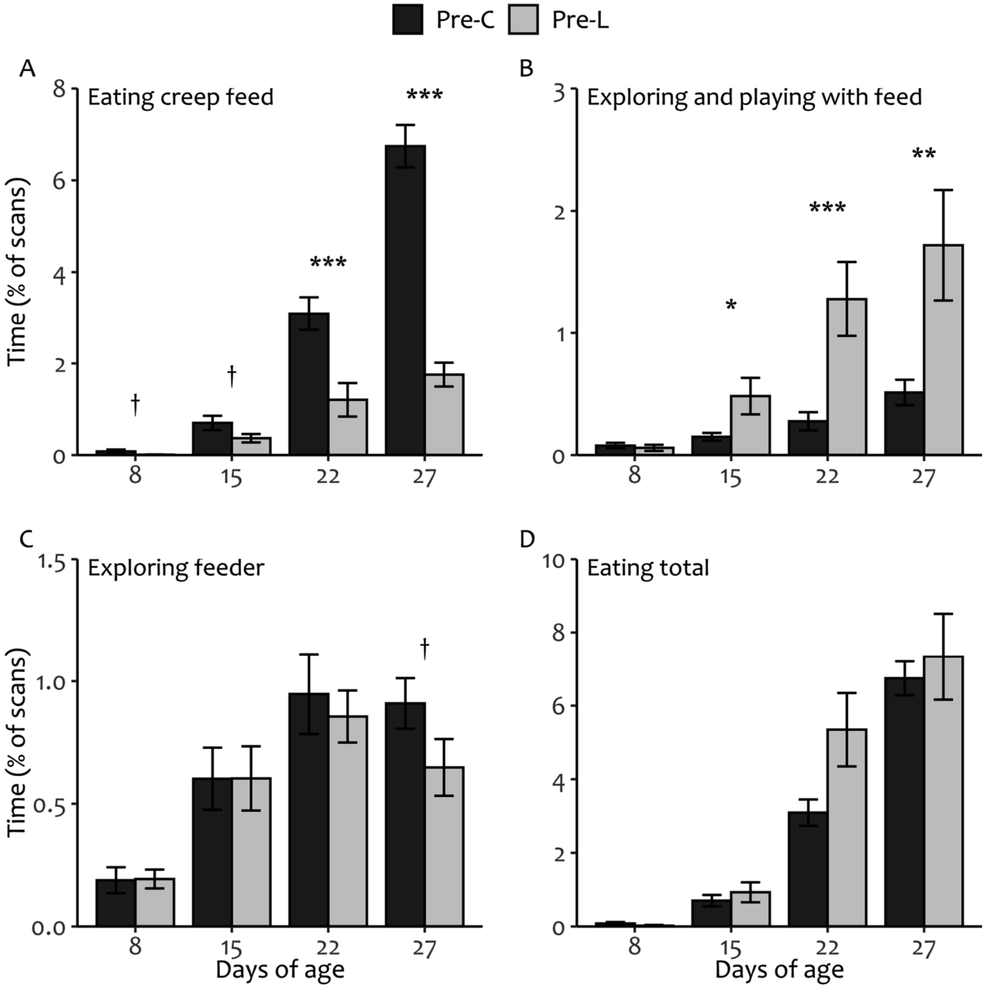


Figure 3. Time spent on feed-directed behaviours scored during the pre-weaning home pen observations of piglets having access to either only creep feed (Pre-C) or creep feed and live black soldier fly larvae (Pre-L). "Eating total" includes the time spent eating creep feed and eating larvae. Per day, treatment effects are indicated as † ($p < 0.1$), * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$). Data are presented as pen means ± SEM.

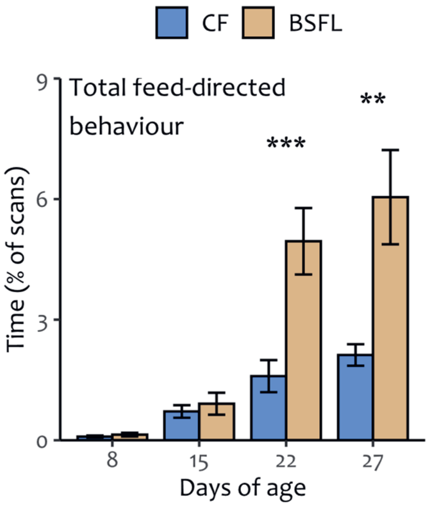


Figure 4. Time spent on all feed-directed behaviours (exploring feeder, exploring or playing with feed, and eating feed) towards creep feed (CF) and black soldier fly larvae (BSFL) as observed in the home pen of Pre-L piglets. Per day, effects of feed type are indicated as ** ($p < 0.01$) or *** ($p < 0.001$). Data are presented as pen means ± SEM.

Performance

Pre-weaning performance

Pre-weaning, piglet birth weight, growth, and weaning weight were not affected by pre-weaning treatment (**Table 5**).

Table 5. Pre-weaning piglet average daily gain and body weight.

Measure	**Pre-C**	**Pre-L**	**P-value**
ADG (g/piglet/day)			
birth-d7	123 ± 9	125 ± 12	0.946
d7-14	217 ± 8	216 ± 13	0.913
d14-21	247 ± 9	258 ± 11	0.447
d21-28	293 ± 11	293 ± 12	0.911
Total, birth-d28	228 ± 7	229 ± 9	0.871
Body weight (kg)			
Birth	1.46 ± 0.03	1.49 ± 0.04	0.706
d28	7.56 ± 0.23	7.71 ± 0.28	0.604

Pre-weaning average daily gain (ADG) and body weight of piglets having access to either only creep feed (Pre-C) or creep feed and live black soldier fly larvae (Pre-L). Data are presented as litter means ± SEM.

Post-weaning performance

No interaction was found between pre- and post-weaning treatment regarding any of the post-weaning performance parameters (**Table 6**).

Pre-weaning treatment influenced piglet growth (**Table 6**). Pre-C piglets tended to grow faster on the first day after weaning and in total from d0-21 after weaning, and they grew faster during d14-21 than Pre-L piglets. Conversely, during d2-7 Pre-L piglets tended to grow faster than Pre-C piglets. Post-weaning treatment also affected piglet growth (**Table 6**), as Post-C piglets grew faster during d1-2 and d2-7 than Post-L piglets.

Feed intake was influenced by pre-weaning treatment (**Table 6**). Pre-C piglets consumed more pellets during d14-21 post-weaning and tended to consume more pellets during the total post-weaning period from d0-21 than Pre-L piglets. Daily larvae dry matter consumption was higher for Pre-L piglets during d2-7, and it tended to be higher for Pre-L piglets during d7-14 and in total from d0-21 compared to Pre-C piglets. Pre-C piglets had a higher total daily dry matter intake (from pellets and larvae) during d14-21, and it tended to be higher in total from d0-21 compared to Pre-L piglets. Feed intake was also affected by post-weaning treatment (**Table 6**). Post-C piglets consumed more pellets than Post-L piglets during all periods. Post-C piglets also consumed more total dry matter than Post-L piglets during all periods except d0-d1.

The number of days with (watery) diarrhoea and the body weight on d21 post-weaning were not affected by pre- and/or post-weaning treatment (**Table 6**).

Gastro-intestinal tract development

Post-mortem analysis indicated that the caecum of LL piglets was longer and tended to be heavier than the caecum of CC piglets (**Table 7**). Treatment did not affect the other segment lengths, empty weights and digesta weights. The proximal stomach digesta pH was higher for LL piglets than CC piglets, and the distal stomach and colon digesta pH did not differ between treatments. Compared to CC piglets, LL piglets had lower glucose and FITC passage through the colon wall, but not through the jejunum wall, and the TRITC passage through the jejunum and colon wall was not affected by treatment (**Table 7**).

Table 6. Post-weaning average daily gain (ADG), final body weight, dry matter intake (DMI) of pellets, BSFL, and both pellets and BSFL combined (total), and days with (watery) diarrhoea of piglets that had access to either only creep feed (Pre-C) or creep feed and live black soldier fly larvae (Pre-L) pre-weaning, and consequently had access to no larvae (Post-C) or had access to live black soldier fly larvae (Post-L) post-weaning. P-values of the effect of pre-weaning treatment (Pre), post-weaning treatment (Post) and their interaction (Pre*Post) are presented, and significant effects ($p < 0.05$) are indicated in **bold** and trends ($p < 0.1$) are indicated in *italic*. Data are presented as pen means ± SEM.

Measure	**Pre-C**		**Pre-L**		**P-value**		
	Post- C	**Post-L**	**Post-C**	**Post-L**	**Pre**	**Post**	**Pre*Post**
ADG (g/pig/day)							
d0-1	93 ± 58	26 ± 31	-41 ± 42	6 ± 35	*0.073*	0.831	0.199
d1-2	284 ± 24	216 ± 23	315 ± 20	221 ± 22	0.402	**<0.001**	0.537
d2-7	149 ± 8	100 ± 17	168 ± 20	145 ± 20	*0.055*	**0.032**	0.408
d7-14	369 ± 15	340 ± 16	343 ± 20	324 ± 22	0.228	0.161	0.777
d14-21	563 ± 19	552 ± 24	482 ± 25	495 ± 33	**0.009**	0.981	0.626
Total d0-21	365 ± 10	333 ± 10	329 ± 16	316 ± 16	*0.060*	0.118	0.497
BW (kg) at d21	15.1 ± 0.2	14.5 ± 0.2	14.6 ± 0.4	14.5 ± 0.4	0.363	0.236	0.927
DMI (g/pig/day) pellets							
d0-1	183 ± 20	115 ± 12	148 ± 19	114 ± 15	0.226	**0.002**	0.268
d1-2	233 ± 17	115 ± 8	212 ± 15	117 ± 10	0.489	**<0.001**	0.371
d2-7	208 ± 9	139 ± 10	210 ± 11	142 ± 14	0.779	**<0.001**	0.949
d7-14	385 ± 13	245 ± 12	357 ± 24	235 ± 18	0.233	**<0.001**	0.600
d14-21	746 ± 27	484 ± 17	647 ± 30	459 ± 25	**0.013**	**<0.001**	0.125
Total d0-21	438 ± 14	281 ± 10	394 ± 16	271 ± 13	*0.059*	**<0.001**	0.239
DMI (g/pig/day) BSFL							
d0-1	-	54 ± 5	-	56 ± 6	0.802	-	-
d1-2	-	61 ± 3	-	66 ± 5	0.374	-	-
d2-7	-	28 ± 3	-	39 ± 3	**0.021**	-	-
d7-14	-	77 ± 4	-	86 ± 4	*0.057*	-	-
d14-21	-	136 ± 0	-	135 ± 0	0.339	-	-
Total d0-21	-	82 ± 1	-	88 ± 2	*0.056*	-	-

Measure	Pre-C		Pre-L		P-value		
	Post- C	Post-L	Post-C	Post-L	Pre	Post	Pre*Post
DMI (g/pig/day) total							
d0-1	183 ± 20	169 ± 13	148 ± 19	169 ± 18	0.243	0.781	0.243
d1-2	233 ± 17	176 ± 8	212 ± 15	183 ± 10	0.614	**0.001**	0.260
d2-7	208 ± 9	167 ± 10	210 ± 11	181 ± 13	0.427	**0.002**	0.569
d7-14	385 ± 13	323 ± 13	357 ± 24	321 ± 19	0.361	**0.002**	0.407
d14-21	746 ± 27	620 ± 17	647 ± 30	594 ± 26	**0.013**	**<0.001**	0.128
Total d0-21	438 ± 14	363 ± 10	394 ± 16	359 ± 14	*0.091*	**<0.001**	0.170
Diarrhoea (# of days)							
Diarrhoea	6.0 ± 0.8	6.2 ± 0.7	5.1 ± 0.6	6.0 ± 1.0	0.355	0.416	0.615
Watery diarrhoea	1.7 ± 0.4	1.6 ± 0.2	1.9 ± 0.3	2.0 ± 1.0	0.903	0.696	0.669

Table 6 continued.

Post-weaning home pen behaviour

The effects of pre-weaning treatment, post-weaning treatment, day, and their two-way interactions on the piglets' time spent on distinct home-pen behaviours post-weaning are shown in **Figure 5**. Significant effects are discussed below.

Ingestive behaviour

Pigs in the Pre-C treatment spent more time eating feed than those in the Pre-L treatment (Pre-C: 9.0 ± 0.3%, Pre-L: 7.8 ± 0.3%), and pigs in the Post-C treatment spent more time eating feed than pigs in the Post-L treatment (Post-C: 9.8 ± 0.3%, Post-L: 7.0 ± 0.3%). On d15 and 20 the time spent eating feed was higher than on d8 (d8: 7.5 ± 0.4%, d15: 8.8 ± 0.4%, d20: 9.0 ± 0.3%, **Figure 5A**). The time spent eating larvae decreased over time; it was higher on d8 than d15 and 20, and it was higher on d15 than on d20 (d8: 9.8 ± 0.6%, d15: 7.6 ± 0.6%, d20: 5.4 ± 0.5%, **Figure 5B**).

Table 7. Post-mortem gastro-intestinal tract measures on d3 post-weaning.

Variable	**CC**	**LL**	**P-value**
Body weight d2 (kg)	8.43 ± 0.13	8.43 ± 0.17	1.000
Segment length			
Small intestine (m)	10.6 ± 1.4	11.0 ± 1.0	0.399
Caecum (cm)	13.5 ± 4.1	16.2 ± 5.2	**0.046**
Colon (cm)	174.2 ± 31.4	176.6 ± 20.4	0.837
Segment empty weight (g)			
Stomach	61.6 ± 7.3	68.0 ± 14.2	0.178
Small intestine	299.7 ± 53.0	324.0 ± 54.0	0.268
Caecum	19.8 ± 4.0	23.2 ± 6.6	*0.080*
Colon	66.7 ± 14.7	69.9 ± 20.3	0.546
Digesta weight (g)			
Stomach	195.2 ± 79.4	181.2 ± 62.6	0.650
Small intestine	179.5 ± 27.5	147.9 ± 19.1	0.486
Caecum	47.9 ± 25.7	59.6 ± 16.3	0.205
Colon	109.7 ± 48.0	101.8 ± 38.5	0.653
Digesta pH			
Proximal stomach	3.96 ± 0.15	4.71 ± 0.29	**0.017**
Distal stomach	3.34 ± 0.24	3.10 ± 0.34	0.563
Colon	6.26 ± 0.06	6.29 ± 0.11	0.801
Glucose passage (µg/cm^2 sac surface)			
Jejunum	10.7 ± 2.8	6.2 ± 1.4	0.164
Colon	4.9 ± 1.5	2.0 ± 0.8	**0.043**
FITC passage (ng/cm^2 sac surface)			
Jejunum	88 ± 7	89 ± 9	0.955
Colon	163 ± 18	112 ± 14	**0.036**
TRITC passage (ng/cm^2 sac surface)			
Jejunum	65 ± 4	71 ± 8	0.472
Colon	96 ± 11	77 ± 6	0.153

Gastro-intestinal tract segment length, segment weight, digesta weight, digesta pH and marker passage through the everted intestinal sacs of piglets that had no access to larvae (CC) or pigs that had access to live black soldier fly larvae during the pre- and post-weaning period (LL). Significant effects ($p < 0.05$) are indicated in **bold** and trends ($p < 0.1$) are indicated in *italic*. Data are presented as pig means ± SEM.

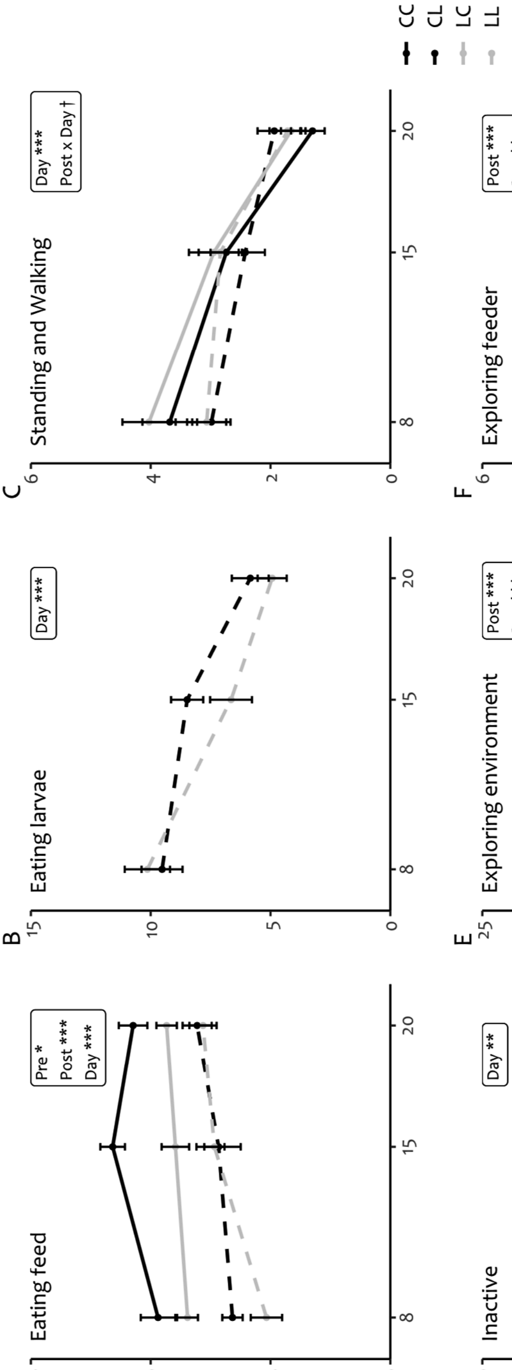
Activity

Activity was not affected by pre- or post-weaning treatment. The time spent standing and walking decreased over time, as it was higher on d8 than on d15 and 20, and it was higher on d15 than on d20 (d8: 3.4 ± 0.2%, d15: 2.7 ± 0.2%, d20: 1.7 ± 0.1%, **Figure 5C**). At the same time, the time spent inactive increased over time, as it was lower on d8 and 15 compared to d20 (d8: 57.8 ± 1.1%, d15: 59.0 ± 1.2%, d20: 61.8 ± 0.9%, **Figure 5D**).

Exploratory behaviour

Pre-weaning treatment did not affect exploratory behaviour. Post-C piglets explored the environment more than Post-L piglets (Post-C: 16.6 ± 0.7%, Post-L: 12.5 ± 0.5%). Overall, piglets spent more time exploring the environment on d8 than on d15 and 20 (d8: 17.4 ± 0.9%, d15: 13.2 ± 0.7%, d20: 13.0 ± 0.6%, **Figure 5E**). Post-L piglets spent more time exploring the experimental feeder (including tubes) than Post-C piglets (Post-C: 1.0 ± 0.1%, Post-L: 4.0 ± 0.2%), and the time spent exploring the enrichment device was higher on d15 than on d8, with d20 in between (d8: 2.1 ± 0.3%, d15: 2.8 ± 0.3%, **Figure 5F**).

Figure 5 (next page). Time spent on behaviours scored during the post-weaning home pen observations of piglets that had access to either only creep feed (Pre-C) or creep feed and live black soldier fly larvae (Pre-L) pre-weaning, and consequently had access to no larvae (Post-C) or had access to live black soldier fly larvae (Post-L) post-weaning, resulting in treatments CC, CL, LC and LL. Any effects of pre-weaning treatment, post-weaning treatment, day, and their 2-way interactions are indicated as † ($p < 0.1$), * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$), and non-significant ($p > 0.1$) effects are not indicated. Data are presented as pen means ± SEM.


A
Eating feed
Pre *
Post ***
Day ***
Time (% of scans)
15
10
5
0
8
15
20
B
Eating larvae
Day ***
15
10
5
0
8
15
20
C
Standing and Walking
Day ***
Post x Day †
6
4
2
0
8
15
20
CC
CL
LC
LL
D
Inactive
Day **
Time (% of scans)
80
60
40
20
0
8
15
20
Days after weaning
E
Exploring environment
Post ***
Day ***
25
20
15
10
5
0
8
15
20
Days after weaning
F
Exploring feeder
(including tubes)
Post ***
Day **
6
4
2
0
8
15
20
Days after weaning

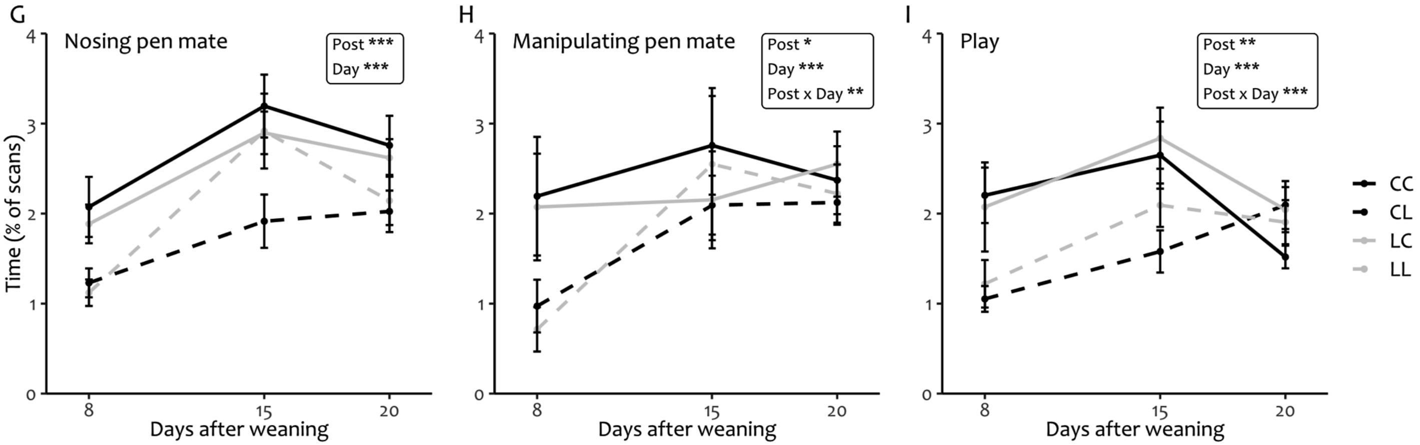


Figure 5 continued.

Pig-directed behaviour

Pig-directed behaviour was not influenced by pre-weaning treatment. Compared to Post-C piglets, Post-L piglets spent less time nosing pen mates (Post-C: 2.6 ± 0.1%, Post-L: 1.9 ± 0.1%). Also, less time was spent on nosing pen mates on d8 than on d15 and 20 (d8: 1.6 ± 0.1%, d15: 2.7 ± 0.2%, d20: 2.4 ± 0.3%, **Figure 5G**). The time spent on oral manipulation of pen mates was affected by post-weaning treatment, day, and their two-way interaction. Post-hoc analysis indicated that only on d8 Post-C piglets spent more time performing this behaviour than Post-L piglets (Post-C: 2.1 ± 0.3%, Post-L: 0.8 ± 0.2%). Post-C piglets did not differ in their time spent on oral manipulation of pen mates over time, while Post-L piglets spent less time on oral manipulation of pen mates on d8 compared to d15 and 20 (d8: 0.8 ± 0.2%, d15 2.3 ± 0.3%, d20: 2.2 ± 0.2%, **Figure 5H**).

Play behaviour

The time spent playing was affected by post-weaning treatment, day, and their two-way interaction, but not by pre-weaning treatment. Post-C piglets spent more time playing than Post-L piglets on d8 (Post-C: 2.1 ± 0.3%, Post-L: 1.1 ± 0.1%) and on d15 (Post-C: 2.7 ± 0.2%, Post-L: 1.8 ± 0.2%), and they spent more time playing on d15 than on d20 (d15: 2.7 ± 0.2%, d20: 1.8 ± 0.1%). Post-L piglets spent more time playing on d15 and 20 than on d8 (d8: 1.1 ± 0.1%, d15: 1.8 ± 0.2%, d20: 2.0 ± 0.2%, **Figure 5I**).

Affective state

Novel Environment Test (NET)

The first factor obtained from the factor analysis on the NET variables had high positive loadings for the time spent moving and the vocalizing frequency, and high negative loadings for the time spent exploring the environment and the latency to explore the feed bowl. The second factor had high positive loadings for the time spent standing and standing alert, and high negative loadings for the time spent exploring the feed bowl (**Table 4**). Pre-weaning treatment, post-weaning treatment and their interaction did not affect the piglets' scores on both factors (Factor 1: CC -0.09 ± 0.18, CL 0.23 ± 0.14, LC 0.06 ± 0.18, LL -0.19 ± 0.29, pre-weaning treatment: p = 0.505, post-weaning treatment: p = 0.864, interaction: p = 0.157; Factor 2: CC 0.06 ± 0.12, CL 0.14 ± 0.23, LC -0.04 ± 0.23, LL -0.16 ± 0.19, pre-weaning treatment: p = 0.324, post-weaning treatment: p = 0.946, interaction: p = 0.621).

Attention Bias Test (ABT)

Comparing behaviour of piglets that did or did not receive a threat during the ABT showed that piglets receiving a threat spent less time moving and tended to spend more time standing alert. No other behaviours were affected by the threat (**Table 8**). During the threat, Pre-L piglets paid more attention to the threat than Pre-C piglets. No other behaviours performed during the threat were affected by pre-weaning treatment, post-weaning treatment, or their interaction (**Table 9**).

Table 8. Behaviour performed in the Attention Bias Test with or without receiving a threat.

Behaviour	With threat	Without threat	P-value
Moving (% of time)	24.0 ± 1.5	29.5 ± 2.1	**0.047**
Standing (% of time)	2.5 ± 0.4	3.3 ± 0.6	0.291
Standing alert (% of time)	48.5 ± 2.6	39.7 ± 3.5	*0.054*
Exploring environment (% of time)	20.5 ± 1.7	22.3 ± 3.0	0.538
Exploring feed bowl (% of time)	4.4 ± 0.8	5.2 ± 1.5	0.603
Latency exploring feed bowl (s)	73.1 ± 9.6	56.8 ± 12.4	0.343
Eliminating (frequency)	0.5 ± 0.1	0.4 ± 0.2	0.327
Vocalizing (frequency)	46.2 ± 4.1	55.9 ± 7.0	0.204

Significant effects (p < 0.05) are indicated in **bold** and trends (p < 0.1) are indicated in *italic*. Data are presented as pen means ± SEM.

The first factor obtained from the factor analysis on piglet behaviour in the ABT during the 150 s after the threat had high positive loadings for the time spent moving, time spent exploring the feed bowl, and vocalizing frequency, and high negative loadings for the time spent paying attention to the threat location, time spent standing alert and latency to explore the feed bowl. The second factor had a high positive loading for time spent exploring the environment and a high negative loading for time spent alert. The third factor had a high positive loading for the time spent standing and the eliminating frequency (**Table 4**). The pre-weaning treatment, post-weaning treatment, and their interaction did not affect the piglets' scores on any of the factors (Factor 1: CC 0.11 ± 0.27, CL -0.11 ± 0.17, LC 0.40 ± 0.24, LL -0.28 ± 0.26, pre-weaning treatment: p = 0.781, post-weaning treatment: p = 0.195, interaction: p = 0.511; Factor 2: CC -0.27 ± 0.37, CL -0.02 ± 0.16, LC -0.03 ± 0.13, LL 0.17 ± 0.26, pre-weaning treatment: p = 0.366, post-weaning treatment: p = 0.229, interaction: p = 0.915; Factor 3: CC -0.24 ± 0.17, CL 0.10 ± 0.32, LC 0.32 ± 0.27, LL -0.08 ± 0.28, pre-weaning treatment: p = 0.428, post-weaning treatment: p = 0.976, interaction: p = 0.111).

Table 9. Behaviour performed in the Attention Bias Test during the 10s threat.

Behaviour	Pre-C		Pre-L		P-value		
	Post- C	Post-L	Post-C	Post-L	Pre	Post	Pre*Post
Attention to threat (% of time)	50.0 ± 5.2	50.2 ± 6.5	67.2 ± 4.2	66.3 ± 5.4	**0.008**	0.760	0.994
Moving (% of time)	45.6 ± 5.5	43.4 ± 7.1	48.7 ± 7.2	44.4 ± 5.5	0.757	0.387	0.992
Standing alert (% of time)	32.7 ± 6.2	47.3 ± 7.0	39.3 ± 7.7	40.1 ± 6.6	0.987	0.246	0.321
Exploring environment (% of time)	13.4 ± 5.6	5.1 ± 2.1	7.4 ± 2.0	6.7 ± 2.1	0.664	0.178	0.284
Exploring feed bowl (% of time)	6.5 ± 3.7	2.9 ± 1.6	2.9 ± 1.9	5.5 ± 3.4	0.897	0.935	0.283
Vocalizing (frequency)	0.6 ± 0.2	1.2 ± 0.5	0.25 ± 0.2	0.8 ± 0.4	0.436	0.296	0.417

Behaviour performed during the 10 s threat of pigs that had access to either only creep feed (Pre-C) or creep feed and live black soldier fly larvae (Pre-L) pre-weaning, and consequently had access to no larvae (Post-C) or had access to live black soldier fly larvae (Post-L) post-weaning. P-values of the effect of pre-weaning treatment (Pre), post-weaning treatment (Post) and their interaction (Pre*Post) are presented, and significant effects ($p < 0.05$) are indicated in **bold**. Data are presented as pen means ± SEM.

Discussion

We investigated the effect of providing piglets with live black soldier fly larvae (BSFL) as edible enrichment during the pre- and/or three weeks post-weaning period. Pre-weaning larvae provisioning did not improve feed intake and body weight gain and had no effect on the indicators of affective state. However, it did lead to several changes in gastro-intestinal tract development around weaning.

Post-weaning larvae provisioning reduced post-weaning feed intake but not total body weight gain, and it reduced oral manipulation of pen fixtures and pen mates.

Pre-weaning

Larvae provisioning from d3 after birth until weaning did not influence the total time spent on eating, the number of piglets eating, and the distribution of total (creep feed and/or larvae) eater types. Previously, it was found that providing a diverse diet (including creep feed, celery, cereal honey loops, and peanuts in shell) improved overall feed intake and time spent eating compared to providing only creep feed (Middelkoop et al., 2019b), and providing creep feed that varied daily in flavour (including various fruity and sweet flavours) increased feed intake and feeder visits compared to providing creep feed with a uniform flavour (Adeleye et al., 2014). In these studies, the degree of dietary diversity was relatively high, as respectively four different feed items and five different flavours were used, compared to two feed items applied in the current study. Under natural conditions, young piglets also sample a large variety of feed items (reviewed by Ballari and Barrios-García, 2014), and a higher degree of dietary diversity may be required to improve pre-weaning feed intake. In line with this, we see that piglets that received larvae before weaning (Pre-L piglets) spent a similar amount of time eating as piglets provided with two types of creep feed simultaneously (Middelkoop et al., 2018), while their time spent eating was substantially lower than that of piglets provided with four feed items simultaneously (Middelkoop et al., 2019b) in studies with a similar set-up and with similar observation periods as the current study. As such, providing only larvae in addition to creep feed was not sufficient to increase the time spent eating before weaning.

Furthermore, the absence of any effect of pre-weaning larvae provisioning on total eating behaviour may in part be due to the relatively high percentage of control (Pre-C) piglets that had consumed creep feed at weaning. Almost 100% of Pre-C piglets had sampled creep feed at weaning, which is higher than previously reported (e.g., ± 34% (Shea and Beaulieu, 2014), ± 48% (de Ruyter et al., 2017), ± 70% (Bruininx et al., 2004) at weaning on d28). The relatively high percentage of eaters per litter can be a function of multiple factors that were previously found to affect (creep) feed intake, such as large pen size (Laskoski et al., 2021), numerous feeding spaces (He et al., 2016; Sulabo et al., 2010c; Wattanakul et al., 2005), early feed provisioning (Sulabo et al., 2010d), higher weaning age (Callesen et al., 2007b), and/or creep feed composition (Callesen et al., 2007a, 2007b; Huting et al., 2021). The large amount of time spent eating of Pre-C piglets may have abated any beneficial effects of larvae provisioning.

While total eating behaviour did not differ, the time spent eating creep feed and the percentage of creep feed eaters per litter were negatively affected by larvae provisioning, particularly close to weaning. Consequently, the distribution of creep feed eater types was more skewed towards worse creep feed eaters in the Pre-L compared to the Pre-C treatment. Previously, providing a diverse diet also decreased consumption of creep feed compared to when only creep feed was provided (Middelkoop et al., 2019b). In that study, piglets preferred exploring the other feed items, and this was correlated to an increased time spent eating these feed items. Other studies also indicated that facilitating exploration, for example by means of a play feeder, draws more piglets to the feeder (Middelkoop et al., 2019a) and can increase feed consumption (Kuller et al., 2010). Indeed, exploring feed items, or "foraging", is often a precursor for feed intake (Studnitz et al., 2007). In the current study, interacting with larvae was also preferred over interacting with creep feed by Pre-L piglets, and Pre-L piglets generally spent more time exploring feed than Pre-C piglets. Larvae presumably facilitate exploration due to their preferred nutritional (high fat/protein) and textural (high moisture content) attributes (Ipema et al., 2021b), and this coincides with an increased time spent eating larvae at the expense of creep feed. Actual consumption of feed items could not be measured in the current study, so the effects on that are unknown. Despite the preference for larvae, of all Pre-L piglets that sampled solid feed before weaning approximately 94% (based on behavioural observations) sampled both creep feed and larvae at least once.

Because creep feed and BSFL have a different nutritional composition, and Pre-C and Pre-L piglets differed in their time spent eating creep feed and larvae, treatment may have influenced nutrient intake. However, pre-weaning piglet growth and weaning weight were not influenced by treatment. Any potential effects on growth caused by differences in nutrient uptake from the provided feed items were likely overruled by the uptake of nutrients from the sow's milk, as this is the piglets' main nutrient source during lactation.

Post-weaning

Some gastrointestinal tract measures on d3 post-weaning were affected by larvae provisioning. First, the proximal stomach digesta of piglets provided with larvae around weaning (LL piglets) had a higher pH than that of piglets without larvae (CC piglets). The proximal region of the stomach acts as a feed reservoir (Kelly, 1980). LL piglets consumed whole larvae that are more rigid than feed pellets and may also be less easily digested because chitin in the larvae integument can hinder protein digestion (Marono et al., 2015). These larvae may have hindered mixing of gastric juices with feed in the proximal stomach. This was also found in previous studies comparing rigid (e.g., roasted almonds) to soft (e.g., cooked rice) feed items (Bornhorst et al., 2014; Nadia et al., 2021). The consequences of a higher proximal stomach digesta pH for nutrient utilization are unclear, as this depends on numerous other factors such as gastric emptying rate and buffering capacity (Bornhorst et al., 2014; Nadia et al., 2021).

The caecum of LL piglets was longer and marginally heavier than that of CC piglets. This could also be attributed to the larvae's larger size and potentially lower digestibility compared to creep feed. If certain feedstuffs cannot be digested in the small intestine, a larger volume of undigested feed will reach the large intestine (van Erp et al., 2020; van Hees et al., 2019). This can affect large intestine development, for example by increasing the size and/or weight of the cecum and colon (Choudhury et al., 2021; van Erp et al., 2020; van Hees et al., 2019) Larvae consumption could have similarly increased cecum fill and thereby promoted cecum growth. However, colon size and weight and digesta weight were not affected by larvae provisioning, and the exact mechanisms causing the observed effect require further investigation.

While colon size was not affected by treatment, passage of FITC through the colon wall was lower in LL piglets compared to CC piglets, though TRITC passage was only numerically reduced. It is expected that the smaller FITC molecule (4 kD) is transported paracellularly, while the larger TRITC molecule (40 kD) is likely transported both trans- and paracellularly. Thus, only paracellular transport seems to be higher in CC piglets, and this can increase the chance of pathogens crossing the intestinal epithelium (as reviewed by Modina et al., 2019; Pluske et al., 2018; Wijtten et al., 2011), posing a health risk. As suggested above, the undigested fraction of larvae may have increased the large intestinal fill in LL piglet. Increasing large intestinal fill was previously found to benefit colonic intestinal barrier function by changing the microbiota composition (Chen et al., 2015) or by promoting epithelial cell differentiation (Mariadason et al., 1999), and the undigested fraction of larvae may have had a similar effect.

Finally, LL piglets had significantly less glucose passage through the colon wall and numerically less glucose passage through the jejunum wall than CC piglets. Intestinal glucose absorption occurs through active NA^+ dependent transport, which happens mainly in the small intestine (Kellett et al., 2008). CC piglets spent more time eating creep feed while LL piglets spent more time eating BSFL, and because creep feed has a much higher carbohydrate level than BSFL, the carbohydrate intake and intestinal carbohydrate level of CC piglets was likely higher. Intestinal carbohydrate levels are positively linked to the expression of glucose transporters in the intestine (Moran et al., 2010), therefore LL piglets may have had lower intestinal glucose transporter expression, possibly explaining the lower glucose passage across the intestinal wall. Around weaning, decreased glucose passage could be an indication of impaired intestinal barrier function (Modina et al., 2019), however this is not supported by the observed FITC and TRITC passage rates.

Overall, the presence or absence of live BSFL around weaning had diverging effects on GIT development and functioning, and most observed differences were minor. Accordingly, pre- and post-weaning BSFL provisioning had no effect on the number of days piglets had (watery) diarrhoea. Post-weaning diarrhoea is a multi-factorial problem (reviewed by Heo et al., 2013), and feed intake has had contrasting effects on diarrhoea occurrence (Callesen et al., 2007a; Carstensen et al., 2005; Middelkoop et al., 2020a, 2019b). Including BSFL fat in weanling piglets' diet also did not affect diarrhoea rate during four weeks post-weaning (Yu et al., 2020a). More research is required to determine the exact mechanisms by which live BSFL consumption affects GIT development, intestinal permeability, and diarrhoea occurrence.

Pre-weaning treatment influenced post-weaning performance. Pre-C piglets tended to grow faster during d0-1, while Pre-L piglets tended to grow faster during d2-7 post-weaning, irrespective of feed intake. Significant differences only occurred during d14-21 post-weaning, where Pre-C piglets ate more and grew faster than Pre-L piglets. The timing of these effects contrasts with several other studies that found benefits of increased pre-weaning feed intake mainly directly after weaning (Berkeveld et al., 2007; Langendijk et al., 2007; Lee et al., 2021; Pluske et al., 2007b), often followed by a reduction in treatment differences over time (Berkeveld et al., 2007; Langendijk et al., 2007; Lee et al., 2021). The inconsistent results directly after weaning may be due to the confounding effect of post-weaning treatment on performance. Also, there was a high variation in growth rate between pens directly after weaning. During week 3 post-weaning piglet performance became less variable, and an improved performance of Pre-C piglets became apparent. However, body weight on d21 was not affected by pre-weaning treatment, likely because of the variability in growth rate and feed intake throughout the three weeks post-weaning. The results suggest that pre-weaning larvae provisioning does not aid the dietary transition at weaning as opposed to providing only creep feed. Even though increased feed exploration pre-weaning was previously found to benefit post-weaning feed intake (Middelkoop et al., 2019a), in the current study the effects of increased exploration facilitated by larvae were likely superseded by the high time spent eating creep feed of Pre-C piglets. Creep feed is more similar in texture and nutritional composition to the weaner diet than larvae, and familiarity with these features presumably eased the weaning transition (Heo et al., 2018) and improved post-weaning feed intake and growth in Pre-C piglets. Similarly, familiarity with larvae before weaning also marginally increased larvae intake after weaning. Due to the relatively low intake of larvae compared to weaner feed, this did not affect post-weaning piglet performance.

Compared to the pre-weaning treatment, the post-weaning treatment had a higher impact on post-weaning performance, as the time spent eating and the pellet and total dry matter intake were continuously higher for Post-C piglets than for Post-L piglets. These results contradict with other studies where enrichment such as extra space, straw and/or peat improved piglet feed intake (Luo et al., 2019; Oostindjer et al., 2010). In contrast to straw and peat, larvae have a high nutritional value and were therefore expected to have a different impact on feed intake and performance. Larvae are very palatable due to their high levels of fat and protein (Makkar et al., 2014), and short preference tests indicated that eating larvae is preferred over eating regular feed pellets (Ipema et al., 2021b). In line with this, both the current and a previous study observed high levels of interaction with larvae, and a simultaneous reduction in time spent eating feed (Ipema et al., 2021a). The high intake of larvae likely increased the feeling of satiety (Ratanpaul et al., 2019), and as such it reduced the motivation to eat pellets and subsequently lowered total feed consumption. Additionally, the consumption rate of larvae seems to be slower than that of pellets, as Post-L piglets spent equal amounts of time eating larvae and pellets, but the dry matter intake of larvae was substantially lower. Therefore, larvae may satisfy the exploratory and eating motivation of piglets at lower intake levels than feed, resulting in a lower overall feed intake. It must be noted that, despite the increased feed intake, Post-C piglets only experienced a temporary higher growth rate, and body weight on d21 post-weaning was not affected by post-weaning treatment. This indicates that Post-L piglets maintained a similar growth rate as Post-C piglets despite the lower feed intake, and suggests that Post-L piglets may have been more efficient in their feed conversion, though this must be confirmed in future studies.

Concerning behaviour, pre-weaning treatment only influenced the time spent eating, whereas post-weaning treatment influenced a range of behaviours. The larger effect on behaviour of the current environment as opposed to the former environment was expected as the presence of larvae mainly influences these behaviours, and this was also observed previously for numerous enrichment items (Oostindjer et al., 2011b; Vanheukelom et al., 2011). Post-C piglets spent more time on exploring the environment and nosing pen mates on all observation days, and they spent more time on manipulating pen mates and playing on some of the observation days. On the other hand, Post-L piglets continuously spent more time on exploring the enrichment device and eating larvae. Overall, larvae provisioning clearly facilitated exploratory behaviours, redirecting exploration away from the pen and pen mates. These results are similar to a study in which small amounts of larvae were provided for 11 days post-weaning (Ipema et al., 2021a). Exploring larvae is likely more satisfying than exploring pen fixtures or other pigs, as larvae have more characteristics that are preferred by pigs, such as being odorous, destructible and edible (van de Weerd et al., 2003). Furthermore, pig-directed oral manipulation has been associated with a higher presence of painful lesions and wounds (Straw and Bartlett, 2001; Ursinus et al., 2014), therefore redirecting exploration away from pigs by providing larvae can benefit piglet welfare.

Some effects of larvae provisioning on behaviour varied over time. Post-C piglets spent more time manipulating pen mates and playing than Post-L piglets only on d8 and d8 and 15 post-weaning, respectively. For Post-L piglets, the time spent eating larvae decreased on d15 and 20 compared to d8, and concurrently the time spent manipulating pen mates and playing increased over time. Therefore, it seems piglets redirected their activity away from larvae and towards their pen mates later in the post-weaning period, explaining the absence of treatment effects during those days. It is unlikely that piglets lost interest in the larvae over time, as most pens consumed the maximum amount of larvae every day in week 3 post-weaning. A more likely explanation is that piglets became more efficient over time in retrieving the larvae from the tubes, as this was also observed in a previous study where piglets had access to tubes containing larvae (Ipema et al., 2021b). In the current study, larvae were provided at the same time every day, and this temporal predictability may have exacerbated beneficial effects on behaviour and welfare (Bassett and Buchanan-Smith, 2007; Dudink et al., 2006), though it may also have diminished interaction with the enrichment device containing larvae in-between provisioning moments. Prolonging the engagement with larvae may require changes in the amount, manner and/or timing of larvae provisioning.

Piglets that received a threat during the Attention Bias Test (ABT) spent less time moving and more time standing alert than piglets that did not receive a threat. Reduced locomotion and increased vigilance have previously been observed in piglets in response to a novel stimulus and have been linked to increased fearfulness (Dalmau et al., 2009; Forkman et al., 2007; Ursinus et al., 2013), suggesting that the negative stimulus used in the ABT was considered a threat. Contrarily, the applied positive stimulus, namely a feed bowl filled with feed pellets mixed with corn and raisins, did not receive as much attention from the pigs compared to a previous study where feed mixed with chocolate peanuts and carrots was provided in an ABT (Luo et al., 2019). This may be a result of the generally low and variable feed intake recently weaned piglets (Dong and Pluske, 2007), causing these piglets to not yet be habituated to the feed and not consider it a positive stimulus. To improve the design of the ABT for recently weaned piglets, providing a different positive stimulus may be required.

Both factors retrieved from the factor analysis on the Novel Environment Test (NET) responses included behaviours that have previously been linked to fearfulness, such as low exploration of the environment and a high frequency of vocalizations in Factor 1, and a high time standing alert in Factor 2 (Donald et al., 2011; Ursinus et al., 2013). Pre- and post-weaning treatment did not influence piglets' responses during the NET, therefore we have no indication that larvae provisioning affected piglets' fearfulness. Environmental enrichment found to decrease piglet fearfulness includes marginally increased space and more toys (Tönepöhl et al., 2012), hanging ropes and tyres (Rodarte et al., 2004), and live BSFL provided during 11 days after weaning (Ipema et al., 2021a). Compared to this last study that also included larvae provisioning, the NET in the current study was performed closer to weaning (d4 instead of d10-11 post-weaning), therefore there was less time for post-weaning larvae provisioning to impact piglet fearfulness, possibly explaining the contradictory results. Pre-L piglets did have extensive experience with larvae provisioning before weaning, however pre-weaning treatment also did not affect NET responses. Previously, pre-weaning dietary diversity also did not influence NET responses at weaning (Middelkoop et al., 2020b). It appears that increasing dietary diversity by providing creep feed and larvae before weaning does not habituate piglets more to novelty than providing only creep feed in the current experimental setting. Under more barren commercial conditions, effects may differ.

Factor 1 retrieved from the ABT reflects the direction of attention bias of the piglets, where positive scores on this factor relate to an attention bias towards the positive stimulus (the feed bowl) and away from the negative stimulus (the threat location). Larvae provisioning before or after weaning did not affect the piglets' scores on this factor, therefore larvae provisioning did not result in a more positive or negative attention bias, reflective of an animal's affective state (C. Lee et al., 2018; Lee et al., 2016). Previously, enrichment had a positive (Douglas et al., 2012) or no (Carreras et al., 2016; Luo et al., 2019) effect on pig's affective state. As mentioned before, the positive reward provided in the ABT may not have been viewed as positive by all pigs, therefore any bias in attention may not have been related to the positive perception of feed, but more to exploration in general. Also, as observed in the NET, larvae provisioning may not have been sufficient to improve the affective state of newly weaned piglets. Pre-L piglets did pay more attention to the threat during the 10 s the threat was present than Pre-C piglets. Previous studies have associated increased attention towards a threat with either increased (Luo et al., 2019) or reduced (Verbeek et al., 2021) anxiousness. As overall responses to the NET and ABT did not differ, it is possible that the increased attention was not linked to the piglet's affective state. Instead, the increased interest in the threat may be due to a more positive association with a disturbance, caused by regularly receiving more preferred larvae as opposed to only less preferred feed pellets (Ipema et al., 2021b) before weaning. As these results are based on a 10 second period, they should be interpreted with caution.

In conclusion, pre-weaning larvae provisioning increased feed-directed exploration, decreased the time spent eating creep feed, and did not affect the overall time spent eating feed before weaning. Continuous larvae provisioning around weaning affected caecal and colonic development and proximal stomach digesta pH. After weaning, larvae provisioning redirected exploration away from pen fixtures and pen mates and towards the larvae. Larvae provisioning also reduced post-weaning feed intake without affecting piglet growth rate and body weight on d21 post-weaning. Affective state assessed in behavioural tests shortly after weaning were not influenced by larvae provisioning. Overall, larvae were easily accepted from a young age onwards, yet they did not have a large impact on the weaning transition. In the current set-up larvae provisioning was more beneficial for piglet welfare post-weaning compared to piglet feed intake pre-weaning. However, the impaired post-weaning feed intake that accompanied larvae provisioning indicates that a different method or amount of larvae provisioning may be more appropriate to support piglet welfare.

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Supplementary data

Table S1. Nutrient composition of creep feed.

Calculated nutrient composition[1]	**Creep feed**
Net energy	1010
Dry matter	872
Crude protein	208
Crude fat	45
Crude ash	61
Crude fibre	43
Starch	497
Sugars	41
Non-starch polysaccharides[2]	148
Calcium	8.6
Phosphorus	6.5
Magnesium	1.8
Potassium	8.5
Sodium	1.8
Chloride	4.5
Standard ileal digestible lysine	12
Standard ileal digestible methionine	4.5
Standard ileal digestible threonine	7.1
Standard ileal digestible tryptophan	2.3

[1] Calculated nutrient composition according to CVB (2016). Nutrients are presented in g/kg dry matter, except net energy (kJ/100 g) and dry matter (g/kg).

[2] Calculated on a dry matter basis as = 1000 - crude protein - crude fat - crude ash - starch - sugars.

Table S2. Ingredient composition of creep feed.

Ingredient composition[1]	%
Wheat	27.1
Barley	25
Maize	20
Soybean meal	16.5
Sunflower meal	6
Soybean oil	1.3
Vitamin and mineral premix	0.5
Limestone fine	1.3
Monocalcium phosphatase	0.9
Salt	0.4
Citric acid	0.1
L-lysine hydrochloride	0.52
DL-methionine	0.15
L-threonine	0.16
L-tryptophan	0.05
L-valine	0.02

[1] Feed colorant Indigo carmine was included in the feed at 5 g/kg feed.

Part 3: The big picture

Chapter 8

Principles for the responsible use of farmed insects as livestock feed

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Insect farming is a new and rapidly expanding agricultural sector with the potential to make livestock production systems, and therefore human diets, more sustainable. However, to realize this, a responsible and effective use of farmed insects as livestock feed is needed.

Main

It is widely acknowledged that food systems need to be transformed to achieve the Sustainable Development Goals (SDGs, Loboguerrero et al., 2020). Production systems of conventional livestock (including farm animals and fish) are among the systems in dire need for transformation. Livestock plays a key role for global food security, however, intensive livestock production is currently not congruent with many of the SDGs (e.g., SDG 12 Responsible consumption and production and SDG 13 Climate Action) as it harms the environment and impairs animal welfare.

In the context of making conventional livestock production more sustainable, the use of farmed insects as a source of feed for livestock is gaining traction (Dicke, 2018). The idea is to feed farmed insects with the massive quantities of by-products, food losses and waste (here referred to as organic residual streams) we produce in current food systems, and to use the resulting insect biomass as livestock feed. The insect biomass, rich in valuable proteins, fats, vitamins, and minerals, is expected to replace conventional feed ingredients that threaten the climate and biodiversity such as soybean and fishmeal. Using farmed insects as feed not only has the potential to contribute to sustainable and circular livestock production, but could also improve livestock welfare by facilitating natural foraging behaviour and promoting good health (e.g., by reducing broiler leg problems, Ipema et al., 2020b).

However, just feeding insects with organic residual streams and then feeding these insects to livestock will not by default solve the environmental and welfare problems of current livestock production systems. Here, we present seven key principles to guide the responsible use of farmed insects as livestock feed. These principles are based on our view of the current developments in the sector integrated with the outcomes of a four-year research project aimed at holistic assessment of the effects of farmed insects on the environmental, welfare and productivity dimensions of conventional livestock production systems.

0. Prioritize waste reduction

The environmental benefits of using insects are founded on their potential to turn organic waste streams, such as manure, industrial and household food waste, into valuable feed or food. However, from an environmental point of view it is always more efficient to avoid the generation of these waste streams. Preventing food waste is out of the scope of insect producers, but should be the first priority for policy makers, governmental agencies, the private sector, and civil society organizations engaged with rerouting food systems towards a sustainable and food secure future (Muscat et al., 2021). It is important to focus efforts on creating and enabling an environment for food systems that generate less waste, in which insects and other circular interventions are used to upcycle only those nutrients contained in unavoidable waste streams.

1. Avoid unnecessary feed competition between farmed insects and conventional livestock

In the circular economy, organic residual streams such as crop residues, food waste, livestock manure, and food by-products can be used for different purposes such as food, feed, fertilizers, biomaterials, and pharmaceuticals. From these purposes, basic human needs such as food, and by extension feed, are generally considered most important (Muscat et al., 2021), and therefore recovering and reusing waste streams into edible biomass should be prioritized over other uses. In this context, it is key to select the optimal pathways to utilize the streams to maximize the production of biomass edible to humans with the lowest environmental impact. While conventional livestock such as ruminants consume different crop residues, and monogastric animals such as pigs have been traditionally fed on by-products from the food industry and swill (Mottet et al., 2017; zu Ermgassen et al., 2016), now farmed insects are becoming a new pathway to do so. If we use farmed insects as feed, we should not feed them with residual streams that can be directly fed to livestock as this will create unnecessary competition for feedstocks and higher environmental footprints. Thus, it is more responsible and effective to concentrate efforts on rearing insects destined for feed with streams not generally used to feed livestock, such as manure and food waste. Still, in contexts where the available residual streams are not suitable for the local livestock or where streams are disposed directly into landfills, insects could play a role in upcycling nutrients and making them suitable for local and overseas livestock consumption.

2. Ensure farmed insects are safe as livestock feed

Many of the abundant organic residual streams that could be consumed by insects, such as manure and food waste, pose safety risks for livestock and humans. Potential hazards in low-value rearing substrates include heavy metals, (myco)toxins, pathogenic microorganisms, pesticides, parasites, and in case of manure, veterinary drugs and hormones (Imathiu, 2020; van der Fels-Klerx et al., 2018). While in some cases it is known that particular components can accumulate in the insect body mass when present in high concentrations in the feed substrate (e.g., heavy metals, van der Fels-Klerx et al., 2016), in other cases insects can break down or excrete the unsafe components (i.e., mycotoxins) resulting in concentrations below maximum residue limits (Bosch et al., 2017). For some hazards (e.g., parasites) the accumulation potential is still unknown and must be investigated to assess the safety risks of using insects as livestock feed. To overcome safety risks, we must invest in developing strategies for waste segregation (i.e., separation of plant and animal waste), pre-treatment of residual organic streams (e.g., pasteurization, fermentation) and larval processing to eliminate hazards (e.g., fasting, drying, boiling, blanching, acidification, fermentation, and/or freezing). These strategies should be evaluated for unwanted consequences for the environment (e.g., high energy use) and insect welfare. If feed safety cannot be guaranteed, then it is important to evaluate the environmental consequences of using the insect biomass for applications outside the food chain (e.g., pet food, biofuels, and pharmaceuticals) versus not producing insects and using residual organic streams for other purposes in the bioeconomy (e.g., biomaterials, bioenergy, fertilizers).

3. Revise regulatory frameworks

As insect farming develops, regulatory frameworks for farming insects should also be adapted according to local contexts and needs. Three key points for responsible insect farming that require close attention are the permitted feedstocks allowed as insect feed, the collection of wild insects for farming purposes, and biosecurity standards for insect production. In regions such as the

European Union (EU), strict regulatory frameworks for food and feed safety exist, and therefore farmed insects can often only be fed with the same feedstocks currently allowed for conventional livestock. Residual organic streams demonstrated to be suitable to feed insects, such as food waste and manure, are not allowed as insect feed in the EU due to potential feed safety concerns, but in other world regions they are. Without changing regulatory frameworks in the EU and other regions with similar restrictions for use of insect as livestock feed, insect producers will be constrained and motivated to use feedstocks that inevitably promote feed competition between insects and conventional livestock. As soon as more experimental evidence on safety is available, regulatory frameworks must be revised and adapted accordingly.

In different parts of the world, the collection of wild insect populations for rearing purposes is common practice, especially at the initial stage of insect farming. Regulatory frameworks are needed to allow the responsible utilization of wild insect populations while safeguarding biodiversity. Kenya is one of the few countries that already installed clear rules and procedures for the collection of insects from the wild for farming purposes (Niassy et al., 2022). These frameworks could be used by other countries and be adapted for new species that might be farmed in the near future. Lastly, insect farming should be practiced meeting biosecurity standards to minimize the risk of disease outbreaks (Maciel-Vergara et al., 2021) and the escape of insects from production facilities. Although the ecological consequences of insect farming are still largely unknown (Berggren et al., 2019), the escape of non-native and possibly invasive farmed insects could threaten local biodiversity, for example by spreading diseases to wild populations or outcompeting native species. It is therefore important to include biosecurity measures as part of regulatory frameworks that promote responsible insect farming.

4. Optimize performance of insects fed low-value streams

Farmed insects fed low-value residual organic streams such as vegetable waste and manure tend to have lower yields and performance parameters compared to those fed on nutrient-rich and more digestible streams (Spranghers et al., 2017). Driven by the current legislation and motivated by market profits, many large-scale insect producers are attaining high insect yields by feeding insects with customised diets made of a mix of different livestock-edible streams that complement each other nutritionally (e.g., by-products from the potato and bio-ethanol industry). Although this would not be a problem if insects are destined for food, it is a problem when insects are destined for feed, as it promotes feed competition with conventional livestock and therefore the non-responsible use of insects. To avoid this, it is key that the insect production sector and relevant stakeholders (e.g., regulatory agencies) also focus on optimizing low-quality feeds to attain higher yields. Examples of strategies to optimally use low-quality organic residual streams include pre-treatment and co-treatment methods (i.e., physical, chemical and biological, Peguero et al., 2022), combining streams to overcome nutrient deficiencies in pure streams, and genetically improving the performance of larvae and their gut microbiome on specific feeds (Fowles and Nansen, 2019). Successful implementation of these strategies not only requires technological innovation, but also institutional changes. For example, genetic breeding and engineering of farmed insects fosters faster reproduction and more efficient bioconversion of feedstocks (Joosse, 2022; Zhan et al., 2019). The history of adoption of genetically engineered crops and other technological developments has taught us that realizing the innovative potential of insects requires investments by both the private sector and the public sector, and building frameworks for responsible stewardship and equitable access to GMO-insects benefits (Pixley et al., 2019).

5. Balance livestock productivity, welfare, and environmental goals of insect feeding strategies

Alongside aiming for positive environmental consequences of feeding insects to conventional livestock, we should also aim to improve livestock productivity and/or welfare by feeding them insects, though there will be trade-offs between these goals. Important variables determining which goals are favoured include insect species, rearing method, degree of processing (e.g., alive, dead, or as meal) and level of inclusion in livestock diets. Generally, livestock productivity can be increased by including low levels (< 10%) of processed insects (i.e., meal), while higher inclusion levels or providing whole insects maintains or decreases productivity (Ipema et al., 2020b; Moula and Detilleux, 2019). Concerning livestock welfare, many insects contain immunomodulatory components such as lauric acid and antimicrobial peptides which can benefit livestock health, but their presence and availability depends on insect rearing substrates and processing methods (Dörper et al., 2020). In contrast to processed insects, whole insects (i.e., fresh or dried without further processing) have been found to stimulate natural behaviours and activity in poultry (e.g., pecking and scratching litter) and pigs (e.g., rooting), which has clear health and welfare benefits. For instance, low activity levels of broilers cause leg problems such as lameness and foot lesions, and by promoting active foraging behaviour providing whole insects reduced broiler leg problems, and these welfare benefits increased when the larvae inclusion level increased from 5 to 10% (Ipema et al., 2020a, 2020b). Similarly, pigs that received larvae spent less time biting other pigs (Ipema et al., 2021a).

Currently, including low levels of processed insects is often favoured over whole insects as this is considered safer and can increase livestock productivity. However, as it is expected that insect production systems and feeding strategies will be optimized over time, in the future feeding higher amounts of whole insects to livestock could increase the benefits for livestock welfare and the environment. Insect feeding strategies should be continuously re-evaluated and adjusted to balance livestock productivity, welfare, and environmental goals of livestock production systems, in order to avoid unexpected trade-offs and to optimally benefit from using farmed insects as livestock feed.

6. Account for insect welfare

There is increasing evidence that many insect species, including those farmed, possess cognitive and emotional abilities and sentience (Lambert et al., 2021). For black soldier fly larvae, researchers have started to focus on the mechanisms by which large groups of larvae collectively consume feed quickly (Shishkov et al., 2019), and on whether they prefer to consume certain substrates over others (Parodi et al., 2020). Ethicians argue that, just as for conventional livestock species, the intrinsic value of insects must be acknowledged and warranted in commercial production systems (Baracchi and Baciadonna, 2020). In these systems, insect welfare can be compromised by for example suboptimal stocking densities and feed quality, starvation, and killing (e.g., by boiling, freezing, or being fed live to conventional livestock). If insects must be reared and killed to benefit conventional livestock welfare, there is a clear trade-off between insect and livestock welfare that must be evaluated. In this evaluation, we must also consider the use of insects directly as food, as this could reduce the overall impact on animal welfare. While some organizations are promoting welfare standards for production of insects (IPIFF, 2019), a change in ethical perspectives on insect production is required to guide and create legislation that ensures good insect welfare in both commercial rearing systems and research.

To conclude, farmed insects have the potential to improve the environmental sustainability and welfare of livestock production systems, but for that to happen an enabling environment is needed. Insects must be optimally fed with residual organic streams unsuitable for livestock, be safe to use as feed, and, if proven safe, regulatory frameworks need to be revised and an optimal balance must be found between livestock productivity, livestock and insect welfare, and environmental sustainability. For a responsible use of insects as feed, the principles we outlined should be adhered to now the sector is starting to grow. Mistakes made in the past (i.e., food-feed competition and compromised livestock welfare) should not be repeated in this emerging agricultural sector.

Chapter 9

General Discussion

Many commercially reared broilers and pigs suffer from welfare issues that are in part a result of the barren, stimulus-poor environment they are housed in. Black soldier fly larvae (BSFL) can be part of a sustainable livestock diet, and they possess several nutritional and physical characteristics that might make them attractive as edible environmental enrichment. Therefore, it was hypothesized that BSFL provisioning could improve the welfare of broilers and pigs (**Chapter 1**). This thesis aimed to determine the impact of BSFL provisioning on the welfare of broilers (**Chapter 2-4**) and pigs during the weaning transition (**Chapter 6-7**). As it was unknown if pigs were interested in insect larvae, it was first determined whether and to what extent pigs prefer BSFL over other feed items and enrichment materials (**Chapter 5**). To place the use of BSFL as livestock feed and enrichment in the broader context of sustainable livestock production, the outcomes of **Chapter 2-7** were synthesized with knowledge on sustainable BSFL production. Based on this, key principles for the responsible use of insects as livestock feed were developed (**Chapter 8**). In this General Discussion (**Chapter 9**) the findings of the previous chapters are discussed, integrated, and placed in the broader context of existing literature. To guide this discussion, an overview of the applied BSFL treatments per chapter is shown in **Table 1**, and the main effects of each treatment are shown in **Table 2**. The effects of BSFL provisioning on the three pillars of welfare (i.e., natural behaviour, health and functioning, and affective states) will be discussed first for broilers and then for pigs, pinpointing novel insights and remaining knowledge gaps. Within this discussion, several correlational analyses will be presented to explore the complex relationship between the various aspects of animal welfare. Finally, directions for future research and the role of insects in sustainable food systems will be addressed.

Table 1. Treatments applied in **Chapter 2-7**.

Treatment name	Daily amount	BSFL form	Provisioning method	Daily provisioning frequency
Broilers				
Chapter 2				
A5-F2	5% of DDMI	Live	Scattered through pen	2×
A5-F4	5% of DDMI	Live	Scattered through pen	4×
A10-F2	10% of DDMI	Live	Scattered through pen	2×
A10-F4	10% of DDMI	Live	Scattered through pen	4×
Chapter 3				
A5-S4	5% of DDMI	Live	Scattered through pen	4×
A5-S7	5% of DDMI	Live	Scattered through pen	7×
A10-S4	10% of DDMI	Live	Scattered through pen	4×
A10-TB	10% of DDMI	Live	In 5 transparent tubes with holes on the floor	1×
Chapter 4				
INC-F	8% of DDMI	Meal & oil	Incorporated in feed pellets	-
D-F	8% of DDMI	Whole dried	On top of feed pellets	4×
D-S	8% of DDMI	Whole dried	Scattered through pen	4×
L-S	8% of DDMI	Live	Scattered through pen	4×
Pigs				
Chapter 5				
BSFL	150 g/pig	Live	In 2 transparent tubes with holes hanging above separate feeder	1×
Chapter 6				
LAR	75 g/pig (d1-4) 150 g/pig (d5-11)	Live	Scattered through pen	2×
Chapter 7				
Pre-L	*Ad libitum*	Live	In separate feeder	On demand
Post-L	20% of DDMI	Live	In 2 transparent tubes with holes hanging above separate feeder	1-3×

BSFL = black soldier fly larvae, DDMI = daily dry matter intake.

Table 2. Effects of BSFL on the three pillars of welfare as determined in **Chapter 2-7.**

Treatment	**Natural behaviour[1]**	**Health and functioning**			**Affective state[3]**
		Health[2]	**Final weight**	**Daily feed intake**	
Broilers					
Chapter 2					
A5-F2	↑	=	=	=	-
A5-F4	↑	↑	=	=	-
A10-F2	↑	↑	↓	=	-
A10-F4	↑	↑	=	=	-
Chapter 3					
A5-S4	↑	=	=	=	=
A5-S7	↑	=	=	=	↑
A10-S4	↑	↓	=	=	=
A10-TB	↑	=	=	=	↑
Chapter 4					
INC-F	=	↑	=	=	-
D-F	=	=	↑	=	-
D-S	↑	↑	↑	=	-
L-S	↑	↑	↑	↓	-
Pigs					
Chapter 5					
BSFL	↑	-	-	-	-
Chapter 6					
LAR	↑	=	=	=	↑
Chapter 7					
Pre-L	↑	=	=	-	=
Post-L	↑	=	=	↓	=

↑ and ↓ indicate improved/increased or impaired/reduced welfare or performance, respectively, = indicates no significant effects, – indicates not studied. A5 = 5% of dry matter, A10 = 10% of dry matter, F2 = scattered through the pen 2× a day, F4/S4 = scattered through the pen 4× a day, S7 = scattered through the pen 7× a day, TB = in transparent tubes with holes, INC = incorporated in feed pellets, D = dried larvae, L = live larvae, F = provided in the feeder, S = scattered through the pen, LAR = larvae provided, Pre-L = larvae provided pre-weaning, Post-L = larvae provided post-weaning.

[1] Includes foraging and exploratory behaviour.

[2] Includes leg health parameters for broilers and days with (watery) diarrhoea for pigs.

[3] Includes fearfulness (**Chapter 3 & 6**) or fearfulness and degree of optimism (**Chapter 7**).

Effects of BSFL provisioning on the natural behaviour of broilers

The extent to which an animal can perform natural behaviour is an important pillar of animal welfare. Semi- wild red junglefowl, the ancestor of the modern chicken, spend the large majority of their active time performing foraging behaviours such as ground pecking and ground scratching to gather feed items (Dawkins, 1989). Modern fast-growing broilers have maintained the motivation to forage even though they have continuous access to feed (Bokkers and Koene, 2004; Pichova et al., 2016), but their fast growth rate and barren rearing environment does not facilitate foraging behaviour (Bessei, 2006). Facilitating foraging behaviour in broilers under commercial housing conditions by providing enrichment materials has proven difficult (Bailie et al., 2013; Baxter et al., 2018c; Norring et al., 2016). In contrast to many other enrichment materials, this thesis showed that live BSFL provisioning consistently promoted foraging behaviour in broilers throughout the entire rearing period (**Table 2**), resulting in higher overall activity levels and more time spent in standing posture (**Chapter 2-4**). These effects were elevated to a small extent by providing higher amounts of BSFL (i.e., 10% vs. 5% of the daily dry matter (DM) intake), while they were elevated to a large extent by prolonging the interaction time with the larvae, either by increasing the frequency of provisioning or by providing larvae in transparent tubes with holes (**Chapter 3**). These results pinpoint the high attractivity of insect larvae, as suggested previously (Makkar et al., 2014; Pichova et al., 2016), and show, for the first time, that this high attractivity is maintained over the whole rearing period.

Broilers that received live BSFL in tubes showed the highest level of foraging behaviour, spending 23.3% of the observed time on these behaviours, compared to controls that spent 7.6% of the observed time on foraging behaviours (**Chapter 3**). Getting larvae out of the tubes required considerable effort. It is expected that this effort increases the reward value of the larvae as animals generally prefer to work for feed over consuming freely available feed (i.e., contra-freeloading, Osborne, 1977), though for broilers this phenomenon has not been widely studied (Lindqvist et al., 2006). The observed increase in foraging behaviour caused by live BSFL provisioning is much higher than in previous studies using environmental enrichment for broilers. Providing only straw bales, straw bales combined with platforms and moving lasers, or straw bales combined with oat hulls failed to increase the time broilers spent foraging (Bailie et al., 2013; Baxter et al., 2018c; da Silva et al., 2021). Scattering all feed pellets of fast-growing broilers through the pen did greatly increase foraging behaviour compared to controls, from on average 1.0% to 42.2% of the observed time, but this reduced the broilers' final weight and, according to the authors, likely resulted in feed losses (Jordan et al., 2011). The results from **Chapter 2-4** suggest that, compared to other commonly used enrichment materials, live BSFL can be more effective environmental enrichment for broilers, improving their welfare regarding the pillar "natural behaviour".

Chapter 4 showed that incorporating BSFL meal and oil in the feed pellets of broilers does not promote foraging behaviour, as expected. Also, providing dried larvae in the feeder four times a day did not promote foraging behaviour or increase behaviour directed towards the feeder. It is possible that larvae provided in this way are too easy to find and consume, and as such this method of BSFL provisioning does not stimulate foraging behaviour. Scattering dried larvae through the pen did facilitate foraging behaviour throughout the rearing period, resulting in overall higher activity levels compared to controls. However, dried larvae stimulated foraging behaviour to a lesser extent than live larvae (12.5% vs. 16.7% of the observed time spent foraging, respectively), likely due to their low moisture content, increased rigidness, absence of movement and/or lower palatability compared to live larvae (as discussed in **Chapter 4**). It is possible that alternative provisioning methods for dried or live larvae that increase the effort required to obtain the larvae cause a similar or higher level of foraging behaviour than observed in **Chapter 2-4**, warranting further investigation.

Effects of BSFL provisioning on the health and functioning of broilers

Leg health and body weight

Apart from natural behaviour, broiler leg health and body weight are also related to welfare. We observed that for some treatments BSFL provisioning reduced the severity of several leg health problems in **Chapter 2 & 4**, with scattering live BSFL at relatively high amounts (i.e., 8-10% vs. 5% of the daily dry matter intake) being the most effective in improving broiler leg health (**Table 2**). However, no leg health benefits were observed in **Chapter 3** where similar high amounts of live BSFL were applied in two of the treatments, even though these treatments did increase activity (**Table 2**). Due to these contrasting results, the hypothesis that BSFL provisioning benefits leg health could not be conclusively confirmed or refuted. Broiler body weight, which is an important factor affecting leg health (Bessei, 2006; Shepherd and Fairchild, 2010) was also inconsistently affected by BSFL provisioning across chapters. However, this does not explain the discrepancy in effects on leg health between **Chapter 3** and **Chapter 2 & 4**, as in both **Chapter 2 & 3** several BSFL provisioning treatments (temporarily) decreased body weights, whereas the opposite was found in **Chapter 4** (**Table 2**). Broiler activity, posture, weight, and leg health have a complex relationship, and this relationship was further explored by performing several correlational analyses (see **Box 1** for methodology and **Table 3A-D** for results). Since many correlations were checked the results should be treated with caution and considered exploratory.

Box 1: Correlational analyses methodology

All analyses were performed using statistical software SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Because BSFL treatments influenced many investigated parameters, all correlation analyses were controlled for treatment. Pearson correlation coefficients were calculated for normally distributed variables, otherwise Spearman correlations were used. Also, ordinal data were always analysed with a Spearman's correlation test. A preliminary analysis performed separately per treatment indicated that treatment affected the magnitude of the R values but not the direction of the correlations within a chapter, therefore data from all treatments were analysed together per chapter.

Table 3. Correlation coefficients of analyses done on parameters of **Chapter 2-4,** all controlling for treatment. Results are shown from the full analyses **(A)**, or the analyses controlled for Weight **(B)**, Activity **(C)**, or Standing **(D)**.

A) Full

Chapter 2	*Activity*	*Standing*	*Weight*	*HB*
Standing^P	0.79***			
Weight^P	-0.39***	-0.51***		
HB^S	-0.12*	-0.16**	0.22***	
GS^S	-0.16**	-0.35***	0.46***	0.13*
Chapter 3	*Activity*	*Standing*	*Weight*	*HB*
Standing^P	0.88***			
Weight^P	-0.47***	-0.52***		
HB^S	-0.17***	-0.24***	0.33***	
GS^S	-0.28***	-0.31***	0.45***	0.28***
Chapter 4	*Activity*	*Standing*	*Weight*	*HB*
Standing^P	0.69***			
Weight^P	-0.02	-0.16**		
HB^S	-0.08	-0.12**	0.10*	
GS^S	0.00	0.00	0.25**	-0.10

B) Controlled for weight

Chapter 2	*Activity*	*Standing*	*HB*
Standing^P	-0.74***		
HB^S	0.04	-0.06	
GS^S	-0.01	-0.16**	0.03
Chapter 3	*Activity*	*Standing*	*HB*
Standing^P	-0.85***		
HB^S	0.02	-0.09	
GS^S	0.09	-0.12*	0.16**
Chapter 4	*Activity*	*Standing*	*HB*
Standing^P	-0.70***		
HB^S	0.08	-0.10	
GS^S	-0.01	0.02	-0.12

C) Controlled for activity

Chapter 2	*Standing*	*Weight*	*HB*
Weight^P	-0.36***		
HB^S	-0.11*	0.19***	
GS^S	-0.35***	0.43***	0.11*
Chapter 3	*Standing*	*Weight*	*HB*
Weight^P	-0.27***		
HB^S	-0.20***	0.30***	
GS^S	-0.15***	0.37***	0.25***
Chapter 4	*Standing*	*Weight*	*HB*
Weight^P	-0.21***		
HB^S	-0.07	0.10	
GS^S	-0.01	0.25**	-0.10

D) Controlled for standing

Chapter 2	*Activity*	*Weight*	*HB*
Weight^P	-0.02		
HB^S	0.00	0.16**	
GS^S	-0.17**	0.35***	0.08
Chapter 3	*Activity*	*Weight*	*HB*
Weight^P	0.01		
HB^S	-0.09	0.26***	
GS^S	0.02	0.35***	0.22***
Chapter 4	*Activity*	*Weight*	*HB*
Weight^P	-0.13*		
HB^S	0.01	0.08	
GS^S	-0.01	0.25**	-0.10

Table 3 continued.
Significant correlations are indicated as * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$). Correlations with $p < 0.001$ are further highlighted in grey to visualize major differences between analyses.
Activity = average percentage of time spent not resting, standing = average percentage of time spent in standing posture, weight = weight at the end of the rearing period, HB = hock burn score, GS = gait score, indicating lameness.
[P] Row contains Pearson’ s correlation coefficients.
[S] Row contains Spearman’ s correlation coefficients.

There are several possible hypotheses on how BSFL provisioning can influence broiler activity and weight. First, the increased activity caused by BSFL provisioning may have a negative effect on broiler body weight gain because physical activity requires energy, resulting in less energy being available for muscle growth and thus body weight gain (Tickle et al., 2018). Second, the increased activity caused by BSFL provisioning can improve leg development, allowing broilers to walk more easily to the feeder and eat, thereby potentially increasing body weight gain (de Jong et al., 2014; Reiter and Bessei, 2009). Third, BSFL may benefit growth due to their nutritional profile (i.e., appropriate fat and protein composition, potential immunomodulating properties, Murawska et al., 2021). A higher body weight, in turn, may impair a broiler's ability to perform active behaviours. In **Chapter 2 & 3** broilers that spent more time on active behaviours and in standing posture had a lower final body weight, therefore it is likely that in these chapters the increased activity caused by BSFL provisioning reduced broiler weight (i.e., the first hypothesis, **Table 3A**). However, correlations with weight were weak (for standing) or absent (for activity) in **Chapter 4** (**Table 3A**)**.** Here, a combination of influences of BSFL provisioning may play a role, and these could possibly cancel each other out, reducing the correlation between weight and activate behaviour/standing posture.

Broilers that spent more time standing and/or being active often had less severe leg health problems, though some correlations were weak or absent for **Chapter 4** (**Table 3A**). Controlling for standing reduced or removed any correlations between activity and leg health, while controlling for activity did not change the correlations between standing and leg health (**Table 3C-D**). This suggests that the time spent standing has a more direct relationship with leg health than the time spent on active behaviour. It makes sense that activity alone has little benefit for leg health as active behaviour can be performed while sitting, which may even promote the development of contact dermatitis (Haslam et al., 2007). In contrast, a standing posture may exert more direct effects on leg health by moderating pressure on the legs and reducing the time feet and legs spend in contact with soiled litter, thereby reducing the risk for lameness and contact dermatitis (de Jong et al., 2014; Reiter and Bessei, 2009). It must be noted that the direction of the relationship may also be reversed, as lameness and contact dermatitis limit the ability of broilers to perform active behaviours and standing postures (Reiter and Bessei, 2009; Weeks et al., 2000). Therefore, when BSFL provisioning reduces the risk of leg problems by increasing activity and/or the time spent in standing posture, this may further facilitate active behaviours and standing postures.

Notably, while the correlations between active behaviour or standing posture and leg health were often strong, they were reduced or removed by controlling for weight (**Table 3B**). In contrast, controlling for activity or standing did not change the correlations between weight and leg health parameters (**Table 3C-D**). This suggests that weight has a more direct relationship with leg health than standing and activity do. Throughout all chapters, heavier broilers had higher hock burn and gait scores indicating they had more severe leg problems than lighter broilers, though in **Chapter 4** the correlations were weak (**Table 3A**). Having a higher weight can directly impair leg health, as this increases pressure on the feet and legs, increasing the risk of contact dermatitis (Shepherd and Fairchild, 2010) and developmental leg problems that result in lameness (Bessei, 2006). In line with this, previous studies also found that higher body weights increased the severity of contact dermatitis (da Costa et al., 2014; Haslam et al., 2007) and lameness (Kestin et al., 2001; Knowles et al., 2008) in poultry. However, leg health can also influence body weight gain. For example inducing foot pad dermatitis caused lower body weight gains in broilers, likely because of difficulty reaching the feed (de Jong et al., 2014). This may explain the relatively better leg health and higher body weight in the BSFL treatments compared to controls in **Chapter 4**.

Overall, it appears that weight has the strongest relationship with leg health, followed by the time spent standing. However, different effects of BSFL provisioning and different correlations between active behaviour, standing posture, weight, and leg health were found across chapters. This suggests that while weight and standing posture can be associated with leg health, other elements moderate this relationship.

Broiler slaughter age may play a role. In **Chapter 4** the broilers were slaughtered at 35 instead of 42 days of age in the other chapters, and at this age they also had lower final body weights. It is possible that the correlation between body weight and leg health becomes weaker at a lower body weight. Indeed, one study that reduced the load on broiler legs found no effect on leg development at 19 days of age (Rutten et al., 2002), and studies that did suggest a link between broiler weight and lameness took place at d40 or later in the rearing period (Kestin et al., 2001; Knowles et al., 2008). The observation that BSFL provisioning improved broiler leg health without lowering the final body weight in **Chapter 4** indicates that BSFL provisioning influences leg health also through other factors.

One such factor is litter quality. Across literature, litter quality has been consistently brought forth as a main determinant for contact dermatitis (de Jong et al., 2014; Shepherd and Fairchild, 2010; Swiatkiewicz et al., 2017), which is intuitive as the moisture and ammonia in dirty litter irritate the skin and cause the lesions that we call contact dermatitis (de Jong et al., 2014). Lameness in poultry has also been associated with bad litter quality, potentially through the direct effects of litter quality on contact dermatitis as this partly determines walking ability (Bradshaw et al., 2002; da Costa et al., 2014; Granquist et al., 2019). Interestingly, in **Chapter 3** BSFL provisioning did not influence leg health and the litter quality of the BSFL treatments was similar or worse than that of controls. In contrast, in **Chapter 4** both leg health and litter quality were improved in several BSFL treatments. This contrast between chapters suggests that the benefits of BSFL provisioning on broiler leg health can be at least in part caused by their effects on litter quality. If litter quality was a main determinant for leg health in **Chapter 4**, this may explain why weight, activity, and standing were only weakly correlated to leg health, as litter quality is influenced by pen mates and can thereby interfere with intra-individual correlations.

Active foraging behaviours such as litter scratching can cause drying of the litter (de Jong and van Harn, 2012), and through this BSFL provisioning could have influenced litter quality. Additionally, broiler feed is known to have a strong effect on litter quality, mainly by affecting water excretion levels (Collett, 2012). In line with this, BSFL meal and oil incorporated in the pellets and dried BSFL provided in the feeder improved litter quality without affecting foraging behaviour in **Chapter 4**. This suggest a distinct role of the composition of the different BSFL forms on litter quality, though the mechanisms of this remain to be studied. Besides using different BSFL forms, the total dietary composition applied across chapters differed. **Chapter 2 & 3** applied the same 2-phase diet and **Chapter 4** applied a different 3-phase diet. Formulation of all diets involved some assumptions regarding the digestibility of whole BSFL, as only the digestibility of BSFL meal and oil had been studied for broilers. It is possible that the digestibility of BSFL was over- or under-estimated in the different chapters, resulting in either more nutrient-rich or nutrient-poor diets being provided to broilers in the BSFL treatments compered to controls. This in turn could have influenced the broilers' growth rate, litter quality, and/or leg health.

Finally, differences between treatment effects in **Chapter 2-4** could have resulted from different housing conditions. All chapters applied different stocking densities (4.5, 11, or 14 broilers/m^2, respectively), and **Chapter 4** applied a different lighting schedule and different litter management (i.e., no litter supplementation) than **Chapter 2 & 3**, and all these factors can influence litter quality, growth rate, and leg health (Dawkins et al., 2004; de Jong and van Harn, 2012; Feddes et al., 2002; Olanrewaju et al., 2006). The higher stocking densities in **Chapter 4** may have made it generally more difficult for broilers to reach the feeder, whereas this was less of a limiting factor in **Chapter 2 & 3**. Therefore, any beneficial effects on leg health in **Chapter 4** may have increased body weight gain as this made accessing and consuming feed easier. Also, the different management strategies are presumably responsible for the generally higher severity of leg problems in **Chapter 4**, which in turn may have caused the different effects of BSFL provisioning and the different correlations between aspects of broiler health and functioning in this chapter compared to **Chapter 2 & 3**.

Overall, the relationship between BSFL provisioning and broiler behaviour, weight, and leg health is very complex and potentially moderated by other factors such as litter quality. The exact mechanisms underlying these relationships require clarification. Since housing conditions may determine the effect of BSFL provisioning on broilers, case-specific studies are required to assess how BSFL provisioning affects broilers in distinct production systems.

To investigate other aspects of broiler health and functioning, BSFL treatment effects on natural antibodies, blood serotonin, and feather corticosterone were investigated in 35-day-old broilers in **Chapter 4**.

Natural antibodies

Plasma IgM and IgG natural antibody titers against keyhole limpet hemocyanin, which are naturally circulating antibodies that play a role in innate immunity

(Coutinho et al., 1995), were not influenced by any form or method of BSFL provisioning (**Chapter 4**). It has been suggested that the humoral and cell-mediated immunological responses of broilers could be mediated by the prebiotic compounds in BSFL (Deng et al., 2008; Xia et al., 2021). Indeed, previously dietary BSFL has had some effects on broiler immunology, for example BSFL meal increased the occurrence of $CD3^{+}CD4^{+}$ T lymphocytes in the spleen (J. Lee et al., 2018), while it decreased the occurrence of $CD3^{+}CD8^{+}$ cytotoxic T lymphocytes in the intestine (de Souza Vilela et al., 2021b). In pigs KLH-IgM titers and $CD4^{+}CD8\alpha^{+}$ T cells were influenced by environmental enrichment (Luo et al., 2020a), and particularly live and dried BSFL provisioning could have had a similar effect for broilers. We did not find, however, that inclusion of BSFL meal and oil, dried BSFL, or live BSFL affected broiler natural antibody production, though whether these BSFL forms influence other aspects of broiler immunology remains to be investigated. Future studies should apply infectious challenges such as *S. Gallinarum* infection, as these activate the specific immune system and are therefore prone to reveal any modulations of this system and, importantly, may reveal the impact of BSFL provisioning on disease susceptibility.

Blood serotonin

Serotonin (5-Hydroxytryptamine, or 5-HT) concentrations in whole blood, which reflect long-term 5-HT system functioning (Shajib and Khan, 2015), were also not influenced by BSFL provisioning (**Chapter 4**). It is known that 5-HT is released by the intestines and can be influenced by diet (Yue et al., 2017), though we could not confirm any effect of dietary BSFL on 5-HT concentrations. Furthermore, lower blood 5-HT has been linked to increased fearfulness and pessimistic affective states in pigs (Stracke et al., 2017; Ursinus et al., 2013) and to increased fearfulness in laying hens (Bolhuis et al., 2009). In broilers there may be a similar connection between whole blood 5-HT and fearfulness. However, as broiler fearfulness was only directly studied in **Chapter 3**, we could not assess the potential association between broiler fearfulness and whole blood 5-HT. As 5-HT concentrations have been linked to affective states and some chapters found benefits on affective states after BSFL provisioning, it is interesting to further explore the effect of BSFL provisioning on 5-HT system functioning.

Feather corticosterone

Prolonged stress can impair broiler health (e.g., lesions on the bursa of Fabricius, Pamok et al., 2009), affective states (Iyasere et al., 2017), and body weight gain (D. Y. Kim et al., 2021). As corticosterone (CORT) production is increased in response to stress, CORT concentrations are often used to quantify stress responses. Measuring feather CORT concentrations as a reflection of chronic stress is a relatively novel method, and the suitability of this method is explored in **Box 2**. Feather CORT concentrations were influenced by BSFL provisioning, though without any significant differences between individual treatments. Numerical differences in feather CORT concentrations between treatments could have been partially attributed to leg health and might have been influenced by contact with faeces, as discussed in **Chapter 4**. A link between feather CORT concentrations and leg health is somewhat substantiated by correlational analyses (method described in **Box 1** on p. 261, corrected for treatment), which indicate that feather CORT concentrations are positively, albeit weakly, correlated to lameness ($R = 0.24$, $p = 0.003$), but not to hock burn ($R = -0.07$, $p = 0.421$) or foot pad dermatitis ($R = -0.07$, $p = 0.394$). Stressors other than leg problems may also play a role in CORT production, and the exact mechanisms through which BSFL provisioning affects broiler stress require further investigation.

Box 2: Feather corticosterone measures

In the past corticosterone (CORT) was often measured through blood sampling. However, this is invasive, can be influenced by stressful capturing/handling procedures, and only provides a snapshot reflecting basal stress levels, a temporary acute stress response, or increased activity (Bortolotti et al., 2008). Recent innovations have shifted interest towards the CORT that is deposited in growing feathers, as measuring this requires less invasive procedures and reflects long-term CORT production, and thus chronic stress (Bortolotti et al., 2008). Broiler feather CORT has previously been measured using ELISA kits (Carbajal et al., 2014) and in **Chapter 4** we successfully applied the novel method suggested by Ataallahi et al. (2021) involving accelerated metal beads that pulverize the feathers and methanol extraction of feather CORT, on a large scale. We did adjust the method for cleaning feathers, as dry wipes were not sufficient for feathers covered in dirt/faeces, and a step involving 10-sec soaking in demi-water was added to this process. This step may be required for future studies involving commercially housed broilers, which tend to get quite dirty over time (e.g., no broilers had a score of zero indicating completely clean feathers in **Chapter 4**). The feather CORT concentrations did vary a lot between birds, which was also found in previous studies (Bortolotti et al., 2009; Carbajal et al., 2014). This may indicate that individuals differ a lot in their stress response, or that the method by which feather CORT is measured has limited accuracy. Indeed, a recent study indicated that feather CORT concentrations can be influenced by exposure to faecal matter (Bartels et al., 2021), which may limit applicability of this method. Overall, further validation and standardization of the applied method is required, such as determining the effect of which feather is analysed and equalizing in what unit feather CORT is presented.

Effects of BSFL provisioning on the affective states of broilers

Balancing affective states towards positive emotions is key for good animal welfare. The increased natural behaviour and/or the improved leg health of broilers after BSFL provisioning may have improved the affective states of these broilers. Broiler fearfulness, reflecting a negative affective state, was only studied in **Chapter 3**. Here, broilers in the treatments with the most frequent (seven times a day) or prolonged (via tubes) access to larvae spent less time in tonic immobility than controls, indicating reduced fearfulness (Forkman et al., 2007). In this chapter BSFL provisioning did not influence broiler leg health, and correlational analyses (method described in **Box 1** on p. 261, corrected for treatment) on the time spent in tonic immobility and on hock burn and lameness show that these parameters were not correlated (R = -0.06, p = 0.425 and R = 0.10, p = 0.211, respectively), suggesting that leg health was not linked to fearfulness here. The two BSFL treatments that reduced fearfulness did also have a profound impact on natural behaviour, and it has been shown that facilitating natural behaviour leads to a more positive affective state as performing such behaviour can be rewarding (Anderson et al., 2021a). In support of this, correlational analyses showed that the time spent in tonic immobility was negatively (though weakly) correlated to the time spent on active behaviours in the home-pen (R= -0.17, p = 0.039), showing that more active broilers were less fearful. The observation that neither the time spent in tonic immobility nor long-term activity levels were affected in the BSFL treatments with less frequent access to BSFL confirms that extended access to live BSFL is most effective in improving broiler welfare. The importance of increased environmental stimulation is in line with previous studies where reduced fearfulness in broilers was observed when they had access to perches and dust baths, but not when only one of these items were present (Baxter et al., 2018a). Because complex environments stimulate interaction with a range of diverse objects, broilers in such environments can learn to approach instead of avoid unfamiliar objects and thereby become less fearful, as suggested previously (Tahamtani et al., 2018).

Fearfulness can be related to other affective states, such as the degree of optimism of an individual. Briefly, the degree of optimism of an animal can be measured by observing their response to an ambiguous cue, where a faster approach suggests a more positive expectation and thus a more optimistic state (Roelofs et al., 2016). Lambs treated with the anxiolytic drug diazepam displayed more optimistic behaviours in response to an ambiguous cue than untreated controls (Destrez et al., 2012). Also, increasing environmental complexity caused broilers to approach ambiguous cues faster than controls housed in barren environments, indicating optimism (Anderson et al., 2021a). Therefore, we attempted to investigate the degree of optimism of broilers by means of an attention bias test (ABT), though this presented some difficulties (**Chapter 3**, unpublished data; evaluated in **Box 3**). Further development of methods for assessing positive affective states of broilers is required, such as optimization of the ABT or applying novel techniques like thermal imaging (Moe et al., 2012). Given that we used only one measure of affective state in one chapter of the broiler part of this thesis, more measures of affective states should be used to determine the overall effect of BSFL provisioning on this.

Box 3: Attention bias test

Background

The affective state of an animal is linked to their cognitive abilities and processes. Affect and cognition are partially regulated by the same brain structures such as the amygdala and the prefrontal cortex (Barrett et al., 2007; Holland and Gallagher, 1999). As affect is a result of experiences and related to expected outcomes, cognitive processes are intrinsically involved in this. Therefore, an animal's cognitive processes can be used to assess their affective state (Lee et al., 2016; Roelofs et al., 2016). As such, behavioural tests that involve the judgement of a situation (i.e., Judgement Bias Test, JBT) have been used to assess the affective state of, among others, sheep (Verbeek et al., 2014), laying hens (Hernandez et al., 2015), broilers (Anderson et al., 2021a), and pigs (Douglas et al., 2012). In these tests, an animal is first trained to associate distinct cues (e.g., sounds, colours) with positive and negative stimuli. Subsequently, the animal is confronted with a novel, ambiguous cue, and the behavioural responses towards this cue are compared to the responses towards the positive and negative cues to determine an animal's expectations and degree of optimism (reviewed by Roelofs et al., 2016).

Box 3 continued.

While the JBT has been applied regularly, it has the downside of needing extensive training. This is not only time-consuming, which is problematic for broilers that have a short lifespan, but can also in itself influence the affective state of an animal (Roelofs et al., 2016). Furthermore, a JBT generally requires an active behavioural response, which can be challenging for animals like broilers that often suffer from leg health problems. To overcome these challenges, a novel test called the Attention Bias Test (ABT) has been developed. In this test, an animal is confronted simultaneously with a positive (e.g., food reward) and negative (e.g., loud noise) stimulus, and the bias in attention towards the stimuli is recorded (Luo et al., 2019).

Attention bias test for broilers

In **Chapter 3** broilers were tested in an ABT on d21-24. One day before the ABT, broilers were habituated to the test arena (1 m^2) including a tray with whole wheat that was provided as a positive stimulus. During habituation, the interest in the wheat was very low, and only two out of the 40 tested birds sampled it. To increase the motivation of broilers to eat during the ABT, all feeders were removed from the pen 2h before testing. During the 5-min ABT, the tray with wheat was located at one side of the test arena, and on the other side the sound of barking dogs played from a speaker for 10 seconds at the start of the test. The attention towards the wheat and the box that had played the sound was measured by noting the direction of the head, and the latency before eating and the time spent eating the wheat were also recorded.

Despite the 2-hour feed deprivation period, none of the tested birds consumed wheat during the ABT. The broilers had to walk approximately 0.5 m towards the wheat and at this age we did not observe any severe leg problems (unpublished data), therefore it is unlikely that birds were unable to reach the wheat. It is more plausible that whole wheat is not a strong positive stimulus for broilers, and as such any attention bias could not be reliably measured in this set-up. In this thesis we observed that live BSFL are attractive to broilers, therefore they could be a more effective positive stimulus.

Box 3 continued.

In a previous study, the presence of mealworms as a positive stimuli only resulted in ten out of 60 broilers (16.7%) eating, though this may be explained by the lack of habituation to the feed item (Anderson et al., 2021b). However, when broilers were tested in groups of 3, this increased to 92 out of 144 birds (63.9%) eating, and the authors suggested that testing in groups is better as it avoids confoundment with social isolation (Anderson et al., 2021b). It would be interesting to include the same broilers individually and in groups in an ABT to determine the effect of social isolation on ABT outcomes. Alternatively, conspecifics could be used as the positive stimulus in an ABT as the motivation for social reinstatement in broilers is high (Guhl, 1968). Furthermore, even though broilers responded more intensely to the sound of dogs barking than to an alarm call from conspecifics in a pilot study (unpublished data), other negative stimuli (e.g., physical presence of a predator) could be more effective and this should be explored.

Overall, while an ABT could be a promising tool to assess the general affective state of broilers, more research is needed to improve the experimental set-up. Besides choosing the optimal positive and negative stimuli, attributes such as the length of the habituation and test period, the number of animals tested together, and the moment of testing must be considered. Also, measuring attention by observing the direction of the head may not be appropriate for animals with (partial) monocular vision, such as broilers. In humans, eye tracking has been done as a means to assess attention (Richards et al., 2014), and while this could work for animals, it requires the animal to be restrained. Finally, additional validation of the ABT is required, for example by correlating ABT responses to other measures of affective states.

Effects of BSFL provisioning on the natural behaviour of pigs

As for broilers, natural behaviour is also a key part of pig welfare. In nature, piglets already start to explore a range of feed items from a few days after birth, and throughout several months they gradually shift towards consuming only solid feed items (Jensen and Recén, 1989; Petersen, 1994). Throughout their life pigs remain highly motivated to forage, often spending more than half their active time on foraging and eating behaviour (Petersen, 1994; Stolba and Wood-Gush, 1989). Commercial practices such as early and abrupt weaning and barren housing limit the possibility for pigs to perform natural behaviour, which can have negative consequences for pig welfare. For example, early and abrupt weaning can impair the health of piglets by causing post-weaning diarrhoea (Lallès et al., 2004), and a lack of suitable rooting substrate can cause pigs to redirect their exploratory behaviour towards other pigs by performing harmful behaviour such as tail and ear biting (Schrøder-Petersen and Simonsen, 2001). Providing pigs with environmental enrichment can stimulate foraging and exploratory behaviour, and the most interesting enrichment materials for pigs include rooting materials that are odorous, manipulable, destructible, chewable, palatable, and edible (Studnitz et al., 2007; van de Weerd et al., 2003). Live BSFL possess all these characteristics, and in **Chapter 5** we concluded that pigs consistently prefer to interact with live BSFL over other feed items (i.e., corn, raisins, feed pellets) and other enrichment materials (i.e., jute sacks, tubes with pellets, and rubber balls on chains). This was determined by a series of preference tests, the suitability of which is discussed in **Box 4**. After confirming that pigs are interested in live BSFL, live BSFL were provided to piglets before and after the weaning transition, and their effect on natural behaviour was observed.

Box 4: Preference tests

The preference tests in **Chapter 5** compared the appetitive behaviour towards and consumption of live BSFL, corn, raisins, and feed pellets provided in a feeder (low workload) or in tubes with holes that had to be rotated to obtain the larvae (high workload), in no-choice (1 item/test) and two-choice (2 items/test) tests. Here, the key strengths and weaknesses of these tests and measures are compared and discussed.

Box 4 continued.

No-choice vs. two-choice

In the no-choice tests one feed item was present per test, and feed preference was indirectly determined by comparing the responses of pigs to the individually presented feed items in different tests. In contrast, in the two-choice tests two feed items were present simultaneously, and the preference for one item over the other was directly observed in each test. In our study and in previous studies with sows both test types resulted in similar preference profiles, indicating that both tests can determine feed preferences (Aubé et al., 2019a, 2019b). However, the differences between the interest in distinct items is often larger in two-choice than in no-choice tests (**Chapter 5**, Aubé et al., 2019b), probably because in a two-choice test spending time interacting with one item directly reduces the available time to interact with the other item. As such, subtle differences in preference may be more easily detected by two-choice tests. Furthermore, the two-choice test excludes the effect of time on feed preference, as feed items are presented simultaneously as opposed to in succession in the no-choice test. A downside of both test types is that only a limited number of items can be compared, even more so for the two-choice tests where the time needed increases exponentially with increasing number of tested items. The two-choice test could be expanded to include more than two feed items simultaneously, though this makes it difficult to observe the preference between individual items.

Low vs. high workload

In **Chapter 5** feed items were provided either in open feeders (low workload), or in tubes with holes suspended above a feeder that had to be pushed to obtain the feed item (high workload). Using tubes means that active appetitive behaviour (i.e., rooting) is required before the consummatory phase (i.e., eating) can begin, which is also often the case for pigs in nature (Berridge, 2004). As this demands more effort to obtain the feed, pigs are expected to be more selective in what feed item they try to access depending on their underlying motivation (Bak Jensen and Pedersen, 2007).

Box 4 continued.

Indeed, presenting the feed in tubes revealed substantially more distinct preference profiles, especially concerning appetitive behaviour. Pigs interacted minimally with empty tubes, indicating that despite their inherent curiosity to explore manipulable items (Studnitz et al., 2007; van de Weerd et al., 2003), their differential interest in the feed items overruled interest in the tubes. A disadvantage of requiring active behaviour is that animals need to be habituated or trained before testing. Behavioural observations indicated, however, that all pigs turned the tubes after eight days of habituation, and further research can reveal whether shorter habituation periods are sufficient.

Appetitive behaviour vs. consumption

The degree of preference for feed items was determined by measuring the appetitive behaviour towards feed items and the consumption of the feed items. Feed composition and texture affect satiety and the feed consumption rate (Aubé et al., 2019a; Solà-Oriol et al., 2009), and when feed items vary in these parameters the consumed amount may not accurately reflect the motivational demand for these feed items (Bak Jensen and Pedersen, 2007). In contrast, the appetitive behaviour towards the (tubes/feeder containing the) feed items is a direct reflection of the motivation of pigs to obtain a feed item (Berridge, 2004), and in our case thus a better indicator of the value of live BSFL as edible environmental enrichment. A downside of both measures is that they can be influenced by pen mates, for example when pigs fight for access to a particular feeding place. This could be overcome by testing pigs individually, though social isolation could influence pig behaviour (Reimert et al., 2013) and extensive habituation would be required.

Overall, for determining the preference for certain edible enrichment items, measuring the appetitive behaviour in two-choice tests using a system that requires a high workload to reach the feed was most appropriate and obtained the most distinct preference profiles in our study.

Box 4 continued

The applied tests could be improved by varying the workload required to access each feed item, as has previously been done in so called consumer-demand tests with operant conditioning structures, allowing for measurements of demand elasticity and the maximum price paid to quantify the strength of motivation (Matthews and Ladewig, 1994). This approach could be extended to double demand operant conditioning structures that identify relative preference (Bak Jensen and Pedersen, 2007; Webb et al., 2014). However, such tests require extensive training and testing and are therefore more time-consuming.

Feed-directed behaviour around weaning

In **Chapter 7** it was observed that piglets that received live BSFL before weaning spent a considerable time interacting with the BSFL (up to 6% of the observed time close to weaning), representing the major portion of their feed-directed behaviours. The presence of live BSFL did not increase creep feed intake nor other parameters related to piglet welfare around weaning. However, it is known that pre-weaning feed intake varies greatly between piglets, even within litters (Collins et al., 2013). Therefore, correlational analyses (method described in **Box 1** on p. 261, corrected for treatment) were performed to explore the relationship between an individual piglet's time spent eating BSFL before weaning and other relevant parameters of welfare around weaning (**Table 4**).

Concerning behaviour, the time spent eating BSFL pre-weaning was positively correlated to the time spent eating creep feed (**Table 4**), indicating that piglets that sample one feed item are likely to also sample the other feed item. This is in line with previous studies that provided a diverse diet, where 87% (two flavours), 90% (two types of feed pellets), and 86% (four feed items) of piglets that sampled one item also sampled all other items (Middelkoop et al., 2019b, 2018). It is likely that the increased exploratory behaviour in the presence of live BSFL stimulated piglets to also sample the creep feed, as exploration is part of the appetitive phase that precedes feed consumption (Berridge, 2004) and increased exploration has previously coincided with increased feed intake (Middelkoop et al., 2019b). Additionally, providing a diverse diet is expected to decrease sensory-specific satiety, promoting higher feed intake in general (Middelkoop et al., 2019b; Rolls et al., 1981), and the combination of BSFL and creep feed could have had a similar effect. Despite the absence of an effect of BSFL treatment on the total feed-directed behaviour, these results suggests that pre-weaning live BSFL provisioning can benefit individual piglets that do consume the larvae, and that live BSFL could be an integral part of a diverse pre-weaning diet aimed at easing the weaning transition. The time spent eating BSFL pre-weaning was significantly correlated to other parameters, and these correlations will be discussed in their associated sections of this General Discussion.

Table 4. Correlation coefficients of analyses done on pre- and post-weaning parameters of piglets that received live BSFL in **Chapter 7**.

Pre-weaning parameters	*Eating BSFL pre-weaning*[1]
Eating creep feed[1,P]	0.29**
Eater type[2,P]	0.34***
Weaning weight[P]	-0.04
Post-weaning parameters	
Eating pellets[1,P]	0.06
Eating BSFL post-weaning[1,P]	0.13
Manipulation[1,S]	-0.03
Final weight[P]	0.09
Number of days with diarrhoea[P]	-0.26*
NET score factor 1[3,P]	0.19
ABT score factor 1[3,P]	0.44*

Significant correlations are indicated as * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$).

[1] Percentage of observed time.

[2] Based on rectal swabs, a higher score indicates creep feed was consumed on more days.

[3] Calculated from the factor analysis on behaviours performed during the Novel Environment Test (NET) and Attention Bias Test (ABT) presented in **Chapter 7-Table 4**.

[P] Row contains Pearson's correlation coefficients.

[S] Row contains Spearman's correlation coefficients.

Behaviour in the home pen

Across **Chapter 5-7** pigs showed great interest in live BSFL. In **Chapter 5** pigs spent a higher percentage of the observed time interacting with hanging tubes containing live BSFL (21.9%) than with hanging tubes containing pellets (5.8%), jute sacks (11.8%), or rubber balls hanging from chains (1.5%). In **Chapter 6** scattering small amounts of live BSFL twice a day during 11 days after weaning increased exploration towards the pen floor compared to controls (33.0% vs. 19.8% of the observed time). In **Chapter 7** pigs actively interacted with hanging tubes with holes that contained live BSFL during three weeks after weaning (11.8% of the observed time). Simultaneously, the time spent on pig-directed oral manipulation was (temporarily or continuously) decreased in all chapters (on average from 2.6% to 1.3% in **Chapter 5** compared to pigs having a rubber ball on a chain, from 2.4% to 0.9% in **Chapter 6,** and from 2.4% to 1.8% in **Chapter 7** after weaning, compared to controls). This indicates that live BSFL can consistently function as effective environmental enrichment for pigs, facilitating intrinsically motivated exploratory behaviours and reducing the motivation of pigs to orally manipulate pen mates. The observed increase in exploratory behaviour and decrease in manipulation of pen mates was previously only observed when extensive enrichment (e.g., a combination of increased space, straw, wood shavings, peat, and branches) was provided (Bolhuis et al., 2006; Oostindjer et al., 2011b), indicating that live BSFL are very effective environmental enrichment. Indeed, compared to commonly used commercial enrichment items such as rubber balls on chains, live BSFL elicit higher interest and can maintain interest over longer periods of time (**Chapter 5**), likely due to their palatability which is known to prolong the interest of pigs in enrichment materials (Holm et al., 2008; Machado et al., 2017).

Both scattering live BSFL through the pen (**Chapter 6**) and providing them in tubes that had to be rotated to access the larvae (**Chapter 7**) caused similarly high levels of exploratory behaviour, suggesting that these methods are both effective ways of providing live BSFL to pigs. Over the 3-week period, pigs became more efficient in retrieving the larvae from the tubes, resulting in a decrease in time spent exploring the larvae and a simultaneous increase in time spent manipulating pen mates (**Chapter 7**). Scattered live BSFL were only provided for 11 days, therefore it is unknown whether piglets become more efficient in retrieving larvae from the litter over time. Commercial application of live BSFL as enrichment will require research into suitable provisioning methods that prolong the time pigs can interact with the larvae. For example, alternating strategies of BSFL provisioning may reduce habituation and increase the beneficial effects of live BSFL provisioning on pig behaviour.

Effects of BSFL provisioning on the health and functioning of pigs

Pig health and functioning is another key aspect of pig welfare around weaning, and the studied parameters related to this include body weight gain, feed intake, creep feed eater types and gastro-intestinal tract (GIT) development around weaning, and post-weaning diarrhoea.

Effects of pre-weaning BSFL provisioning

Pre-weaning live BSFL provisioning did not influence piglet body weight gain and weaning weight (**Chapter 7**). This was expected as pre-weaning feeding strategies rarely influence pre-weaning body weight gain (Bruininx et al., 2002; Middelkoop et al., 2019b), because the sow's milk is the main nutrient supply of nursing piglets. While pre-weaning feed intake could not be measured due to spillage and contamination by faeces, the total time spent eating did not differ between pre-weaning treatments (**Chapter 7**). Based on this it is expected that the total pre-weaning feed intake also did not differ between treatments. In line with these results, a diverse diet consisting of two feed items also stimulated exploratory behaviours without increasing the number of piglets that consumed feed or their production performance (Middelkoop et al., 2018). Similarly, a play feeder stimulated exploration without affecting pre-weaning performance, though in this study post-weaning feed intake and body weight gain were increased (Middelkoop et al., 2019a), which was not the case in **Chapter 7.**

While it appears that pre-weaning live BSFL provisioning did not stimulate pre-weaning feed intake or ease the weaning transition, individual piglets may still benefit from it. Correlational analyses indicated that piglets that spent more time eating BSFL were classified as better creep feed eaters based on them having blue coloured rectal swabs more often (**Table 4**), meaning they ate the blue coloured creep feed on more observation days. The large majority (89%) of piglets that were observed to eat creep feed on one observation day also ate creep feed on all consecutive observation days, suggesting that eating BSFL stimulated earlier intake of creep feed. In previous studies, good/early creep feed eaters pre-weaning had a higher feed intake and growth rate than bad/late eaters for three days after weaning (Carstensen et al., 2005; Pluske et al., 2007b), which may indicate a less negative impact of the weaning transition.

While the effect of eater type on post-weaning performance was not analysed, we did study GIT development on d3 post-weaning of a small selection (n = 12 per treatment) of piglets with either access to live BSFL before and after weaning or no access to BSFL before and after weaning (**Chapter 7**). Live BSFL provisioning around weaning was found to influence GIT development as these piglets had an increased caecum length and proximal stomach digesta pH, and decreased passage of glucose and fluorescein isothiocyanate through the colon wall. It is possible that (some of) these differences indicate improved GIT functioning around weaning caused directly by BSFL consumption and/or indirectly by the stimulative effect of BSFL consumption on (earlier) creep feed consumption. In line with this, correlation analyses show that piglets that spent more time eating BSFL before weaning had post-weaning diarrhoea during fewer days (**Table 4**), and reduced post-weaning diarrhoea has previously been linked to improved GIT functioning and health (reviewed by Heo et al., 2013; Pluske et al., 2018). Some potential mechanisms for the observed GIT changes have been discussed in **Chapter 7**, though the small number of piglets and the measurements only being done on one time point limit understanding of the mechanisms through which live BSFL provisioning affected these parameters. Furthermore, while these results are interesting, it must be noted that the correlation coefficients were relatively small (**Table 4**). In **Chapter 7** the time spent on eating feed items was recorded, but individual feed intake could not be determined. Future studies should measure individual feed intake to further explore how live BSFL provisioning influences pig health and functioning around weaning.

Effects of post-weaning BSFL provisioning

Post-weaning live BSFL provisioning did not influence the faecal consistency (**Chapter 6**) and days with (watery) diarrhoea (**Chapter 7**). Therefore, the hypothesis that post-weaning live BSFL provisioning benefits piglet health during the weaning transition could not be confirmed. We did observe a large difference in the occurrence of diarrhoea between studies. In the first 11 days after weaning, 13.6% of the observations indicated diarrhoea in **Chapter 6**, and 20.9% of the observations indicated diarrhoea in **Chapter 7**. In **Chapter 7** more barren housing conditions were applied (i.e., absence of bedding) and pens had double the stocking density compared to **Chapter 6.** Such conditions were previously found to increase diarrhoea prevalence (Lallès et al., 2004; Weary et al., 2008). It is possible that in even more intensive systems pigs experience more welfare problems, and pigs in such systems could benefit more from BSFL provisioning.

Post-weaning live BSFL provisioning also did not influence the total daily feed intake, body weight gain, or final weight of pigs when provided for 11 days post-weaning (**Chapter 6**). In contrast, live BSFL provisioning for 21 days post-weaning temporarily lowered the body weight gain and reduced the total daily feed intake, without affecting the final weight of pigs (**Chapter 7**). The amount of larvae provided was nearly doubled in **Chapter 7** compared to **Chapter 6**. Therefore, it is possible that the reduced growth rate and feed intake in **Chapter 7** are a result of the increased stomach fill and higher satiety levels after consuming larger amounts of larvae, which would make the piglets less interested in feed pellets. A low feed intake and reduced growth rate during the weaning transition can be detrimental for piglet health, for example by decreasing gastric motility which increases the risk of diarrhoea (Heo et al., 2013; Moeser et al., 2017). However, as the lower body weight gain and feed intake did not coincide with a decrease in final body weight or an increase in days with post-weaning diarrhoea in this study, it is doubtful that piglet health was compromised by live BSFL provisioning.

In **Chapter 6 & 7** the feed pellet composition was not adjusted for larvae intake, as very little was known about the ability of young piglets to digest whole BSFL. As a result, the unbalanced diets between treatments may have influenced the feed intake and body weight gain of piglets, and these effects could have overruled any effects of live BSFL provisioning. Digestibility of BSF larvae and prepupae meal has been studied in post-weaning pigs, showing that the amino acid and energy digestibility coefficients are high and similar to comparable protein sources that are commonly used in pig diets such as soybean and fishmeal (Crosbie et al., 2020; Spranghers et al., 2018). Whether these digestibility coefficients are similar for whole live BSFL remains to be determined. The chitin in BSFL could hinder nutrient digestibility, especially in young piglets that produce less chitin-degrading enzymes (Kawasaki et al., 2021). As the nutrient composition including the chitin level of BSFL depends on their age (Liu et al., 2017), the digestibility of larvae of different ages must be determined, and younger BSFL may be more suitable for inclusion in the diet of younger piglets.

Effects of BSFL provisioning on the affective states of pigs

Pig fearfulness was assessed through a Novel Environment Test (NET) immediately followed by a Novel Object Test (NOT) on d10-11 after weaning in **Chapter 6**, and a NET on d4 after weaning in **Chapter 7**, in which the behavioural responses to an unfamiliar environment and/or object were determined. Live BSFL provisioning caused piglets to respond less neophobic towards a novel object in the NOT in **Chapter 6**. In accordance with this, piglets with access to edible substrates (i.e., straw, wood shavings, branches, and peat) pre-weaning were previously found to be less neophobic towards novel feed items on d25 before weaning (Oostindjer et al., 2011a). Interaction with preferred edible enrichment materials that are frequently replenished might cause piglets to form an association between novelty and a reward, causing them to expect a positive outcome when confronted with a novel object and thereby reducing neophobia.

In contrast to the NOT, reduced fearfulness was not observed in response to a novel environment per se in the NET. Similarly, moderate enrichment (i.e., more toys, additional feeder type, and more conspecifics while maintaining the space per pig) also affected piglets' responses to a NOT but not to a NET (Tönepöhl et al., 2012), and providing straw, ropes, or shredded paper as enrichment also did not change behavioural responses of pigs during a NET (de Jong et al., 2000; Lewis et al., 2006). It is possible that a novel environment does not elicit a strong fear response, as in a previous study more fear-related behaviour and higher serotonergic responses were observed during a NOT than during a NET (Ursinus et al., 2013). Also, de Jong et al. (2000) noticed a behavioural shift during their 5-min NET, where barren-housed pigs initially seemed more fearful than enriched-housed pigs but these differences disappeared over time. Therefore, it may be useful to shorten fear-related behavioural tests to observe more distinct initial fear responses before any habituation to the test situation occurs. It is also possible that access to live BSFL only influences stimulus-specific neophobia and not overall fearfulness, as discussed in **Chapter 6**.

Live BSFL provisioning did not influence the degree of optimism of pigs as determined by responses in an attention bias test (ABT) on d5 post-weaning (**Chapter 7**, background described in **Box 3** on p. 272). However, the correlation analyses indicated that piglets that spent more time eating live BSFL pre-weaning scored higher on factor 1 of the ABT (**Table 4**). This factor had high positive loadings for the time spent moving, time spent exploring the feed bowl, and vocalizing frequency, and high negative loadings for the time spent paying attention to the threat location, time spent standing alert, and latency to explore the feed bowl (**Chapter 7-Table 4**). Thus, a piglet with a high positive score on this factor paid more attention to the positive stimulus (i.e., the feed bowl) and less attention to the negative stimulus (i.e., the threat), indicating a more optimistic affective state (Crump et al., 2018). It is possible that consuming high amounts of live BSFL was rewarding for these piglets and thereby directly improved their affective state, which was also observed in response to extensive environmental enrichment previously (Douglas et al., 2012). Additionally, live BSFL provisioning may have eased the weaning transition, resulting in reduced weaning stress and consequently a more positive affective state after weaning. The increased attention toward the feed bowl may also be a direct result of the reduced neophobia due to regular live BSFL provisioning as observed in the NOT in **Chapter 6**. These results indicate that the affective state of some piglets may benefit from live BSFL provisioning. The suitability of an ABT to measure the affective state of pigs is explored in **Box 5**.

Overall, live BSFL provisioning had some benefits for a pig's affective state. In **Chapter 6 & 7** most piglets (except piglets in the LL treatment in **Chapter 7**) only had access to live BSFL for a short period of time post-weaning (4-11 days), and it is possible that long-term access to enrichment is more beneficial for a pig's affective state. Indeed, larger effects on behavioural responses to a NET were found when piglets had access to cognitive enrichment for a long period of time (Puppe et al., 2007), and prolonged exposure to an enriched environment caused stronger optimistic responses in a judgement bias test (Douglas et al., 2012). Therefore, prolonged access to live BSFL may be more beneficial for pig affective states, and this warrants further investigation. As for broilers, future studies should include multiple, integrated measures of affective states to gain conclusive information on the overall effect of BSFL provisioning on pig welfare.

Box 5: Attention bias test for pigs

In the attention bias test (ABT) in **Chapter 7** the piglets' regular feed pellets mixed with corn and raisins was applied as a positive stimulus, and only four out of the 64 piglets (6.3%) ate during the ABT. This indicates that eating behaviour was not a suitable measure of attention bias. Directly after weaning, feed intake of piglets is generally low (Dong and Pluske, 2007), therefore positive stimuli other than feed items may be more appropriate in this period. For example, pigs are highly motivated to interact with enrichment items such as straw or toys (van de Weerd et al., 2003) and with conspecifics (Jansen et al., 2009), therefore these could provide a more appropriate positive stimulus for pigs in an ABT, especially close to weaning. In addition to optimizing the stimuli, the ABT also requires further validation. A Pearson's correlation test (corrected for treatment) revealed a positive correlation between piglet scores on ABT factor 1 and NET factor 1 ($R = 0.49$, $p < 0.001$, **Chapter 7**). Like ABT factor 1, NET factor 1 had high positive loadings for time spent moving and frequency of vocalizations, and a high negative loading for the latency to explore the feed bowl (**Chapter 7-Table 4**). The observation that piglet scores for these two factors are positively correlated therefore indicates that piglets are consistent in their behaviour across these tests, and also supports the internal validity of the ABT for measuring affective state (Forkman et al., 2007). Further standardization and validation of this test are required to be able to consistently apply ABTs to determine pigs' affective states.

Conclusions

Currently, commercially reared broilers and pigs often experience welfare problems, and there is a consumer-driven demand for the protection and improvement of their welfare (European Commission, 2016). The effectiveness of a novel type of environmental enrichment, namely black soldier fly larvae (BSFL), aimed at improving broiler and pig welfare was studied in this thesis.

For broilers, all treatments in which live BSFL were provided increased natural foraging behaviour (**Chapter 2-4**). Scattering live BSFL through the pen two or four times a day temporarily increased the overall activity level of broilers. Moreover, scattering live BSFL seven times a day or providing live BSFL in tubes that had to be manipulated to access them increased activity levels throughout the whole rearing period, and these treatments also reduced broiler fearfulness (**Chapter 3**). Scattering dried BSFL only temporarily increased foraging behaviour without influencing overall activity levels (**Chapter 4**). Scattering live BSFL reduced the severity of lameness and/or hock burn in some treatments in **Chapter 2**, and it reduced the severity of foot pad dermatitis and hock burn in **Chapter 4**, though leg health, tibia development, and breast myopathies were not influenced by any method of live larvae provisioning in **Chapter 3**. Scattering dried BSFL or incorporating BSFL meal and oil in the feed pellets reduced the severity of foot pad dermatitis but not that of hock burn and lameness (**Chapter 4**), and the effect of these BSFL forms on affective states was not studied.

Pigs were more interested in live BSFL than in several other feed items (i.e., corn, raisins, feed pellets) and enrichment materials (i.e., tubes containing feed pellets, jute sacks, rubber balls on chains, **Chapter 5**). Live BSFL provisioning increased piglets' feed-directed exploration and play before weaning, but contrary to expectations it did not influence pre-weaning creep feed and total feed intake, or other parameters linked to pig welfare around weaning (**Chapter 7**). During the observed 11 days (**Chapter 6**) or three weeks (**Chapter 7**) after weaning, live BSFL provisioning increased exploratory behaviours and consequently reduced the oral manipulation of pen mates. Pre-weaning live BSFL provisioning affected GIT development around weaning (i.e., increased caecum length and proximal stomach digesta pH and decreased passage of glucose and fluorescein isothiocyanate through the colon wall, **Chapter 7**), but it did not influence post-weaning diarrhoea (**Chapter 6 & 7**). Furthermore, providing live BSFL after weaning reduced neophobia (**Chapter 6**), though it did not influence overall fearfulness and the degree of optimisms of pigs (**Chapter 7**). While the effect of BSFL provisioning around weaning on easing the weaning transition appears to be minimal, individual piglets that spent much time eating BSFL pre-weaning seemed to eat creep feed on more days, have fewer days with post-weaning diarrhoea, and show more optimistic behaviour during an attention bias test. This may indicate that these piglets handle the weaning transition better than piglets that spent less time eating BSFL before weaning.

To conclude, in this thesis we showed that live BSFL provisioning can be effective environmental enrichment and improve multiple facets of the welfare of broilers (**Chapter 2-4**) and pigs (**Chapter 5-7**).

Directions for future research

Throughout this General Discussion, several directions for future research have already been highlighted, aimed at furthering the understanding of the complex relationship between BSFL provisioning, animal welfare, production performance, full diet composition, and housing conditions. Currently, we have many tools available to study animal behaviour and animal health. Behaviour can be assess through for example instantaneous scan sampling (**Chapter 2-7**) or automated video tracking (van der Zande et al., 2021), and health parameters can be scored by validated visual scoring systems (Butterworth, 2009; Pedersen and Toft, 2011) or biophysical and chemical analyses (Goodlad et al., 1991; Güz et al., 2021; Luo et al., 2020a). In contrast, there are limited tools available to study the affective state of an animal, especially concerning positive affective states. Physiological parameters related to positive affective states are scarcely investigated, therefore behavioural observations (either in the home-pen or in cognitive bias tests) are predominantly used to assess these states (Roelofs et al., 2016). However, behaviour depends highly on factors such as age, health, and social status, and behavioural assessments of affective states are often subjective if the link between the behaviour and the affective state is not validated (Whittaker and Marsh, 2019). Integrating behavioural observations with physiological parameters (e.g., hormone and thermal dynamics) is expected to improve the accuracy of measures of affective states, and such parameters need to be identified, focussing mainly on parameters that can be assessed in a non-invasive way (e.g., by thermal imaging). More knowledge combined with validation and automation of observations will make measures of positive affective states more objective and reliable. Only when this is done can we further explore associations between affective states and other parameters such as behaviour, health, gut functioning, and production performance, and determine the impact of BSFL provisioning on affective states.

This thesis focussed on fast-growing broilers and on pigs during the weaning transition, though BSFL provisioning could have more widespread uses as environmental enrichment. For example, while slow-growing broilers experience fewer leg health problems than fast-growing broilers (Rayner et al., 2020) they still experience health problems and are housed in barren environments. A such, their behaviour and leg health could benefit from environmental enrichment (Güz et al., 2021). Other poultry may also benefit from BSFL provisioning. For example, layer hens and turkeys often perform feather pecking behaviours, and the motivation behind these damaging behaviours is like that of pigs orally manipulating pen mates (Brunberg et al., 2016). BSFL provisioning reduced damaging behaviours in pigs, and as such BSFL may also reduce feather pecking. In line with this one study found that providing live BSFL to layer hens improved their feather condition, possibly indicating reduced feather pecking (Star et al., 2020), and for turkeys live BSFL provisioning was found to reduced aggressive pecking (Veldkamp and van Niekerk, 2019). Based on our results it is worthwhile to further study the effect of whole BSFL provisioning on poultry welfare. For pigs, the motivation to explore is present throughout their lifetime (Vermeer et al., 2017), therefore BSFL provisioning to grower or finisher pigs may be beneficial for their welfare and this requires attention.

The focus of most chapters has been on live BSFL. However, we also found evidence that dried BSFL or BSFL meal and oil incorporated in feed pellets can benefit broilers, albeit to a lower extent. Dried BSFL may be more commercially viable because they are less susceptible to degradation and microbiological spoilage due to their high DM content, and they are easier to transport and to store long-term than live larvae (Kröncke et al., 2019). Therefore, it is worth to also study these BSFL forms as feed and/or enrichment, not only for broilers but also for other animals. Providing dried BSFL by using the most effective provisioning methods found for live BSFL (i.e., regularly scattering small amounts or providing larvae in tubes) may provide similar benefits for livestock welfare as live BSFL. Also, for broilers a combination of providing live larvae early in the rearing period and dried larvae later in the rearing period may be beneficial from a food safety and practical point of view, and the welfare consequences of such combinations should be determined. Furthermore, while most chapters attempted to emulate semi-commercial conditions, studies on large-scale intensive commercial farms are lacking, and they are required to determine the impact BSFL can have on the commercial livestock sector.

Finally, our focus has been on improving the welfare of conventional livestock, though in **Chapter 8** we highlight the need to also consider insect welfare. There is already some evidence that insects have cognitive and emotional abilities (Lambert et al., 2021), and more research is needed to determine how we can minimize the negative impact of commercial rearing practices on insect welfare. Large numbers of insects are required to benefit the welfare of one broiler or pig, creating a clear trade-off, and we may even conclude that the costs of diminished insect welfare are too big to justify using insects to benefit conventional livestock welfare.

Insects in sustainable food systems

There is a need for our entire society to become more sustainable, as reflected by the broadly scoped and numerous Sustainable Development Goals. Being sustainable means meeting the needs of the current population without compromising the ability of future generations to meet their needs, and insects can play an important role in sustainable food systems (**Chapter 8**, Dicke, 2018). Ultimately, using insects as human food instead of livestock feed would be more sustainable, as this would (partially) replace conventional livestock and thereby reduce pressure on the environment. However, the current acceptance of insects as food is low in many regions. For example, an American survey indicated that consumers more easily accept the use of insect products when they are incorporated in livestock or pet feed as opposed to human food (Higa et al., 2021). Therefore, at the moment the most benefits from insect in a sustainable society are likely gained from using them as livestock feed.

BSFL provisioning can promote sustainability by improving animal welfare. For BSFL to be used as commercial environmental enrichment, provisioning methods must be optimized. Across chapters, stimulating animals to work for access to BSFL was found to obtain the most animal welfare benefits, likely because this mimics the natural situation of performing appetitive behaviour before consuming feed, which is rewarding (Studnitz et al., 2007). However, the tested methods (i.e., regular scattering through the pen or filling of tubes) were often time-consuming and labour-intensive. By developing BSFL provisioning methods that match the natural motivations of an animal and subsequently automating these methods, live BSFL can be used to benefit the welfare of livestock across large-scale commercial rearing systems. It must be noted that while BSFL provisioning alleviated several welfare issues, impaired welfare still occurred regularly even when BSFL were provided. For example, in **Chapter 4** live BSFL provisioning reduced the severity of hock burn, but 40% of the studied broilers still showed signs of hock burn. Therefore, it is important to make further changes to livestock production systems, for example by providing BSFL in concert with other enrichment materials and/or management changes. It is possible that current intensive livestock production systems do not have enough flexibility to apply the changes needed to greatly reduce welfare problems. If this is the case, we may need to completely adjust the way that we rear livestock, for example by switching to only free-range, low-intensity systems, to promote only good welfare in sustainable livestock production systems.

Currently, limited information on the safety of insects as feed, as well as suboptimal legislation and high costs of insect production hinder utilization of insect larvae as livestock feed and enrichment (explored in **Chapter 8**). Rearing insects on low-quality biological waste streams (e.g., manure) is environmentally sustainable, prevents feed-feed competition with traditional livestock that cannot consume these waste streams, and is cost-effective (**Chapter 8**, Veldkamp et al., 2012). However, little is known about the accumulation of potentially harmful compounds in insects reared on these waste streams (van der Fels-Klerx et al., 2018), and as a result EU legislation prohibits the use of biological waste streams as rearing substrate for insects used as farm animal feed. Using higher-quality feedstuffs as insect rearing substrate not only limits environmental sustainability, it also increases the cost of insect production, adding to the already high current costs due to the absence of optimized large-scale insect production facilities (Cadinu et al., 2020). The observed welfare benefits of BSFL provisioning to broilers and pigs in this thesis and to layer hens and turkeys in other works (Star et al., 2020; Veldkamp and van Niekerk, 2019) supports the use of insect larvae as livestock feed and enrichment. This may drive research into feed safety, faster optimization of insect rearing systems, and faster legal changes required to use insect larvae as effective and sustainable environmental enrichment for livestock. Furthermore, the principles for the responsible use of insects as livestock feed outlined in **Chapter 8** promote the use of low-quality waste streams as insect rearing substrate and highlight the need to balance economic, environmental, and welfare aspects of livestock production systems. These principles should be used to guide optimization of sustainable livestock production systems that incorporate insects as livestock feed and enrichment. Several of the principles could also be applied to the implementation of insects as human food, further promoting a sustainable society.

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Summary

Samenvatting

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About the author

Publications

Education and training certificate

Summary

Including insects in the diet of livestock has been suggested to increase the environmental sustainability of livestock production systems. Black soldier fly larvae (*Hermetia illucens*, BSFL) are very suitable for this as they can consume organic waste streams and can be reared with a relatively small negative environmental impact. BSFL also have the potential to benefit livestock welfare, though this potential remains to be explored. Many commercially reared broilers and pigs experience welfare problems. Broilers have a high growth rate and are housed in stimulus-poor environments at high stocking densities, which limits their ability to perform essential natural foraging behaviours such as ground pecking and ground scratching. As a result, broilers often suffer from (leg) health problems (e.g., contact dermatitis and lameness) and may experience negative affective (i.e., emotional) states. Pigs are also very motivated to explore their environment through exploratory behaviour such as rooting and chewing, but these behaviours are not facilitated by barren commercial pens. Consequently, pigs frequently redirect exploratory behaviour towards pen mates by damaging behaviour such as tail or ear biting, and they may experience negative affective states. Furthermore, the early and abrupt commercial weaning process does not allow piglets to become sufficiently familiar with solid feed items before weaning. This combined with additional stressors around weaning (e.g., relocation to a different pen and in many cases being mixed with unfamiliar piglets) can lead to health problems and reduced production performance post-weaning. Also, directly after weaning piglets show increased damaging behaviour towards pen mates, especially when housed in pens without relevant distractions. Providing broilers and pigs with whole BSFL might stimulate natural foraging and exploratory behaviour and consequently may improve broiler and pig welfare. That was investigated in this PhD thesis.

This thesis is divided in three parts. In the first part the effect of BSFL provisioning on broiler welfare was investigated. Within this, BSFL provisioning methods that caused the highest welfare benefits were determined. In the second part the effect of live BSFL provisioning on pig welfare around the weaning transition was studied. Additionally, in this part the interest of pigs in live BSFL was compared to other feed items and enrichment materials. For both broilers and pigs three key

aspects of welfare were considered: natural behaviour, health and biological functioning, and affective states. Finally, the third part considered the big picture. This was done by creating principles for the responsible use of insects in sustainable livestock production systems, and by providing a synthesis and discussion of all chapters, giving directions for future research, and reflecting on the role of insects in sustainable food systems.

Black soldier fly larvae for broilers

In **Chapter 2** live BSFL were provided to broilers. This happened in different amounts (i.e., 5% or 10% of the dietary dry matter (DM) replaced with BSFL) and at different frequencies (i.e., scattering BSFL through the pen two or four times a day across the bedding), resulting in four combinations of amounts and frequencies that were compared to a control treatment without BSFL provisioning. Broilers were housed at final stocking densities of 14 kg/m^2. All combinations of amounts and frequencies increased the time broilers spent on foraging behaviours and their overall activity level compared to controls. Broilers that received live BSFL four times a day were most active and spent more time in standing posture than controls throughout the rearing period. Broilers that received 5% of their diet as live BSFL across four provisioning moments had less severe hock burns than controls. Broilers that received 10% of their diet as live BSFL had less severe hock burns and lameness, and when BSFL were provided twice a day they also had lower final weights than controls. Overall, provisioning of live BSFL stimulated the natural behaviour of broilers and, in some cases, improved their leg health.

In **Chapter 3** the most effective live BSFL provisioning strategies from **Chapter 2** (i.e., replacing 5% or 10% of the dietary DM across four provisioning moments) were provided to broilers kept at commercial final stocking densities (33 kg/m^2). Two additional strategies aimed at prolonging the interaction with live BSFL were included in this study: 5% of the dietary DM replaced with live BSFL that were scattered through the pen seven times a day, or 10% of the dietary DM replaced with live BSFL provided in transparent tubes on the floor that needed to be manipulated by the broilers to get the larvae out. Like in **Chapter 2**, all broilers that received live BSFL showed more foraging behaviour and were more active than controls receiving no BSFL. The two new strategies prolonged the interaction time with live BSFL and consequently broilers in these treatments had the highest foraging and activity levels, and they spent more time in standing posture than controls throughout the rearing period. Broilers in these two treatments also appeared less fearfulness than controls in a behavioural test, and hence they appeared to experience a less negative affective state. Broilers that received 10% of the diet as live BSFL across four provisioning moments had less wide tibia and the litter on which they were housed was less friable than that of controls. Other parameters of health (including abnormalities in the legs, tibias, and muscles) and litter quality were not influenced by live BSFL provisioning. The body weight gain of broilers in all live BSFL treatments except for the 5% provided across four provisioning moments was temporarily reduced, though their total feed intake and final weight did not differ from that of controls. Overall, live BSFL provisioning again increased natural behaviour, and prolonged access to live BSFL also resulted in a less negative affective state.

The aim of **Chapter 4** was to disentangle the effects of different BSFL forms (i.e., processed in different ways) and different provisioning methods on broiler welfare. To this aim, broilers received 8% of the dietary DM as BSFL meal and oil incorporated in the pellets provided in the feeder, dried whole BSFL provided in the feeder, dried whole BSFL scattered through the pen, live BSFL scattered through the pen, or they did not receive BSFL. Dried and live BSFL were provided four times a day. Compared to controls, scattering dried or live BSFL through the pen increased the time spent on foraging behaviour and overall activity, but only live BSFL increased the time broilers spent in standing posture. Broilers that received dried or live BSFL had temporarily higher body weight gains early in the rearing period and they had higher final body weights, despite the finding that broilers receiving live BSFL had a lower total feed intake than controls. Compared to controls, including BSFL as meal and oil incorporated in the feed and scattering dried or live BSFL through the pen reduced the severity of foot pad dermatitis, and live BSFL additionally reduced the severity of hock burn. All BSFL treatments improved litter quality. Lameness, cleanliness, and concentrations of natural antibodies and serotonin in the blood were not influenced by BSFL treatments. Overall, all BSFL treatments benefitted one or more aspects of broiler welfare, with scattering live BSFL causing the most welfare benefits.

In conclusion, live BSFL seem the most appropriate BSFL form to improve broiler welfare, as providing live BSFL consistently promoted natural behaviour. Also, prolonged interaction with live BSFL reduced broiler fearfulness, and some of the studies found health benefits of BSFL provisioning. Dried BSFL only substantially improved broiler welfare when scattered through the pen and not when provided in a feeder, confirming the importance of the BSFL provisioning method for the effects on welfare. Even though behaviour was not influenced by dried BSFL provided in the feeder and of BSFL meal and oil incorporated in the feed, these treatments did benefit litter quality, growth rates (for the former treatment), and leg health (for the latter treatment). These benefits are likely caused by the nutritional qualities of BSFL, though the mechanisms of this were not studied in this thesis. Additional correlational analyses on welfare parameters of individual broilers suggested that leg health is more related to weight than to time spent active and/or in standing posture, though other factors such as litter quality and stocking density influence these parameters. As such, validation of the observed effects in distinct production systems is important.

Black soldier fly larvae for pigs

In **Chapter 5** the preference of pigs for live BSFL over other feed items (i.e., corn, raisins, feed pellets) and enrichment materials (i.e., feed pellets provided in rotatable tubes, jute sacks, or rubber balls on chains) was investigated. Pigs consistently consumed more live BSFL than other feed items and interacted longer with live BSFL provided in a feeder or in tubes than with the other feed items and enrichment materials. Furthermore, when pigs had access to the enrichment materials during five days, live BSFL in rotatable tubes promoted the most exploratory behaviours. Also, they reduced damaging behaviours towards pen mates compared to when rubber balls on chains were provided. The research described in this chapter demonstrated the expected high interest of pigs in live BSFL.

In **Chapter 6** piglets received live BSFL for 11 days after weaning (75 g/pig on d1-4, 150g/pig on d5-11) provided by scattering them through the pen twice a day across the bedding (wood shavings). The control group received equal volumes of wood shavings twice a day. Live BSFL provisioning increased the time spent on floor-directed exploration and decreased the time spent on damaging behaviours towards pen mates, fighting, and eating pellets. It also temporarily decreased the pellet intake, but the total feed and energy intake, body weight gain, final weight, and the faecal consistency did not differ between treatments. Piglets that received live BSFL did not differ from controls in fear-related behaviours in a novel environment, however they did display less neophobic behaviours when a novel object was introduced to this environment. Providing small amounts of live BSFL during 11 days after weaning thus improved several aspects of piglet welfare.

In **Chapter 7** piglets received either only creep feed (control group) or creep feed and live BSFL *ad libitum* for four weeks until weaning, and for three weeks post-weaning piglets either had no access to live BSFL (control group) or they could rotate tubes that released live BSFL at levels up to 20% of their expected daily DM intake. This resulted in four combinations of pre- and post-weaning treatments. Piglets that received live BSFL before weaning were more interested in them than in the creep feed, therefore they interacted less with the creep feed than the controls. The total time spent on feed-directed behaviour was not influenced by live BSFL provisioning. Having access to live BSFL both before and after weaning influenced gastro-intestinal tract development as measured on d3 post-weaning. After weaning, the piglets that had received live BSFL before weaning had a marginally lower feed intake and body weight gain than pre-weaning controls. Piglets with access to live BSFL post-weaning had a lower total feed intake than controls. They also spent less time on exploring the environment and nosing pen mates throughout the post-weaning period. Moreover, they spent less time on damaging behaviours and playing during the first weeks of the post-weaning period compared to controls. Pre- and/or post-weaning live BSFL provisioning did not influence post-weaning final weight, the number of days with diarrhoea, and the responses of pigs in behavioural tests aimed at observing fearfulness (i.e., novel environment test) or degree of optimism (i.e., attention bias test). Overall, it could not be concluded that pre-weaning live BSFL provisioning benefitted piglet welfare during the weaning transition. However, post-weaning live BSFL provisioning did benefit piglet welfare as less damaging behaviour towards pen mates was observed.

In conclusion, live BSFL provisioning around the weaning transition benefitted pig behaviour, as the live larvae were always explored and consumed for long periods of time, and this reduced damaging behaviours towards pen mates. Live BSFL provisioning had some benefits for a pig's affective state, though this was not observed in all chapters. While no strong effects of pre-weaning live BSFL provisioning were found, correlational analyses showed that piglets that spent more time eating live BSFL before weaning also ate creep feed during more observation days, showed a more optimistic response during the attention bias test, and had post-weaning diarrhoea during fewer days. This indicates that individual piglets that did consume high amounts of live BSFL may have benefitted from this during the weaning transition.

The big picture

Insects can become an integral part of sustainable livestock production systems, and several principles for the responsible use of insects in these systems were created in **Chapter 8**. The proposed principles are avoiding unnecessary feed competition between farmed insects and conventional livestock; ensuring farmed insects are safe as livestock feed; revising regulatory frameworks; optimizing performance of insects on low-value waste streams; balancing livestock productivity, welfare, and environmental goals of insect feeding strategies; and accounting for insect welfare. The results from all chapters may contribute to the question whether insects can be used as feed and enrichment for livestock and how this can be done responsibly. Additional research on this topic is required, for example into the use of insect larvae as enrichment for other animals and into feed safety of different insect forms.

Conclusion

The results from this PhD thesis indicate that BSFL can be used as effective environmental enrichment for both broilers and pigs. Especially natural foraging behaviour was greatly stimulated by live BSFL provisioning across chapters. Furthermore, some aspects of broiler and pig health and affective states also improved due to BSFL provisioning, depending on the applied forms and provision methods. These results verify the importance of environmental stimulation for broilers and pigs, and they support the necessity to change current commercial housing conditions where such stimulation is often minimal.

Samenvatting

Het toevoegen van insecten aan het dieet van landbouwhuisdieren zou de duurzaamheid van de veehouderij mogelijk kunnen verhogen. Zwarte soldatenvlieg larven (*Hermetia illucens*, ZSL) lijken hiervoor zeer geschikt omdat ze kunnen groeien op organische reststromen en omdat hun kweek relatief weinig negatieve invloed heeft op het milieu. Het verstrekken van ZSL zou mogelijk ook positieve effecten op het welzijn van landbouwhuisdieren kunnen hebben, maar dit is nog niet onderzocht. Veel commercieel gehouden vleeskuikens en varkens ervaren welzijnsproblemen. Vleeskuikens groeien snel en worden meestal gehouden in een stimulus-arme omgeving met een hoge bezettingsdichtheid, waardoor ze hun essentiële foerageergedrag scharrelen minder goed uit kunnen voeren. Hierdoor hebben vleeskuikens vaak gezondheidsproblemen aan hun poten (bv. voetzoollaesies en kreupelheid) en ervaren ze mogelijk een negatieve emotionele toestand. Varkens zijn ook zeer gemotiveerd om hun omgeving te ontdekken via exploratieve gedragingen zoals wroeten en kauwen, maar in de stimulus-arme hokken waarin ze commercieel gehouden worden kunnen ze deze gedragingen niet goed uitvoeren. Daardoor richten varkens hun exploratieve gedragingen vaak op hokgenoten in de vorm van beschadigend gedrag zoals staart- of oorbijten, en ervaren ze mogelijk een negatieve emotionele toestand. Daarnaast worden biggen in de gangbare varkenshouderij vaak vroeg en abrupt gespeend, waardoor ze weinig ervaring op kunnen doen met het eten van vast voedsel voor spenen. In combinatie met de andere stressoren rondom spenen (bv. verplaatsen naar een ander hok en in veel gevallen gemengd worden met onbekende biggen) kan dit zorgen voor gezondheidsproblemen en verlaagde voedselopname en groei na spenen. Ook vertonen biggen direct na spenen meer beschadigend gedrag richting hokgenoten, vooral als ze gehuisvest worden in hokken zonder enige afleiding. Het aanbieden van hele ZSL aan vleeskuikens en varkens zou hun natuurlijke foerageer- en exploratiegedrag kunnen stimuleren, en als gevolg hiervan mogelijk hun welzijn kunnen verbeteren. Dat is onderzocht in dit proefschrift.

Dit proefschrift is opgedeeld in drie delen. In het eerste deel werd het effect van het aanbieden van ZSL op het welzijn van vleeskuikens onderzocht. Hierbij werd bepaald welke methoden voor het verstrekken van larven zorgde voor de grootste welzijnsvoordelen. In het tweede deel werd het effect van het aanbieden van ZSL op het welzijn van biggen rond het speenproces onderzocht. In dit deel werd onder meer de interesse van varkens in levende ZSL vergeleken met andere voeder items en verrijkingsmaterialen. Bij vleeskuikens en varkens werden drie belangrijke aspecten van welzijn bestudeerd: natuurlijk gedrag, gezondheid en biologisch functioneren, en de emotionele toestand van een dier. Als laatste werd in deel drie het grotere geheel beschouwd. Dit is gedaan door een lijst met principes voor het verantwoordelijke gebruik van insecten in duurzame veehouderij op te stellen. Daarnaast werd de samenhang tussen verschillende hoofdstukken bediscussieerd, werden er richtingen voor toekomstig onderzoek aangegeven en werd gereflecteerd op de rol van insecten in duurzame voedselsystemen.

Zwarte soldatenvlieg larven voor vleeskuikens

In **Hoofdstuk 2** werden levende ZSL aangeboden aan vleeskuikens. Dit gebeurde in verschillende hoeveelheden (d.w.z., 5% of 10% van het droge stof (DS) gehalte van het dieet vervangen door ZSL) en in verschillende frequenties (d.w.z., ZSL twee of vier keer per dag door het hok gestrooid over het strooisel). Dit resulteerde in vier combinaties van hoeveelheid en frequentie die vergeleken werden met een controlebehandeling waarin geen ZSL werden verstrekt. Vleeskuikens waren gehuisvest bij een uiteindelijke bezettingsdichtheid van 14 kg/m^2. Alle combinaties van hoeveelheid en frequentie verhoogde de tijd die vleeskuikens spendeerden aan foerageergedrag en hun totale activiteit ten opzichte van de controlegroep. Vleeskuikens die vier keer per dag levende ZSL kregen waren het meest actief en stonden meer tijdens de hele groeiperiode dan controledieren. Vleeskuikens die 5% van hun dieet als levende ZSL kregen verdeeld over vier momenten hadden minder last van brandhakken dan controledieren. Vleeskuikens die 10% van hun dieet als levende ZSL kregen hadden minder last van brandhakken en kreupelheid, en als ze tweemaal daags levende ZSL kregen hadden ze ook een lager eindgewicht dan controledieren. In het algemeen stimuleerde het verstrekken van levende ZSL het foerageergedrag van vleeskuikens, en soms verbeterde het hun pootgezondheid.

In **Hoofdstuk 3** werden de beste strategieën voor het aanbieden van levende ZSL van **Hoofdstuk 2** (d.w.z., 5% of 10% van de DS van het dieet vervangen door levende

ZSL die vier keer per dag door het hok gestrooid werden) aangeboden aan vleeskuikens die gehuisvest waren in een gangbare uiteindelijke bezettingsdichtheid (33 kg/m^2). Ook twee nieuwe strategieën gericht op het verlengen van de interactie met levende ZSL werden meegenomen in dit onderzoek: 5% van de DS van het dieet vervangen door levende ZSL die zeven keer per dag door het hok gestrooid werden, of 10% van het dieet vervangen door levende ZSL aangeboden in doorzichtige buizen op de vloer die door kuikens zelf bewogen moesten worden om de larven te verkrijgen. Net als in **Hoofdstuk 2** vertoonden alle vleeskuikens die levende ZSL kregen meer foerageergedrag en waren ze actiever dan controledieren die geen ZSL kregen. De twee nieuwe strategieën verlengden de interactie met de levende ZSL, en zodoende waren deze vleeskuikens het meest aan het foerageren en het meest actief. Ook stonden ze meer tijdens de hele groeiperiode vergeleken met controledieren. Vleeskuikens in deze twee behandelingen bleken in een gedragstest minder angstig dan controledieren en leken dus een minder negatieve emotionele toestand te ervaren. Vleeskuikens die 10% van het dieet als levende ZSL kregen verdeeld over vier momenten hadden minder brede scheenbenen en het strooisel waarop ze waren gehuisvest was minder rul dan bij controledieren. Andere kenmerken van gezondheid (waaronder afwijkingen aan de poten, scheenbenen, en spieren) en strooiselkwaliteit werden niet beïnvloed door het aanbieden van levende ZSL. De groei van alle vleeskuikens die levende ZSL kregen, met uitzondering van de '5% vier keer per dag door het hok gestrooid'-groep, was tijdelijk verlaagd, maar de totale voedselopname en het eindgewicht verschilden niet van controledieren. In het algemeen verhoogde het aanbieden van levende ZSL wederom het foerageergedrag, en het verlengen van de interactie met larven zorgde ook voor een minder negatieve emotionele toestand.

Het doel van **Hoofdstuk 4** was om de effecten van ZSL verstrekt in verschillende vormen (d.w.z., op verschillende manieren verwerkt) en op verschillende manieren op het welzijn van vleeskuikens los te koppelen. Hiervoor kregen vleeskuikens 8% van de DS van het dieet als ZSL-meel en -olie gemengd in de voerpellets aangeboden in de voerbak, als gedroogde hele ZSL aangeboden in de voerbak, als gedroogde hele ZSL gestrooid door het hok, als levende ZSL gestrooid door het hok, of ze kregen geen ZSL. De gedroogde en levende ZSL werden vier keer peer dag gegeven. Vergeleken met controledieren verhoogde het strooien van gedroogde of levende ZSL foerageergedrag en totale activiteit, maar alleen het verstrekken van levende ZSL verhoogde de tijd in staande houding. Vleeskuikens die gedroogde of levende ZSL kregen hadden aan het begin van de groeiperiode tijdelijk een snellere groei en ze hadden ook een hoger eindgewicht, ondanks dat de vleeskuikens die levende ZSL kregen een lagere voeropname hadden dan controledieren. Vergeleken met controledieren hadden vleeskuikens die ZSL-meel en -olie gemengd in de voerpellets of gedroogde of levende ZSL gestrooid door het hok kregen minder last van voetzoollaesies, en als ze levende ZSL kregen hadden ze ook minder last van brandhakken. Alle ZSL-behandelingen verbeterde de strooiselkwaliteit. Kreupelheid, reinheid, en gehaltes van natuurlijke antilichamen en serotonine in het bloed werden niet beïnvloed door ZSL-behandelingen. In het algemeen waren alle ZSL-behandelingen voordelig voor één of meer welzijnsparameters, en het strooien van levende ZSL leverde de meeste welzijnsvoordelen op.

Samenvattend lijken levende ZSL de meest geschikte ZSL-vorm om vleeskuikenwelzijn te verbeteren, aangezien het aanbieden van levende larven stelselmatig het natuurlijke gedrag stimuleerde. Daarbij zorgde verlengde interactie met levende ZSL voor lagere angstigheid, en werden in een aantal van de studies gezondheidsvoordelen van het vertrekken van ZSL gevonden. Gedroogde ZSL verhoogde vleeskuikenwelzijn alleen als ze door het hok gestrooid werden en niet wanneer ze aangeboden werden in de voerbak, wat bevestigt dat de methode van aanbieden belangrijk is voor de effecten op welzijn. Ondanks dat gedrag niet beïnvloed werd door gedroogde ZSL aangeboden in de voorbak en door ZSL-meel en -olie gemengd in de voerpellets hadden deze behandelingen wel een positief effect op de strooiselkwaliteit, groei (voor de eerste behandeling) en gezondheid (voor de tweede behandeling). Deze voordelen komen waarschijnlijk door de voedingswaarde van ZSL, maar de werking hiervan is niet onderzocht in dit proefschrift. Extra correlatieanalyses op welzijnsparameters van individuele vleeskuikens suggereerden dat pootgezondheid meer gerelateerd is aan gewicht dan aan activiteit en de tijd in staande houding, alhoewel andere factoren zoals strooiselkwaliteit en bezettingsdichtheid deze parameters ook beïnvloeden. Daarom is validatie van de gevonden effecten in verschillende opfoksystemen belangrijk.

Zwarte soldatenvlieg larven voor varkens

In **Hoofdstuk 5** werd de voorkeur van varkens voor levende ZSL ten opzichte van andere voeder items (mais, rozijnen, voerpellets) en verrijkingsmaterialen (voerpellets in draaibare buizen, jute zakken, of rubberballen aan kettingen) onderzocht. Varkens aten stelselmatig meer levende ZSL dan de andere voeder items en ze waren langer bezig met levende ZSL aangeboden in een voerbak of in buizen dan met de andere voeder items en verrijkingsmaterialen. Wanneer varkens toegang hadden tot de verrijkingsmaterialen gedurende 5 dagen stimuleerde levende ZSL in draaibare buizen het meest exploratief gedrag. Daarnaast verlaagde ze beschadigend gedrag richting hokgenoten vergeleken met wanneer varkens toegang hadden tot rubberballen aan kettingen. Het onderzoek beschreven in dit hoofdstuk toonde de verwachte hoge interesse van varkens in levende ZSL aan.

In **Hoofdstuk 6** kregen biggen levende ZSL gedurende 11 dagen na spenen (75 g/big op d1-4, 150 g/big op d5-11), aangeboden door deze tweemaal daags te strooien door het hok over het strooisel (houtkrullen). De controledieren kregen hetzelfde volume aan houtkrullen twee keer per dag. Het aanbieden van levende ZSL verhoogde de tijd die besteed werd aan exploratie van de vloer, en het verlaagde de tijd die besteed werd aan beschadigend gedrag richting hokgenoten, vechten, en het eten van voer. Het verlaagde ook tijdelijk de voeropname, maar de totale voer- en energieopname, groei, het eindgewicht, en de fecale consistentie verschilden niet tussen behandelingen. Biggen die levende ZSL kregen verschilden niet van controledieren in angst-gerelateerd gedrag in een nieuwe omgeving, maar ze leken wel minder bang voor een onbekend object dat werd geïntroduceerd in deze omgeving. Het aanbieden van kleine hoeveelheden levende ZSL gedurende 11 dagen na spenen verbeterde dus verschillende welzijnsaspecten van biggen.

In **Hoofdstuk 7** kregen biggen alleen vast voer (controlegroep) of vast voer en levende ZSL *ad libitum* gedurende vier werken voor spenen, en gedurende drie weken na spenen kregen biggen alleen vast voer (controlegroep) of ze konden buizen draaien waaruit levende ZSL vielen tot 20% van hun verwachte dagelijkste DS opname. Dit resulteerde in vier combinaties van behandelingen rondom spenen. Biggen die levende ZSL kregen voor spenen hadden hiervoor meer belangstelling dan voor het vaste voer en waren daarom minder lang bezig met het exploreren van dit voer dan controledieren. De totale tijd die biggen besteedden aan voergericht gedrag werd niet beïnvloed door het aanbieden van levende ZSL. Het verstrekken van levende ZSL voor en na spenen had invloed op de ontwikkeling van het maag-darmkanaal op d3 na spenen. Biggen die voor spenen levende ZSL kregen hadden na spenen een iets lagere voeropname en groei dan controledieren. Biggen die na spenen levende ZSL kregen hadden een lagere totale voedselopname dan controledieren. Ze besteedden ook minder tijd aan het exploreren van de omgeving en hadden minder neuscontact met andere biggen na spenen. Bovendien besteedden ze minder tijd aan beschadigend gedrag richting hokgenoten en aan spelen tijdens de eerste weken na spenen vergeleken met controledieren. Het aanbieden van levende ZSL voor en/of na spenen had geen invloed op het eindgewicht, aantal dagen met diarree, en de reacties van biggen in gedragstesten die gericht waren op het waarnemen van angst (d.w.z., een nieuwe omgeving test) of de mate van optimisme (d.w.z., een attentie-bias test). Er kan dus niet geconcludeerd worden dat het aanbieden van levende ZSL voor spenen positieve effecten had op biggen tijdens het speenproces, maar het verstrekken van levende ZSL na het spenen verhoogde wel het welzijn van biggen aangezien dit leidde tot minder beschadigend gedrag richting hokgenoten.

Samenvattend had het aanbieden van levende ZSL rond het speenproces een positief effect op het gedrag van varkens aangezien de levende larven altijd langdurig geëxploreerd en gegeten werden, en dit zorgde voor minder beschadigend gedrag richting hokgenoten. Het verstrekken van levende ZSL had enige positieve effecten op de emotionele toestand van varkens, maar dit werd niet in elk hoofdstuk gevonden. Hoewel er geen sterke effecten van het aanbieden van levende ZSL voor spenen werden gevonden, toonde correlatieanalyses aan dat biggen die meer tijd spendeerden aan het eten van levende ZSL voor spenen ook vast voer aten tijdens meer observatiedagen, een meer optimistische reactie vertoonden tijdens de attentie-bias test, en minder dagen diarree hadden na spenen. Dit geeft aan dat individuele biggen die veel levende ZSL aten voor spenen hier mogelijk voordeel van hadden tijdens het speenproces.

Het grote geheel

Insecten kunnen een volwaardige bijdrage leveren aan een duurzame veehouderij, en een lijst met principes voor het verantwoordelijke gebruik van insecten in veehouderijsystemen is opgesteld in **Hoofdstuk 8**. Deze principes omvatten het voorkomen van onnodige voedselcompetitie tussen gekweekte insecten en conventionele landbouwhuisdieren; ervoor zorgen dat gekweekte insecten veilig zijn als voedsel voor landbouwhuisdieren; aanpassen van kaders van regelgeving; en het optimaliseren van productie van insecten op afvalstromen met lage voedingswaardes. Verder moeten productiviteit en welzijn van landbouwhuisdieren en milieudoelen in balans worden gebracht wanneer insecten verstrekt worden, en moet rekening gehouden worden met het welzijn van insecten. De resultaten van alle hoofstukken kunnen bijdragen aan de vraag of insecten as voer en verrijking voor landbouwhuisdieren kunnen worden gebruikt en hoe dat verantwoord kan. Extra onderzoek naar dit onderwerp is nodig, bijvoorbeeld naar het gebruik van insectenlarven als verrijking voor andere diersoorten en naar de voedselveiligheid van insecten aangeboden in verschillende vormen.

Conclusie

De resultaten van dit proefschrift geven aan dat ZSL gebruikt kunnen worden als effectief verrijkingsmateriaal voor vleeskuikens en varkens. Vooral natuurlijk foerageergedrag wordt enorm gestimuleerd door levende ZSL aan te bieden zoals blijkt in alle hoofdstukken. Daarnaast worden sommige aspecten van de gezondheid en de emotionele toestand van vleeskuikens en varkens ook verbeterd door ZSL, afhankelijk van de verschillende vormen en methodes van aanbieden. Deze resultaten bevestigen de essentie van een stimulerende omgeving voor vleeskuikens en varkens, en ze onderschrijven het belang van een noodzakelijke verandering in commerciële huisvestingssystemen waar omgevingsverrijking vaak minimaal is.

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Allyson

About the author

Allyson Ipema was born on the 23rd of June in Heemskerk, the Netherlands. She started her BSc in Biology at Wageningen University in 2012, which she finished with her BSc thesis on the effects of monoculture crops on parasitoids. After this she continued at Wageningen University in 2015 with the MSc in Biology, specializing in animal adaptation and behavioural biology. During this, she studied the effects of parenting styles in the dog-owner relationship on dog behaviour for her MSc thesis at the Behavioural Ecology Group. Hereafter she investigated how a low birth weight of piglets influenced their judgement bias during her internship at the University of Utrecht, the Netherlands. She graduated in 2017 and started her PhD at the Adaptation Physiology Group of Wageningen University in May 2018. During her PhD she studied the effects of black soldier fly larvae provisioning on broiler and pig welfare, and the results of this research are presented in the current thesis. After her PhD she plans to write a project proposal to study positive affective states in pigs, and she hopes to execute this research at the Adaptation Physiology Group.

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* Authors contributed equally to the study

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Education and training certificate

Approved by the Wageningen Institute of Animal Sciences.

Description	Year
The Basic Package (1.8 ECTS)	
WIAS - Introduction Day	2018
WGS - Scientific Integrity & Ethics in Animal Science	2018
Disciplinary Competences (11.1 ECTS)	
WIAS - Writing literature survey	2018
PE&RC - Introduction to R for Statistical Analysis	2018
University of Utrecht - Species-specific Laboratory Animals Course: Pigs	2018
Training health observations in growing-finishing pigs (MSc level)	2021
Workshop: Cross-fertilization Between Neuroscience and Animal Welfare	2021
WGS - RMarkdown	2021
Research Master Cluster research proposal reviews	2021
WIAS - Advanced Statistics course Design of Experiments	2022
Professional Competences (7.3 ECTS)	
WGS - Brain Training	2018
WGS - Scientific Writing	2019
WGS - Supervising BSc and MSc Thesis Students	2019
WGS - Presenting with Impact	2020
WGS - Brain-friendly Working and Writing	2020
WGS - Efficient Writing Strategies	2021
WGS - Last Stretch of the PhD Programme	2021
WGS - Writing Propositions for Your PhD	2021
WSG - Communication with the Media and with the General Public	2021
WIAS - The Final Touch: Writing the General Introduction and Discussion	2021
Committee member selection Animal Production Systems Chairholder	2021
Presentation Skills (4 ECTS)	
Oral - WIAS Annual Conference	2020
Oral - Regional conference of the ISAE Benelux	2020
Oral - 8th International Conference of the WAFL	2021
Oral - Regional conference of the ISAE Benelux	2021
Teaching Competences (6 ECTS)	
Supervising 1 BSc and 8 MSc thesis students	2019-2022
Total 30.2 ECTS (One ECTS equals a study load of 28 hours)	

Colophon

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