Gut microbiota and feather pecking

Jerine van der Eijk 2019

Gut microbiota and feather pecking

Integrating microbiota, behaviour, stress, serotonin and the immune system

Jerine van der Eijk

Propositions

1. Selection for feather pecking creates behaviourally active and immunologically responsive chickens.
   (this thesis)

2. Homologous microbiota transplantation reduces fearfulness.
   (this thesis)

3. The focus on statistical significance distracts from biological relevance.

4. Solitary housing of rabbits is detrimental to their welfare and should be prohibited.

5. The most important part of productivity is timely leisure.

6. Propositions go against a scientist's nature.

Propositions belonging to the thesis, entitled

Gut microbiota and feather pecking – integrating microbiota, behaviour, stress, serotonin and the immune system

Jerine Alexandra Johanna van der Eijk

Wageningen, 13 December 2019

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Jerine Alexandra Johanna van der Eijk

Thesis committee

Promotors
Prof. Dr M. Naguib
Professor of Behavioural Ecology
Wageningen University & Research

Prof. Dr B. Kemp
Professor of Adaptation Physiology
Wageningen University & Research

Co-promotors
Prof. Dr T.B. Rodenburg
Professor of Animal Welfare
Utrecht University

Dr A. Lammers
Researcher, Adaptation Physiology
Wageningen University & Research

Other member
Prof. Dr W.J.J. Gerrits, Wageningen University & Research
Dr A. Harlander, University of Guelph, Canada
Prof. Dr C.J. Nicol, The Royal Veterinary College, United Kingdom
Prof. Dr H.F.J. Savelkoul, Wageningen University & Research

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Jerine Alexandra Johanna van der Eijk

Thesis

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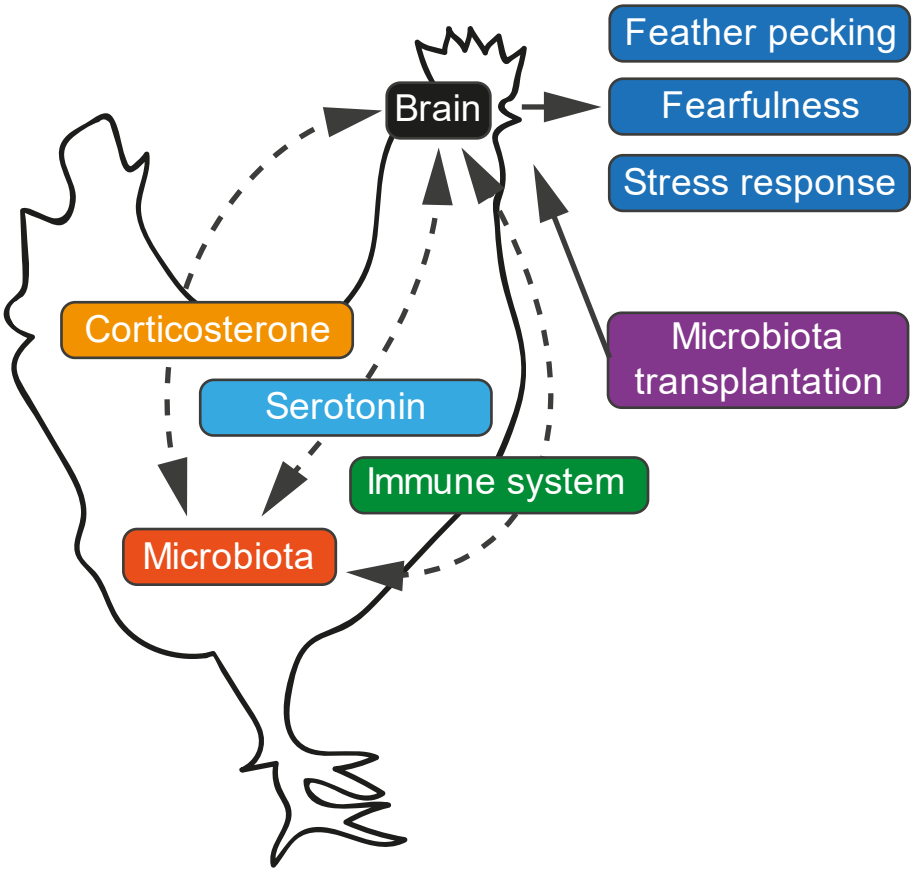
Chapter 1

General introduction



Early-life factors can have a profound impact on an animal's behavioural development. An important moment early in life is the rapid microbial colonization of the gut, leading to the establishment of the gut microbiota. There is striking evidence that microbiota influences host behaviour and physiology, for example anxiety, stress, and the serotonergic and immune systems. Through these effects microbiota could alter an animal's ability to cope with environmental and social challenges, such as those encountered in animal production systems, and could thereby affect the development of damaging behaviours in production animals.

Fearfulness, stress, and the serotonergic and immune systems have been related to severe feather pecking, a damaging behaviour in chickens which involves pecking and pulling out feathers of conspecifics, thereby negatively affecting animal welfare and productivity. Furthermore, high and low feather pecking selection lines differed in gut microbial metabolites and microbiota composition determined from caecal droppings. These findings suggest a link between the gut microbiota and feather pecking. Yet, it is unknown whether the gut microbiota can influence the development of feather pecking. Therefore, the aim of this thesis was to identify effects of gut microbiota on the development of feather pecking.

In this general introduction I will first describe gut microbiota effects on brain development and functioning, and the potential pathways through which gut microbiota affects the brain. Then, I will give an overview of gut microbiota effects on behaviour and physiology as found in rodents and poultry, where I will focus on behavioural and physiological characteristics that have been related to feather pecking. Lastly, I will give an overview of these behavioural and physiological characteristics, indicate how they have been related to feather pecking and provide the research problem, aim and scope of the thesis.

Microbiota

Humans and other animals share a mutualistic relationship with a multitude of microorganisms, also known as the microbiota. These microorganisms are present on all body surfaces, including the skin, gastrointestinal, respiratory and urogenital tract (Costello et al., 2009). Studies identifying interactions between microbiota and its host have mainly focused on the gut microbiota, as it is the most densely colonized body surface, containing approximately 100 trillion bacteria in humans (Ley et al., 2006). The gut microbiota can be influenced by many (host) factors, including genetics, diet, sex, stress and the immune system (Kers et al., 2018; Lozupone et al., 2012). Yet, numerous studies show the gut microbiota influences its host as well, as it plays a role in digestion, immunity and metabolism (Sommer and Bäckhed, 2013).

In the last decade, the gut microbiota has become a topic of interest in behavioural research, as accumulating data suggests it is involved in brain functioning and influences behaviour. For the remainder of this thesis I will refer to the gut microbiota as microbiota

Microbiota affects the brain

Microbiota is involved in brain development and functioning as indicated by altered expression of genes related to brain development and functioning in germ-free animals (i.e. without microbiota) compared to conventional animals (i.e. with microbiota) (Borre et al., 2014; Dinan and Cryan, 2017). For example, germ-free mice had reduced mRNA levels of brain-derived neurotrophic factor, a protein involved in synaptic plasticity, in the hippocampus and amygdala (Clarke et al., 2013; Gareau et al., 2011; Heijtz et al., 2011; Sudo et al., 2004), although one study reported the opposite (Neufeld et al., 2011). Microbiota further influences functioning of the central serotonergic system, which is involved in regulating many types of behaviours, and of the major stress regulatory system, the hypothalamic-pituitary-adrenal (HPA) axis. For example, germ-free mice had reduced mRNA levels of serotonin (5-Hydroxytryptamine or 5-HT) 1a receptor (Neufeld et al., 2011) and 5-HT transporter in the hippocampus, although no difference in mRNA levels of 5-HT1a, 5-HT2c and 5-HT6 receptors or tryptophan hydroxylase (enzyme for 5-HT synthesis) in the hippocampus was also found (Clarke et al., 2013). With regard to the HPA axis, germ-free mice had reduced mRNA levels of glucocorticoid receptor in the hippocampus and increased mRNA levels of corticotrophin-releasing factor in the hypothalamus (Crumeyrolle-Arias et al., 2014; Sudo et al., 2004). Corticotrophin-releasing factor stimulates production of adrenocorticotropic hormone (ACTH), which in turn increases production and release of corticosterone (CORT), the major stress hormone. The affected brain regions, mainly the amygdala, hippocampus and hypothalamus, are involved in regulating anxiety, fear, memory, learning and stress (Colombo and Broadbent, 2000; Puglisi-Allegra and Andolina, 2015; Saint-Dizier et al., 2009), indicating that microbiota could affect these processes.

Pathways through which microbiota affects the brain

The underlying mechanisms through which microbiota affects the brain remain largely unknown, but it is clear that multiple complex pathways exist, including metabolic, immune and neural pathways (Collins et al., 2012; Cryan and Dinan, 2012) (see Figure 1).

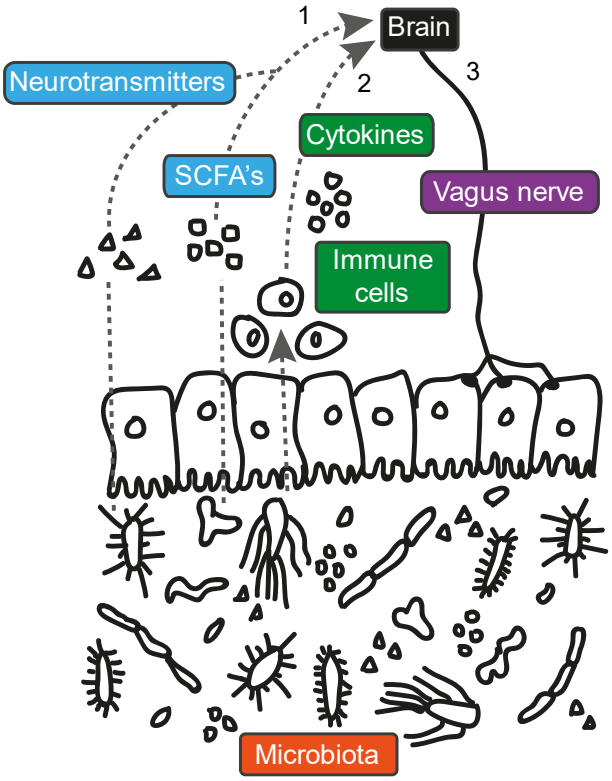


Figure 1. Pathways through which microbiota affects the brain. 1) metabolic, for example through the production of neurotransmitters or short-chain fatty acids (SCFAs); 2) immune, through activation of immune cells that produce cytokines; and 3) neural, through activation of the vagus nerve.

Firstly, microbiota influences metabolism, producing or utilizing metabolic precursors of hormones and neurotransmitters or directly producing the active metabolites themselves (Wall et al., 2014). For example, microbiota modulates the availability of tryptophan (precursor for 5-HT) (Clarke et al., 2013; Lee and Lee, 2010) or produces neuroactive metabolites, such as 5-HT, dopamine, γ-amino butyric acid and histamine (Barrett et al., 2012; Özogul, 2004; Özogul et al., 2012). Microbiota further produces short-chain fatty acids (SCFAs) (LeBlanc et al., 2017) and SCFAs, such as butyrate, acetate and propionate, are known to have neuroactive properties (MacFabe et al., 2011, 2007; Stilling et al., 2016).

Secondly, microbiota influences the immune system by activating various immune cells, particularly innate immune cells, such as macrophages, neutrophils and dendritic cells (Juul-Madsen et al., 2014; Kaspers and Kaiser, 2014). Upon activation these cells produce cytokines, the key signalling molecules of the immune system (Kaiser and Stäheli, 2014). Peripherally produced cytokines can act on the brain (Dantzer et al., 2008), altering HPA axis sensitization, serotonergic and dopaminergic neurotransmission (Dantzer et al., 1999; Miller et al., 2013).

Lastly, microbiota influences the brain through the vagus nerve, the primary nerve connecting the enteric nervous system to the central nervous system (Forsythe et al., 2014). Indeed, several studies have shown that microbiota effects on the brain depend on vagal activation (Bercik et al., 2011b; Bravo et al., 2011; Goehler et al., 2008). A potential mechanism through which microbiota modulates the activity of the vagus nerve is via the production of neuroactive metabolites (Bravo et al., 2011; Goehler et al., 2005).

Thus, multiple pathways exist through which microbiota affects brain functioning and thereby microbiota could influence behaviour. Indeed, many studies have shown that microbiota affects behaviour.

Microbiota affects behaviour

Rodent studies

In the last decade there has been a vast amount of studies that identified effects of microbiota on behaviour. Most studies to date focus on comparing germ-free rodents to conventional or specific pathogen free rodents, showing that microbiota influences behavioural characteristics, such as anxiety, stress and activity (Cryan and Dinan, 2012; Dinan and Cryan, 2012). However, these germ-free models are rather extreme, which makes it difficult to translate findings to 'normally' occurring situations. Yet, more evidence is provided by studies that manipulate microbiota in rodents, via for example anti-, pro-, pre- or synbiotic treatment. Antibiotics inhibit the growth of or destroy microorganisms or both; probiotics contain viable, beneficial microorganisms; prebiotics contain non-digestible food ingredients that promote the growth or activity of beneficial microorganisms or both; and synbiotics contain both pro- and prebiotics (Schrezenmeir and de Vrese, 2001). Manipulating microbiota via these treatments influences behavioural characteristics, such as anxiety and stress (Cryan and Dinan, 2012; Dinan and Cryan, 2012; Joseph and Law, 2019). However, it should be noted that different types of pro-, pre- or synbiotics can target different aspects of host behaviour and physiology. Overall, studies using germ-free animals or manipulating microbiota show that microbiota affects behavioural characteristics. Interestingly, there is striking evidence that behavioural profiles of donors are adopted by recipients via microbiota transplantation. When germ-free mice from one mouse strain received microbiota from another mouse strain, they showed exploratory behaviour similar to that of the donor strain (Bercik et al., 2011a; Collins et al., 2013). Rats depleted of microbiota via antibiotic treatment (i.e. pseudo germ-free) and receiving microbiota from depressed patients showed more anhedonia- and anxiety-like behaviours compared to rats receiving microbiota from control patients

(Kelly et al., 2016). Furthermore, pseudo germ-free mice receiving microbiota from rats with an anhedonia-like phenotype showed a more anhedonia-like phenotype, while the opposite was found for mice receiving microbiota from rats without anhedonia (Yang et al., 2019). These findings point to interesting effects of microbiota transplantation on behaviour, where recipients seem to adopt behavioural characteristics of donors. In summary, it is clear that microbiota influences a range of behaviours in rodents, but three recent studies also point to effects of microbiota on behaviour in poultry.

Poultry studies

Germ-free quails showed reduced fearfulness compared to colonized quails (Kraimi et al., 2018) and similarly probiotic treatment reduced fearfulness in quails (Parois et al., 2017). Moreover, microbiota transplantation resulted in quails adopting the fear-related behaviour of donors early in life, although this was reversed later in life (Kraimi et al., 2019). These findings suggest that microbiota can influence behavioural characteristics in poultry as well. However, are these effects on behaviour immediate or do they also persist after treatment ended?

Immediate or long-term effects of microbiota on behaviour?

Most studies to date identified behavioural responses during treatment, using anti-, pro-, pre- or synbiotic treatment (Barrera-Bugueño et al., 2017; Bravo et al., 2011; Burokas et al., 2017; Desbonnet et al., 2015; Liu et al., 2016a; Liu et al., 2016b; McKernan et al., 2010; McVey Neufeld et al., 2018; Savignac et al., 2015, 2014), and using microbiota transplantation (Kelly et al., 2016). Yet, only a limited number of studies identified microbiota effects on behaviour after treatment ended. Antibiotic treatment increased anxiety-like behaviour 5 days after treatment ended (Ceylani et al., 2018), but did not affect anxiety-like behaviour 1 week (Hoban et al., 2016) or 6 weeks after treatment ended in rodents (O'Mahony et al., 2014). Microbiota transplantation altered anhedonia-like behaviour 4 days after treatment ended (Yang et al., 2019) and exploratory behaviour 3 weeks after treatment ended in rodents (Bercik et al., 2011a). Furthermore, microbiota transplantation altered fearfulness 4 weeks after treatment ended in quails (Kraimi et al., 2019). Thus, most studies to date focused on identifying microbiota effects on behaviour during treatment and effects of antibiotic treatment on behaviour do not seem to persist 1 week after treatment ended. Yet, microbiota transplantation seems to have long-term effects on behavioural responses in rodents and poultry, which might be related to

effects being more persistent when treatment is given during a sensitive period, such as the early-life period.

Early-life as a sensitive period for microbiota to affect brain and behaviour?

Early in life both the brain and the gut microbiota are rapidly developing and in this period the gut microbiota could have long-term effects on brain and behaviour (Borre et al., 2014). Indeed, sensitive periods seem to exist during which microbiota colonization of germ-free mice can restore brain and behavioural characteristics to that of conventionalized or specific pathogen free mice. Colonization of germ-free mice at 3 weeks of age restored anxiety-like behaviour, but not central 5-HT and 5-HIAA (5-HT metabolite) levels at 6 - 9 weeks of age (Clarke et.al., 2013) or neuronal activity of the amygdala at 10 weeks of age (Stilling et al., 2015). Furthermore, colonization of germ-free mice at 5 – 6 weeks of age restored central mRNA levels of 5-HT7 receptor, but not monoamine oxidase B (enzyme that metabolizes 5-HT to 5-HIAA), 5-HT1f and 5-HT3b receptors, nor did it restore anxiety-like behaviour at 8 – 9 weeks of age (Pan et al., 2019). In addition, there is evidence for a sensitive period of the stress system, as colonization at 6 weeks of age did restore stress-induced ACTH and CORT levels at 9 weeks of age, but colonization at 14 weeks of age did not restore these levels at 17 weeks of age (Sudo et al., 2004). Thus, early in life there seems to be a sensitive period or even a critical window where microbiota colonization can restore brain and behavioural characteristics in germ-free animals to that of conventionalized conspecifics. This indicates that the early-life period is a crucial moment for microbiota to affect brain and behaviour. Yet, next to microbiota effects on behaviour it also affects physiology.

Microbiota affects physiology

Rodent studies

Microbiota influences physiological systems, such as the stress, serotonergic and immune systems (Bauer et al., 2006; Foster et al., 2017; Gensollen et al., 2016; O'Mahony et al., 2015; Sommer and Bäckhed, 2013).

Stress is comprised of a behavioural and physiological response. Next to the effects of microbiota on the behavioural stress response, it further influences the physiological stress response. Germ-free rodents generally have higher stress-induced ACTH and CORT levels compared to conventional or specific pathogen free rodents (Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Sudo et al., 2004). However, manipulating microbiota via anti- or prebiotics did not alter stress-induced CORT level (Barrera-Bugueño et al., 2017; Desbonnet et al., 2015; Hoban et al.,

2016) and synbiotics reduced stress-induced CORT level (Barrera-Bugueño et al., 2017). Findings for probiotics are inconsistent with studies showing higher (Sudo et al., 2004), lower (Bravo et al., 2011) or no effect on stress-induced CORT level (Barrera-Bugueño et al., 2017; Savignac et al., 2014). Thus, microbiota can affect the physiological stress response, although effects depend on the treatment used and there is inconsistency in findings for probiotic treatments.

As indicated previously, microbiota affects the central serotonergic system, but increasing evidence suggests it can also affect the peripheral serotonergic system. In general, germ-free rodents have lower mRNA levels of tryptophan hydroxylase in the gut, lower plasma and serum 5-HT levels, but higher mRNA levels of the serotonin transporter and of 5-HT3a receptor in the gut (Hata et al., 2017; Sjögren et al., 2012; Wikoff et al., 2009; Yano et al., 2015). Findings with regard to 5-HT levels in the gut of germ-free mice are inconsistent, as both higher (Hata et al., 2017) and lower 5-HT levels have been reported (Yano et al., 2015). Manipulating microbiota via antibiotic treatment decreased serum 5-HT level, mRNA level of tryptophan hydroxylase and 5-HT level in the gut (Ge et al., 2017; Yano et al., 2015), while heat killed probiotics increased mRNA level of tryptophan hydroxylase and 5-HT level in the gut (Hara et al., 2018). Thus, next to its effects on the central serotonergic system, microbiota affects the peripheral serotonergic system as well, where having no gut microbiota or being depleted of gut microbiota generally reduces peripheral 5-HT level, while probiotic treatment seems to increase peripheral 5-HT level.

With regard to the immune system, microbiota has been shown to regulate the development of lymphoid structures, the production of cytokines and chemokines, modulate the differentiation of immune cell subsets and educate the adaptive immune system (Sommer and Bäckhed, 2013; Zhao and Elson, 2018). However, it is beyond the scope of this general introduction to discuss all findings with regard to microbiota effects on the immune system and since there are multiple studies that identified microbiota effects on immune characteristics in chickens I will focus on discussing those in the next section.

In summary, it is clear that gut microbiota influences the stress, serotonergic and immune systems in rodents, but several studies also point to effects of the gut microbiota on these physiological systems in chickens.

Chicken studies

With regard to the stress system, most studies to date identified effects of gut microbiota on basal CORT level in chickens. Manipulating microbiota via probiotic treatment did not alter basal CORT level in unpleasantly handled broilers

(Meimandipour et al., 2011), broilers (Cengiz et al., 2015; Yan et al., 2018) or laying hens, but it reduced basal ACTH level in laying hens (Lei et al., 2013). Similarly, prebiotic treatment did not alter basal CORT level in broilers (Houshmand et al., 2012). Yet, pro-, pre and synbiotic treatment reduced basal CORT level in heat-stressed broilers (Cheng et al., 2018; Sohail et al., 2012, 2010). Thus, findings are inconsistent with regard to microbiota effects on basal CORT level, potentially because of different pro- and prebiotics used, but these are first indications that microbiota could alter the physiological stress response in chickens. However, no study to date identified effects of microbiota on stress-induced CORT level. Thus, it remains unknown whether microbiota affects the physiological stress response in chickens.

For the peripheral and central serotonergic systems, manipulating microbiota via antibiotic treatment reduced gut content 5-HT level, although this was dependent on the antibiotic used (Gadde et al., 2018). Similarly, probiotic treatment reduced plasma 5-HT level in dominant laying hens (Hu et al., 2018) and increased central 5-HT level in broilers (Yan et al., 2018). However, probiotic treatment did not affect plasma 5-HT level, nor did it alter central 5-HIAA level or 5-HT turnover (5-HIAA/5-HT) in broilers (Yan et al., 2018). Thus, microbiota seems to affect the peripheral and central serotonergic systems in chickens, but findings are inconsistent even within treatments used.

Many studies have identified effects of microbiota on the immune system in chickens (Broom and Kogut, 2018). Therefore, I will focus on studies that identified effects of microbiota on immune characteristics that were measured in this thesis. Nitric oxide production by monocytes was measured as indicator for innate pro-inflammatory immune functioning (Murray and Wynn, 2011; Uehara et al., 2015) and specific antibody level was measured as part of the adaptive immune system. Furthermore, natural (auto)antibody level was measured, as natural antibodies play an essential role in both innate and adaptive immunity, for example by maintaining homeostasis, increasing disease resistance and linking the two types of immunity (Berghof et al., 2019; Lammers et al., 2004; Ochsenbein and Zinkernagel, 2000; Panda and Ding, 2015). Natural antibodies are antibodies that can bind antigen without intentional prior exposure to that antigen, where natural antibodies bind to non-self-antigen and natural autoantibodies bind to self-antigen (Avrameas, 1991; Baumgarth et al., 2005). Immune cell subsets were included to identify whether potential differences in immune characteristics were reflected by differences in immune cell subsets.

Manipulating microbiota via antibiotic treatment reduced specific antibody level in broilers and laying hens (Hong et al., 2012; Simon et al., 2016), although also no effects were found on specific antibody level in broilers and laying hens (Hong et al., 2012; Shang et al., 2015; Simon et al., 2016) nor on natural antibody level in laying hens (Shang et al., 2015; Simon et al., 2016). Thus, antibiotic treatment seems to reduce the specific antibody response, but findings are inconsistent potentially because of different antigens used. Furthermore, antibiotic treatment seems to have no effect on natural antibody level. In broilers, probiotic treatment increased natural and specific antibody levels (Haghighi et al., 2006, 2005), but was also found to have no effect on specific antibody level to various antigens (Haghighi et al., 2005; Qorbanpour et al., 2018; Wu et al., 2019a) or on immune cell subsets (Wu et al., 2019a). Yet, probiotic treatment altered specific antibody level and nitric oxide production by macrophages *in vitro*, but effects were dependent on the probiotic strain used (Brisbin et al., 2015, 2011). Thus, probiotic treatment seems to increase natural antibody level, but effects on specific antibody level and nitric oxide production by macrophages were dependent on the probiotic used. Furthermore, probiotic treatment seems to have no effect on immune cell subsets. Prebiotic treatment did not alter immune cell subsets (Kim et al., 2011) or specific antibody levels to various antigens in broilers (Alizadeh et al., 2017; Houshmand et al., 2012). Similarly, synbiotic treatment did not alter specific antibody level in broilers (Alizadeh et al., 2017). Thus, both pre- and synbiotic treatments do not seem to affect immune characteristics in chickens.

In summary, microbiota affects behavioural and physiological characteristics in poultry as well. However, it should be noted that bidirectional communication exists between the gut microbiota and the brain (Grenham et al., 2011), potentially through the stress, serotonergic and immune systems (de Weerth, 2017; O'Mahony et al., 2015; Powell et al., 2017). Overall, it is clear that microbiota affects behavioural and physiological characteristics, such as anxiety, the stress response, and the serotonergic and immune systems. Through its effects on these behavioural and physiological characteristics gut microbiota could alter an animal's ability to cope with environmental and social challenges and could thereby affect the development of damaging behaviours in production animals. Different types of damaging behaviours exist, but the most common one in laying hens is feather pecking.

Feather pecking

Feather pecking (FP) is a major behavioural problem in the egg laying industry and involves hens pecking and pulling at feathers of conspecifics. Different types of FP

have been defined: gentle FP, which consists of nibbling or gentle pecks at the feathers and causes little or no damage; and severe FP, which consists of forceful pecks and pulls of feathers and can cause serious damage to the recipient and could even develop into cannibalistic pecking (Savory, 1995). Gentle and severe FP are suggested to have different motivational backgrounds. Exploratory FP, a type of gentle FP, is thought to derive from an explorative social behaviour (Riedstra and Groothuis, 2002), while severe FP is thought to derive from a redirected ground pecking behaviour (Blokhuis, 1989; Rodenburg et al., 2013). Furthermore, gentle FP is often performed by the majority of chickens, especially early in life, while severe FP is performed by only a few individuals and peaks during the egg laying period (Bright, 2009; Newberry et al., 2007). When studying severe FP it is therefore essential to not only identify differences between lines that differ in FP (i.e. FP genotypes), but also between individuals within these lines, as they can become a feather pecker, feather pecker-victim, victim or neutral (i.e. FP phenotypes) (Daigle et al., 2015). Furthermore, severe FP is a multifactorial behaviour, meaning it can be influenced by a multitude of factors, including individual factors (such as genetics, neurobiology or the immune system), physical and social environmental factors (such as feed or group dynamics) (Brunberg et al., 2016). Since severe FP is multifactorial, many behavioural and physiological characteristics have been related to the development of severe FP. The most common ones being fearfulness, stress and the serotonergic system (de Haas and van der Eijk, 2018; Rodenburg et al., 2013). Furthermore, the immune system has been suggested to be related to severe FP as well (Parmentier et al., 2009). I will now discuss findings for each of these behavioural and physiological characteristics in relation to severe FP, where I will refer to severe FP as FP.

Fearfulness

Fearfulness can be defined as the tendency of an animal to be easily frightened in response to potentially dangerous stimuli (Boissy, 1995; Jones, 1996). When comparing commercial lines that differ in FP or feather damage, as indicator of FP (Bilcik and Keeling, 1999), FP is usually related to high fearfulness. Commercial lines with a high FP tendency were more fearful in various behavioural tests compared to lines with a low FP tendency (de Haas et al., 2013; Dudde et al., 2018; Mahboub et al., 2004; Uitdehaag et al., 2008b). Furthermore, a strong negative correlation was found between fearfulness at a young age and high FP at adult age (Rodenburg et al., 2004a), indicating that fearful chicks are more likely to develop FP at adult age. Similarly, fear of humans during the rearing period was suggested

as predictor for feather damage at adult age (de Haas et al., 2014a). In summary, these studies indicate that FP is related to high fearfulness and that high fearfulness could even lead to FP in commercial lines.

Selection on egg production traits resulted in high (HP) and low (LP) FP lines (Korte et al., 1997). Similar to findings for commercial lines, HP chicks were more fearful in an open field test than LP chicks, although no difference was found in tonic immobility duration (Jones et al., 1995), which is considered a validated measure for innate fearfulness (Forkman et al., 2007). Interestingly, in lines divergently selected on FP, resulting in a high FP (HFP) and a low FP (LFP) line (Kjaer et al., 2001), the relationship between fearfulness and FP seems to be opposite to that described for commercial and other experimental lines. HFP chicks were less fearful compared to LFP chicks, although no difference was found at adolescent age (Kops et al., 2017). At adult age, HFP birds were less fearful in various behavioural tests compared to LFP birds (Bögelein et al., 2014; de Haas et al., 2010), but no difference in fearfulness at adult age has also been reported (Rodenburg et al., 2010). Overall, the FP selection lines show an opposite relation between fearfulness and FP compared to that found in commercial and other experimental lines, especially at young age.

It should be noted that studies mentioned so far mainly compared lines that differ in FP or feather damage. Yet, as indicated previously, it is important to study FP phenotypes, especially since high fearfulness could be a result of being feather pecked (i.e. victim) (Hughes and Duncan, 1972), although no relation between receiving FP and fearfulness has also been reported (Bögelein et al., 2014). For the performance of FP (i.e. feather pecker or feather pecker-victim) either a positive, negative or no relation has been found with fearfulness (Bögelein et al., 2014; Jensen et al., 2005; Vestergaard et al., 1993). These findings indicate that relationships between performing or receiving FP and fearfulness are inconsistent and further research is needed to identify fearfulness in FP phenotypes.

In summary, FP is related to high fearfulness in commercial lines, but this relation is opposite in the FP selection lines. Furthermore, findings for fearfulness in FP phenotypes are inconsistent.

Stress response

Feather pecking has been suggested to be related to increased stress sensitivity, yet there seems to be some inconsistency in findings. Commercial lines with a high FP tendency showed less active stress responses (Uitdehaag et al., 2011, 2009), higher stress-induced CORT levels (Fraisse and Cockrem, 2006), or no difference in behavioural or physiological stress responses compared to lines with a low FP

tendency (Uitdehaag et al., 2011, 2008c). Interestingly, CORT supplementation increased FP (El-lethey et al., 2001). These findings suggest there is some support for increased stress sensitivity being related to FP in commercial lines.

When comparing experimental lines, the opposite was found with HP birds having lower stress-induced CORT levels and heart rate variability compared to LP birds (Korte et al., 1999, 1997; van Hierden et al., 2002). In the FP selection lines, HFP birds had more active stress responses, higher heart rate, lower heart rate variability and higher stress-induced CORT levels compared to LFP birds (Kjaer and Guémené, 2009; Kjaer and Jørgensen, 2011; Kops et al., 2017). Thus, there is inconsistency in findings with regard to increased stress sensitivity being related to FP in experimental lines.

When focusing on individual FP behaviour, feather peckers had more active stress responses compared to non-peckers, but no difference was found in stress-induced CORT levels (Jensen et al., 2005). Similarly, stress-induced CORT levels did not differ between FP phenotypes (Daigle et al., 2015). Yet, poor plumage condition (i.e. victims) was related to lower stress-induced CORT levels (Jensen et al., 2005). Thus, findings are inconsistent with regard to FP phenotypes differing in stress sensitivity.

In summary, stress sensitivity seems to be related to FP, but findings with regard to the direction of this relation are inconsistent.

Serotonergic system

The central serotonergic system has been indicated to be involved in the development of FP (de Haas and van der Eijk, 2018). The central and peripheral serotonergic system show similar characteristics in their transporters and receptors (Yubero-Lahoz et al., 2013) and whole blood 5-HT was correlated with central 5-HT, 5-HIAA and 5-HT turnover in chickens (Uitdehaag et al., 2011). Therefore, whole blood 5-HT level was used in this thesis as an indicator of central 5-HT level, although it should be noted that 5-HT cannot cross the blood-brain barrier (Pietraszek et al., 1992). Commercial lines with a high FP tendency had lower whole blood 5-HT compared to lines with a low FP tendency (de Haas et al., 2014b, 2013; Uitdehaag et al., 2011), suggesting that FP is related to low peripheral 5-HT in commercial lines.

In the FP selection lines, HFP birds had higher plasma 5-HT levels than LFP birds (Buitenhuis et al., 2006), which is opposite to what is found in commercial lines. However, this discrepancy might be explained by the method used (plasma vs. whole blood), as whole blood 5-HT more likely reflects storage concentration of 5-HT,

while plasma 5-HT reflects unbound 5-HT (Shajib and Khan, 2015). When taking into account the central serotonergic system, HFP chicks had lower central serotonergic activity compared to LFP chicks in several brain regions (Kops et al., 2017). Similarly, HP chicks had reduced central serotonergic activity compared to LP chicks in several brain regions (van Hierden et al., 2004a, 2002). Interestingly, at adult age the differences between the FP selection lines had disappeared or were opposite to what was found at young age (Kops et al., 2017). Low central serotonergic activity might thus predispose chickens to develop FP, while at an adult age FP seems to be related to high central serotonergic activity (de Haas and van der Eijk, 2018). This shift in serotonergic activity might be linked to performing or receiving FP.

Indeed FP phenotypes were shown to differ in central serotonergic activity, where feather peckers had higher central serotonergic activity compared to neutrals (Kops et al., 2013). For the peripheral serotonergic system, neutrals had higher whole blood 5-HT levels compared to victims and feather pecker-victims (Daigle et al., 2015). Thus, findings for FP phenotypes are limited and more research is needed to identify peripheral 5-HT level in FP phenotypes.

In summary, low central serotonergic activity at a young age and low peripheral 5-HT level are related to FP, but findings for 5-HT in FP phenotypes are limited.

Immune system

In recent years, there is increasing evidence for a relation between the immune system and FP. High natural antibody level and genetic mutations in cytokine genes of cage mates were associated with an individual's feather damage (Biscarini et al., 2010; Sun et al., 2014), suggesting that high natural antibody level and cytokines may be related to the performance of FP. Furthermore, a line with a high FP tendency showed upregulation of genes related to immune system processes in the brain compared to a line with a low FP tendency (Habig et al., 2014, 2012) and a negative genetic correlation was found between FP and the specific antibody response (Buitenhuis et al., 2004). Interestingly, activation of the specific immune response at a young age increased feather damage at adult age, suggesting that stimulation of the specific immune response predisposes chickens for FP (Parmentier et al., 2009). These findings suggest that a more responsive immune system or activation of the immune system is related to FP in commercial lines.

In the FP selection lines, HFP birds had higher specific antibody levels, while LFP birds had a higher number of white blood cells and higher expression of MHC class I molecules on T and B cells (Buitenhuis et al., 2006). These results indicate that FP

seems to be related to a more responsive immune system in the FP selection lines as well.

First indications show that FP phenotypes might differ in immune system functioning, as feather peckers showed altered expression of genes involved in immune defence compared to neutrals in the brain (Brunberg et al., 2011). However, further research is needed to identify whether FP phenotypes actually differ in immune characteristics.

In summary, these findings indicate that the immune system is (genetically) related to FP, where FP seems to be related to a more responsive immune system. However, to date no studies have identified immune characteristics in FP phenotypes.

Microbiota and feather pecking

Since fearfulness, stress, and the serotonergic and immune systems have been related to FP, microbiota could influence the development of FP through its effects on these behavioural and physiological characteristics. Indeed, there are first indications for a relation between microbiota and FP. The FP selection lines differed in gut microbial metabolites (i.e. products produced by the gut microbiota), where HFP birds had higher n-butyrate and propionate, but lower i-butyrate and acetate in the gut content compared to LFP birds (Meyer et al., 2013). Furthermore, the FP selection lines were shown to differ in microbiota composition determined from caecal droppings, where HFP birds had higher relative abundance of bacteria within the order Clostridiales, but lower relative abundance of the genus *Lactobacillus* compared to LFP birds (Birkl et al., 2018). Yet,

it is unknown whether gut microbiota influences the development of feather pecking

Why is it important to study feather pecking?

FP is a major behavioural problem in the egg laying industry. The prevalence of FP was between 40 and 80% in non-cage systems (Blokhuis et al., 2007; Huber-Eicher and Sebö, 2001) and between 39% and 52% in organic systems (Bestman et al., 2017; Bestman and Wagenaar, 2003). Thus, FP occurs frequently thereby reducing animal welfare and productivity. However, two aspects of FP, 1) only a few individuals perform it and 2) it is a multifactorial behaviour, make it difficult to control or prevent FP and despite over four decades of research there is no clear control or preventive measure. Yet, it is now more important than ever to find solutions to control or prevent FP because of the bans on conventional cages (2012,

European Union) and on beak trimming (2018, the Netherlands). In the egg laying industry, chickens are now housed in large groups and have sharp beaks, which ultimately leads to feather peckers having more victims at their disposal and inflicting more damage on their victims. Indeed, FP and mortality percentages due to cannibalism, as a consequence of FP, are often higher in loose housing systems (free range or non-cage systems) (Fossum et al., 2009; Rodenburg et al., 2008; Weeks et al., 2016). Furthermore, non-beak trimmed chicken flocks showed more FP, more feather damage and higher mortality than beak trimmed chicken flocks (Blokhuis et al., 2007; Gilani et al., 2013; Kaiser and Stäheli, 2014; Lambton et al., 2013, 2010; Morrissey et al., 2016; Riber and Hinrichsen, 2017; Sepeur et al., 2015; Weeks et al., 2016). These findings clearly show the importance of finding alternative solutions to control or even prevent FP.

Since microbiota affects behavioural and physiological characteristics that have been indicated to be involved in the development of FP or related to FP, it could be a factor that influences the development of FP. A better understanding of microbiota effects on the development of FP might contribute to developing alternative solutions to control or even prevent FP.

Aim and scope of this thesis

The main aim of this thesis was to identify gut microbiota effects on the development of FP. Genetic lines specifically selected for high FP (HFP) and low FP (LFP) were used as a model system to identify effects on FP (see Figure 2 for a schematic overview of the model and PhD thesis).

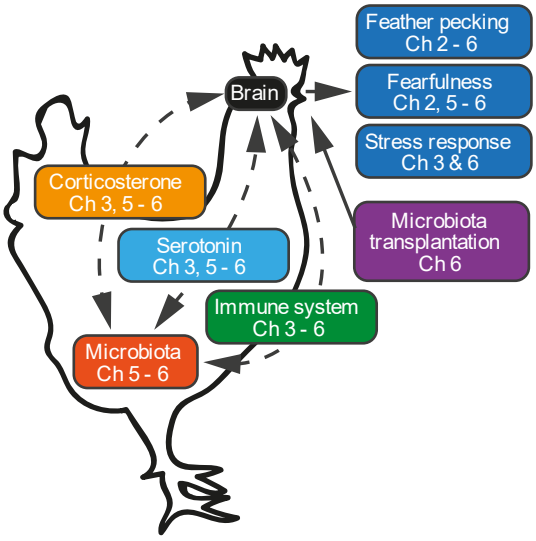


Figure 2. Schematic overview of model and chapters of PhD thesis.

First, I identified characteristics of the FP selection lines with emphasis on behaviour (**chapter 2**: FP, fearfulness; **chapter 3**: the stress response) and physiology (**chapter 3**: corticosterone, serotonin and natural antibodies; **chapter 4**: immune characteristics) shown to be influenced by gut microbiota and to be related to FP, and on microbiota composition (**chapter 5**). Behavioural and physiological

characteristics were further related to FP phenotypes (**chapter 2, 3** and **5**). Based on previous findings I hypothesized that HFP birds would show more FP, lower fearfulness, more active stress responses, higher corticosterone, lower peripheral serotonin, higher natural and specific antibody levels. Furthermore, I hypothesized that HFP birds would differ from LFP birds in microbiota composition, especially with regard to genera of the order Clostridiales and *Lactobacillus* (see Figure 3 for schematic overview of hypothesis). As previous findings for FP phenotypes were inconsistent or unknown, I had no a priori hypothesis for FP phenotypes.

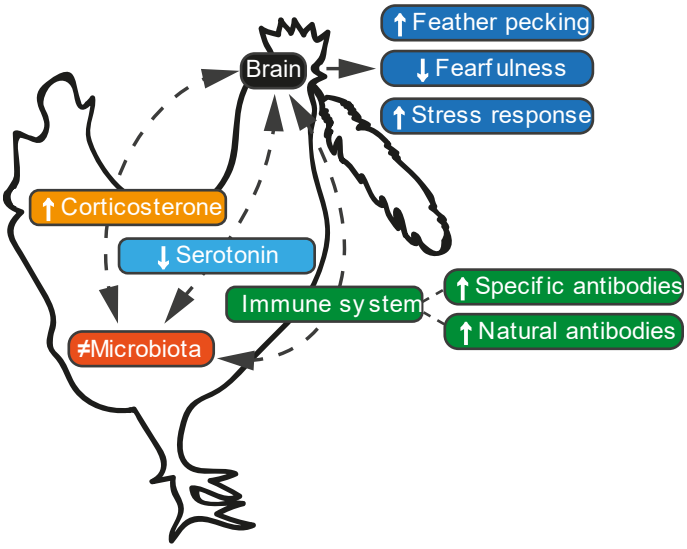
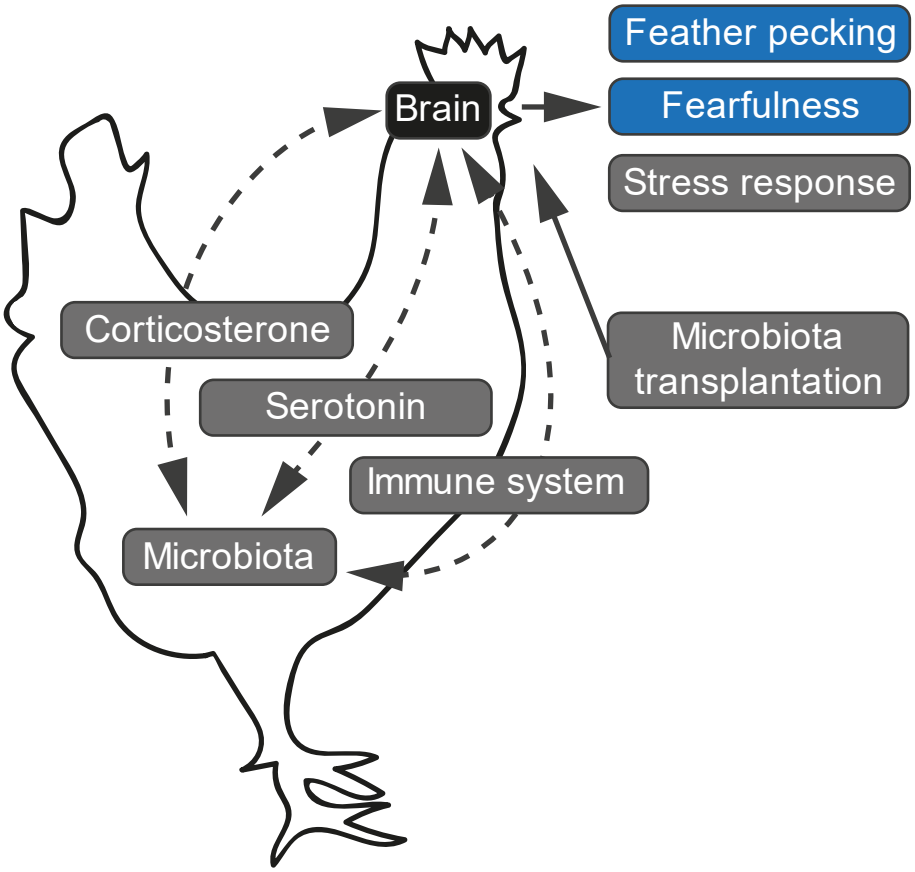


Figure 3. Schematic overview of hypothesis for the comparison between HFP vs. LFP birds for behavioural and physiological characteristics as determined in **chapter 2, 3, 4** and **5**.

Second, to determine whether microbiota influences the development of FP, effects of early-life microbiota transplantation on FP and on behavioural and physiological characteristics influenced by gut microbiota and related to FP were identified (**chapter 6**). For this experiment HFP and LFP chicks received one of three treatments, either a control treatment, microbiota from the HFP or LFP line, directly post hatch and during the first two weeks post hatch. I hypothesized that recipients would adopt a similar behavioural profile as that of the donor line. Thus, LFP birds receiving HFP microbiota would show more FP, lower fearfulness and more active stress responses compared to LFP birds receiving LFP microbiota and *vice versa* for HFP birds receiving LFP microbiota compared to HFP birds receiving HFP microbiota. In **chapter 7**, a synthesis of this thesis is given and the major findings are discussed.

Chapter 2

Feather pecking genotype and phenotype affect behavioural responses of laying hens



Jerine A.J. van der Eijk, Aart Lammers, Peiyun Li, Joergen B. Kjaer, T. Bas Rodenburg

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Abstract

Feather pecking (FP) is a major welfare and economic issue in the egg production industry. Behavioural characteristics, such as fearfulness, have been related to FP. However, it is unknown how divergent selection on FP affects fearfulness in comparison to no selection on FP. Therefore, we compared responses of birds selected on low (LFP) and high feather pecking (HFP) with birds from an unselected control line (CON) to several behavioural tests (i.e. novel object (NO), novel environment (NE), open field (OF) and tonic immobility (TI)) at young and adult ages. Furthermore, the relation between actual FP behaviour (i.e. FP phenotypes) and fearfulness is not well understood. Therefore, we compared responses of birds with differing FP phenotypes. Feather pecking phenotypes of individual birds were identified via FP observations at several ages. The number of severe feather pecks given and received was used to categorize birds as feather peckers, feather pecker-victims, victims or neutrals. Here we show that HFP birds repeatedly had more active responses (i.e. they approached a NO sooner, vocalized sooner and more, showed more flight attempts and had shorter TI durations), which could indicate lower fearfulness, compared to CON and LFP birds at both young and adult ages. Within the HFP line, feather peckers had more active responses (i.e. they tended to show more flight attempts compared to victims and tended to walk more compared to neutrals), suggesting lower fearfulness, compared to victims and neutrals. Thus, in this study high FP seems to be related to low fearfulness, which is opposite to what previously has been found in other experimental and commercial lines. This stresses the need for further research into the genetic and phenotypic correlations between FP and fearfulness in various populations of chickens, especially in commercial lines. Findings from experimental lines should be used with caution when developing control and/or prevention methods that are to be applied in commercial settings. Furthermore, activity and/or coping style might overrule fearfulness within the HFP line, as HFP birds and feather peckers within the HFP line had more active responses. This might indicate a complex interplay between fearfulness, activity and coping style that could play a role in the development of FP.

Introduction

Feather pecking (FP) is a major behavioural problem in the egg production industry and involves laying hens pecking and pulling at feathers of conspecifics. Different types of FP have been defined: gentle feather pecking (GFP) consists of nibbling or gentle pecks at the feathers and causes little or no damage; and severe feather pecking (SFP) consists of forceful pecks and pulls of feathers and can thus cause serious damage to the recipient and can even develop into cannibalistic pecking (Savory, 1995). Preventing or controlling FP is difficult as it is influenced by many factors, both environmental and genetic (Rodenburg et al., 2013).

Certain behavioural characteristics, such as fearfulness, have been related to FP. Fearfulness can be defined as the tendency of an animal to be easily frightened in response to potentially dangerous stimuli (Boissy, 1995; Jones, 1996). Selection on egg production traits resulted in a high (HP) and low (LP) FP line (Korte et al., 1997). HP chicks showed a longer duration of freezing, and vocalized and walked later in an open field (OF) test than LP chicks, but no difference was found in tonic immobility (TI) duration (Jones et al., 1995). In a commercial line comparison, fewer Rhode Island Red (RIR) birds moved away from a novel object (NO) than White Leghorn (WL) birds at adult age and WL birds had more feather damage, indicating that WL birds were more fearful and showed more FP than RIR birds (Uitdehaag et al., 2008a). On an individual level Rodenburg et al. (2004a) found a strong negative correlation between OF activity at a young age and high levels of FP at adult age, indicating that fearful chicks are more likely to develop FP as adult birds. This is supported by de Haas et al. (2014a) on farm level who showed that fear of humans during the rearing period is a predictor for feather damage at adult ages. These findings indicate that FP is related to high fearfulness in young and adult birds.

In lines divergently selected on FP, resulting in a high (HFP) and a low (LFP) FP line (Kjaer et al., 2001), first indications were found that they differ in fearfulness. However, the relationship between fearfulness and FP seems to be the opposite to that described above. Kops et al. (2017) found that HFP chicks vocalized and walked sooner in an isolation test, approached a NO faster and more chicks approached the NO compared to LFP chicks and similar results were found in a human approach (HA) test, suggesting HFP chicks were less fearful compared to LFP chicks. Lines did not differ in the number of steps or vocalizations, or in the latency to vocalize in an OF test at adolescent age (Kops et al., 2017). In a novel maze, HFP birds walked a longer distance, spent a larger proportion of time walking and vocalized sooner compared to LFP birds at adult age (de Haas et al., 2010). Bögelein et al. (2014) found that adult HFP birds had a shorter TI duration, shorter latency to step and

vocalize in an OF test and shorter latency to emerge in an emerge test compared to LFP birds. The findings from these studies suggest that HFP birds are less fearful compared to LFP birds at an adult age. Another study, however, found no differences between the HFP and LFP line in TI, HA or NO test at an adult age (Rodenburg et al., 2010). Taken together, there is inconsistency on whether the FP selection lines differ with regard to fearfulness, especially at an adult age. At a young age HFP chicks seem to be less fearful and show more active responses compared to LFP chicks. Thus, the FP selection lines show a different relation between FP and fearfulness compared to commercial lines and other experimental lines (i.e. HP and LP lines). Yet, other factors such as coping style and/or activity could play a role in the behavioural responses of the FP selection lines as suggested by previous studies (de Haas et al., 2010; Kjaer, 2009; Kops et al., 2017).

In order to better understand the development of FP it is crucial to identify how actual FP behaviour is related to behavioural characteristics, since animals can become feather peckers, feather pecker-victims, victims or neutrals (i.e. FP phenotypes). Only a few studies to date have related actual FP behaviour to fearfulness. Vestergaard et al. (1993) found a positive correlation between TI duration and rate of SFP given, indicating that feather peckers are more fearful. Jensen et al. (2005) showed that adult feather peckers were faster at approaching both novel food and a NO compared to non-feather peckers, but feather peckers and non-feather peckers did not differ in TI duration. In the FP selection lines, Bögelein et al. (2014) found low correlations between FP and several fear criteria, suggesting that fear might not be related to FP. Thus, FP phenotypes seem to differ in fearfulness, but the direction of the relation remains unclear and may depend on the genotype used.

As it is unknown how divergent selection on FP affects fearfulness in comparison to no selection on FP, we compared responses of HFP and LFP birds with those of birds from an unselected control line (CON) to several behavioural tests at young and adult ages. Furthermore, as the relation between actual FP behaviour (i.e. FP phenotypes) and fearfulness is not well understood, we compared the responses of birds with differing phenotypes. Therefore, the aim of this study was to investigate fearfulness in relation to FP genotype (divergent selection on FP and no selection on FP) and FP phenotype (actual FP behaviour). We hypothesized that HFP birds would be less fearful than LFP and CON birds at both young and adult ages. Based on previous findings the relation between fearfulness and FP phenotypes remains unclear, so we had no a priori hypothesis for differences in fearfulness between FP phenotypes.

Material and Methods

Animals and Housing

White Leghorn birds from the 18th generation of an unselected control (CON) line and lines selected on high (HFP) respectively low feather pecking (LFP) were used (see Kjaer et al., 2001 for a detailed description of the selection procedure). The HFP and LFP line were divergently selected on FP for seven generations and were maintained in subsequent generations. The parent stock was between 38 and 43 weeks of age at the time of egg collection. A total of 456 birds were produced in two batches of eggs that were incubated at an average egg shell temperature of 37.3 ºC and average relative humidity of 55.6 %. The two batches had the same housing conditions and experimental set-up with 4 pens per line, but with two weeks between batches. Only non-beak-trimmed female birds were used for the experiment. Birds were housed per line in 24 floor pens (height 2 m, length 2 m, width 1 m) in groups of 19 birds. At 1 day, 5 weeks and 10 weeks of age group size was reduced (n = 16-17 birds per pen, n = 10-15 birds per pen and n = 8-12 birds per pen, respectively). At 20 weeks of age, group size was levelled out at 8-9 birds per pen, with a total of 63 LFP, 72 HFP and 71 CON birds. All birds were individually marked with a small neck tag with a colour/number combination (Roxan, Selkirk, Scotland) for individual identification. At 3 and 4 weeks of age, birds were colour marked on the neck and/or back for individual identification (colours: black, purple, green, blue and orange). The same colours were used in a previous study where no effect on FP was found (Rodenburg et al., 2003). At 7 weeks of age, the birds were equipped with a light weight backpack with a number for individual identification.

At all times, water and feed were provided *ad libitum.* Birds received a standard rearing diet 1 until 8 weeks of age, a standard rearing diet 2 from 8 until 16 weeks of age and a standard laying diet from 16 weeks of age onwards. Each pen was provided with wood shavings on the floor, a perch installed 5 cm above the floor from 3 to 5 weeks of age and a perch installed 45 cm above the floor from 6 weeks of age onwards. Post hatch, temperature was kept around 33°C and gradually lowered to 24°C at 4 weeks of age. From 19 weeks of age onwards, temperature was kept around 21°C. The light regime was 23L:1D post hatch, and was weekly, gradually reduced to 8L:16D at 4 weeks of age. From 15 weeks of age, the light regime was weekly extended with 1 h until 13L:11D at 20 weeks of age. At 22 weeks of age, the light regime was increased to 16L:8D. Light intensity for each pen was measured with a Voltcraft MS-1300 light meter (Conrad Electric Benelux, Oldenzaal, the Netherlands) and ranged between 34.8-68.2 LUX (average 48.1 LUX) during the first 3 weeks of life. At 3 weeks of age the light intensity was

lowered, ranging between 2.74-7.09 LUX (average 4.68 LUX) to reduce the risk of cannibalism. Straw was provided in racks from 3 to 20 weeks of age to enrich the environment and reduce the risk of cannibalism. At 20 weeks of age straw racks were removed. A wooden nest box was placed in front of the pen at 15 weeks of age. Visual barriers of approximately 1.5 m high were placed between pens at the start of the experiment to prevent birds in adjacent pens of seeing each other. Birds received vaccinations against Marek's disease (day 0, intramuscular (i.m.)), Infectious Bronchitis (day 0, 14, 56 and 108, via spray), Newcastle Disease (day 7, 28, 70 via spray and day 84 i.m.), Infectious Bursal Disease/Gumboro (day 25, via drinking water), Avian Encephalomyelitis and Pox Diphteria (day 84, via wing web injection) and Infectious Laryngo Tracheitis (day 84, via eye drops). The experiment was approved by the Central Authority for Scientific Procedures on Animals according to Dutch law (no: AVD104002015150).

Behavioural Observations and Tests

Feather pecking behaviour was observed between 3 and 29 weeks of age. Birds were subjected to four behavioural tests that are related to fearfulness: novel object test, novel environment test, open field test and tonic immobility test. The novel object test and tonic immobility test were performed twice. A timeline of the feather pecking observations and behavioural tests performed at specific ages is provided in Figure 1. The order for testing and observations was always randomized on pen level. Order for testing during the open field test and tonic immobility test were randomized on individual level. The experimenters were blinded to the lines.

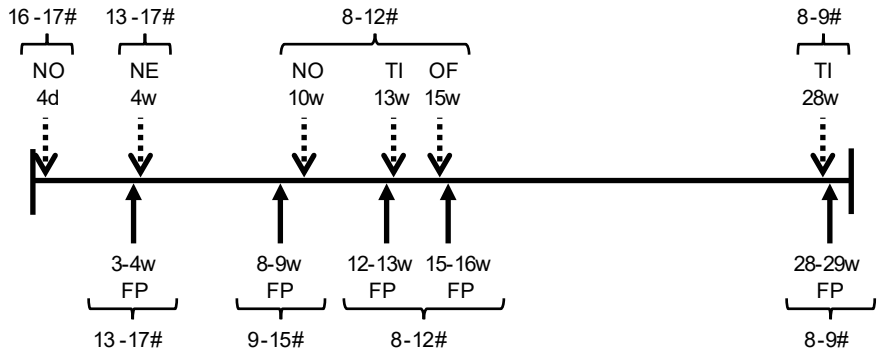

Figure 1. Timeline of feather pecking observations (below line) and behavioural tests (above line) performed at specific ages in days (d) or weeks (w) and the range of group sizes in pens (#). FP = feather pecking observations, NO = novel object test, NE = novel environment test, TI = tonic immobility test and OF = open field test.

Feather Pecking Observations

Feather pecking behaviour was observed on an individual level from week 3-4, 8-9, 12-13, 15-16 and 28-29. In week 3-4 birds were observed by direct observation. Each observation lasted 30 min, either in the morning (8:30 h-12:00 h) or in the afternoon (12:30 h-16:00 h), after a 5 min habituation time. In week 8-9, 12-13, 15-16 and 28-29 behaviour was observed from video recordings. Each observation lasted 15 min, either in the morning (10:40 h-10:55 h) or in the afternoon (14:40 h-14:55 h). The Observer XT 10 programme (Noldus Information Technology B.V., Wageningen, the Netherlands) was used for video analysis of FP, categorized according to Table 1 (derived from Newberry et al., 2007) in gentle feather pecks (subdivided into exploratory gentle feather pecks (EFP) and bouts of stereotyped gentle feather pecking (StFP)) and severe feather pecks (SFP). Feather pecking behaviours were summed over two subsequent weeks, thus including one morning and one afternoon observation with a total observation period of 60 min for week 3-4 and a total observation period of 30 min for all other time points. The summed number of SFP, either given or received, was used to identify FP phenotypes. Classification of phenotypes was adapted from Daigle et al. (2015). When a bird gave more than one SFP it was defined as a feather pecker (P). When a bird received more than one SFP it was defined as a victim (V). When a bird gave and received more than one SFP it was defined as a feather pecker-victim (P-V). When a bird gave and received zero or one SFP it was defined as a neutral (N).

Table 1. Ethogram of the feather pecking observations (after Newberry et al., 2007).

Behaviour	**Description**
Exploratory Feather Pecking (EFP)	Bird makes gentle beak contact with the feathers of another bird without visibly altering the position of the feathers. The recipient makes no apparent response. Each peck is recorded.
Stereotyped Feather Pecking Bout (StFP)	Bird makes ≥ 3 gentle pecks at intervals ≤ 1 s at a single body region. Each series of pecks (bout) is recorded. Bout ends when birds separate, or when pecking is directed to another target on the same, or another, bird.
Severe Feather Pecking (SFP)	Bird grips and pulls or tears vigorously at a feather of another bird with her beak, causing the feather to lift up, break or be pulled out. The recipient reacts to the peck by vocalizing, moving away or turning towards the pecking bird. Each peck is recorded.

Novel Object Test

At 4 days and 10 weeks of age, the response to a novel object (NO) was tested. At 4 days of age, the NO was a wooden block (height 8 cm, length 5 cm, width 2.5 cm) wrapped with coloured tape (green, white, black, yellow, and red) (n = 24, see de Haas et al., 2014a for a detailed description of the test method). The test started 10 sec. after the experimenter had placed the NO on the floor in the centre of the home pen (n = 24). The latency for three different birds to approach the NO at a distance of < 25 cm and the number of birds that were within < 25 cm of the NO was recorded every 10 sec for the 2 min test duration. At 10 weeks of age, the NO test was repeated (n = 24). The NO was a plastic stick (length 50 cm, diameter 3.5 cm) wrapped with coloured tape (red, white, green, black, and yellow) (Welfare Quality®, 2009). The same experimenter tested all pens at 4 days and 10 weeks of age.

Novel Environment Test

At 4 weeks of age, the response to a novel environment (NE) was tested for a duration of 1 min (n = 397, see de Haas et al., 2014a for a detailed description of the test method). All birds from a pen were taken and transported in a cardboard box to a room near the testing rooms. The average time difference between the first and last bird to be tested was 25 min. Birds were taken out of the cardboard box to one of two test locations, where birds were placed inside a white bucket (height 57 cm, length 32 cm, width 22 cm). The bucket was covered with a wire mesh to prevent birds from escaping. The experimenter was out of sight of the bird while testing, but was able to record latency to vocalize, number of vocalizations and number of flight attempts. After testing, birds were returned to a second cardboard box and when all birds from a pen were tested they were returned to their home pen. Together, two experimenters tested all birds where each experimenter tested approximately half of the birds alone.

Open Field Test

At 15 weeks of age, birds were individually subjected to an open field (OF) test for a duration of 5 min (n = 244, see Rodenburg et al., 2009 for a detailed description of the test method). Birds were individually transported to and from the test room in a cardboard box. A square wooden enclosure (height 1.22 m, length 1.15 m, width 1 m) was used. Wire mesh prevented birds from escaping. The front of the enclosure consisted of Plexiglas. A video-camera was placed approximately 1.0 m in front of

the Plexiglas. A bird was placed in the middle of the OF at the start of the test. The experimenter was out of sight of the bird while testing, but was able to record latency to step and number of steps from a monitor and latency to vocalize and number of vocalizations. One experimenter tested all birds.

Tonic Immobility Test

At 13 weeks of age, birds were individually subjected to a tonic immobility (TI) test for a maximum duration of 5 min (n = 248, see Jones and Faure 1981 for a more detailed description of the test method). The TI test was performed on two consecutive days in the afternoon and birds were randomly assigned to a test day with half of a pen being tested on the first day and the other half on the second day. Half of the birds in a pen were taken and transported in a cardboard box to a room near the testing rooms. The average time difference between the first and last bird to be tested was 15 min. Birds were taken out of the cardboard box to one of two test locations, where they were placed in supine position in a metal cradle with their head suspended from the side of the cradle. The right hand of the experimenter was placed on the breast of the bird, while the left hand gently forced the bird's head down lightly while cupping its head. Each bird was restrained in this position for 10 sec. When after releasing, the bird remained in this position, TI duration was recorded until the bird returned to upright position. If this happened within 10 sec after release, TI was induced again, with a maximum of three attempts at inducing TI. Eye contact with the bird was avoided, but the experimenter was visible for the bird during the test. The experimenter recorded the number of induction attempts needed and the duration of TI (latency to self-righting). After testing, birds were returned to a second cardboard box and when all birds from a cardboard box had been tested they were returned to their home pen. Together, three experimenters tested all birds where each experimenter tested approximately a third of the birds alone.

At 28 weeks of age, the tonic immobility test was repeated (n = 205). The average time difference between the first and last bird to be tested was 12 min. Together, two experimenters tested all birds where each experimenter tested approximately half of the birds alone.

Statistical Analysis

SAS Software version 9.3 was used for statistical analysis (SAS Institute Inc., Cary, USA). Linear mixed models for line effects were tested for each age separately and consisted of fixed effects of line and batch and the random effect of pen within line, except for the NO test, which was tested at pen level. Phenotype effects were tested

only in the HFP line as on average less than 10% of birds was categorized as P, P-V or V within the LFP and CON lines (see Table 3). Linear mixed models for phenotype effects in the HFP line consisted of fixed effects of FP phenotype and batch and the random effect of pen. Phenotype effects were tested for each behavioural test separately using the most recent FP phenotype categorization (for example, FP phenotypes based on FP observations from week 3 and 4 were used to identify phenotype effects in the NE test). Phenotype effects in the NO test at 4 days of age were not tested as we could not identify FP phenotypes at that age. Test time (morning 8:00 h-12:30 h or afternoon 12:30 h-18:00 h) was added as fixed effect for the NE test and the OF test. Experimenter was added as fixed effect for the NE test and the TI test. Testing order was included as fixed effect for the TI test. The model residuals were visually examined for normality. Variables were square root transformed (i.e. percentage of birds that approached the NO; latency to vocalize and frequency of vocalizations in the NE test; latency to vocalize and step, frequency of steps and vocalizations in the OF test; and TI duration) to obtain normality of model residuals. A Kruskal Wallis test was used to analyse line effects for latency to approach the NO and post hoc comparisons were made with Dunn's test. A generalized linear mixed model with a Binary distribution was used to test line and phenotype effects in the HFP line for flight attempts in the NE test. A generalized linear mixed model with a Poisson distribution was used to test line effects for all FP behaviours. A backward regression procedure was used when fixed effects (i.e. test time, experimenter or testing order) had $P > 0.1$. Post hoc pairwise comparisons were corrected by Tukey–Kramer adjustment. P-values < 0.05 were considered to be significant. P-values between 0.05 and 0.1 were considered to indicate a tendency. All data is presented as (untransformed) mean ± standard error of the mean (SEM).

Results

Line Effects

Feather Pecking Observations

An overview of the line effects on feather pecking behaviour at different ages is given in Table 2. Line effects were found for exploratory feather pecks (EFP) given at 8-9 ($F_{2,20} = 5.36$, $P < 0.05$), 12-13 ($F_{2,20} = 3.62$, $P < 0.05$) and line tended to affect EFP given at 15-16 weeks of age ($F_{2,20} = 3.35$, $P < 0.1$). LFP birds showed less EFP at 8-9 weeks of age compared to HFP and CON birds ($P < 0.05$), but HFP and CON birds did not differ in EFP at this age. HFP birds showed more EFP at 12-13 and tended to show more EFP at 15-16 weeks of age compared to CON birds ($P < 0.05$

and $P < 0.1$, respectively), but LFP birds did not differ in EFP compared to HFP and CON birds at both ages.

Line effects were also found for stereotyped feather pecking bouts (StFP) given at 3-4 ($F_{2,20} = 6.18$, $P < 0.01$), 8-9 ($F_{2,20} = 10.09$, $P < 0.01$) and 12-13 weeks of age ($F_{2,20} = 4.96$, $P < 0.05$). HFP birds tended to show more StFP at 3-4 ($P < 0.1$) and showed more StFP at 8-9 weeks of age ($P < 0.01$) compared to LFP birds. Furthermore, HFP birds showed more StFP at 3-4 ($P < 0.01$) and 8-9 weeks of age ($P < 0.05$) compared to CON birds, but LFP and CON birds did not differ in StFP at these ages. CON birds showed less StFP at 12-13 weeks of age compared to HFP and LFP birds ($P < 0.05$), but HFP and LFP birds did not differ in StFP at this age.

Finally, line effects were found for severe feather pecks (SFP) given at 3-4 ($F_{2,20} = 4.25$, $P < 0.05$), 8-9 ($F_{2,20} = 7.38$, $P < 0.01$), 15-16 ($F_{2,20} = 7.31$, $P < 0.01$) and 28-29 weeks of age ($F_{2,19} = 14.09$, $P < 0.01$). HFP birds showed more SFP at 3-4 ($P < 0.05$), 8-9 ($P < 0.05$), 15-16 ($P < 0.01$) and 28-29 weeks of age ($P < 0.01$) compared to LFP birds. HFP birds showed more SFP at 8-9 and 28-29 weeks of age ($P < 0.01$) and tended to show more SFP at 15-16 weeks of age compared to CON birds ($P < 0.1$). LFP and CON birds did not differ in SFP at all ages.

Feather Pecking Phenotypes

Birds were categorized as feather pecker (P), feather pecker – victim (P-V), victim (V) or neutral (N). The number (and percentage) of hens within each category at different ages is given in Table 3. On average the largest percentage of hens was categorized as N across all ages in all three lines (HFP 51.7%; CON 80.8%; LFP 85.2%). The smallest percentage of hens was categorized as P-V in all three lines (HFP 10.5%; CON 2.7%; LFP 2.1%). The remainder of hens was categorized as P (HFP 14.9%; CON 8.1%; LFP 7.7%) and V (HFP 23.0%; CON 8.4%; LFP 5.1%).

Table 2. Feather pecking behaviour (exploratory feather pecking (EFP), stereotyped feather pecking (StFP) (bouts) and severe feather pecking(SFP)) of the high (HFP), control (CON) and low feather pecking (LFP) lines at different ages.

Variables	HFP	CON	LFP	P-value
Age (3-4 weeks)	**n = 131**	**n = 126**	**n = 125**	
EFP	2.89 ± 0.26	2.51 ± 0.26	2.35 ± 0.57	ns
StFP (bouts)	4.45 ± 1.00^{a}	0.99 ± 0.17^{b}	1.59 ± 0.46^{ab}	< 0.01
SFP	2.37 ± 1.27^{a}	0.44 ± 0.14^{ab}	0.30 ± 0.07^{b}	< 0.05
Age (8-9 weeks)	**n = 110**	**n = 103**	**n = 101**	
EFP	2.82 ± 0.32^{a}	3.03 ± 0.36^{a}	1.76 ± 0.29^{b}	< 0.05
StFP (bouts)	3.02 ± 0.47^{a}	1.42 ± 0.26^{b}	1.05 ± 0.19^{b}	< 0.01
SFP	2.40 ± 0.51^{a}	0.50 ± 0.13^{b}	0.55 ± 0.19^{b}	< 0.01
Age (12-13 weeks)	**n = 88**	**n = 81**	**n = 79**	
EFP	7.45 ± 0.99^{a}	4.64 ± 0.71^{b}	5.27 ± 0.70^{ab}	< 0.05
StFP (bouts)	0.98 ± 0.27^{a}	0.20 ± 0.07^{b}	0.76 ± 0.18^{a}	< 0.05
SFP	2.55 ± 0.33	1.98 ± 0.39	1.34 ± 0.24	ns
Age (15-16 weeks)	**n = 86**	**n = 81**	**n = 77**	
EFP	6.70 ± 0.71	4.37 ± 0.51	4.83 ± 0.48	ns
StFP (bouts)	0.53 ± 0.16	0.47 ± 0.14	0.52 ± 0.14	ns
SFP	2.74 ± 0.78^{a}	0.99 ± 0.23^{ab}	0.49 ± 0.17^{b}	< 0.01
Age (28-29 weeks)	**n = 71**	**n = 70**	**n = 63**	
EFP	4.62 ± 0.66	3.89 ± 0.46	3.43 ± 0.70	ns
StFP (bouts)	0.70 ± 0.25	0.54 ± 0.16	0.60 ± 0.23	ns
SFP	6.25 ± 1.87^{a}	0.63 ± 0.14^{b}	0.48 ± 0.14^{b}	< 0.01

Average number of pecks or bouts per bird per hour (age 3-4 weeks: 60 min total observation time per bird; age 8-9, 12-13, 15-16 and 28-29 weeks: 30 min total observation time per bird). Differing superscript letters (a,b) indicate significant differences ($P < 0.05$) between lines.

Table 3. The number (and percentage) of hens per phenotype category (feather pecker (P), feather pecker-victim (P-V), victim (V) and neutral (N)) within the high (HFP), control (CON) and low feather pecking (LFP) lines based on the number of severe feather pecks (SFP) given or received at different ages.

	P	**P-V**	**V**	**N**
Criteria	Give > 1 SFP Receive 0 or 1 SFP	Give > 1 SFP Receive > 1 SFP	Give 0 or 1 SFP Receive > 1 SFP	Give 0 or 1 SFP Receive 0 or 1 SFP
Age (3-4 weeks)				
HFP	16 (12.2%)	13 (9.9%)	34 (26.0%)	68 (51.9%)
CON	7 (5.6%)	2 (1.6%)	10 (7.9%)	107 (84.9%)
LFP	7 (5.6%)	5 (4.0%)	4 (3.2%)	109 (87.2%)
Age (8-9 weeks)				
HFP	19 (17.3%)	3 (2.7%)	16 (14.6%)	72 (65.5%)
CON	6 (5.8%)	1 (1.0%)	5 (4.9%)	91 (88.4%)
LFP	5 (5.0%)	0 (0.0%)	4 (4.0%)	92 (91.1%)
Age (12-13 weeks)				
HFP	19 (21.6%)	8 (9.1%)	17 (19.3%)	44 (50.0%)
CON	12 (14.8%)	8 (9.9%)	11 (13.6%)	50 (61.7%)
LFP	13 (16.5%)	4 (5.1%)	9 (11.4%)	53 (67.1%)
Age (15-16 weeks)				
HFP	13 (15.1%)	7 (8.1%)	23 (26.7%)	43 (50.0%)
CON	7 (8.6%)	1 (1.2%)	9 (11.1%)	64 (79.0%)
LFP	4 (5.2%)	1 (1.3%)	4 (5.2%)	68 (88.3%)
Age (28-29 weeks)				
HFP	6 (8.5%)	16 (22.5%)	20 (28.2%)	29 (40.9%)
CON	4 (5.7%)	0 (0.0%)	3 (4.3%)	63 (90.0%)
LFP	4 (6.3%)	0 (0.0%)	1 (1.6%)	58 (92.1%)

Behavioural Tests

Novel Object Test

Line effects were found for the average percentage of birds that approached the novel object (NO) and the latency for three birds to approach the NO at 4 days ($F_{2,20}$ = 17.73, $P < 0.01$ and $X^2 = 15.55$, $P < 0.01$, respectively) and 10 weeks of age ($F_{2,20}$ = 7.03, $P < 0.01$ and $X^2 = 11.39$, $P < 0.01$, respectively). More HFP birds approached the NO and they approached it faster at 4 days of age compared to LFP and CON birds ($P < 0.01$). At 10 weeks of age, more HFP birds approached the NO and they

approached it faster compared to LFP birds ($P < 0.01$) and more HFP birds tended to approach the NO and they tended to approach it faster compared to CON birds ($P < 0.1$) (Figures 2A and B). LFP and CON birds did not differ in their response to the NO at both ages.

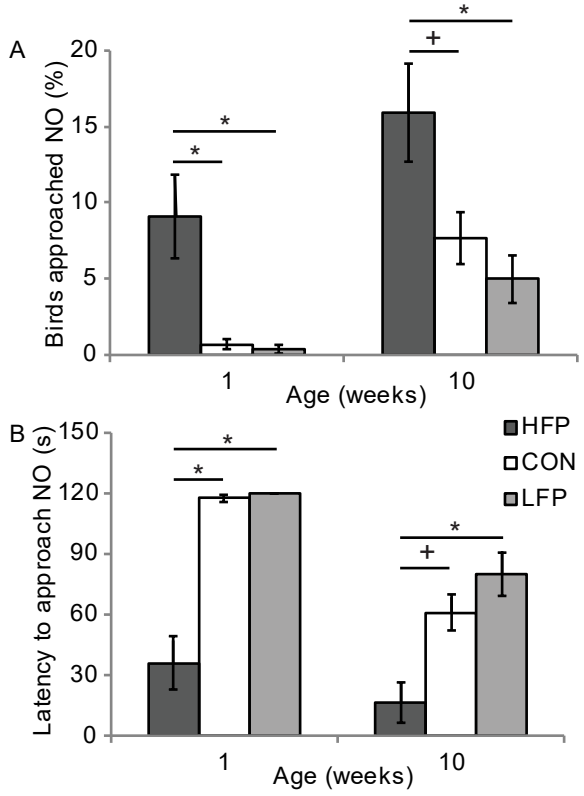

Figure 2. A) Mean percentage (± SEM) of birds approaching the novel object (NO) and B) mean latency (± SEM) for three birds to approach the NO in the NO test at 4 days (indicated as 1 week of age) and 10 weeks of age for the high (HFP, n = 8), control (CON, n = 8) and low feather pecking (LFP, n = 8) lines. $^{+}$ show tendencies ($P < 0.1$) and * show significant differences ($P < 0.05$) between lines.

Novel Environment Test

Line effects were found for latency to vocalize ($F_{2,20} = 13.21$, $P < 0.01$), vocalization frequency ($F_{2,20} = 24.69$, $P < 0.01$) and number of flight attempts ($F_{2,20} = 11.48$, $P < 0.01$) in the novel environment (NE) test at 4 weeks of age. HFP birds vocalized sooner and more compared to LFP and CON birds ($P < 0.01$) (Figures 3A and B). HFP birds showed more flight attempts compared to LFP ($P < 0.01$) and CON birds ($P < 0.05$) (Figure 3C). LFP and CON birds did not differ in their latency to vocalize, vocalization frequency or number of flight attempts.

Open Field Test

Line tended to affect the latency to first step ($F_{2,20} = 3.21$, $P < 0.1$) and line affected latency to vocalize ($F_{2,20} = 4.95$, $P < 0.05$) in the open field (OF) test at 15 weeks of age. HFP birds walked sooner compared to CON birds ($P < 0.05$) and vocalized

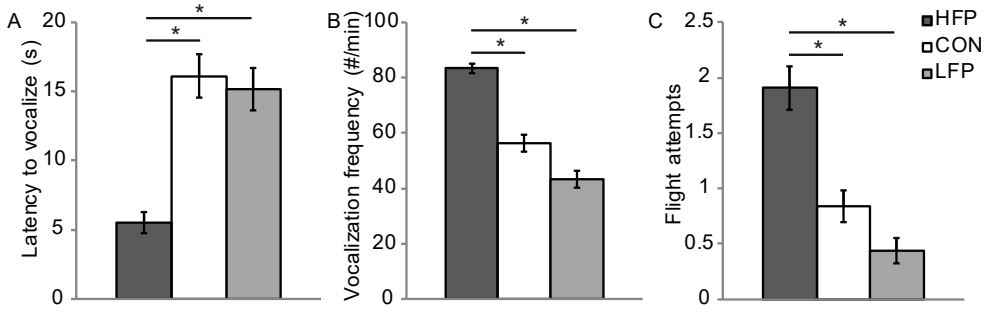

Figure 3. A) Mean latency (± SEM) to vocalize, B) mean vocalization frequency (± SEM) and C) mean number of flight attempts (± SEM) in the novel environment test at 4 weeks of age for the high (HFP, n = 132), control (CON, n = 128) and low feather pecking (LFP, n = 128) lines. * show significant differences ($P < 0.05$) between lines.

sooner compared to LFP birds ($P < 0.05$) (Figure 4A). LFP birds did not differ in latency to first step compared to HFP and CON birds. CON birds did not differ in latency to vocalize compared to HFP and LFP birds. Line tended to affect step frequency ($F_{2,20} = 3.30$, $P < 0.1$) and vocalization frequency ($F_{2,20} = 3.34$, $P < 0.1$).HFP birds tended to show more steps compared to CON birds ($P < 0.1$), while LFP birds did not differ in step frequency compared to HFP and CON birds. CON birds vocalized more compared to LFP birds ($P < 0.05$), while HFP birds did not differ in vocalization frequency compared to LFP and CON birds (Figure 4B).

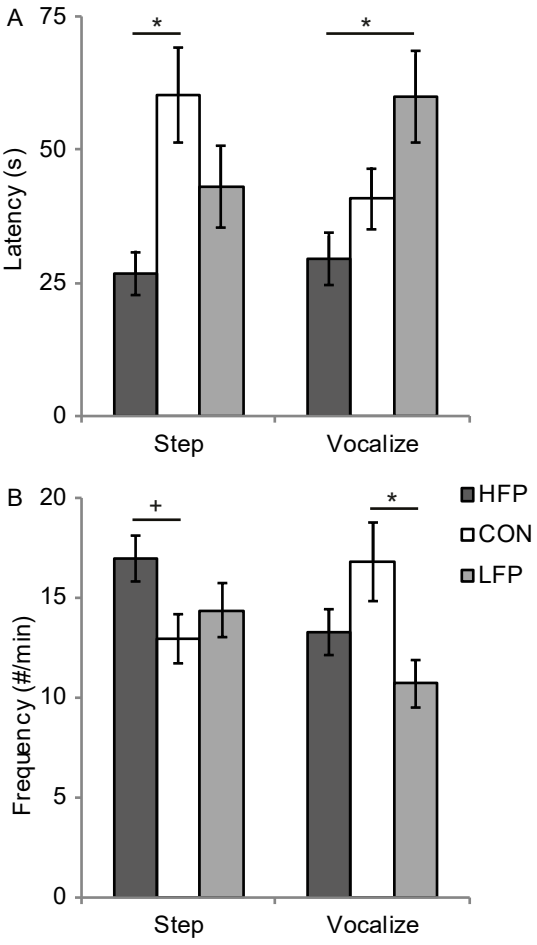

Figure 4. A) Mean latencies (± SEM) to first step and to vocalize and B) mean step and vocalization frequencies (± SEM) in the open field test at 15 weeks of age for the high (HFP, n = 86), control (CON, n = 81) and low feather pecking (LFP, n = 77) lines. $^{+}$ show tendencies ($P < 0.1$) and * show significant differences ($P < 0.05$) between lines.

Tonic Immobility Test

Line affected tonic immobility (TI) duration at 13 ($F_{2,20}$ = 12.89, P < 0.01) and 28 weeks of age ($F_{2,19}$ = 6.35, P < 0.01). HFP birds had a shorter TI duration compared to LFP and CON birds at 13 weeks of age (P < 0.01), while LFP and CON birds did not differ. LFP birds had a longer TI duration than HFP birds (P < 0.01) and tended to have a longer TI duration than CON birds (P < 0.1) at 28 weeks of age, while HFP and CON birds did not differ (Figure 5).

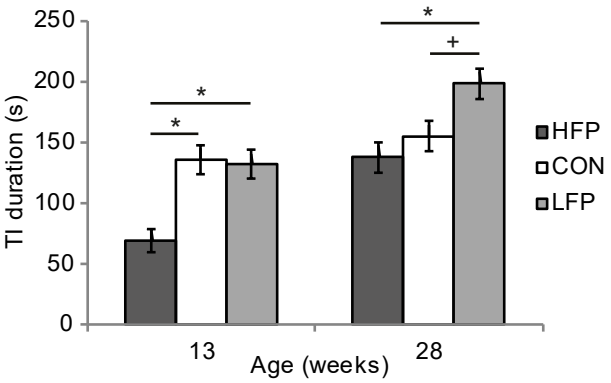

Figure 5. Mean tonic immobility (TI) durations (± SEM) in the TI test at 13 and 28 weeks of age for the high (HFP, n = 88 (13 weeks) and n = 72 (28 weeks)), control (CON, n = 81 (13 weeks) and n = 70 (28 weeks)) and low feather pecking (LFP, n = 79 (13 weeks) and n = 63 (28 weeks)) lines. $^{+}$ show tendencies (P < 0.1) and * show significant differences (P < 0.05) between lines.

Phenotype Effects in the HFP Line

Phenotype affected the number of flight attempts ($F_{3,119}$ = 3.18, P < 0.05) during the NE test. Victims (V) showed more flight attempts compared to neutrals (N) (P < 0.05) and tended to show fewer flight attempts compared to feather peckers (P) (P < 0.1). Feather pecker-victims (P-V) did not differ from P, V or N (Figure 6A). Phenotype tended to affect step frequency ($F_{3,75}$ = 2.64, P < 0.1) during the OF test. P tended to walk more compared to N (P < 0.1), while all other phenotype combinations did not differ (Figure 6B). We found no phenotype effects in the NO or TI test.

Discussion

The aim of this study was to investigate fearfulness in relation to feather pecking (FP) genotype (divergent selection on FP and no selection on FP) and FP phenotype (actual FP behaviour). Our results show that FP genotypes differ in their responses to several behavioural tests at young and adult ages. The high FP (HFP) line showed more active responses (i.e. approached a novel object sooner, vocalized sooner and more, showed more flight attempts and had shorter tonic immobility durations), which could suggest lower fearfulness, compared to the unselected control (CON) and low FP (LFP) line. Our results give first indications that FP phenotypes within

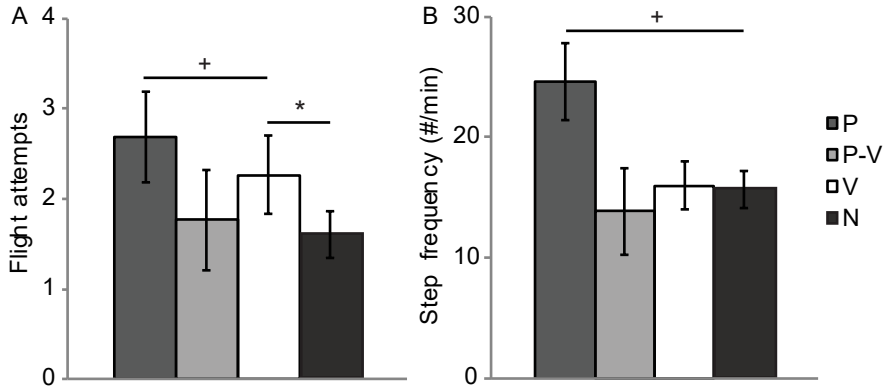

Figure 6. A) Mean number of flight attempts (± SEM) of feather peckers (P, n = 16), feather pecker-victims (P-V, n = 13), victims (V, n = 34) and neutrals (N, n = 68) of the high feather pecking line in the novel environment (NE) test at 4 weeks of age and B) mean step frequency (± SEM) of feather peckers (P, n = 13), feather pecker-victims (P-V, n = 7), victims (V, n = 23) and neutrals (N, n = 43) of the high feather pecking line in the open field test at 15 weeks of age. $^{+}$ show tendencies ($P < 0.1$) and * show significant differences ($P < 0.05$) between phenotypes.

the same genetic line (HFP line) differ in their responses. Feather peckers tended to show more active responses (i.e. they tended to show more flight attempts compared to victims and tended to walk more compared to neutrals), which could suggest lower fearfulness, compared to victims at a young age and compared to neutrals at an adolescent age. Neutrals showed more passive responses (i.e. less flight attempts), which could suggest higher fearfulness, compared to victims at a young age.

Line Effects

Feather Pecking Observations

Our findings indicate that selection for FP results in altered FP behaviour compared to no selection or selection against FP. LFP birds showed less exploratory feather pecking (EFP) compared to CON and HFP birds at a young age, whereas HFP birds showed more EFP compared to CON birds at adolescent ages. Furthermore, HFP birds showed more stereotyped feather pecking bouts (StFP) compared to CON and LFP birds at young ages, whereas CON birds showed less StFP compared to HFP and LFP birds at an adolescent age. We found no differences between the lines in EFP or StFP at adult ages. At both young and adult ages, HFP birds showed more severe feather pecking (SFP) compared to LFP and CON birds.

The HFP and LFP lines were divergently selected on a combination of severe and gentle feather pecking. However, selection did not favour gentle feather pecking,

because gentle pecks in series were counted as a single bout (like for StFP in the present study). This could have resulted in a higher selection pressure on SFP than on gentle feather pecking (identified as EFP and StFP in the present study)(Kjaer et al., 2001) and this might explain why we see more consistent differences in SFP and less consistent or no differences in EFP and StFP. Furthermore, gentle and severe feather pecking are regarded as behaviours with a different motivational background (Kjaer and Vestergaard, 1999). Gentle feather pecking typically decreases with age (Rodenburg et al., 2004a) which could explain why we see no differences in EFP and StFP at adult ages. Previous studies showed similar differences in FP between the HFP and LFP line (Bessei et al., 2013; Bögelein et al., 2015, 2014; Kjaer, 2009; Kjaer et al., 2001; Kjaer and Guémené, 2009; Kops et al., 2017; Piepho et al., 2017). For the first time we show that the LFP and CON line did not differ greatly in FP, especially not in SFP. The LFP and CON line also had similar percentages of birds categorized as feather peckers. Thus, selection for FP is more effective in increasing FP than selection against FP is in reducing FP. This is supported by Piepho et al. (2017) who showed that there are still some extreme feather peckers present in the LFP line. This can be explained by the change in phenotypic variability seen after some generations of selection when the mean level of FP becomes low (Kjaer et al., 2001). Feather pecking is a threshold trait (Kjaer and Jørgensen, 2011) and when the general level of FP is low, most birds will not show any FP even if they differ in their genetic propensity to perform FP. This makes it impossible to distinguish feather peckers from neutrals for selection and the selection for less FP is no longer effective.

Behavioural Tests

The present findings indicate that birds selected for FP show consistent responses in a set of behavioural tests at both young and adult ages and differ from birds that were unselected or selected against FP. Responses to the novel object (NO) (i.e. more birds approached a NO and they approached it sooner) indicate reduced fearfulness (Forkman et al., 2007) in HFP birds compared to CON and LFP birds. In the novel environment (NE) test, HFP birds seem to be less fearful (i.e. vocalized sooner and more and showed more flight attempts) compared to CON and LFP birds as silence and inactivity have been related to high fearfulness (Forkman et al., 2007; Jones, 1996; Suarez and Gallup, 1983). HFP birds seem to be less fearful (i.e. walked sooner and tended to walk more) compared to CON birds in the open field (OF) test, while LFP birds seem to be more fearful (i.e. vocalized less) compared to CON and more fearful (i.e. vocalized later) compared to HFP birds. In the tonic immobility

(TI) test at adolescent age, HFP birds were less fearful (i.e. shorter TI duration) compared to CON and LFP birds as long TI durations have been related to high fearfulness (Forkman et al., 2007; Jones, 1996). Further, LFP birds were more fearful (i.e. longer TI duration) compared to HFP birds and seem to be more fearful (i.e. tended to have longer TI duration) compared to CON birds at adult age. In general, HFP birds appeared less fearful compared to CON and LFP birds in all behavioural tests, especially at young ages. For the first time, we show that CON and LFP birds did not differ in fearfulness at young ages, but LFP birds seem to be more fearful compared to CON birds at adult ages. Overall, selection for FP can alter behavioural characteristics other than FP (i.e. fearfulness) at young and adult ages. Selection against FP seems to alter fearfulness at an adult age.

These results are consistent with previous findings where young (< 16 weeks) HFP chicks were indicated as being less fearful compared to LFP chicks (Kops et al., 2017) and where responses of adult (> 33 weeks) HFP birds suggest that they were less fearful compared to LFP birds (Bögelein et al., 2014; de Haas et al., 2010). However, Rodenburg et al. (2010) found no differences in fearfulness between the HFP and LFP line at an adult age (> 25 weeks) when housed in conventional cages. In other experimental and commercial lines, high FP (indicated by actual FP behaviour or feather damage) has been related to high fearfulness (high vs. low FP line: Jones et al., 1995 (< 5 weeks); Rodenburg et al., 2004a ; White Leghorn vs. Rhode Island Red: Uitdehaag et al., 2008a (>23 weeks)) and de Haas et al., 2014a found the same relation in commercial flocks (ISA Brown and Dekalb White). Even though cause and effect can be discussed in some of these studies, it indicates that genetic correlations between FP and fearfulness might have opposite directions in different genotypes. Thus, findings from the FP selection lines should be used with caution when developing control and/or prevention methods that are to be applied in commercial settings. Furthermore, the responses seen in the behavioural tests in the present study might not only be affected by fear. Fear-related responses are complex and it is unlikely that a particular behaviour is only related to fear (Forkman et al., 2007). Several other factors could have influenced birds' responses, such as coping style, activity, exploration and social motivation (Forkman et al., 2007; Jones, 1996; Koolhaas et al., 1999). For example, in the NE and OF test social isolation can also induce vocal responses, especially in isolated young chicks that seek safety by calling for conspecifics (Gallup and Suarez, 1980; Jones et al., 1995).

Previous studies have indicated that FP might be related to coping style (de Haas et al., 2010; Jensen et al., 2005; Kops et al., 2017; Korte et al., 1997; van Hierden et al., 2002). Coping style is defined as a coherent set of behavioural and physiological

stress responses which is consistent over time and situations (proactive vs. reactive, Koolhaas et al., 1999). Although we did find a consistent difference in behavioural responses between lines over time, with HFP birds showing a more proactive coping style than LFP and CON birds, physiological responses should be considered as well. Kjaer and Guémené (2009) showed that HFP birds had higher corticosterone levels after manual restraint compared to LFP birds, while CON birds had intermediate corticosterone levels, suggesting that HFP birds are more reactive and LFP birds are more proactive. However, preliminary results showed no difference in corticosterone levels between the HFP and LFP lines after manual restraint (van der Eijk et al., 2017). Furthermore, HFP birds had a higher heart rate and lower heart rate variability compared to LFP birds (Kjaer and Jørgensen, 2011), suggesting that HFP birds are more proactive and LFP birds are more reactive. Thus, there is inconsistency between behavioural and physiological findings with regard to coping style in the FP selection lines and further research is needed to indicate whether HFP and LFP birds can be classified into different coping styles. Studies should include behavioural, physiological and neuroendocrine characteristics as coping styles differ in these aspects (Koolhaas et al., 1999).

The present and previous studies show that HFP birds had more active responses to several behavioural tests compared to LFP birds (Bögelein et al., 2014; de Haas et al., 2010; Kops et al., 2017). For the first time, we show that HFP birds had more active responses to several behavioural tests compared to CON birds. Kjaer (2009) showed that HFP birds had higher home-pen locomotor activity compared to LFP and CON birds. Similar results were found in an individual NE test where HFP birds walked a longer distance than LFP birds (de Haas et al., 2017a). Kjaer (2009) suggested that FP in the HFP line might be linked to changes in intrinsic motivation, which either directly or indirectly leads to higher locomotor activity and could thus be a result of a genetically based hyperactivity disorder. When HFP birds are indeed more active in general because of changes in their intrinsic motivation this might result in a more active response to any type of behavioural test. A higher general level of activity in the behavioural tests may suggest that HFP birds are less fearful while this might not be the case. Even responses to the TI test, which is considered a validated test for fearfulness (Forkman et al., 2007), might be affected by activity and/or coping style. Especially when birds have their eyes open but remain lying down during a TI test, latency to self-righting might be more related to activity and/or coping style than to fearfulness as was suggested in pigs by Erhard and Mendl (1999). The comparable responses of LFP and CON birds indicate that selection against FP might not alter fearfulness or intrinsic motivation. Based on the present

findings we suggest that activity and/or coping style might overrule fearfulness within the HFP line, suggesting a complex interplay between fearfulness, activity and coping style that might play a role in the development of FP. Such an interplay between fearfulness, activity and coping style has been suggested before to affect behavioural responses of calves to several behavioural tests (van Reenen et al., 2005, 2004).

Phenotype Effects in the HFP Line

The present findings give first indications that birds which differ in actual FP behaviour (i.e. FP phenotypes) within the same genetic line (HFP line) seem to differ in fearfulness. Previous studies either found a positive (Vestergaard et al., 1993), negative (Jensen et al., 2005) or no relation (Bögelein et al., 2014) between fearfulness and actual FP behaviour. Here we show that feather peckers tended to show more flight attempts compared to victims, while victims showed more flight attempts compared to neutrals in the NE test. In the OF test, feather peckers tended to walk more compared to neutrals. These findings suggest that feather peckers were less fearful (i.e. tended to show more flight attempts) compared to victims at young age and less fearful (i.e. tended to walk more) compared to neutrals at adolescent age. Neutrals seem to be more fearful (i.e. less flight attempts) compared to victims at young age and compared to feather peckers (i.e. tended to walk less) at adolescent age. These findings suggest that victims were more fearful compared to feather peckers and neutrals more fearful compared to feather peckers and victims. The higher fearfulness in victims might be a consequence of being feather pecked as also indicated by earlier studies (Hughes and Duncan, 1972; Rodenburg et al., 2010). It should be noted, that we found no phenotype effects in the TI test, which is considered a validated test for fearfulness (Forkman et al., 2007). Yet, we did find phenotype effects in the NE and OF test, where behavioural responses could also be related to coping style, activity, etc. (Forkman et al., 2007; Jones, 1996; Koolhaas et al., 1999). A similar line of reasoning, as for the differences seen between the FP selection lines, might be true for the differences seen between feather peckers and other FP phenotypes. Feather peckers might be more active in general and have a more proactive coping style compared to other FP phenotypes. In order to classify FP phenotypes into a certain coping style physiological responses should be considered as well. First indications have been found that phenotypes can differ with regard to their physiology. Brunberg et al. (2011) identified differences in brain gene expression when comparing feather peckers to victims and control birds. Furthermore, phenotypes were shown to differ in serotonergic neurotransmission

parameters in several brain areas, although no or small differences were found in dopaminergic neurotransmission parameters (Kops et al., 2013). However, Daigle et al. (2015) found no differences in corticosterone or whole blood serotonin levels after manual restraint between phenotypes. First indications have been found that phenotypes can differ in activity. Feather peckers walked a longer distance than victims in a NE test (de Haas et al., 2017b), suggesting that feather peckers are more active. Furthermore, Newberry et al. (2007) found that birds that performed more foraging behaviour when young were more likely to become feather peckers as adults, indicating that feather peckers might be more active. To shed more light on whether FP phenotypes differ in activity levels and whether they can be classified into different coping styles, further research is needed.

A limitation in our study is that we observed FP behaviour for a limited amount of time which might have led to FP behaviour not being observed. However, continuous observation is impractical. Daigle et al. (2015) showed that around half of the birds were classified with the same phenotype at three out of five ages, suggesting that birds are able to switch phenotypes and are not consistent over time. Unfortunately, we could not identify phenotype consistency as several birds (specifically feather peckers and neutrals) were sacrificed during the experiment for other purposes. However, the strength of this study was that we identified phenotype effects using the most recent FP phenotype categorization that was based on FP observations closest to a particular behavioural test. We emphasize the importance of identifying FP phenotypes as they seem to differ in their responses to several behavioural tests.

Conclusion

Feather pecking genotypes and feather pecking phenotypes within the same genetic line differ in their responses to several behavioural tests at both young and adult ages. The high FP line and feather peckers within the high FP line showed more active responses, suggesting lower fearfulness.

Selection for FP has been effective in increasing FP behaviour and altering other behavioural characteristics (i.e. activity, fearfulness), whereas selection against FP has been less effective in reducing FP and altering other behavioural characteristics.

High FP seems to be related to low fearfulness, which is opposite to what has been found in other experimental and commercial lines. This stresses the need for further research into the genetic and phenotypic correlations between FP and fearfulness in various populations of chickens.

Activity and/or coping style might overrule fearfulness within the high FP line, suggesting a complex interplay between fearfulness, activity and coping style that might play a role in the development of FP.

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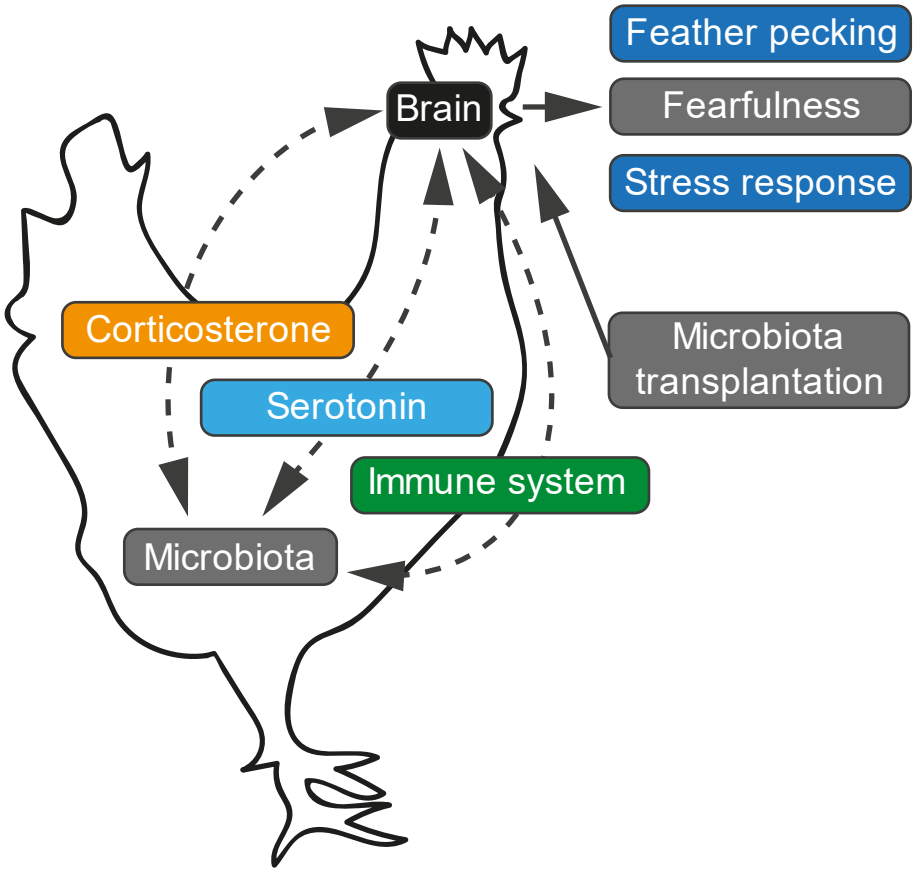
Chapter 3

Stress response, peripheral serotonin and natural antibodies in feather pecking genotypes and phenotypes and their relation with coping style



Jerine A.J. van der Eijk, Aart Lammers, Joergen B. Kjaer, T. Bas Rodenburg

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Abstract

Feather pecking (FP), a serious welfare and economic issue in the egg production industry, has been related to coping style. Proactive and reactive coping styles differ in, among others, the stress response, serotonergic activity and immune activity. Yet, it is unknown whether genetic lines divergently selected on FP (i.e. FP genotypes) or individuals differing in FP (i.e. FP phenotypes) can be categorized into coping styles. Therefore, we determined peripheral serotonin (5-HT) levels, natural antibody (NAb) titers, behavioural and corticosterone (CORT) responses to manual restraint (MR) in FP genotypes (high FP (HFP), low FP (LFP) and unselected control (CON) line) and FP phenotypes (feather pecker, feather pecker-victim, victim and neutral). We further examined the consistency of and relationships between behavioural and physiological measures. FP genotypes differed in behavioural responses to MR, 5-HT levels and NAb titers, but not in CORT levels after MR. HFP birds had less active responses at adolescent age, but more active responses at adult age compared to LFP and CON birds. The CON line had higher 5-HT levels at adolescent age, while the HFP line had lower 5-HT levels than the other lines at adult age. Overall, the HFP line had lower IgM NAb titers, while the LFP line had lower IgG NAb titers compared to the other lines. FP phenotypes differed in behavioural responses to MR and 5-HT levels, but not in CORT levels after MR or NAb titers. Within the HFP line, feather peckers tended to have less active responses compared to neutrals at adolescent age, while victims had more active responses compared to the other phenotypes at adult age. Feather peckers had higher 5-HT levels than neutrals at adult age. Behavioural and CORT responses to MR were not consistent over time, suggesting that responses to MR might not reflect coping style in this study. Furthermore, proactive behavioural responses were correlated with reactive physiological measures and vice versa. Thus, it was not possible to categorize FP genotypes or FP phenotypes into specific coping styles.

Introduction

Feather pecking (FP) is a serious welfare and economic issue in the egg production industry. It involves hens pecking and pulling at feathers of conspecifics, thereby negatively affecting welfare and production. Previous studies have indicated that FP might be related to coping style (Korte et al., 1997; Rodenburg et al., 2004b). Coping style can be defined as a coherent set of behavioural and physiological stress responses which is consistent over time and situations (proactive vs. reactive, (Koolhaas et al., 1999)). In several animal species coping styles are shown to differ in behavioural and physiological responses, where a proactive coping style is, among others, associated with active behavioural responses, low baseline activity and stress reactivity of the hypothalamic-pituitary-adrenal (HPA)-axis, low central serotonergic activity, low humoral immunity, high cellular immunity and innate immune activity compared to a reactive coping style (de Boer et al., 2017; Koolhaas, 2008; Koolhaas et al., 2010; Reimert et al., 2014).

We here focus on the stress response and serotonergic system, as these have been indicated to be involved in FP (de Haas and van der Eijk, 2018; Rodenburg et al., 2013; van Hierden et al., 2004a). We further focus on the immune system as it has been related to FP (Buitenhuis et al., 2004; Parmentier et al., 2009), specifically on natural antibodies (NAb), antibodies that can bind antigens without prior exposure to that antigen (Baumgarth et al., 2005). Natural antibodies play an essential role in both innate and adaptive immunity, for example by maintaining homeostasis, increasing disease resistance and linking the two types of immunity (Berghof, 2018; Lammers et al., 2004; Lutz et al., 2009; Ochsenbein and Zinkernagel, 2000). Some indications have been found that NAb, specifically NAb binding keyhole limpet hemocyanin (KLH), might be related to FP. Certain genetic mutations were associated with both NAb titers and feather damage (as indicator of FP, (Bilcik and Keeling, 1999)) (Biscarini et al., 2010; Sun et al., 2013), and an associative effect of NAb titers on feather damage was detected (Sun et al., 2014). These findings reveal a genetic basis for a relation between NAb and FP.

Laying hens divergently selected on FP, resulting in high (HFP) and low FP (LFP) lines (Kjaer et al., 2001), differ in their responses to behavioural tests. HFP birds respond more actively compared to LFP birds (de Haas et al., 2010; Kjaer, 2009; Kops et al., 2017; **chapter 2**) and compared to unselected control (CON) birds (**chapter 2**), suggesting that HFP birds have a more proactive coping style. These FP selection lines further differ in their stress response and serotonergic activity. HFP birds had higher corticosterone (CORT) levels after manual restraint (MR) (Kjaer and Guémené, 2009) and vocalized sooner and more, but struggled later and

less compared to LFP birds during MR (Kops et al., 2017). Furthermore, HFP birds had lower central serotonergic activity at young age, but higher central serotonergic activity compared to LFP birds at adult age (Kops et al., 2017). To date, no studies have identified NAb titers in these FP selection lines, but a previous study gave first indications that HFP birds differ from LFP birds in immune reactivity and competence (Buitenhuis et al., 2006). These findings indicate that divergent selection on FP affects stress responses, serotonergic activity and immune competence. However, results remain inconsistent with regard to lines being categorized as proactive or reactive. This might be explained by the fact that these studies identified differences between genetic lines, but individuals within a genetic line could be proactive or reactive copers.

To get a better understanding of the relation between FP and coping style it is important to identify the coping style of individual birds and relate this to their FP behaviour, since birds can become feather peckers, feather pecker-victims, victims or neutrals (i.e. FP phenotypes). Feather peckers and victims within the HFP line seemed to respond more actively to behavioural tests (**chapter 2**), indicating that these birds might have a proactive coping style. Only a few studies to date have related actual FP behaviour to the stress response, serotonergic- and immune-systems. FP phenotypes have been shown to differ in serotonergic activity, but the direction of the relation is dependent on brain area studied (Kops et al., 2013). FP phenotypes further differed in whole blood serotonin (5-HT) levels, but not in CORT levels after MR (Daigle et al., 2015). This is supported by a study where FP phenotypes did not differ in CORT levels after MR, but they did differ in behavioural responses to MR (Jensen et al., 2005). To date, no studies have identified NAb titers in FP phenotypes, but genes associated with the immune system were either upregulated or downregulated in the brain when comparing FP phenotypes (Brunberg et al., 2011). These findings indicate that FP phenotypes might differ in immune competence, serotonergic activity and behavioural stress responses, yet no findings indicate that FP phenotypes differ in physiological stress responses. Similar to the findings from the FP selection lines, results remain inconsistent with regard to FP phenotypes being categorized as proactive or reactive.

Although differences in FP have been analysed in relation to the stress response, serotonergic- and immune-systems, no studies to our knowledge have examined these variables in conjunction. Furthermore, most studies to date have compared genetic lines differing in FP, but only a few have compared individuals differing in FP with regard to these variables. Therefore, the aim of this study was to investigate behavioural responses and physiological measures, with a focus on the stress

response, serotonergic- and immune-systems, in relation to FP genotype (HFP, LFP and CON lines) and FP phenotype (feather pecker, feather pecker-victim, victim and neutral). Whole blood 5-HT level was used as indicator for central 5-HT (Uitdehaag et al., 2011), CORT level after MR was used as indicator for HPA-axis activity (Kjaer and Guémené, 2009) and KLH-binding NAb titer was used as a general indicator for immune competence (Star et al., 2007). The MR test was performed twice, at an adolescent and adult age, to examine consistency in individual differences. We further examined the relation between behavioural responses and physiological measures within FP genotypes and FP phenotypes. Based on previous findings where HFP birds and feather peckers within the HFP line responded more actively to several behavioural tests, we hypothesized that HFP birds would have a more proactive coping style compared to LFP and CON birds. Furthermore, we hypothesized that feather peckers within the HFP line would have a more proactive coping style compared to other phenotypes.

Material and Methods

Animals and Housing

White Leghorn birds from the 18th generation of an unselected control (CON) line and lines selected on high (HFP) respectively low feather pecking (LFP) were used (see Kjaer et al., 2001 for the selection procedure). The HFP and LFP line were divergently selected on feather pecking (FP) for seven generations and were maintained in subsequent generations. A total of 456 birds were produced in two batches of eggs that were incubated at an average egg shell temperature of 37.3 ºC and average relative humidity of 55.6 %. The two batches had the same housing conditions and experimental set-up with 4 pens per line, but with two weeks between batches (see **chapter 2** for more details). The experiment was approved by the Central Authority for Scientific Procedures on Animals according to Dutch law (no: AVD104002015150).

Behavioural Observations and Tests

Feather pecking was observed between 3 and 29 weeks of age. Birds were subjected to a manual restraint test at 14 and 24 weeks of age. The order for observations was always randomized on pen level. The order for testing was randomized on individual level. The experimenters were blinded to the lines and phenotypes.

Feather Pecking Observations

Feather pecking was observed on an individual level from week 3-4, 8-9, 15-16, 18-19, 24-25 and 28-29. In week 3-4, birds were observed by direct observation. Each week birds were observed for 30 min, either in the morning (8:30 h-12:00 h) or in the afternoon (12:30 h-16:00 h), after a 5 min habituation time. Thus, in week 3-4 the total observation time was 60 min. In week 8-9, 15-16, 18-19, 24-25 and 28-29, behaviour was observed from video recordings. Each week birds were observed for 15 min, either in the morning (10:40 h-10:55 h) or in the afternoon (14:40 h-14:55 h), with a total observation time of 30 min over two weeks. Feather pecking was categorized according to Table 1 in exploratory FP, bouts of stereotyped FP and severe FP (derived from Newberry et al., 2007). Feather pecking behaviours were summed over two subsequent weeks and the summed number of severe FP, either given or received, was used to identify FP phenotypes. Classification of FP phenotypes was adapted from Daigle et al. (2015). When a bird gave more than one, but received zero or one severe FP it was defined as a feather pecker (P). When a bird gave zero or one, but received more than one severe FP it was defined as a victim (V). When a bird gave and received more than one severe FP it was defined as a feather pecker-victim (P-V). When a bird gave and received zero or one severe FP it was defined as a neutral (N) (see Supplementary Information and **chapter 2** for feather pecking results).

Table 1. Ethogram of the feather pecking observations (after Newberry et al., 2007).

Behaviour	**Description**
Exploratory Feather Pecking	Bird makes gentle beak contact with the feathers of another bird without visibly altering the position of the feathers. The recipient makes no apparent response. Each peck is recorded.
Stereotyped Feather Pecking Bout	Bird makes ≥ 3 gentle pecks at intervals ≤ 1 s at a single body region. Each series of pecks (bout) is recorded. Bout ends when birds separate, or when pecking is directed to another target on the same, or another, bird.
Severe Feather Pecking	Bird grips and pulls or tears vigorously at a feather of another bird with her beak, causing the feather to lift up, break or be pulled out. The recipient reacts to the peck by vocalizing, moving away or turning towards the pecking bird. Each peck is recorded.

Manual Restraint Test

At 14 weeks of age, birds were individually subjected to a manual restraint (MR) test in the same room as their home pens (n = 247) (see Bolhuis et al., 2009 for test method). For both batches, the MR test was performed on two days. Birds were caught individually from their pens and placed on their right side on a table covered with cardboard, with the right hand of the experimenter covering the bird's back and the left hand gently stretching the bird's legs. Birds were restrained in this position for 5 min. The latencies to vocalize and to struggle and the number of vocalizations and struggles were recorded. Together, five experimenters tested the birds, where each experimenter tested approximately one fifth of the birds alone. Distribution of birds over experimenters and time of day was random for pens and lines. Fifteen min after the start of the MR test, blood samples were drawn from the wing vein for assessment of the peak in plasma corticosterone (CORT) level (Fraisse and Cockrem, 2006), whole blood serotonin (5-HT) level and plasma natural antibody (NAb) titers.

At 24 weeks of age, the MR test was repeated using the same method as described above (n = 206), with the following modifications. Birds were caught individually from their pens and placed in a cardboard box. Birds were then moved to one of two testing rooms. Together, three experimenters tested the birds, where each experimenter tested approximately one third of the birds alone.

Blood Collection and Analyses

Blood was collected from all birds at 4, 9, 14, 19, 24 and 29 weeks of age. Blood was taken from the wing vein using a heparinized syringe and kept on ice after blood sampling. In the laboratory, whole blood samples (1 mL) for determination of 5-HT were stored at -20 °C until further analysis. Blood samples for CORT and NAb were centrifuged at 5250 x g for 10 min at room temperature and the obtained plasma was stored at -20 °C until further analysis.

Plasma Corticosterone

Samples from week 14 and 24 were used for determination of plasma CORT concentrations via a radioimmunoassay kit (MP Biomedicals, LLC, Orangeburg, USA) as described previously (Buyse et al., 1987).

Whole Blood Serotonin

Samples from week 14 and 24 were used for determination of whole blood 5-HT concentration (nmol/mL) via a fluorescence assay as described previously (Bolhuis

et al., 2009). The centrifugation steps were performed at 931 x g and fluorescence was determined in a Perkin-Elmer 2000 Fluorescence spectrophotometer (PerkinElmer Inc., Waltham, USA) at 295 and 540 nm.

Plasma IgM and IgG Natural Antibody Titers

Samples from all weeks were used for determination of IgM and IgG NAb titers against keyhole limpet hemocyanin (KLH). Strictly, birds produce IgY and not IgG. However, since bird IgY shares homology in function with mammal IgG we refer to IgY as IgG in this study (Ratcliffe, 2006). NAb titers against KLH were determined by an indirect enzyme-linked immunosorbent assay (ELISA) as described previously (Berghof et al., 2015), with the following modifications. Serial dilutions of plasma were made in four steps starting at dilution 1:40,000 in phosphate buffer saline (PBS) containing 0.05% Tween 20 and 1% horse plasma (100 µL in each well). Peroxidase conjugated goat-anti-chicken IgM (catalogue A30-102P, Bethyl Laboratories Inc., Montgomery, USA; dilution 1:20,000) or goat-anti-chicken IgG (catalogue A30-104P, Bethyl Laboratories Inc., Montgomery, USA; dilution 1:20,000) was used as secondary antibody (100 µL in each well). Substrate buffer was added (100 µL in each well) and after 20 min the reaction was stopped with 50 µL of 1.25M H_2SO_4. Extinctions were measured with a Thermo Scientific Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) at 450 nm. Titers were expressed as log2 values of the dilutions that gave an extinction closest to 50% of Emax, where Emax represents the highest mean extinction of a standard positive (pooled) plasma present on every plate.

Statistical Analysis

SAS Software version 9.3 was used for statistical analysis (SAS Inst. Inc., Cary, NC, USA). Linear mixed models for line effects tested per age consisted of fixed effects line and batch and the random effect pen within line. Linear mixed models for line effects on NAb titers (IgM and IgG) consisted of fixed effects line * age, line, age and batch. The random effect consisted of pen within line with a repeated statement for age with chicken ID as subject and an unstructured covariance structure. The unstructured covariance structure gave the best fitting model. Phenotype effects were tested only in the HFP line as on average < 10% of birds was categorized as feather pecker, feather pecker-victim or victim within the LFP and CON lines (see Supplementary Information). Linear mixed models for phenotype effects tested per age consisted of fixed effects phenotype and batch and the random effect pen. Test time (morning 8:00 h-12:30 h or afternoon 12:30 h-18:00 h) and experimenter were

added as fixed effects for the MR test (including behavioural responses, CORT and 5-HT levels). The model residuals were visually examined for normality. Variables were square root transformed (i.e. latency to struggle and vocalize, vocalization and struggle frequency, 5-HT level) or log transformed (i.e. CORT level) to obtain normality of model residuals. A generalized linear mixed model with a Poisson distribution was used to test line effects per age for all FP behaviours. A backward regression procedure was used when fixed effects (i.e. test time or experimenter) had a P-value > 0.1. Post hoc pairwise comparisons were corrected by Tukey–Kramer adjustment. Principal component analysis (PCA) was used to establish data reduction for each age separately (14 and 24 weeks of age). The four behavioural measures during MR were included in the PCA for both ages: square root transformed latencies and frequencies of struggles and vocalizations. Only principal components with eigenvalues equal to or larger than 1 were considered for further analyses. PCA loadings were considered significant when loadings were > 0.4 or < -0.4. Pearson correlations were calculated to determine the relationships between behavioural and physiological measures and to establish whether individual differences were consistent over time. P-values < 0.05 were considered to be significant. P-values between 0.05 and 0.1 were considered to indicate a tendency. All data is presented as (untransformed) mean ± standard error of the mean (SEM).

Results

Line Effects

Manual Restraint Test

At 14 weeks of age, line effects were found for latency to struggle ($F_{2,20} = 5.91$, $P < 0.01$) and struggle frequency ($F_{2,20} = 4.26$, $P < 0.05$) during manual restraint (MR). High feather pecking (HFP) birds struggled later and less compared to unselected control (CON) birds ($P < 0.05$). HFP birds struggled later ($P < 0.05$) and tended to struggle more compared to low feather pecking (LFP) birds ($P < 0.1$), while LFP and CON birds did not differ in latency to struggle or struggle frequency (Figures 1A and C). We found no line effects on latency to vocalize or vocalization frequency.

At 24 weeks of age, line effects were found for latency to vocalize ($F_{2,19} = 8.60$, $P < 0.01$) and vocalization frequency ($F_{2,19} = 9.28$, $P < 0.01$). HFP birds vocalized sooner and more compared to LFP ($P < 0.05$ and $P < 0.01$, respectively) and CON birds ($P < 0.01$ and $P < 0.05$, respectively) (Figures 1B and D). LFP and CON birds did not differ in latency to vocalize or vocalization frequency. No line effects were found on latency to struggle or struggle frequency.

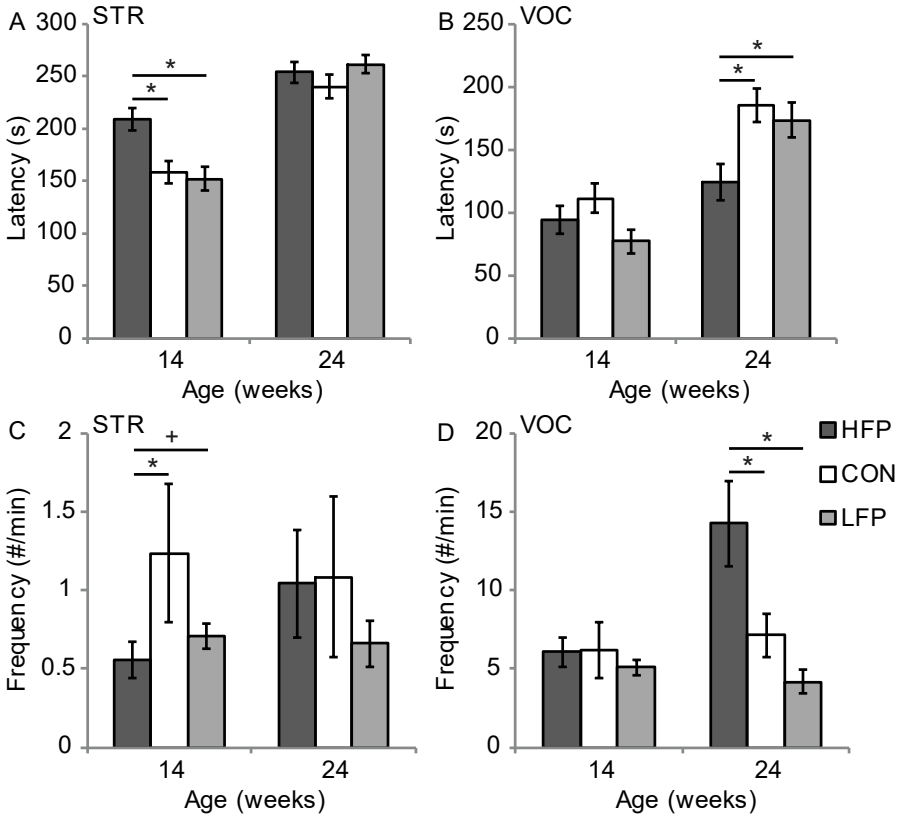

Figure 1. A) Mean latency (± SEM) to struggle (STR), B) mean latency (± SEM) to vocalize (VOC), C) mean struggle frequency (± SEM) and D) mean vocalization frequency (± SEM) during manual restraint at 14 and 24 weeks of age for the high (HFP, n = 87 (14 weeks) and n = 72 (24 weeks)), control (CON, n = 81 (14 weeks) and n = 70 (24 weeks)) and low feather pecking (LFP, n = 79 (14 weeks) and n = 63 (24 weeks)) lines. $^{+}$ show tendencies ($P < 0.1$) and * show significant differences ($P < 0.05$) between lines.

Corticosterone

No line effects were found for corticosterone (CORT) levels after MR at 14 (HFP = 5.35 ng/mL, CON = 4.54 ng/mL and LFP = 5.29 ng/mL) or 24 weeks of age (HFP = 4.22 ng/mL, CON = 5.45 ng/mL and LFP = 4.02 ng/mL).

Serotonin

Line effects were found for whole blood serotonin (5-HT) levels at 14 ($F_{2,20} = 18.24$, $P < 0.01$) and 24 weeks of age ($F_{2,19} = 8.26$, $P < 0.01$). CON birds had higher 5-HT levels compared to LFP and HFP birds ($P < 0.01$), while HFP and LFP birds did not differ in 5-HT levels at 14 weeks of age. At 24 weeks of age, HFP birds had lower

5-HT levels compared to LFP ($P < 0.05$) and CON birds ($P < 0.01$), while LFP and CON birds did not differ in 5-HT levels (Figure 2).

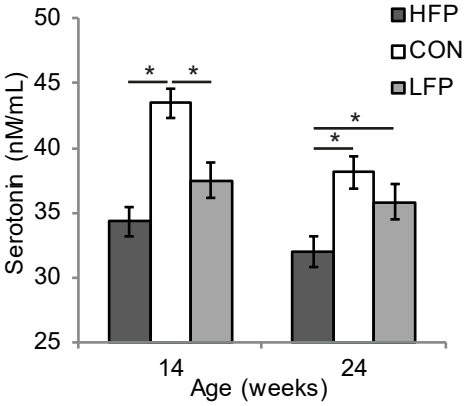

Figure 2. Mean whole blood serotonin level (± SEM) at 14 and 24 weeks of age for the high (HFP, n = 84 (14 weeks) and n = 68 (24 weeks)), control (CON, n = 81 (14 weeks) and n = 68 (24 weeks)) and low feather pecking (LFP, n = 74 (14 weeks) and n = 57 (24 weeks)) lines. * show significant differences ($P < 0.05$) between lines.

IgM and IgG Natural Antibody Titers

A line * age interaction effect was found for both IgM natural antibody (NAb) titers ($F_{10,1537} = 9.47$, $P < 0.01$) and IgG NAb titers ($F_{10,1535} = 3.70$, $P < 0.01$) against keyhole limpet hemocyanin (KLH). Overall, HFP birds had lower IgM titers compared to CON and LFP birds (HFP = 5.76, CON = 6.32 and LFP = 6.38, $P < 0.01$), but CON and LFP birds did not differ significantly. Furthermore, all lines differed significantly from each other for IgG titers, with HFP birds having intermediate, CON birds having the highest and LFP birds having the lowest IgG titers (HFP = 6.08, CON = 6.60 and LFP = 5.46, $P < 0.01$). For specific comparisons of IgM and IgG titers between lines per age see Figures 3A and 3B, respectively.

Phenotype Effects in the HFP Line

Manual Restraint Test

Phenotype effects were found for vocalization frequency ($F_{3,75} = 2.81$, $P < 0.05$) during MR at 14 weeks of age. Neutrals tended to vocalize more compared to feather peckers ($P < 0.1$) (Figure 4D). We found no phenotype effects for latency to struggle, latency to vocalize or struggle frequency (Figures 4A-C).

At 24 weeks of age, phenotype effects were found for latency to struggle ($F_{3,58} = 3.67$, $P < 0.05$), latency to vocalize ($F_{3,59} = 3.27$, $P < 0.05$) and vocalization frequency ($F_{3,61} = 4.61$, $P < 0.01$). Victims struggled sooner compared to feather peckers ($P < 0.05$) and tended to struggle sooner compared to feather pecker-victims and neutrals ($P < 0.1$) (Figure 4A). Victims vocalized sooner compared to feather pecker-victims ($P < 0.05$) and tended to vocalize sooner compared to feather peckers ($P < 0.1$) (Figure 4B). Victims vocalized more compared to all other phenotypes ($P < 0.05$) (Figure 4D). We found no phenotype effects for struggle frequency (Figure 4C).

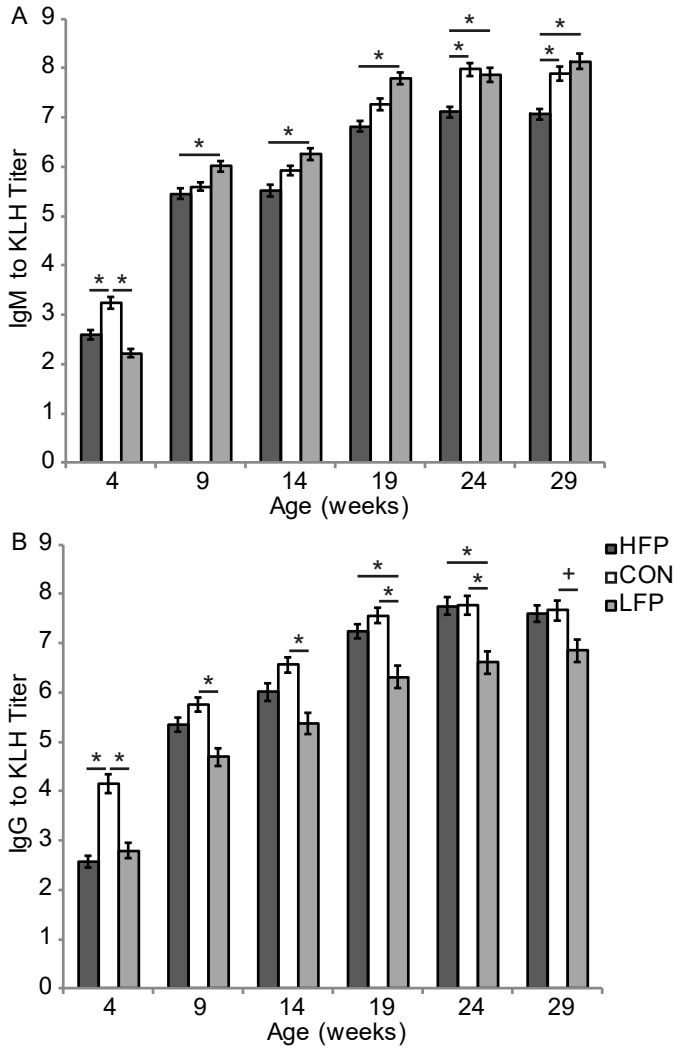

Figure 3. A) Mean natural antibody titers of IgM (± SEM) and B) mean titers of IgG (± SEM) against keyhole limpet hemocyanin (KLH) at 4, 9, 14, 19, 24 and 29 weeks of age for the high (HFP), control (CON) and low feather pecking (LFP) lines. $^{+}$ show tendencies ($P < 0.1$) and * show significant differences ($P < 0.05$) between lines.

Corticosterone

No phenotype effects were found for CORT levels after MR at 14 (feather peckers = 4.85 ng/mL, feather pecker-victims = 4.59 ng/mL, victims = 5.41 ng/mL and neutrals = 5.64 ng/mL) or 24 weeks of age (feather peckers = 6.79 ng/mL, feather pecker-victims = 3.45 ng/mL, victims = 4.49 ng/mL and neutrals = 3.26 ng/mL).

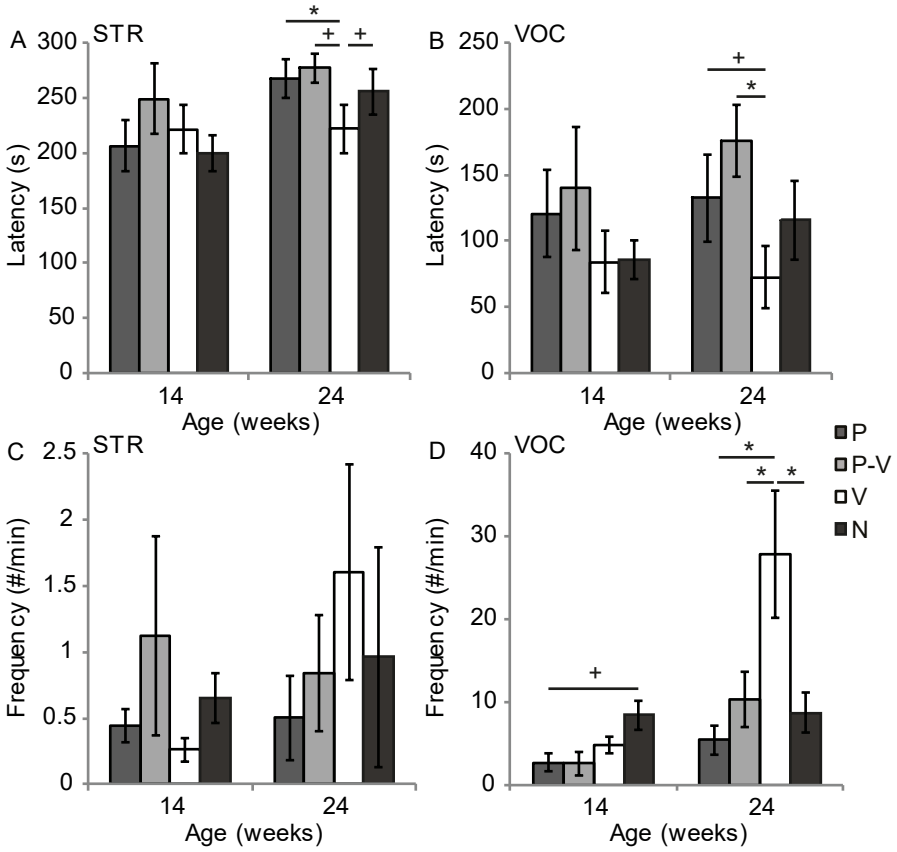

Figure 4. A) Mean latency (± SEM) to struggle (STR), B) mean latency (± SEM) to vocalize (VOC), C) mean struggle frequency (± SEM) and D) mean vocalization frequency (± SEM) during manual restraint at 14 and 24 weeks of age for feather peckers (P, n = 13 (14 weeks) and n = 11 (24 weeks)), feather pecker-victims (P-V, n = 7 (14 weeks) and n = 22 (24 weeks)), victims (V, n = 23 (14 weeks) and n = 21 (24 weeks)) and neutrals (N, n = 43 (14 weeks) and n = 18 (24 weeks)). $^{+}$ show tendencies ($P < 0.1$) and * show significant differences ($P < 0.05$) between phenotypes.

Serotonin

No phenotype effects were found for whole blood 5-HT levels at 14 weeks of age. Phenotype effects were found for 5-HT levels at 24 weeks of age ($F_{3,56} = 3.48$, $P < 0.05$), where feather peckers had higher 5-HT levels compared to neutrals ($P < 0.05$) (Figure 5).

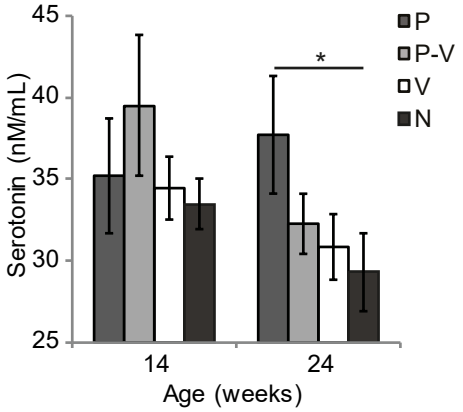

Figure 5. Mean whole blood serotonin level (± SEM) at 14 and 24 weeks of age for feather peckers (P, n = 13 (14 weeks) and n = 11 (24 weeks)), feather pecker-victims (P-V, n = 7 (14 weeks) and n = 20 (24 weeks)), victims (V, n = 22 (14 weeks) and n = 20 (24 weeks)) and neutrals (N, n = 41 (14 weeks) and n = 17 (24 weeks)). * show significant differences (P < 0.05) between phenotypes.

IgM and IgG Natural Antibody Titers

Unfortunately, we could not test for phenotype * age interaction effects on IgM or IgG NAb titers as birds switched between phenotypes. No phenotype effects were found for IgM or IgG NAb titers against KLH at 4, 9, 14, 19, 24 or 29 weeks of age.

Principal Component Analysis

At 14 and 24 weeks of age PCA produced one principal component with eigenvalue larger than 1 (2.00 and 1.94, respectively). All behavioural responses to MR loaded highly on the first principal component at both ages (the percentage of variance explained was 50% and 48%, respectively). We used this behavioural component to identify consistency in behavioural responses to MR over time and to identify relations with physiological measures. At both ages, the behavioural component had high negative loadings for latencies to struggle and vocalize, and high positive loadings for struggle and vocalization frequencies. Thus, chickens with high component scores struggled and vocalized sooner and more and vice versa.

Consistency of Measures over Time

We will focus on presenting Pearson correlation coefficients that were significant (P < 0.05) and above 0.2, as correlation coefficients below 0.2 are thought to show almost negligible relationships (Sprinthall, 2003). We identified consistency of measures over time within FP genotypes (HFP, CON and LFP). Unfortunately, we were unable to identify consistency over time within FP phenotypes as birds switched between phenotypes. Between 14 and 24 weeks of age, individual differences in 5-HT level, IgM and IgG NAb titers were consistent over time for the HFP line (correlations 0.52, 0.25 and 0.47, respectively). Furthermore, IgM and IgG NAb titers were consistent over time for the LFP line (correlations 0.46 and 0.44, respectively) and CON line (correlations 0.27 and 0.32, respectively). However,

scores of the behavioural component and CORT levels were not consistent between 14 and 24 weeks of age for any of the lines (Table 2).

Table 2. Consistency[a] over time of individual differences in behavioural component score and physiological measures as identified in high (HFP), control (CON) and low feather pecking (LFP) lines at 14 and 24 weeks of age.

Measures	**Correlations between 14 & 24 weeks of age**		
	HFP	**CON**	**LFP**
Behavioural component[b]	0.22	0.03	0.07
Corticosterone	0.07	0.06	0.01
Serotonin	0.52**	0.16	0.24
Natural antibody IgM	0.25*	0.27*	0.46**
Natural antibody IgG	0.47**	0.32**	0.44**

[a] Pearson correlations across measures at 14 and 24 weeks of age.

[b] Behavioural component was extracted by principal component analysis of four behavioural responses to manual restraint at both 14 and 24 weeks of age.

*P < 0.05.

**P < 0.01.

Relations between Behavioural and Physiological Measures

Line Effects

At 14 weeks of age, the behavioural component was correlated with 5-HT level in CON birds (-0.23), indicating that CON birds which struggled and vocalized sooner and more during MR had low 5-HT levels. At 24 weeks of age, the behavioural component was correlated with 5-HT level, IgM and IgG NAb titers in CON birds (0.26, -0.29 and -0.34, respectively), indicating that CON birds which struggled and vocalized sooner and more during MR had high 5-HT levels, but low IgM and low IgG NAb titers. Behavioural component scores were not correlated with any of the physiological measures for the HFP or LFP lines at both ages.

Phenotype Effects in the HFP line

At 14 weeks of age, we found no significant correlations between the behavioural component and physiological measures for FP phenotypes. At 24 weeks of age, the behavioural component was correlated with CORT level in feather peckers (0.81), suggesting feather peckers that struggled and vocalized sooner and more had high CORT levels. We found no further significant correlations between the behavioural component and physiological measures for FP phenotypes.

Discussion

In this study, we investigated behavioural responses and physiological measures, with a focus on the stress response, serotonergic- and immune-systems, in relation to feather pecking (FP) genotype (high FP (HFP), low FP (LFP) and unselected control (CON) line) and FP phenotype (feather pecker, feather pecker-victim, victim and neutral). Tests were performed at adolescent and adult age to examine consistency of individual differences within FP genotypes. We further examined relationships between behavioural responses and physiological measures within FP genotypes and within FP phenotypes of the HFP line.

Feather Pecking Genotype and Phenotype

Stress Response

HFP birds responded passively (i.e. struggled later and less) at adolescent age and actively (i.e. vocalized sooner and more) at adult age during manual restraint (MR). This is consistent with previous findings where HFP birds struggled later and less, but vocalized sooner and more compared to LFP birds at adolescent age (Kops et al., 2017) and where HFP birds responded more actively to several behavioural tests at various ages (de Haas et al., 2010; Kops et al., 2017; **chapter 2**). Within the HFP line, feather peckers tended to respond passively (i.e. vocalized less) compared to neutrals at adolescent age and victims responded actively (i.e. struggled sooner, vocalized sooner and more) compared to the other phenotypes at adult age during MR. In a previous study, feather peckers were more active during a MR test compared to non-peckers at adult age (Jensen et al., 2005), which is opposite to what we find here. Previously, we also found that feather peckers tended to respond more actively compared to victims and neutrals, and victims responded more actively compared to neutrals in other behavioural tests (**chapter 2**). Yet, FP genotypes and FP phenotypes did not differ in corticosterone (CORT) levels after MR, thus providing no physiological support for our behavioural findings. Furthermore, this suggests that divergent selection on FP does not affect HPA-axis activity and that FP phenotypes do not differ in HPA-axis activity, indicating that FP genotypes and FP phenotypes might not differ in stress sensitivity. Previously, HFP birds were found to have higher CORT levels after MR compared to LFP birds with CON birds having intermediate levels at adult age (Kjaer and Guémené, 2009), suggesting that HFP birds are more reactive. This discrepancy between studies might be explained by the fact that we used birds from the 18th generation, while the previous study used birds from the 6th generation. These birds were selected as parents of the 7th generation, thus containing extreme individuals with regard to FP (Kjaer and Guémené, 2009).

Furthermore, the FP selection lines were maintained for subsequent generations which could have caused physiological effects to become less pronounced. In addition, HFP birds had increased heart rate and reduced heart rate variability compared to LFP birds (Kjaer and Jørgensen, 2011), suggesting that HFP birds are more proactive. When comparing other lines, selected on egg production traits but also differing in FP, the opposite was found with high FP being related to low CORT levels after MR (Buitenhuis et al., 2004; Korte et al., 1997; van Hierden et al., 2002). Furthermore, no differences in CORT levels were found between FP phenotypes in previous studies (Daigle et al., 2015; Jensen et al., 2005). Thus, there is inconsistency in findings with regard to the relation between high FP and CORT levels within FP genotypes, whereas FP phenotypes do not seem to differ in CORT levels after MR.

It should be noted that behavioural and physiological responses to MR in this study might not be indicative of a stress response, as CORT levels after MR were generally low (average 4.8 ng/mL). Previous studies found peaks above 6.5 ng/mL (Bolhuis et al., 2009; Fraisse and Cockrem, 2006; Kjaer and Guémené, 2009). Low CORT levels might be explained by the fact that we performed multiple behavioural tests (see **chapter 2**), causing birds to become habituated to handling. In repeatedly handled birds CORT levels reduced faster after handling compared to unhandled birds (Freeman and Manning, 1979). Thus, our MR test possibly did not induce a strong stress response, making behavioural and physiological findings difficult to interpret in relation to the stress response. Based on our findings we suggest that divergent selection on FP affects behaviours other than FP (i.e. activity) and that FP phenotypes differ in their behavioural responses.

Serotonergic System

CON birds had higher whole blood serotonin (5-HT) levels compared to HFP and LFP birds at adolescent age, while HFP birds had lower whole blood 5-HT levels compared to CON and LFP birds at adult age. A previous study found the opposite relationship, with HFP birds having higher plasma 5-HT levels than LFP birds (Buitenhuis et al., 2006). This discrepancy with our study might be explained by the methods used (plasma vs. whole blood), as whole blood 5-HT more likely reflects storage concentration of 5-HT, while plasma 5-HT reflects unbound 5-HT (Shajib and Khan, 2015). Previous studies support our findings, where lines with a high FP tendency had lower whole blood 5-HT levels at adult ages (> 40 weeks) (de Haas et al., 2014b, 2013; Uitdehaag et al., 2011), suggesting that high FP is related to low peripheral 5-HT levels. Although FP phenotypes did not differ in whole blood 5-HT at adolescent age, feather peckers within the HFP line had higher whole blood 5-HT

levels compared to neutrals at adult age. Previously the opposite was found where neutrals had higher whole blood 5-HT compared to victims and feather pecker-victims at adult age (Daigle et al., 2015). The peripheral and central serotonergic system show similar characteristics in their transporters and receptors (Yubero-Lahoz et al., 2013) and whole blood 5-HT was correlated with central 5-HT, 5-HIAA (5-HT metabolite) and 5-HT turnover (5-HIAA/5-HT) in chickens (Uitdehaag et al., 2011). However, caution is needed when extrapolating whole blood 5-HT levels to central 5-HT levels as 5-HT cannot cross the blood-brain barrier (Pietraszek et al., 1992). Yet, in a previous study the FP selection lines were shown to differ in central serotonergic activity, where HFP chicks had lower central serotonergic activity compared to LFP chicks in several brain areas. At adult age the differences had disappeared or were opposite to what was found at young age (Kops et al., 2017). Low central serotonergic activity might thus predispose chickens to develop FP, while at an adult age high FP seems to be related to high central serotonergic activity (de Haas and van der Eijk, 2018). This shift in activity might be linked to performing or receiving FP as FP phenotypes were shown to differ in central serotonergic activity, where feather peckers had higher central serotonergic activity compared to neutrals (Kops et al., 2013).

It is interesting to note that we found a similar opposite relation between FP and whole blood 5-HT level, with HFP birds having lowest 5-HT but feather peckers within the HFP line having highest 5-HT. The actual performance of FP might increase peripheral 5-HT levels, possibly due to feather eating. HFP birds are more prone to eat feathers compared to LFP birds (Harlander-Matauschek and Bessei, 2005; Harlander-Matauschek and Häusler, 2009) and feather peckers showed more feather eating compared to non-peckers (McKeegan and Savory, 2001). Ingestion of feathers may increase peripheral 5-HT by providing structural components as the gut releases 5-HT in reaction to sensory perception of the mucosal layer (Coates et al., 2017). However, this relation between feather eating and increased peripheral 5-HT remains speculative and further research is needed. Based on our findings we suggest that divergent selection on FP affects whole blood 5-HT, potentially via mutations and/or alterations in expression of genes involved in the serotonergic system as previously found in relation to feather damage (Biscarini et al., 2010) and in the FP selection lines (Flisikowski et al., 2009; Wysocki et al., 2013). This is supported by the finding that whole blood 5-HT level was consistent between ages in the HFP line, but not in the CON and LFP lines. We further show that FP phenotypes differ in whole blood 5-HT. Since birds in our study already started to feather peck at a young age, we cannot distinguish between cause or consequence of FP in relation to whole

blood 5-HT. Therefore, it would be interesting to identify whole blood 5-HT levels in birds prior to and after the development of FP.

Immune System

Overall, HFP birds had lower IgM NAb titers compared to CON and LFP birds, while LFP birds had lower IgG NAb titers compared to CON birds with HFP birds having intermediate titers. FP phenotypes did not differ in IgM or IgG NAb titers. Thus, we only found differences between FP genotypes but not between FP phenotypes. This could suggest that there are genes simultaneously involved in FP and the immune system as indicated by previous studies (Biscarini et al., 2010; Buitenhuis et al., 2003) even in the FP selection lines (Buitenhuis et al., 2006; Hughes and Buitenhuis, 2010). Indeed, both NAb titers and the performance of FP have been shown to be heritable traits (Berghof et al., 2015; Kjaer and Sørensen, 1997). This is further supported by our finding that both IgM and IgG NAb titers are consistent over time. Findings from a previous study in the FP selection lines, suggest that HFP birds differ from LFP birds in immune reactivity and competence (Buitenhuis et al., 2006). Furthermore, when conspecifics within a cage had higher IgG NAb, the individual might have more feather damage (Sun et al., 2014). This is consistent with our study where HFP birds had higher IgG NAb titers compared to LFP birds, although CON birds did not differ from HFP birds in IgG NAb titers. Interestingly, the HFP line had lower IgM NAb titers, while the LFP line had lower IgG NAb titers compared to the other lines. Previously, it was suggested that IgG NAb are dependent upon exogenous antigen stimulation, while IgM NAb are not (Holodick et al., 2017). Thus, IgM NAb may be more under genetic influence, while IgG NAb may reflect immunomodulating environmental influences. This is further supported by a study that found high genetic correlations, but low phenotypic correlations between IgM and IgG NAb (Berghof et al., 2015). In the FP selection lines, this could mean that lower IgM NAb titers in the HFP line might be explained by alterations in their genetic make-up, while the lower IgG NAb titers in the LFP line might be explained by a difference in environmental influences or immune responsiveness to environmental influences. As lines were exposed to similar environmental conditions, we suggest that the LFP line had reduced immune responsiveness to environmental influences compared to the HFP and CON lines. Previously, the HFP line had higher responses to infectious bursal disease virus compared to the LFP and CON lines (Buitenhuis et al., 2006), suggesting that the HFP line had increased specific antibody responsiveness. Together with our findings this might indicate that HFP birds show increased immune responsiveness (i.e. they

are more responsive to the environment) than LFP birds. In this study we focused on NAb titers, yet further research is needed to identify whether the FP selection lines differ in immune responsiveness by for example, measuring innate and cellular responses to environmental challenges. Furthermore, high NAb titers (both IgM and IgG) have been related to increased survival in laying hens and NAb titer has been suggested as an indicator for general disease resistance (Star et al., 2007; Sun et al., 2011; Wondmeneh et al., 2015). Therefore, divergent selection on FP could potentially affect survival and health via altering NAb titers.

Coping style

Although previous studies have found differences in coping styles between lines which differ in FP tendency (Korte et al., 1999, 1997), we did not find such a clear relation here for FP genotypes or FP phenotypes. Behavioural responses to MR (as indicated by the behavioural component) and CORT levels were inconsistent between ages, suggesting that behavioural and physiological responses to MR in this study might not reflect coping style. Furthermore, for both FP genotypes and FP phenotypes proactive behavioural responses were correlated with reactive physiological measures (either NAb titers, CORT or 5-HT levels) and vice versa. Thus, we cannot categorize FP genotypes or FP phenotypes into specific coping styles.

A limitation in our study is that we observed FP behaviour for a limited amount of time which might have led to FP behaviour not being observed. However, continuous observation is impractical and the strength of this study was that we identified phenotype effects using the most recent FP phenotype categorization that was based on FP observations closest to the MR test at 14 or 24 weeks of age and to blood sampling at 4, 9, 19 and 29 weeks of age. We emphasize the importance of identifying FP phenotypes as they seem to differ in their behavioural responses and in whole blood 5-HT levels.

Conclusion

Divergent selection on feather pecking (FP) affects behavioural characteristics other than FP (i.e. activity), serotonergic- (i.e. peripheral serotonin) and immune-systems (i.e. natural antibodies), but FP genotypes did not differ in HPA-axis activity (i.e. corticosterone) in the present study.

Feather pecking phenotypes seem to differ in behavioural responses and the serotonergic system (i.e. peripheral serotonin), but differences in HPA-axis activity (i.e. corticosterone) or immune system (i.e. natural antibodies) were not found.

The present study could not support the categorization of FP genotypes or FP phenotypes into specific coping styles.

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Supplementary to Chapter 3

Feather Pecking Observations

An overview of the line effects on feather pecking behaviour at different ages is given in Table 1. Lines did not differ in exploratory feather pecks (EFP) or stereotyped feather pecking bouts (StFP) at 18-19 and 24-25 weeks of age. Line effects were found for severe feather pecks (SFP) given at 18-19 ($F_{2,20} = 11.90$, $P < 0.01$) and 24-25 weeks of age ($F_{2,19} = 10.16$, $P < 0.01$). HFP birds showed more SFP at 18-19 ($P < 0.01$) and 24-25 weeks of age ($P < 0.01$) compared to LFP birds. HFP birds showed more SFP at 24-25 weeks of age ($P < 0.05$) and tended to show more SFP at 18-19 weeks of age compared to CON birds ($P < 0.1$). LFP and CON birds only differed in SFP at 18-19 weeks of age, with CON birds showing more SFP compared to LFP birds ($P < 0.05$).

Table 1. Feather pecking behaviour (exploratory feather pecking (EFP), stereotyped feather pecking (StFP) (bouts) and severe feather pecking(SFP)) of the high (HFP), control (CON) and low feather pecking (LFP) lines at different ages.

Variables	HFP	CON	LFP	P-value
Age (18-19 weeks)	**n = 86**	**n = 81**	**n = 77**	
EFP	8.81 ± 1.50	6.96 ± 0.84	5.51 ± 0.64	ns
StFP (bouts)	1.63 ± 0.32	1.28 ± 0.35	1.27 ± 0.23	ns
SFP	5.88 ± 2.34[a]	1.19 ± 0.30[a]	0.16 ± 0.07[b]	< 0.01
Age (24-25 weeks)	**n = 72**	**n = 70**	**n = 63**	
EFP	4.14 ± 0.61	3.86 ± 0.57	2.99 ± 0.44	ns
StFP (bouts)	0.67 ± 0.19	0.26 ± 0.11	0.22 ± 0.08	ns
SFP	7.67 ± 2.03[a]	2.09 ± 0.44[b]	1.08 ± 0.27[b]	< 0.01

Average number of pecks or bouts per bird per hour (30 min total observation time per bird). Differing lowercase letters (a,b) show significant differences ($P < 0.05$) between lines.

Feather Pecking Phenotypes

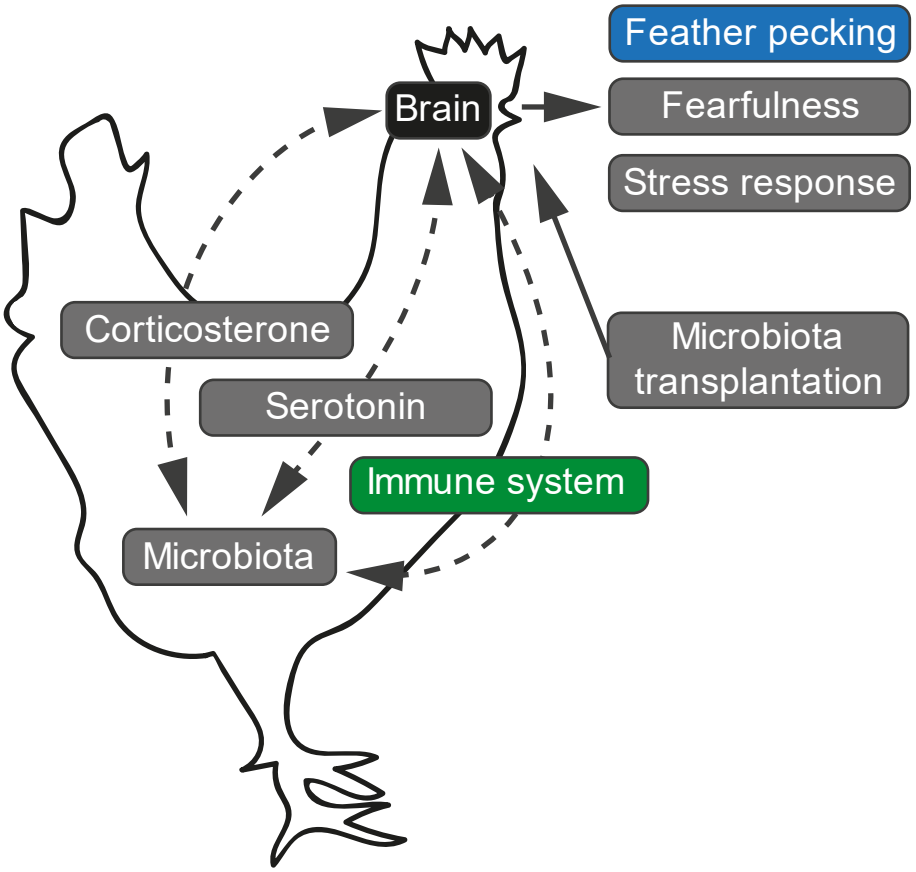
Birds were categorized as feather pecker, feather pecker – victim, victim and neutral. The number (and percentage) of hens within each category at different ages is given in Table 2. On average the largest percentage of hens was categorized as neutrals across all ages in all three lines (HFP 47.8%; CON 77.6%; LFP 85.2%). The remainder of hens was categorized as feather pecker (HFP 14.5%; CON 9.4%; LFP 7.5%), feather pecker - victim (HFP 13.7%; CON 3.3%; LFP 1.9%) and victim (HFP 24.1%; CON 9.6%; LFP 5.4%).

Table 2. The number (and percentage) of hens per phenotype category (feather pecker (P), feather pecker-victim (P-V), victim (V) and neutral (N)) within the high (HFP), control (CON) and low feather pecking (LFP) lines based on the number of severe feather pecks (SFP) given or received at different ages.

	P	**P-V**	**V**	**N**
Criteria	Give > 1 SFP Receive 0 or 1 SFP	Give > 1 SFP Receive > 1 SFP	Give 0 or 1 SFP Receive > 1 SFP	Give 0 or 1 SFP Receive 0 or 1 SFP
Age (3-4 weeks)				
HFP	16 (12.2%)	13 (9.9%)	34 (26.0%)	68 (51.9%)
CON	7 (5.6%)	2 (1.6%)	10 (7.9%)	107 (84.9%)
LFP	7 (5.6%)	5 (4.0%)	4 (3.2%)	109 (87.2%)
Age (8-9 weeks)				
HFP	19 (17.3%)	3 (2.7%)	16 (14.6%)	72 (65.5%)
CON	6 (5.8%)	1 (1.0%)	5 (4.9%)	91 (88.4%)
LFP	5 (5.0%)	0 (0.0%)	4 (4.0%)	92 (91.1%)
Age (12-13 weeks)				
HFP	19 (21.6%)	8 (9.1%)	17 (19.3%)	44 (50.0%)
CON	12 (14.8%)	8 (9.9%)	11 (13.6%)	50 (61.7%)
LFP	13 (16.5%)	4 (5.1%)	9 (11.4%)	53 (67.1%)
Age (15-16 weeks)				
HFP	13 (15.1%)	7 (8.1%)	23 (26.7%)	43 (50.0%)
CON	7 (8.6%)	1 (1.2%)	9 (11.1%)	64 (79.0%)
LFP	4 (5.2%)	1 (1.3%)	4 (5.2%)	68 (88.3%)
Age (18-19 weeks)				
HFP	10 (11.6%)	11 (12.8%)	21 (24.4%)	44 (51.2%)
CON	9 (11.1%)	2 (2.5%)	8 (9.9%)	62 (76.5%)
LFP	1 (1.3%)	0 (0.0%)	1 (1.3%)	75 (97.4%)
Age (24-25 weeks)				
HFP	11 (15.3%)	22 (30.6%)	21 (29.2%)	18 (25.0%)
CON	10 (14.3%)	5 (7.1%)	11 (15.7%)	44 (62.9%)
LFP	8 (12.7%)	2 (3.2%)	7 (11.1%)	46 (73.0%)
Age (28-29 weeks)				
HFP	6 (8.5%)	16 (22.5%)	20 (28.2%)	29 (40.9%)
CON	4 (5.7%)	0 (0.0%)	3 (4.3%)	63 (90.0%)
LFP	4 (6.3%)	0 (0.0%)	1 (1.6%)	58 (92.1%)

Chapter 4

Chicken lines divergently selected on feather pecking differ in immune characteristics



Jerine A.J. van der Eijk, Michel B. Verwoolde, Ger de Vries Reilingh, Christine A. Jansen, T. Bas Rodenburg, Aart Lammers

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Abstract

It is crucial to identify whether relations between immune characteristics and damaging behaviours in production animals exist, as these behaviours reduce animal welfare and productivity. Feather pecking (FP) is a damaging behaviour in chickens, which involves hens pecking and pulling at feathers of conspecifics. To further identify relationships between the immune system and FP we characterized high FP (HFP) and low FP (LFP) selection lines with regard to nitric oxide (NO) production by monocytes, specific antibody (SpAb) titers, natural (auto)antibody (N(A)Ab) titers and immune cell subsets. NO production by monocytes was measured as indicator for innate pro-inflammatory immune functioning, SpAb titers were measured as part of the adaptive immune system and N(A)Ab titers were measured as they play an essential role in both innate and adaptive immunity. Immune cell subsets were measured to identify whether differences in immune characteristics were reflected by differences in the relative abundance of immune cell subsets.

Divergent selection on FP affected NO production by monocytes, SpAb and N(A)Ab titers, but did not affect immune cell subsets. The HFP line showed higher NO production by monocytes and higher IgG N(A)Ab titers compared to the LFP line. Furthermore, the HFP line tended to have lower IgM NAAb titers, but higher IgM and IgG SpAb titers compared to the LFP line. Thus, divergent selection on FP affects the innate and adaptive immune system, where the HFP line seems to have a more responsive immune system compared to the LFP line. Although causation cannot be established in the present study, it is clear that relationships between the immune system and FP exist. Therefore, it is important to take these relationships into account when selecting on behavioural or immunological traits.

Introduction

In vertebrates the immune system and behaviour are deeply connected. Individual differences in behavioural patterns are associated with variation in pathogen and parasite exposure (Barber and Dingemanse, 2010; Lymbery, 2015), which could lead to differences in immune characteristics. In turn, the immune system has been shown to be involved in brain development and regulation of behaviours (Bilbo and Schwarz, 2012, 2009; Grindstaff, 2016), and could thereby influence behavioural characteristics. Behavioural and immune characteristics may also be linked through factors that simultaneously affect these characteristics, such as genetics, early-life experiences or the gut microbiota (Lopes, 2017).

It is crucial to identify whether these relations between behavioural and immune characteristics also exist in production animals, as selection on behavioural characteristics could influence animal health and selection on immune characteristics could influence animal behaviour. Especially of interest are damaging behaviours as these behaviours reduce animal welfare and productivity (Brunberg et al., 2016). Feather pecking (FP) is such a damaging behaviour in chickens, which involves hens pecking and pulling at feathers of conspecifics. Chickens have a high motivation to explore and forage which could lead to the expression of FP in animal husbandry (Dixon et al., 2008; Rodenburg et al., 2013). In addition, chickens with a higher motivation to explore and forage are more likely to be exposed to pathogens, as they explore more of their environment, food sources and interact more with conspecifics (Barber and Dingemanse, 2010), but they are also more likely to develop FP. This points to a potential relationship between the immune system and FP.

Indeed, multiple studies have found relationships between the immune system and FP. Genetic mutations in cytokine Interleukin 4 (IL4), IL9, nuclear factor-kappa-B (NFKB) and chemokine (CCL4) genes of cage mates were associated with feather damage of individuals (Biscarini et al., 2010), where feather damage is an indicator of severe FP (Bilcik and Keeling, 1999). Genetic mutations in the IL4 and IL9 genes were further associated with IgM and IgG natural antibodies (NAb) titers (Sun et al., 2013), where NAb are antibodies that can bind antigen without prior intentional exposure to that antigen (Avrameas, 1991; Baumgarth et al., 2005). These associations were all associative genetic effects on feather damage (the genetic effect of the genotype of cage mates on an individual's feather damage), suggesting that IL4, IL9, NFKB and CCL4 genes and NAb titers may be related to the propensity to perform FP. This is supported by the finding that when cage mates had higher IgG NAb, the individual had more feather damage (Sun et al., 2014). Furthermore, a

strong genetic correlation was found between FP and the specific antibody (SpAb) response (Buitenhuis et al., 2004). These findings suggest a (genetic) relation between the immune system and FP.

Further evidence for a relationship between the immune system and FP comes from lines divergently selected on FP. The high FP (HFP) line had a higher specific antibody (SpAb) response to vaccination, while the low FP (LFP) line had a higher relative abundance of white blood cells, T helper cells, double positive T cells and higher expression of MHC class I molecules on T and B cells (Buitenhuis et al., 2006). The HFP line further had lower IgM NAb, but higher IgG NAb compared to the LFP line (**chapter 3**). These findings suggest that the FP selection lines differ in immune responsiveness and give further support for a relationship between the immune system and FP.

To further identify relationships between the immune system and FP we characterized the FP selection lines with regard to immune characteristics. We identified nitric oxide (NO) production by monocytes, specific antibody (SpAb) titers, natural (auto)antibody (N(A)Ab) titers and immune cell subsets of the HFP and LFP lines. NO production by blood derived monocytes was measured *ex vivo*, as indicator for innate pro-inflammatory immune functioning (Murray and Wynn, 2011; Uehara et al., 2015). SpAb titers to human serum albumin (HuSA) were measured as part of the adaptive immune system. N(A)Ab titers were measured as they play an essential role in both innate and adaptive immunity, for example by maintaining homeostasis, increasing disease resistance and linking the two types of immunity (Berghof et al., 2019; Lammers et al., 2004; Ochsenbein and Zinkernagel, 2000; Panda and Ding, 2015), where NAb bind to non-self-antigen and NAAb bind to self-antigen. We further included immune cell subsets to identify whether differences in immune characteristics were reflected by differences in the relative abundance of immune cell subsets. We hypothesized that the HFP line would have a more responsive immune system (i.e. higher NO production, SpAb and IgG N(A)Ab titers), as it previously had a higher specific immune response and IgG NAb compared to the LFP line (Buitenhuis et al., 2006; **chapter 3**). We further expected higher NO production in the HFP line, as macrophages from a line selected on high antibody response produced more NO compared to a line selected on low antibody response in chickens (Guimarães et al., 2011).

Material and Methods

Animals and housing

Offspring from White Leghorn birds from the 18th generation of lines selected on high (HFP) respectively low feather pecking (LFP) were used (see Kjaer et al., 2001 for the selection procedure). The HFP and LFP line were divergently selected on feather pecking (FP) for seven generations and were maintained in subsequent generations. The parent stock was between 50 and 54 weeks of age at the time of egg collection. A total of 120 birds were produced in one batch of eggs that were incubated at an average egg shell temperature of 37.8 ºC and average relative humidity of 54.9 %. Non-beak-trimmed female birds were used that had a neck tag (Roxan) with a unique number. At 3 and 4 weeks of age, birds were colour marked on the neck and/or back for individual identification (colours: black, purple, green and orange). From 7 weeks of age onwards, birds were equipped with a light weight backpack with a number for individual identification. Birds were housed per line in 10 pens and in groups of 12. At 8 weeks of age group size was reduced by 2 birds (n = 9-10 birds per pen).

At all times, water and feed were provided *ad libitum.* Birds received a standard rearing diet 1 from hatch until 8 weeks of age, a standard rearing diet 2 from 8 until 16 weeks of age and a standard laying diet from 16 weeks of age onwards until the end of the experiment. Each floor pen (h: 2 m, l: 2 m, w: 1 m) had wood shavings on the floor, a platform installed 45 cm above the floor and visual barriers of 1.5 m high to prevent birds in adjacent pens of seeing each other. Post hatch, temperature was kept around 33°C and gradually lowered to 21°C at 5 weeks of age. The light regime was 23L:1D post hatch, and was weekly, gradually reduced to 8L:16D at 4 weeks of age. From 15 weeks of age, the light regime was weekly extended with 1 h until 16L: 8D at 22 weeks of age. Light intensity in pens ranged between 25.8 and 68.2 LUX (average 49.8 LUX) during the first 6 weeks of life, thereafter light intensity was reduced and ranged between 3.5 and 5.8 LUX (average 4.8 LUX) as measured with a Voltcraft MS-1300 light meter (Conrad Electric Benelux). A wooden nest box was placed in front of the pen at 15 weeks of age. The experimental set-up (housing conditions, vaccinations, etc.) was designed to reflect commercial conditions as FP is an issue in the egg laying industry. The experiment was approved by the Central Authority for Scientific Procedures on Animals according to Dutch law (no: AVD104002015150).

Feather pecking observations

FP behaviour was observed on individual level in week 4-5, 9-10, 14-15, 19+21, 24-25 and 28-29. In week 4-5 birds were observed by direct observation. Each observation lasted 30 min, either in the morning (8:30 h-12:00 h) or in the afternoon (12:30 h-16:00 h), after a 2 min habituation period. For all other weeks FP was observed from video recordings. Each observation lasted 15 min, either in the morning (10:40 h-10:55 h) or in the afternoon (14:40 h-14:55 h). The Observer XT 10 program (Noldus Information Technology) was used for video analysis of FP, categorized into gentle FP (subdivided into exploratory FP and bouts of stereotyped FP) and severe FP (Table 1). Inter-observer reliability for video analysis was high for all FP behaviours (Pearson correlations: exploratory FP = 0.99, stereotyped FP = 0.96 and severe FP = 0.98). The order for observations was randomized at pen level and observers were blinded to the lines. FP behaviours were summed over two subsequent weeks, thus including one morning and one afternoon observation with a total observation period of 60 min for week 4-5 and a total observation period of 30 min for all other weeks.

Table 1. Ethogram of the feather pecking (FP) observations (after Newberry et al., 2007).

Behaviour	**Description**
Exploratory FP	Bird makes gentle beak contact with the feathers of another bird without visibly altering the position of the feathers. The recipient makes no apparent response. Each peck is recorded.
Stereotyped FP Bout	Bird makes ≥ 3 gentle pecks at a single body region at intervals of ≤ 1 s. Each series of pecks (bout) is recorded. Bout ends when birds separate, or when pecking is directed to another target on the same, or another, bird.
Severe FP	Bird grips and pulls or tears vigorously at a feather of another bird with her beak, causing the feather to lift up, break or be pulled out. The recipient reacts to the peck by vocalizing, moving away or turning towards the pecking bird. Each peck is recorded.

Vaccinations, immune challenge, blood collection and analysis

All birds received vaccinations against Marek's disease (day 0 intramuscular), Infectious Bronchitis (day 0 and week 2 eye drops, week 8 and 15 spray), Newcastle Disease (week 1, 4, 10 spray and week 12 injection), Infectious Bursal Disease (day 25 drinking water), Avian Encephalomyelitis and Pox Diphtheria (week 12 wing web) and Infectious Laryngo Tracheitis (week 12 eye drops). At 8 weeks of age, 15

birds per line (3 per pen) were intra-tracheally immunized with Human Serum Albumin (HuSA) (0.5 mg/kg, Sigma-Aldrich A3782) using a blunted needle (Parmentier et al., 2008).

Blood was collected from all birds at 5, 10, 15, 20, 25 and 30 weeks of age. Blood was additionally collected from immunized birds at day 0, prior to HuSA immunization (8 weeks of age), day 4, day 7 (9 weeks of age) and day 14 (10 weeks of age) post HuSA immunization (see Figure 1 for overview). Blood was taken from the wing vein using a heparinized syringe and kept on ice. In the laboratory, blood samples for natural (auto)antibody (N(A)Ab) titers and specific antibody (SpAb) titers were centrifuged at 5250 x g for 10 min at room temperature and plasma was stored at -20 °C until further analysis. Blood samples for leukocyte isolation were not kept on ice and analysed directly in the laboratory.

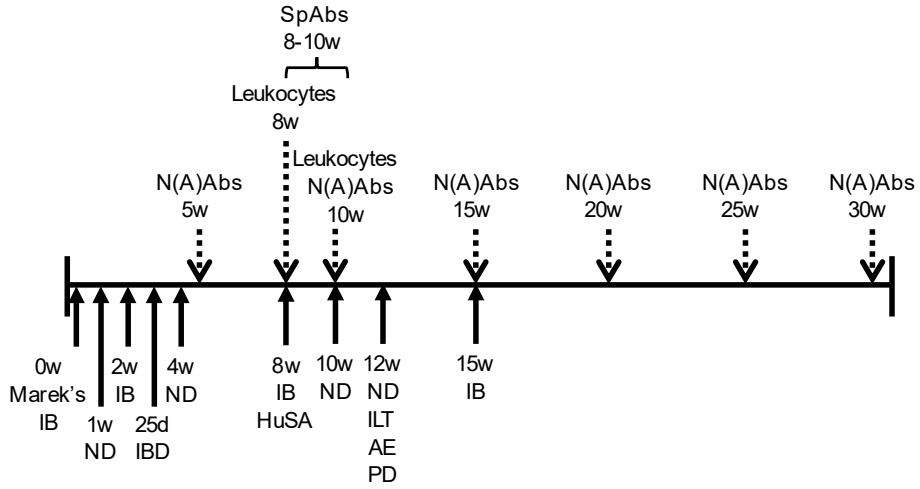

Figure 1. Timeline of blood sampling (above line), vaccinations and immune challenge (below line) performed at specific ages in days (d) or weeks (w). N(A)Abs = natural (auto)antibodies, Leukocytes = leukocyte isolation, SpAbs = specific antibodies, Marek's = Marek's disease, IB = Infectious Bronchitis, ND = Newcastle Disease, IBD = Infectious Bursal Disease, HuSA = Human Serum Albumin immunization, ILT = Infectious Laryngo Tracheitis, AE = Avian Encephalomyelitis, PD = Pox Diphtheria.

Plasma IgM and IgG natural (auto)antibody titers

Samples from all birds at 5, 10, 15, 20, 25 and 30 weeks of age were used for determination of IgM and IgG NAb titers against keyhole limpet hemocyanin (KLH) and for determination of IgM and IgG NAAb titers against phosphorylcholine conjugated to bovine serum albumin (PC-BSA) and Herring sperm DNA. N(A)Ab titers against KLH (2 µg/mL, Sigma-Aldrich H8283), PC-BSA (1 µg/mL, LGC

Biosearch Technologies PC-1011-10) and Herring sperm DNA (5 µg/mL, Sigma-Aldrich D6898) were determined by an indirect enzyme-linked immunosorbent assay (ELISA) as described previously (Berghof et al., 2015) with the following modifications. Plasma samples were added on plates in a four step dilution starting at dilution 1:40 in phosphate buffer saline (PBS) containing 0.05% Tween 20 and 1% horse plasma (100 µL in each well). Peroxidase conjugated goat-anti-chicken IgM (Bethyl Laboratories A30-102P, dilution 1:20,000) or goat-anti-chicken IgG (Bethyl Laboratories A30-104P, dilution 1:20,000) was used as secondary antibody (100 µL in each well). Substrate buffer (tetramethylbenzidine and 0.05% H_2O_2) was added (100 µL in each well) and after 20 min the reaction was stopped with 50 µL of 1.25M H_2SO_4. Extinctions were measured with a Thermo Scientific Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific) at 450 nm. Titers were expressed as log2 values of the dilutions that gave an extinction closest to 50% of Emax where Emax represents the highest mean extinction of a standard positive (pooled) plasma present on every plate.

Plasma IgM and IgG specific antibody titers

Samples from immunized birds at day 0, prior to HuSA immunization, day 4, day 7 and day 14 post HuSA immunization were used for determination of IgM and IgG SpAb titers against HuSA (4 µg/mL, Sigma-Aldrich A3782). SpAb titers against HuSA were determined by an indirect ELISA as described above, with the following modifications. Peroxidase conjugated goat-anti-chicken IgM (Bethyl Laboratories A30-102P, dilution 1:20,000) or goat-anti-chicken IgG (Bethyl Laboratories A30-104P, dilution 1:40,000) was used as secondary antibody (100 µL in each well).

Leukocyte isolation and stimulation experiment

Samples from immunized birds at 8 and 10 weeks of age were used for leukocyte isolation and *ex vivo* stimulation. Samples were diluted 1:1 in RPMI 1640 (Gibco). Diluted blood was loaded onto a Histopaque-1119 gradient (Sigma-Aldrich Histopaque-1119) and centrifuged at 700 x g for 40 min at room temperature. The interphase containing the leukocytes was collected, washed 2 times and re-suspended in complete culture medium RPMI 1640 (Gibco) supplemented with HEPES, Glutamax, 10% heat-inactivated chicken serum (Gibco) and 0.5% antibiotics (Final concentration of 50 U/mL penicillin and 50 µg/mL streptomycin, Gibco). Leukocytes were seeded at a concentration of $1*10^6$ cells per well to a 96-wells flat-bottom plate (Greiner CELLSTAR) with a total volume of 100 µL per well. The cells were incubated overnight at 41°C in 5% CO_2 and 95% humidity. The next day, non-

adherent cells were washed away with pre-warmed culture medium. Adherent cells were stimulated with 200 µL per well culture medium (control), lipopolysaccharides (LPS) from *E. coli* serotype O55:B5 (10 µg/mL, Sigma-Aldrich L6529), recombinant chicken interferon gamma (IFN-γ) (100 ng/mL, Kingfisher Biotech) or a combination of LPS + IFNγ with the same concentrations for 48 h for the nitric oxide production assay.

Flow cytometry

Subsets of innate and adaptive immune cells in the blood were analysed by flow cytometry. All antibodies were obtained from Southern biotech, except for the mouse-anti-chicken CD40 and the secondary antibodies goat-anti-mouse IgG2a - APC and IgG3-PE which were obtained from Biorad and BD biosciences, respectively. The chicken natural killer (NK) cell markers were kindly provided by professor T. Göbel, LMU Munich.

500,000 isolated leukocytes were washed with FACS buffer (PBS w/o CaMg (Gibco) supplemented with 0.5% BSA and 0.005% Sodium azide (Sigma-Aldrich)). The cells were transferred to a 96-wells round-bottom plate and stained with monoclonal antibodies specific for chicken immune cells. Mix 1 includes a combination of mouse-anti-chicken β2M FITC (clone F21-F21, IgG1), mouse-anti-chicken CD3-PE (clone CT3; IgG1), mouse-anti-chicken CD4-biotin (clone CT4, IgG1) and mouse-anti-chicken CD8α-APC (CT8, IgG1). In mix 2 a combination of mouse-anti-chicken TCR1-FITC (TCRγδ, IgG1), mouse-anti-chicken MHC II-PE (CIa, IgM) and mouse-anti-chicken CD40 (AV79, IgG2a) was used. In mix 3 mouse-anti-chicken-Bu-1-FITC (AV20, IgG1) was used together with mouse-anti-chicken CD3 APC (CT3, IgG1) and the mouse-anti-chicken NK markers 28-4 (IgG3) (Göbel et al., 2001) and 20E5-biotin (IgG1) (Jansen et al., 2010). Cells were incubated with antibodies for 20 min at 4°C. Next, cells were washed 2 times in FACS buffer. Afterwards, streptavidin-Percp (BD biosciences) was added to the samples stained with mix 1, goat-anti-mouse IgG2a-APC to samples stained with mix 2, a combination of streptavidin-Percp and goat-anti-mouse IgG3-PE to cells stained with mix 3, or a combination of all secondary antibodies was added to samples without a primary antibody mix as negative control (Sup. Table 1). Cells were stained for 20 min at 4°C and washed in PBS. Next, cells were re-suspended in PBS and an equal volume of 4% paraformaldehyde (Merck) was added to fixate the cells. Cells were incubated for 10 min at room temperature, washed 1 time using FACS buffer and re-suspended in FACS buffer. Flow cytometry was performed using a

FACS Canto flow cytometer (BD Biosciences) and at least 100,000 lymphocytes were collected. Data were analysed using FlowJo software (Threestar).

Nitric oxide production assay

The nitric oxide (NO) production assay was performed 48 h after the *ex vivo* stimulation. The reactive nitrogen oxide intermediate NO was indirectly measured by quantifying the production of the more stable nitrite (NO_2-), using Griess reagents (Parmentier et al., 2010). The assay is a colorimetric assay and quantifies the accumulation of NO in the culture medium. A volume of 50 µL culture supernatant was transferred to a 96-wells flat-bottom plate (Greiner CELLSTAR) and combined with 50 µL of the Griess reagent. The Griess reagent consists of a 1:1 mixture: Griess reagents solution A (2% Sulphanilamide in 5% H_3PO_4) and Griess reagents solution B (0.2% N-(1-naphthyl)ethylenediamine dihydrochloride in H_2O). The plate was incubated for 10 min at room temperature. NO concentration was determined by measuring the optical density (OD_{540}) with a Thermo Scientific Multiskan GO microplate spectrophotometer. The amount is determined by a calibration line using two fold dilutions of a sodium nitrite solution ($NaNO_2$) in the range of 100 µM – 0 µM.

Statistical analysis

SAS Software version 9.4 was used for statistical analysis (SAS Inst.). Linear mixed models for line effects on N(A)Ab titers consisted of fixed effects line * age, line and age. The random effect consisted of pen within line, a repeated statement for age with chicken ID as subject and an unstructured covariance structure. The unstructured covariance structure gave the best fitting model (model 1). Linear mixed models for line effects on SpAb titers consisted of fixed effects line * age, line and age. The random effect consisted of pen within line (model 2). Linear mixed models for line effects on NO production and SpAb titers tested per age consisted of fixed effects line and the random effect pen within line (model 3). The model assumptions were visually examined. NO production at 10 weeks of age was log transformed to obtain normality of model residuals. Post hoc pairwise comparisons were corrected by Tukey–Kramer adjustment. A Kruskal Wallis test was used to identify line effects on FP behaviour, the relative abundance of immune cell subsets and NO production at 8 weeks of age. All data is presented as (untransformed) mean ± standard error (SE).

Model 1) $\gamma_{ijkl} = \mu + LINE_i + AGE_j + LINE_i * AGE_j + (pen_k/line_i) + id_l + e_{ijkl}$

γ = N(A)Ab titers
μ = overall mean
line = fixed effect of line (i = HFP or LFP)
age = fixed effect of age, week at which blood was collected for measurement (j = 5, 10, 15, 20, 25 or 30)
pen/line = random effect of line within pen (k = 1-10)
id = random effect of chicken id (l = 1-120) with repeated observations assumed to be distributed as $\sim N(0,T\sigma^2_{id})$ in which T is the unstructured covariance matrix with the chicken id as the subject and age as the repeated effect, σ^2_{id} is the variance between chickens
e = residual effect

Model 2) $\gamma_{ijk} = \mu + LINE_i + AGE_j + LINE_i * AGE_j + (pen_k/line_i) + e_{ijk}$

γ = SpAb titers
μ = overall mean
line = fixed effect of line (i = HFP or LFP)
age = fixed effect of age, days post immunization at which blood was collected for measurement (j = 0, 4, 7 or 14)
pen/line = random effect of line within pen (k = 1-10)
e = residual effect

Model 3) $\gamma_{ij} = \mu + LINE_i + (pen_j/line_i) + e_{ij}$

γ = NO production or SpAb titers
μ = overall mean
line = fixed effect of line (i = HFP or LFP)
pen/line = random effect of line within pen (j = 1-10)
e = residual effect

Results

Divergent selection on feather pecking affects feather pecking behaviour

Feather pecking (FP) behaviour was observed at an individual level throughout the experiment to identify whether divergent selection on FP actually resulted in differences in FP. FP was categorized into gentle FP (subdivided into exploratory

and stereotyped FP) and severe FP, where gentle FP usually does not result in damage and severe FP is the problematic behaviour in terms of damage to the recipient (Rodenburg et al., 2013). An overview of line effects on the different types of FP is given in Table 2.

Table 2. Feather pecking (FP) behaviour (exploratory FP, stereotyped FP (bouts) and severe FP) of the high (HFP) and low feather pecking (LFP) lines at different ages.

Variables	**HFP**	**LFP**	**P-value**	**$\chi 2$**
Age (4-5 weeks)	**n = 59**	**n = 59**		
Exploratory FP	3.91 ± 0.54	1.36 ± 0.22	< 0.01	21.44
Stereotyped FP (bouts)	1.01 ± 0.19	0.35 ± 0.07	< 0.01	7.91
Severe FP	0.57 ± 0.13	0.30 ± 0.07	< 0.1	3.72
Age (9-10 weeks)	**n = 48**	**n = 49**		
Exploratory FP	4.08 ± 0.89	2.00 ± 0.58	< 0.05	6.02
Stereotyped FP (bouts)	0.50 ± 0.16	0.24 ± 0.13	ns	2.20
Severe FP	0.30 ± 0.30	0.08 ± 0.08	ns	0.00
Age (14-15 weeks)	**n = 46**	**n = 47**		
Exploratory FP	3.52 ± 0.66	1.19 ± 0.39	< 0.01	12.76
Stereotyped FP (bouts)	0.17 ± 0.08	-	< 0.05	4.13
Severe FP	0.43 ± 0.15	0.09 ± 0.06	< 0.05	4.09
Age (19+21 weeks)	**n = 45**	**n = 42**		
Exploratory FP	3.60 ± 1.44	0.74 ± 0.23	< 0.05	4.71
Stereotyped FP (bouts)	0.20 ± 0.10	0.07 ± 0.04	ns	0.93
Severe FP	0.38 ± 0.19	0.05 ± 0.05	< 0.1	3.44
Age (24-25 weeks)	**n = 43**	**n = 39**		
Exploratory FP	6.47 ± 1.80	0.87 ± 0.31	< 0.01	14.42
Stereotyped FP (bouts)	0.56 ± 0.23	0.10 ± 0.10	< 0.05	4.19
Severe FP	1.07 ± 0.38	0.05 ± 0.05	< 0.05	5.58
Age (28-29 weeks)	**n = 43**	**n = 36**		
Exploratory FP	2.98 ± 0.59	0.72 ± 0.33	< 0.01	10.80
Stereotyped FP (bouts)	0.42 ± 0.14	0.06 ± 0.06	< 0.05	4.83
Severe FP	1.07 ± 0.47	0.11 ± 0.08	< 0.05	4.95

Average number of pecks or bouts per bird per hour (age 4-5 weeks: 60 min total observation time per bird; age 9-10, 14-15, 19+21, 24-25 and 28-29 weeks: 30 min total observation time per bird).

HFP birds showed more exploratory FP at all ages and showed more stereotyped FP at 4-5, 14-15, 24-25 and 28-29 weeks of age compared to LFP birds ($P < 0.05$). Furthermore, HFP birds tended to show more severe FP at 4-5 and 19+21 weeks of age ($P < 0.1$) and showed more severe FP at 14-15, 24-25 and 28-29 weeks of age compared to LFP birds ($P < 0.05$). These results indicate that divergent selection on FP indeed altered FP behaviour, with the HFP line showing more FP compared to the LFP line.

Divergent selection on feather pecking does not affect immune cell subsets

Leukocytes were analysed by flow cytometry to identify the relative abundance of immune cell subsets in the FP selection lines. An example of the gating strategy is shown in Sup. Figure 1 and an overview of the results is shown in Sup. Table 2. We found no differences between lines for any of the immunological cell types (relative abundance or expression), except for the expression (mean fluorescent intensity) of MHC class I on T helper cells (CD4) ($\chi^2 = 4.99$, $P < 0.05$) and on CD4+ CD8α+ ($\chi^2 = 4.79$, $P < 0.05$), which were lower in the HFP line compared to the LFP line. These results suggest that the FP selection lines did not differ in relative abundance of immune cell subsets.

Divergent selection on feather pecking affects nitric oxide production by blood derived monocytes

Nitric oxide (NO) production by blood derived monocytes was measured *ex vivo* as indicator for innate pro-inflammatory immune functioning (Murray and Wynn, 2011; Uehara et al., 2015). At 8 and 10 weeks of age, the HFP line had higher NO production by blood derived monocytes stimulated *ex vivo* with lipopolysaccharides (LPS) ($\chi^2 = 5.04$, $P < 0.05$ and $F_{1,8} = 23.81$, $P < 0.01$, respectively) or LPS + interferon gamma (IFNγ) ($\chi^2 = 7.68$, $P < 0.01$ and $F_{1,8} = 11.30$, $P < 0.01$, respectively) compared to the LFP line, but no differences were found for the control (medium) or stimulation with IFNγ (Figure 2). When comparing the control to stimulation with LPS, IFNγ or LPS + IFNγ, we found that in the HFP line NO production increased by addition of LPS ($P < 0.05$) or LPS + IFNγ ($P < 0.01$) at 8 weeks of age ($F_{3,44} = 88.36$, $P < 0.01$). In the LFP line, NO production only increased by addition of LPS + IFNγ ($P < 0.01$) compared to the control ($F_{3,33} = 11.16$, $P < 0.01$). At 10 weeks of age, NO production increased for both lines by addition of LPS or LPS + IFNγ ($P < 0.01$), but not by addition of IFNγ compared to the control (HFP: $F_{3,55} = 105.53$, $P < 0.01$ and LFP: $F_{3,51} = 21.02$, $P < 0.01$). These results suggest that divergent selection on FP affects monocyte activity, as we found no differences in the relative abundance

of CD40+ MHC II+ cells, which includes monocytes, with the HFP line having higher monocyte activity compared to the LFP line.

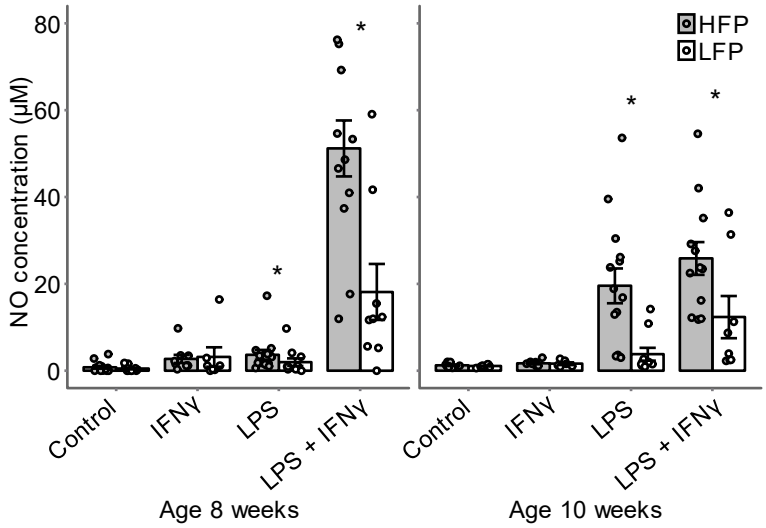

Figure 2. Mean nitric oxide (NO) concentration (± SE) by blood derived monocytes after stimulation with medium (Control), interferon gamma (IFNγ), lipopolysaccharides (LPS) or LPS + IFNγ for immunized high (HFP, n=9-15) and low feather pecking (LFP, n=7-14) birds at 8 and 10 weeks of age. * indicates significant differences ($P < 0.05$) between lines.

Divergent selection on feather pecking affects IgM and IgG specific antibody titers

We measured specific antibody (SpAb) titers to human serum albumin (HuSA) in the FP selection lines to identify differences in the specific humoral immune response as part of the adaptive immune system. No significant line * age interactions were found on IgM or IgG SpAb titers to HuSA. When identifying differences between lines for ages separately, we found that the HFP line tended to have higher IgM ($F_{1,8} = 5.14$, $P < 0.1$) (Figure 3A) and IgG SpAb titers ($F_{1,8} = 3.50$, $P < 0.1$) (Figure 3B) compared to the LFP line at day 14 post immunization. These findings suggest that divergent selection on FP affects the specific immune response, where the HFP line seems to have a higher specific immune response compared to the LFP line.

Divergent selection on feather pecking affects IgG natural (auto)antibody titers, but not IgM natural (auto)antibody titers

We measured natural (auto)antibody (N(A)Ab) titers in the FP selection lines as they play an essential role in both innate and adaptive immunity (Ochsenbein and

Zinkernagel, 2000; Panda and Ding, 2015), with NAb binding to non-self-antigen and NAAb binding to self-antigen.

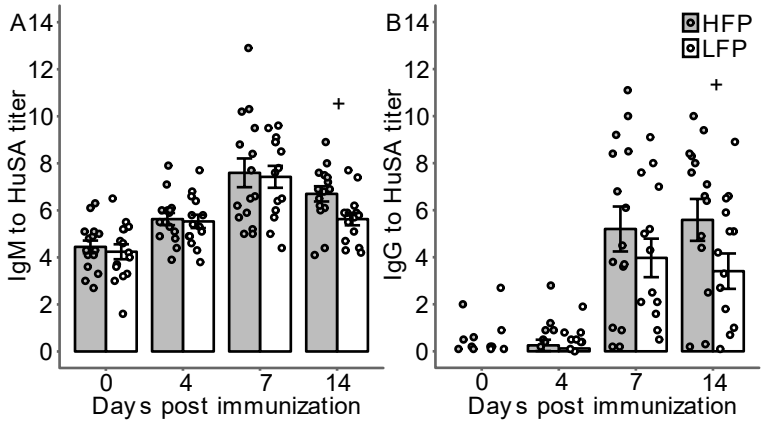

Figure 3. A) Mean specific antibody titers of IgM (± SE) and B) IgG to human serum albumin (HuSA) of immunized high (HFP, n=15) and low feather pecking (LFP, n=14-15) birds at day 0 (prior to immunization), 4, 7 and 14 post immunization. + indicates tendencies ($P < 0.1$) between lines.

Significant line * age interactions were seen on IgM and IgG NAb titers to keyhole limpet hemocyanin (KLH) (IgM: $F_{5,498} = 10.78$, $P < 0.01$; IgG: $F_{5,497} = 7.94$, $P < 0.01$; Figures 4A and B). Overall, no differences between lines were found for IgM to KLH, but the HFP line did have higher IgG to KLH ($P < 0.01$) compared to the LFP line.

Significant line * age interactions were also seen on IgM and IgG NAAb titers to phosphorylcholine conjugated to bovine serum albumin (PC-BSA)(IgM: $F_{5,495} = 5.62$, $P < 0.01$; IgG: $F_{5,494} = 3.37$, $P < 0.01$; Figures 4C and D) and to Herring DNA (IgM: $F_{5,495} = 5.35$, $P < 0.01$; IgG: $F_{5,495} = 3.02$, $P < 0.05$; Figures 4E and F). Overall, the HFP line tended to have lower IgM to PC-BSA and Herring DNA ($P < 0.1$), but had higher IgG to PC-BSA and Herring DNA ($P < 0.01$) compared to the LFP line. For specific comparisons of IgM and IgG N(A)Ab titers between lines per age see Figure 4.

These results suggest that divergent selection on FP affects N(A)Ab titers of the IgG isotype, where the HFP line had higher IgG N(A)Ab titers compared to the LFP line with even almost four fold higher titers at adult ages. Yet, divergent selection on FP does not seem to affect N(A)Ab titers of the IgM isotype, although the HFP line had lower IgM N(A)Ab titers compared to the LFP line at specific ages.

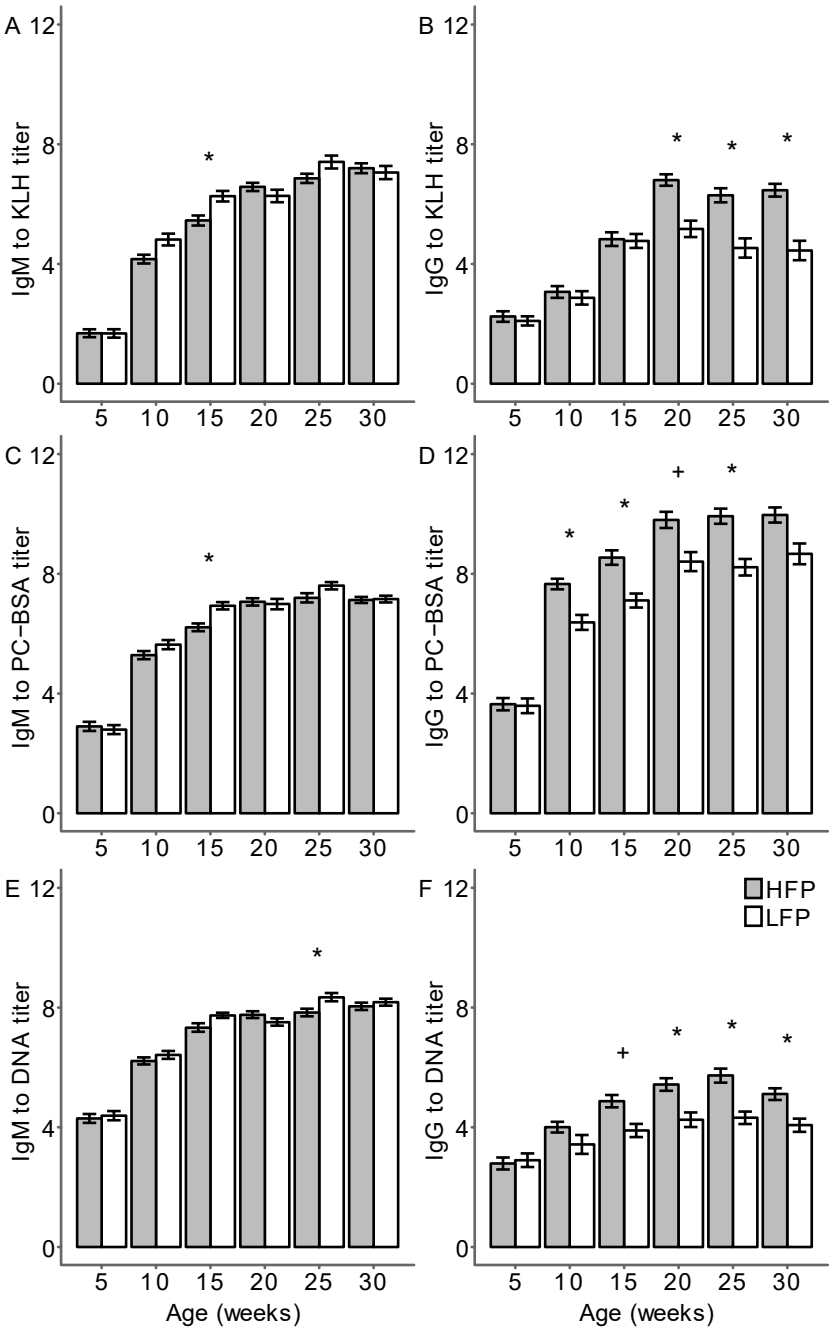

Figure 4. Mean natural (auto)antibody titers (± SE) of IgM (A, C and E) and IgG (B, D and F) to keyhole limpet hemocyanin (KLH) (A and B); phosphorylcholine conjugated to bovine serum albumin (PC-BSA) (C and D) and Herring DNA (E and F) of the high (HFP) and low feather pecking (LFP) lines at 5, 10, 15, 20, 25 and 30 weeks of age. + indicates tendencies ($P < 0.1$) and * indicates significant differences ($P < 0.05$) between lines.

Discussion

To further identify relationships between the immune system and feather pecking (FP) we characterized lines divergently selected on FP with regard to immune characteristics. We identified nitric oxide (NO) production by monocytes, specific antibody (SpAb) titers, natural (auto)antibody (N(A)Ab) titers and immune cell subsets of the high FP (HFP) and low FP (LFP) lines. NO production by monocytes was measured as indicator for innate pro-inflammatory immune functioning, SpAb titers were measured as part of the adaptive immune system and N(A)Ab titers were measured as they play an essential role in both innate and adaptive immunity. We further included immune cell subsets to identify whether differences in immune characteristics were reflected in the relative abundance of immune cell subsets.

We hypothesized that the HFP line would have a more responsive immune system (i.e. higher NO production, SpAb and IgG N(A)Ab titers) compared to the LFP line.

Divergent selection on FP affected FP behaviour, where the HFP line showed more FP compared to the LFP line. Divergent selection on FP further affected NO production by monocytes, SpAb and N(A)Ab titers, but not the relative abundance of immune cell subsets. The HFP line had higher NO production by blood derived monocytes compared to the LFP line. We assumed NO was mainly produced by viable monocytes, as the majority of the cell population would consist of monocytes since previous studies showed that the number of viable thrombocytes decreases rapidly within the first 24 h and decreases even further in the next 48 h (DaMatta et al., 1999; He et al., 2008, 2006). Furthermore, the HFP line tended to have higher IgM and IgG SpAb titers, lower IgM NAAb titers and had higher IgG N(A)Ab titers compared to the LFP line. Previously, it was suggested that IgG NAb are dependent upon exogenous antigen stimulation, while IgM NAb are not (Holodick et al., 2017). This indicates that differences in IgM NAb titers might be explained by genetic alterations, while differences in IgG NAb titers might be explained by a difference in environmental influences or immune responsiveness to environmental influences. As both lines were exposed to similar environmental conditions, we suggest that the HFP line had an increased immune responsiveness to environmental influences compared to the LFP line. This is further supported by the higher SpAb titers in the HFP line. Overall, these findings suggest that the HFP line had a more responsive immune system (both innate and adaptive immune system) compared to the LFP line.

Our findings are supported by previous studies in the FP selection lines, where the HFP line had lower IgM NAb, but higher IgG NAb and SpAb compared to the LFP line (Buitenhuis et al., 2006; **chapter 3**), suggesting a more responsive immune

system in the HFP line. Yet, Buitenhuis et al. (2006) did find differences in immune cell subsets, where the HFP line had a lower relative abundance of T helper cells (CD4) and double positive T cells (CD4+CD8+) compared to the LFP line. Further support for our findings comes from previous studies in chickens, where a genetic correlation was found between FP and SpAb response (Buitenhuis et al., 2004) and high NAb titers were suggested to be related to the propensity to perform FP (Sun et al., 2014, 2013). Thus, overall high FP seems to be related to increased immune responsiveness.

It should be noted that we did not include the unselected control line in the present study. Therefore we cannot compare the effects of selection for or against FP with the effects of no selection on FP. Previously, we found that the LFP line had the lowest IgG NAb titers, the HFP line had intermediate IgG NAb titers and the unselected control line had the highest IgG NAb titers (**chapter 3**). This suggests that selection against FP reduces immune responsiveness compared to no selection, rather than that selection for FP increases immune responsiveness compared to no selection. Yet, the HFP line also had higher SpAb responses compared to the unselected control and LFP lines one week post vaccination (Buitenhuis et al., 2006). Overall, our and previous findings suggest that divergent selection on FP affects the responsiveness of the innate and adaptive immune system.

Still, these findings in the FP selection lines could be caused by differences in behaviour, differences in immune system functioning or factors simultaneously affecting behaviour and immune characteristics, such as genetics, gut microbiota, serotonin or stress. We will now briefly discuss each of these options.

<u>Differences in behaviour lead to differences in immune characteristics</u>

Chickens have a high motivation to explore and forage (Cooper and Albentosa, 2003; Rodenburg et al., 2013) and FP is considered to be a redirected foraging behaviour (Blokhuis, 1986; Dixon et al., 2008). Chickens with a higher motivation to explore and forage are more likely to be exposed to pathogens, as they explore more of their environment, food sources and interact more with conspecifics (Barber and Dingemanse, 2010; Kortet et al., 2010).

Previously, HFP birds showed more explorative pecking (de Haas et al., 2010), pecked more in an operant conditioning test to obtain mealworms (Hausler and Harlander-Matauschek, 2008) and showed a higher number of responses in a Skinner box to gain access to feathers and wood shavings compared to LFP birds (Harlander-Matauschek et al., 2006). These findings indicate a higher motivation to explore and

forage in the HFP line which could lead to increased immune responsiveness. Indeed, more explorative birds invested more in innate immune function, such as complement activity and NAbs (Zylberberg et al., 2014). However, the opposite has also been found, where more explorative birds had lower NAb (Jacques-Hamilton et al., 2017). High locomotor activity can further lead to increased exposure to pathogens, for example more active fish had a higher parasite load compared to non-active fish (Poulin et al., 1991). HFP birds showed higher locomotor activity (Kjaer, 2009) and more active responses to various behavioural tests (Kops et al., 2017; **chapter 2**). Thus, the differences in immune responsiveness between the FP selection lines could be caused by increased locomotor activity, exploration and foraging, which leads to increased exposure to pathogens, thereby potentially altering immune system functioning.

Differences in immune responsiveness lead to differences in behavioural characteristics

Having a more responsive immune system might make HFP birds more at risk of developing FP compared to LFP birds. The increased immune responsiveness might result in HFP birds responding stronger to immune challenges or vaccinations with the synthesis and release of pro-inflammatory cytokines. Increased monocyte activity in the HFP line, as indicated by higher NO production, suggests increased production of pro-inflammatory cytokines (Dale et al., 2008; Tizard, 2009). Although we measured NO production *ex vivo*, our *in vitro* findings suggest higher potential of macrophages (i.e. differentiated monocytes) to produce NO and pro-inflammatory cytokines in the HFP line. HFP birds had higher NAAb, indicating increased cell damage (Lutz et al., 2009) potentially caused by higher NO production and pro-inflammatory activity of macrophages (Tamir et al., 1996). HFP birds also had higher IgG N(A)Ab and SpAb, suggesting increased inflammation as IgG has pro-inflammatory activity (Aschermann et al., 2010) and this increased inflammation might be caused by pro-inflammatory cytokines (Wigley and Kaiser, 2003). Furthermore, nuclear-factor-kappa-B (NFKB) and chemokine (CCL4) genes were suggested to be related to the propensity to perform FP (Biscarini et al., 2010). Interestingly, NFKB can induce transcription of inducible nitric oxide synthase (iNOS), the enzyme responsible for NO production (MacMicking et al., 1997), and plays a role in pro-inflammatory cytokine production and release (Li and Verma, 2002). CCL4 (also known as macrophage inflammatory protein-1β (Petrenko et al., 1995)) has been suggested as marker for macrophages that produce pro-

inflammatory cytokines (Mantovani et al., 2004). These findings point to a potential relation between pro-inflammatory cytokines and FP.

Peripherally produced pro-inflammatory cytokines can act on the brain via various routes (Dantzer et al., 2008) where they reduce serotonergic and dopaminergic neurotransmission (Miller et al., 2013). A deficient serotonergic system was suggested to predispose chickens to develop FP (de Haas and van der Eijk, 2018) and young HFP birds had lower central serotonergic and dopaminergic activity compared to young LFP birds (Kops et al., 2017). Pro-inflammatory cytokines can further alter hypothalamus-pituitary-adrenal axis sensitization, thereby increasing susceptibility to stressors (Dantzer et al., 1999; Schmidt et al., 2003). Stress sensitivity has been suggested to play a role in the development of FP (Rodenburg et al., 2013) and the HFP line had higher stress sensitivity compared to the LFP line (Kjaer and Guémené, 2009; Kjaer and Jørgensen, 2011). Thus, increased immune responsiveness might result in the development of FP via the production of pro-inflammatory cytokines which act on the brain and alter neurotransmission. However, we did not measure pro-inflammatory cytokine levels in this study, as detection of avian cytokines is still limited by the lack of specific antibodies and reliable tests for cytokine production (Kaiser and Stäheli, 2014; Wigley and Kaiser, 2003).

A first indication for a role of the immune system in FP was found, where activation of the specific immune response at a young age increased feather damage at adult age, suggesting that stimulation of the specific immune response predisposes chickens for FP (Parmentier et al., 2009). Whether differences in immune responsiveness could be causal to FP or are a consequence of increased locomotor activity, foraging or exploration remains to be elucidated.

Factors simultaneously affecting behavioural and immune characteristics, such as genetics, gut microbiota, serotonin and stress

The differences seen in immune responsiveness between the FP selection lines could be caused by genes simultaneously involved in FP and the immune system, as indicated by previous studies (Biscarini et al., 2010; Buitenhuis et al., 2006, 2003; Hughes and Buitenhuis, 2010). Apart from the findings in the FP selection lines, other genetic associations have been found between the immune system and feather damage or FP (Biscarini et al., 2010; Buitenhuis et al., 2004; Sun et al., 2014). Furthermore, divergent selection on NAb resulted in more feather damage in the high NAb line compared to the low NAb line (Ba et al., 2017), suggesting selection for high NAb results in more FP. A chicken line with less feather damage showed

downregulation of genes related to immune system processes in the cerebrum compared to a chicken line with more feather damage (Habig et al., 2014, 2012), suggesting low FP is related to downregulation of immune functioning. Feather peckers showed upregulation or downregulation of hypothalamic gene expression involved in immunomodulation compared to neutrals (Brunberg et al., 2011). These studies provide evidence for a genetic link between the immune system and FP.

Another factor that has been shown to influence both behaviour and the immune system is the gut microbiota (Collins et al., 2012; Cryan and Dinan, 2012; Sommer and Bäckhed, 2013). Interestingly, the FP selection lines were shown to differ in microbiota composition determined from caecal droppings and luminal gut microbiota composition, where the HFP line had a higher relative abundance of Clostridiales but lower relative abundance of *Lactobacillus* compared to the LFP line (Birkl et al., 2018; **chapter 5**). Administration of *Lactobacillus* species altered SpAb responses (Brisbin et al., 2011) and *Lactobacillus* species were found to alter NO production by macrophages *in vitro* (Brisbin et al., 2015). However, we did not identify microbiota composition in the present study and it should be noted that the relative abundance of *Lactobacillus* was not determined at the species level, but at genus level in previous studies. Yet, the orders of Clostridiales and Lactobacillales were associated with the development of NAb repertoire in mice (Bello-Gil et al., 2019), suggesting these orders may regulate production of NAb. Whether the line differences in FP and immune responsiveness are due to differences in microbiota composition remains to be determined.

The serotonergic system, which is involved in regulating many types of behaviour, and the hypothalamic-pituitary-adrenal axis, which is the major stress regulatory system, have been indicated to be involved in the development of FP (de Haas and van der Eijk, 2018; Rodenburg et al., 2013) and can further influence the immune system. For example, serotonin and glucocorticoid receptors are present on various immune cells, such as lymphocytes, monocytes, macrophages and dendritic cells (Padgett and Glaser, 2003; Wu et al., 2019b). Furthermore, serotonin seems to play a role in functioning of monocytes and macrophages, gut inflammation and autoimmunity (Wu et al., 2019b) and it is well established that stress can alter immune function as mediated by glucocorticoids (i.e. corticosterone) (Padgett and Glaser, 2003). However, stress-induced corticosterone levels and whole blood serotonin levels did not differ between the FP selection lines in the present study (data not shown). Therefore, these systems might not explain the line differences in immune responsiveness seen in the present study.

Conclusion

In conclusion, divergent selection on FP affects nitric oxide production by blood derived monocytes, specific antibody titers and natural (auto)antibody titers, but not the relative abundance of immune cell subsets. Thus, divergent selection on FP affects different arms of the immune system, where the HFP line had a more responsive immune system (i.e. higher nitric oxide production, higher IgM and IgG specific antibody titers and higher IgG natural (auto)antibody titers) compared to the LFP line. Although causation cannot be established in the present study, it provides further evidence that relationships between the immune system and FP exist either through behavioural differences, immunological differences, genetics, gut microbiota, serotonin or stress. Based on our and previous findings in the FP selection lines, genetics and immunological differences seem to be the most likely explanations for relationships found. Thus, it is important to take these relationships into account when selecting on certain behavioural or immunological traits.

Acknowledgements

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Supplementary to Chapter 4

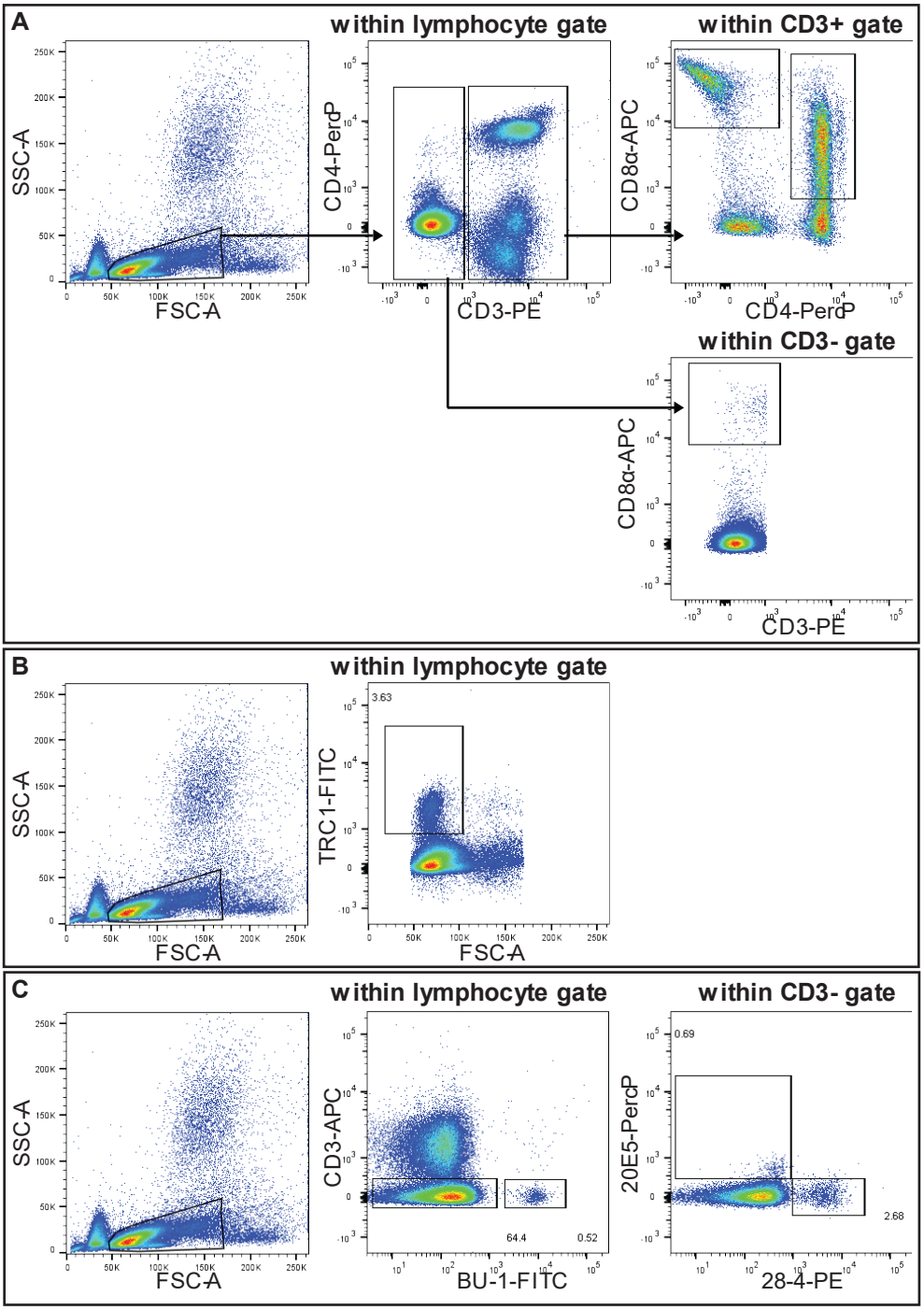
Table 1. Overview of primary and secondary antibody mixes used for flow cytometry.

	Primary antibody mix	Secondary antibody mix
Mix 1	mouse-anti-chicken β2M FITC (clone F21-F21, IgG1) mouse-anti-chicken CD3-PE (clone CT3; IgG1) mouse-anti-chicken CD4-biotin (clone CT4, IgG1) mouse-anti-chicken CD8α-APC (CT8, IgG1)	streptavidin-Percp
Mix 2	mouse-anti-chicken TCR1-FITC (TCRγδ, IgG1) mouse-anti-chicken MHC II-PE (CIa, IgM) mouse-anti-chicken CD40 (AV79, IgG2a) UNL	goat-anti-mouse IgG2a-APC
Mix 3	mouse-anti-chicken-Bu-1-FITC (AV20, IgG1) mouse-anti-chicken CD3 APC (CT3, IgG1) mouse-anti-chicken NK markers 28-4 (IgG3) UNL 20E5-biotin (IgG1)	streptavidin-Percp goat-anti-mouse IgG3-PE
Mix 4		streptavidin-Percp goat-anti-mouse IgG2a-APC goat-anti-mouse IgG3-PE

Table 2. Relative immune cell abundance and expression (mean fluorescence intensity: MFI) for the high feather pecking (HFP) and low feather pecking (LFP) lines.

Variables	HFP (n = 15)	LFP (n = 13-15)	P-value
% Lymphocytes (T, B & NK cells)	82.60 ± 1.75	72.17 ± 4.83	ns
% CD3- (B and NK cells)	63.09 ± 1.64	60.67 ± 4.22	ns
% CD3- CD8α+ (NK cells)	0.52 ± 0.07	0.54 ± 0.22	ns
% 20E5+ NK cells	0.25 ± 0.03	1.23 ± 0.80	ns
% 28-4+ NK cells	2.45 ± 0.31	3.57 ± 1.61	ns
% CD40+ MHC II+	3.33 ± 0.45	3.84 ± 0.73	ns
% large CD40+ MHC II+	0.83 ± 0.07	0.93 ± 0.12	ns
% small CD40+ MHC II+	1.93 ± 0.31	3.27 ± 0.85	ns
% γδ T cells	4.95 ± 0.46	4.77 ± 0.64	ns
% CD3+ (Total T cells)	36.52 ± 1.63	38.98 ± 4.19	ns
% CD4 (T Helper cells)	31.21 ± 2.26	35.51 ± 1.84	ns
% CD4+ CD8α+	1.19 ± 0.14	1.10 ± 0.12	ns
% CD4+ CD8α-	30.51 ± 2.20	35.08 ± 1.69	ns
% CD8α+ (Cytotoxic T cells)	23.20 ± 1.19	21.75 ± 0.70	ns
% BU-1+ (B cells)	1.62 ± 0.35	1.77 ± 0.49	ns
MFI CD4	6706.07 ± 114.82	6635.08 ± 167.53	ns
MFI CD8α	53770.87 ± 1842.91	54123.54 ± 1268.37	ns
MFI CD4+ CD8α+	5822.93 ± 93.32	5739.54 ± 159.10	ns
MFI γδ	608.27 ± 21.37	660.00 ± 62.37	ns
MFI MHC I on CD4	1334.60 ± 90.89	1838.62 ± 158.87	< 0.05
MFI MHC I on CD8α	1479.93 ± 94.22	1691.92 ± 125.67	ns
MFI MHC I on CD4+ CD8α+	1714.40 ± 106.93	2182.15 ± 171.57	< 0.05

NK: Natural killer, MHC: Major histocompatibility complex


A
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within CD3+ gate
within CD3- gate
SSC-A
FSC-A
CD4-PerCP
CD3-PE
CD8α-APC
CD4-PerCP
CD8α-APC
CD3-PE
B
within lymphocyte gate
SSC-A
FSC-A
TRC1-FITC
3.63
FSC-A
C
within lymphocyte gate
within CD3- gate
SSC-A
FSC-A
CD3-APC
64.4
0.52
BU-1-FITC
20E5-PerCP
0.69
2.68
28-4-PE

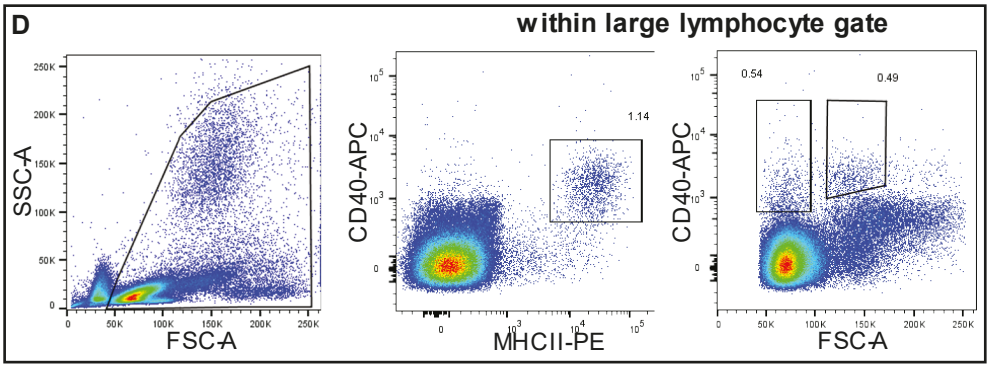
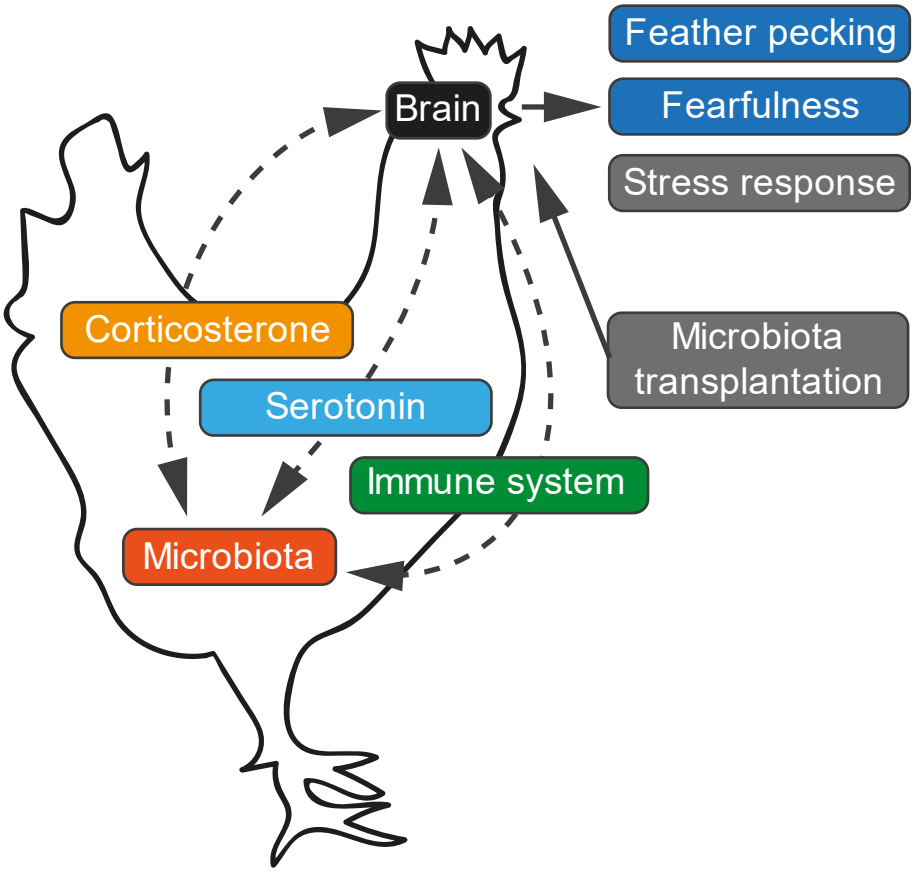


Figure 1. Gating strategy to identify different subsets of immune cells in blood. Lymphocytes were gated based on forward and side scatter. A) Lymphocytes were divided into CD3 negative non T cells (CD3-) and CD3 positive T cells (CD3+). Within the non T cell population CD8α+ cells were selected. CD3 positive T cells were further separated into CD8α+ T cells, CD4+CD8α- T cells and CD4+CD8α+ T cells. B) Within the lymphocyte population, cells expressing TCR1, which is a marker expressed on γδ T cells, were selected. C) Within the lymphocyte population BU-1+ B cells were selected. Furthermore, within the non B and T cell population 20E5+ and 28-4+ cells were selected which represent different subsets of chicken natural killer (NK) cells. D) Within the large lymphocyte gate, which includes multiple cell types, such as lymphocytes, monocytes, thrombocytes, and heterophils, MHCII+CD40+ cells were selected which represent a population of antigen presenting cells (APC), large and small MHCII+CD40+ were selected (all plots are from a high feather pecking individual).

Chapter 5

Differences in gut microbiota composition of laying hen lines divergently selected on feather pecking



Jerine A.J. van der Eijk, Hugo de Vries, Joergen B. Kjaer, Marc Naguib, Bas Kemp, Hauke Smidt, T. Bas Rodenburg, Aart Lammers

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Abstract

Feather pecking (FP), a damaging behaviour where laying hens peck and pull at feathers of conspecifics, is multifactorial and has been linked to numerous behavioural and physiological characteristics. The gut microbiota has been shown to influence host behaviour and physiology in many species, and could therefore affect the development of damaging behaviours, such as FP. Yet, it is unknown whether FP genotypes (high FP (HFP) and low FP (LFP) lines) or FP phenotypes (i.e. individuals differing in FP, feather peckers and neutrals) differ in their gut microbiota composition. Therefore, we identified mucosa-associated microbiota composition of the ileum and caecum at 10 and 30 weeks of age. At 30 weeks of age, we further identified luminal microbiota composition from combined content of the ileum, caeca and colon. FP phenotypes could not be distinguished from each other in mucosa-associated or luminal microbiota composition. However, HFP neutrals were characterized by a higher relative abundance of genera of Clostridiales, but lower relative abundance of *Lactobacillus* for the luminal microbiota composition compared to LFP phenotypes. Furthermore, HFP neutrals had a higher diversity and evenness for the luminal microbiota compared to LFP phenotypes. FP genotypes could not be distinguished from each other in mucosa-associated microbiota composition. Yet, FP genotypes could be distinguished from each other in luminal microbiota composition. HFP birds were characterized by a higher relative abundance of genera of Clostridiales, but lower relative abundance of *Staphylococcus* and *Lactobacillus* compared to LFP birds. Furthermore, HFP birds had a higher diversity and evenness for both caecal mucosa-associated and luminal microbiota compared to LFP birds at adult age. In conclusion, we here show that divergent selection on FP can (in)directly affect luminal microbiota composition. Whether differences in microbiota composition are causal to FP or a consequence of FP remains to be elucidated.

Introduction

The gut microbiota (i.e. the microorganisms in the gut) has been shown to influence both host behaviour and physiology in numerous ways, where many studies compared conventional and germ-free (microbiota-deficient) rodents (Cryan and Dinan, 2012; Sommer and Bäckhed, 2013). Infection with enteric pathogens or altering microbiota composition, via for example anti-, pre- or probiotic treatment, has been shown to affect anxiety, activity, stress sensitivity, and the serotonergic, dopaminergic and immune systems in rodents (Ait-Belgnaoui et al., 2014; Bercik et al., 2011a, 2010; Bravo et al., 2011; Desbonnet et al., 2010; Esmaili et al., 2009; Goehler et al., 2008). The gut microbiota might have similar effects in birds. Germ-free quails showed reduced fearfulness compared to colonized quails (Kraimi et al., 2018). Probiotic treatment reduced fearfulness and improved memory in quails (Parois et al., 2017), reduced plasma serotonin levels in dominant hens, but not in subordinate hens (Hu et al., 2018), and enhanced serum and intestinal natural antibody titers (Haghighi et al., 2006). However, probiotic treatment did not affect corticosterone levels in laying hens (Lei et al., 2013), but broilers infected with *Clostridium perfringens* showed increased corticosterone levels compared to non-infected birds (Calefi et al., 2016). These findings suggest that gut microbiota influences behaviour and physiology in poultry and could therefore influence an animal's ability to cope with environmental challenges, such as those encountered in animal husbandry.

Feather pecking (FP) is a damaging behaviour in laying hens where they peck and pull at feathers of conspecifics. Feather pecking is multifactorial and has been linked to numerous behavioural characteristics, such as activity, fearfulness, stress sensitivity, but also to physiological characteristics, such as the serotonergic, dopaminergic and immune systems (Rodenburg et al., 2013). Since similar behavioural and physiological systems affected by the gut microbiota have been linked to FP, the gut microbiota could affect the development of FP. For example, lines selected on high (HFP) and low FP (LFP) differ in stress response, locomotor activity, responses to various behavioural tests, central serotonergic and dopaminergic activity, immune competence, immune reactivity, natural antibody titers and peripheral serotonin levels (Buitenhuis et al., 2006; Kjaer, 2009; Kjaer and Guémené, 2009; Kops et al., 2017; **chapter 2, 3** and **4**). Moreover, HFP and LFP birds differ in intestinal microbial metabolites and in microbiota composition determined from caecal droppings (Birkl et al., 2018; Meyer et al., 2013). Yet, it is unknown whether the FP selection lines (i.e. FP genotypes) differ in microbiota composition of specific gut sections. Furthermore, individuals within a line can

become feather peckers, feather pecker-victims, victims or neutrals (i.e. FP phenotypes), but it is unknown whether FP phenotypes differ in microbiota composition of specific gut sections. Identifying microbiota composition from different gut sections (i.e. ileum, caecum or colon) is crucial as microbiota composition of faecal samples is variable because faeces can originate from different gut sections (Sekelja et al., 2012), which differ in microbiota composition (Awad et al., 2016; Lu et al., 2003). It is further important to identify microbiota composition from different locations within the gut sections (i.e. luminal and mucosa-associated microbial communities) as mucosa-associated microbiota composition has been shown to differ from luminal microbiota composition (Awad et al., 2016; Olsen et al., 2008). Furthermore, communication between the gut microbiota and the host occurs via several pathways (Collins et al., 2012; Cryan and Dinan, 2012), and the intestinal epithelium plays an important role in these communication pathways as it is the primary interface for host – microbiota crosstalk (Artis, 2008). Therefore, mucosa-associated microbiota might have more influence on the host compared to luminal microbiota as it is in closer proximity to the host (Ouwerkerk et al., 2013).

The aim of this study was to identify differences in gut microbiota composition in relation to FP genotype (HFP and LFP) and FP phenotype (feather peckers and neutrals). We focused on feather peckers and neutrals to identify factors related to the performance of FP and to neither performing nor receiving FP. We identified mucosa-associated microbiota composition from intestinal scrapings of the ileum and caecum and luminal microbiota composition from combined content of the ileum, caeca and colon. Based on findings from Birkl et al. (2018) we hypothesized that FP genotypes would differ in gut microbiota composition with regard to the relative abundance of genera of Clostridiales and of *Lactobacillus*. We further hypothesized that FP phenotypes would differ with regard to enteric pathogens and the abundance of beneficial commensal bacteria as these bacteria can alter fearfulness, serotonergic activity and stress sensitivity and might therefore affect the development of FP. We further determined whether behaviour (fearfulness) and physiology (serotonin, corticosterone and natural antibodies) were related to gut microbiota composition. We focused on these variables as studies show that they can be affected by gut microbiota and have been linked to FP (de Haas and van der Eijk, 2018; Rodenburg et al., 2013; Sun et al., 2014).

Materials and Methods

Animals and Housing

White Leghorn birds from the 18th generation of lines selected on high (HFP) and low feather pecking (LFP) were used (see Kjaer et al., 2001 for selection procedure). The HFP and LFP lines were divergently selected on feather pecking (FP) for seven generations and were maintained in subsequent generations. The parent stock was between 38 and 43 weeks of age at the time of egg collection. A total of 304 chicks were collected from two batches of eggs that were incubated at an average egg shell temperature of 37.3 ºC and average relative humidity of 55.6 %. The two batches had the same housing conditions and experimental set-up with four pens per line, but with two weeks between batches (see **chapter 2** for more details). The experiment was approved by the Central Authority for Scientific Procedures on Animals according to Dutch law (no: AVD104002015150).

Feather Pecking Observations

Severe FP was observed at an individual level at eight to nine and 28 to 29 weeks of age and was defined as follows: "A bird grips and pulls or tears vigorously at a feather of another bird with her beak, causing the feather to lift up, break or be pulled out. The recipient reacts to the peck by vocalizing, moving away or turning towards the pecking bird. " (derived from Newberry et al., 2007). Severe FP was observed from video recordings, and each observation lasted 15 min, either in the morning (10:40 h-10:55 h) or in the afternoon (14:40 h-14:55 h). The number of severe feather pecks, either given or received, was summed over two subsequent weeks, thus including one morning and one afternoon observation with a total observation period of 30 min, and was used to identify FP phenotypes (adapted from Daigle et al., 2015). When a bird gave more than one and received zero or one severe feather peck it was defined as a feather pecker. When a bird gave and received zero or one severe feather peck it was defined as a neutral. We did not include victims or feather pecker-victims in this study.

Tonic Immobility Test

At 28 weeks of age, birds were individually subjected to a tonic immobility test for a maximum duration of 5 min. The tonic immobility test is considered a validated test for fearfulness in poultry (Forkman et al., 2007). Tonic immobility duration was recorded until the bird returned to upright position (see **chapter 2** for more details).

Blood Collection and Analyses

Blood was collected from all birds at 4, 9, 14, 19, 24 and 29 weeks of age. Blood was taken from the wing vein using a heparinized syringe. Blood samples were used for determination of plasma IgM and IgG natural antibody titers against keyhole limpet hemocyanin using an indirect ELISA (see **chapter 3** for more details). Blood samples taken at 24 weeks of age were used for determination of plasma corticosterone concentrations via a radioimmunoassay kit (MP Biomedicals, LLC, Orangeburg, state) as described previously (Buyse et al., 1987). Blood samples taken at 24 weeks of age were further used for determination of whole blood serotonin concentration (nmol/mL) via a fluorescence assay as described previously (Bolhuis et al., 2009).

Microbiota Sampling

At 10 and 30 weeks of age 20 birds per line (2 to 3 birds per pen) were sacrificed via cervical dislocation to collect gut microbiota from intestinal samples. We collected intestinal scrapings from a ± 2 cm midsection of the ileum (between Meckel's diverticulum and caeca) and from the caeca, as the ileum is mainly involved in nutrient absorption and immune modulation, while in the caeca microbial fermentation occurs (Moran, 1982; Svihus et al., 2013). We removed the gut content and scraped off the mucosa using sterile scalpel blades. Samples were stored in cryovials at -80 °C until further analysis. At 30 weeks of age we collected combined luminal content of the ileum, caeca and colon. Combined luminal content was further collected for transplantation purposes of our subsequent study. Five mL of sterile saline was added per g of gut content, and this was mixed for 10 s on a Vortex. Samples were centrifuged at low speed (58 x g) for 3 min to remove large particles and supernatant was collected in clean tubes. Sterile 85% glycerol was added to a final concentration of 15%. Samples were stored in cryovials at -80 °C until further analysis.

Microbiota Analysis

Total bacterial DNA was extracted from intestinal samples using a customized Maxwell 16 Total RNA protocol (Promega Corp., Madison, WI) with Stool Transport and Recovery Buffer (STAR; Roche Diagnostics Corp., Indianapolis, IN). Briefly, 100 mg of stool or digesta was homogenized with 0.25 g of sterilized 0.1 mm zirconia beads and three 2.5 mm glass beads in 300 µL STAR buffer for 3 x 1 min at 5.5 m s^{-1} using a bead beater (Precellys 24, Bertin Technologies, Montigny-le-Bretonneux, France), with a waiting step of 15 s in between. Samples were

incubated with shaking at 300 rpm for 15 min at 95°C and pelletized by 5 min centrifugation at 4°C and 16100 x g. Supernatant was collected and the pellets were processed again (from bead beating onwards) using 200 µL fresh STAR buffer. Samples were incubated at 95°C and centrifuged as before. Supernatant was collected, pooled with the first supernatant, and 250 µL of the combined supernatant was used for purification with Maxwell 16 Tissue LEV Total RNA Purification Kit, catalogue no.AS1220 (Promega Corp.) customized for DNA extraction in combination with the STAR buffer. DNA was eluted with 50 µL of DNAse- and RNAse-free water (Qiagen, Hilden, Germany). DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and adjusted to 20 ng μL^{-1} with DNAse- and RNAse-free water. PCR amplification was carried out with primers directed to the V5-V6 region of the bacterial 16S rRNA gene, namely BSF784F (5'-RGGATTAGATACCC) and 1064R (5'-CGACRRCCATGCANCACCT). PCR reactions were done in duplicate, each in a total volume of 50 µL and containing 20 ng of template DNA. Each sample was amplified with a uniquely barcoded primer pair (10 µM each per reaction), 1x HF buffer (Thermo Fisher Scientific, Waltham, MA), 1 µL dNTP Mix (10 mM each; Roche Diagnostics GmbH, Mannheim, Germany), 1 U Phusion Hot Start II High Fidelity DNA Polymerase (Thermo Fisher Scientific), and 36.5 µL of DNAse- and RNAse-free water. The amplification program included 30 s initial denaturation at 98°C, followed by 25 cycles (with the exception of ileal mucosal DNA samples which were processed with 30 cycles to yield sufficient amplicon fragments) of denaturation at 98°C for 10 s, annealing at 42°C for 10 s, elongation at 72°C for 10 s, and a final extension at 72°C for 7 min. PCR product presence and size (≈280 bp) was confirmed by gel electrophoresis using a 1% agarose gel. Seventy unique barcode tags were used in each library and two artificial (mock) communities were included in addition to a water (no template) control. PCR products were purified using the HighPrep PCR kit (MagBio Genomics Inc., Gaithersburg, MD), and DNA concentrations were measured using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific). A total of 100 ng of each barcoded PCR product was added to an amplicon pool that was subsequently concentrated using the HighPrep PCR kit to a volume of 20 µL. The DNA concentration of the amplicon pool was measured using the Qubit dsDNA BR Assay Kit and the libraries were sent for Illumina HiSeq sequencing (Eurofins Genomics, Ebersberg, Germany). Data was processed and analysed using NG-Tax, an in-house bioinformatics pipeline, as described by Ramiro-Garcia et al. (2016), which resulted in a minimum of 1945 reads and a maximum of 948018 reads per sample. Alpha diversity (i.e. richness, diversity and

evenness indices) was calculated with CANOCO 5 software package (Biometris, Wageningen, the Netherlands). Richness (S) was defined as the number of species in a sample. The Shannon diversity index (H) describes the diversity of the microbiota and was calculated using the formula below. Evenness estimates the similarity of species abundance (H / log(S)) (Finotello et al., 2018).

$$H = \sum_{i=1}^{S} - (P_i * \ln P_i)$$

P_i = fraction of the entire population made up of species i
$\sum$ = sum from species 1 to species S

Statistical Analysis

Multivariate analysis was applied for data interpretation. In order to relate changes in total microbial composition to explanatory variables, redundancy analysis (RDA) was used as implemented in the CANOCO 5 software package (Biometris). FP genotype (HFP and LFP) and the interaction between FP genotype and FP phenotype (feather pecker and neutral) were introduced as nominal variables (see Table 1 for sample sizes). The relative contribution of 198 genus-level phylogenetic groups identified by 16S rRNA gene sequencing were used as response variables. Average natural antibody IgG and IgM titers were introduced as quantitative variables at 10 (averages from 4 and 9 weeks of age) and 30 weeks of age (averages from 4 to 29 weeks of age). Tonic immobility duration (fearfulness), corticosterone and serotonin levels were included as quantitative variables for the analyses of 30 weeks of age. Analyses were performed for each age (10 or 30 weeks of age) and gut section (ileum, caecum or luminal content) separately. The Monte Carlo Permutation test (number of permutations 499) with forward selection was applied to test for significance of the effect of FP genotype, FP genotype * FP phenotype, tonic immobility, serotonin, corticosterone, and natural antibodies IgM and IgG on microbiota composition. Batch was included as covariate. P-values were corrected using Bonferroni correction. We performed Kruskal-Wallis tests in SAS Software version 9.3 (SAS Inst. Inc., Cary, NC) to identify differences between FP genotypes and FP genotype * FP phenotype in individual microbial groups, richness, diversity and evenness indices. Post hoc comparisons were made with the Dwass, Steel, Chritchlow-Fligner method.

Table 1. The number of birds sampled for gut microbiota (and total number of birds in study) per phenotype (feather pecker (P) or neutral (N)) within the high (HFP) and low feather pecking (LFP) lines for each gut section (ileum, caecum or luminal content) at 10 and 30 weeks of age.

Gut section	**Ileum**		**Caecum**		**Luminal content**	
Phenotype	**P**	**N**	**P**	**N**	**P**	**N**
Age (10 weeks)						
HFP	4 (19)	2 (72)	5 (19)	5 (72)	-	-
LFP	3 (5)	4 (92)	4 (5)	4 (92)	-	-
Age (30 weeks)						
HFP	6 (6)	10 (29)	6 (6)	10 (29)	5 (6)	10 (29)
LFP	4 (4)	10 (58)	4 (4)	10 (58)	4 (4)	10 (58)

Results

Microbiota Composition

Multivariate redundancy analysis showed that FP genotype * FP phenotype groups could not be distinguished from each other in mucosa-associated or luminal microbiota composition. It is interesting to note that LFP feather peckers contributed to explaining the observed variation in mucosa-associated microbiota composition of the ileum at 10 weeks of age ($P = 0.036$). Furthermore, LFP feather peckers and neutrals contributed to explaining the observed variation in luminal microbiota composition ($P = 0.040$ and $P = 0.036$, respectively). However, after Bonferroni correction these effects were no longer significant. FP genotypes could not be distinguished from each other in mucosa-associated microbiota composition. However, the RDA diagram for luminal microbiota composition showed that HFP and LFP birds could be distinguished from each other (Figure 1). Monte Carlo Permutation testing showed that the HFP and LFP lines significantly contributed to explaining the observed variation in microbiota composition ($P = 0.012$ and $P = 0.020$, respectively). The HFP line was correlated positively with the genera *Anaerotruncus*, *Butyricicoccus*, *Desulfovibrio*, *Eubacterium*, *Faecalibacterium*, *Sellimonas*, *Succinatimonas* and *Sutterella* as well as unclassified genus-level groups within families *Alcaligenaceae* and *Ruminococcaceae,* and negatively with *Lactobacillus*.

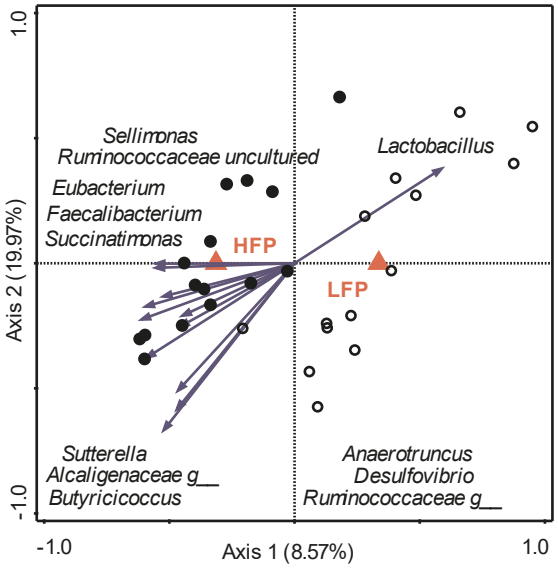

Figure 1. Triplot for partial RDA analysis of luminal microbiota composition (combined content of the ileum, caeca and colon) at 30 weeks of age. Nominal environmental variables high feather pecking (HFP) line and low feather pecking (LFP) line are represented by red triangles. Samples are grouped by line: HFP (black filled circles) and LFP (black open circles). Microbial groups contributing at least 20% to the horizontal explanatory axis are represented as vectors. The horizontal axis explains 8.57% of the total variance in the dataset. Groups that could not be assigned to a specific genus are classified by the family name appended with ";g_".

To further identify differences in mucosa-associated microbiota composition we determined whether FP genotypes or FP genotype * FP phenotype groups differed in relative abundance of microbial groups. At 10 weeks of age only one microbial group differed significantly between FP genotypes for the caecum and none for the ileum (for details see Table 2). At 30 weeks of age the relative abundance of one microbial group differed significantly between FP genotypes for the ileum and multiple microbial groups differed significantly for the caecum. For the luminal content (combined content of the ileum, caeca and colon) the relative abundance of multiple microbial groups differed significantly between FP genotypes (for details see Table 3). Of these the genera *Anaerotruncus, Butyricicoccus*, *Desulfovibrio, Eubacterium*, *Faecalibacterium*, *Lactobacillus*, *Ruminococcaceae, Sellimonas*, *Sutterella* and genus-level taxa assigned to families *Alcaligenaceae* and *Ruminococcaceae* were identified as microbial groups contributing at least 20% to the horizontal explanatory axis in the RDA analysis (see Figure 1). No differences were found between FP genotype * FP phenotype groups, except for one microbial group for the caecum and three microbial groups for the luminal content at 30 weeks of age (for details see Table 4).

Table 2. Average relative contribution (%) of genus-level groups different between the high feather pecking (HFP) and low feather pecking (LFP) lines in mucosa-associated microbiota of the ileum and caecum at 10 and 30 weeks of age.

	P-value		
	HFP vs. LFP	**HFP**	**LFP**
Age (10 weeks)			
Ileum		**n=6**	**n=7**
-	-	-	-
Caecum		**n=10**	**n=8**
Subdoligranulum	0.013 ↑	6.91 ± 1.42	2.50 ± 0.85
Age (30 weeks)			
Ileum		**n=16**	**n=14**
Aliidiomarina	0.049 ↑	0.15 ± 0.09	0.0 ± 0.0
Bacillaceae;g__	0.016 ↑	0.79 ± 0.17	0.32 ± 0.07
Caldalkalibacillus	0.013 ↑	2.21 ± 0.50	0.74 ± 0.15
Idiomarina	0.009 ↑	0.12 ± 0.06	0.01 ± 0.01
Nesterenkonia	0.018 ↑	3.81 ± 0.72	1.46 ± 0.25
Tetragenococcus	0.043 ↑	0.14 ± 0.05	0.02 ± 0.01
Trichococcus	0.049 ↑	0.14 ± 0.07	0.0 ± 0.0
Turicibacter	0.018 ↓	0.06 ± 0.03	0.44 ± 0.18
Caecum		**n=16**	**n=14**
Anaerotruncus	0.038 ↑	0.79 ± 0.08	0.53 ± 0.08
Butyricicoccus	0.020 ↑	2.31 ± 0.66	1.29 ± 0.51
Flavonifractor	0.023 ↑	1.88 ± 0.90	0.25 ± 0.07

P-values are from Kruskal-Wallis test. Values represent means ± SEM. ↑ and ↓ respectively indicate higher and lower relative abundance when comparing the HFP line to the LFP line. The genus-level groups with a relative abundance lower than 0.1% in both lines are not shown. Groups that could not be assigned to a specific genus are by classified by the family name appended with ";g_"

Table 3. Average relative contribution (%) of genus-level groups different between the high feather pecking (HFP) and low feather pecking (LFP) lines in luminal microbiota at 30 weeks of age.

	P-value		
	HFP vs. LFP	**HFP**	**LFP**
Luminal		**n=15**	**n=14**
Alcaligenaceae;g__	0.029 ↑	0.18 ± 0.07	0.01 ± 0.01
Anaerotruncus	0.005 ↑	0.37 ± 0.05	0.16 ± 0.04
Butyricicoccus	0.001 ↑	1.09 ± 0.19	0.40 ± 0.15
Clostridiales;g__	0.019 ↑	0.46 ± 0.10	0.14 ± 0.05
Deferribacteraceae;g__	0.033 ↑	0.34 ± 0.14	0.03 ± 0.02
Desulfovibrio	0.026 ↑	0.39 ± 0.08	0.17 ± 0.06
Erysipelatoclostridium	0.016 ↑	1.07 ± 0.20	0.42 ± 0.09
Eubacterium	0.010 ↑	0.17 ± 0.07	0.0 ± 0.0
Faecalibacterium	0.001 ↑	5.31 ± 0.76	2.21 ± 0.58
Fusicatenibacter	0.023 ↑	1.62 ± 0.25	0.88 ± 0.27
Lactobacillus	0.001 ↓	20.0 ± 3.09	41.84 ± 4.84
Olsenella	0.038 ↑	0.76 ± 0.23	0.29 ± 0.11
Peptococcus	0.026 ↑	0.44 ± 0.06	0.28 ± 0.08
Ruminiclostridium	0.015 ↑	1.19 ± 0.13	0.66 ± 0.13
Ruminococcaceae;g__	0.008 ↑	1.88 ± 039	0.64 ± 0.18
Sellimonas	0.005 ↑	0.20 ± 0.05	0.02 ± 0.02
Staphylococcus	0.028 ↓	0.30 ± 0.18	1.50 ± 0.92
Sutterella	0.010 ↑	0.89 ± 0.12	0.48 ± 0.08
Ruminococcaceae;g__ uncultured	0.003 ↑	2.26 ± 0.40	0.91 ± 0.15

P-values are from Kruskal-Wallis test. Values represent means ± SEM. ↑ and ↓ respectively indicate higher and lower relative abundance when comparing the HFP line to the LFP line. The genus-level groups with a relative abundance lower than 0.1% in both lines are not shown. Groups that could not be assigned to a specific genus are by classified by the family name appended with ";g_"

Table 4. Average relative contribution (%) of genus-level groups differing between feather peckers (P) and neutrals (N) of the high feather pecking (HFP) and low feather pecking (LFP) lines in mucosa-associated microbiota of the caecum and luminal microbiota at 30 weeks of age.

	P-value	HFP		LFP	
		P	N	P	N
Caecum		**n=6**	**n=10**	**n=4**	**n=10**
Anaerostipes	0.017	0.0 ± 0.0^{a}	0.11 ± 0.07^{ab}	0.47 ± 0.18^{b}	0.29 ± 0.25^{ab}
Luminal		**n=5**	**n=10**	**n=4**	**n=10**
Lactobacillus	0.003	28.33 ± 5.08^{ab}	15.87 ± 3.29^{a}	47.13 ± 3.33^{b}	39.72 ± 6.65^{b}
Butyricicoccus	0.006	0.84 ± 0.16^{ab}	1.22 ± 0.28^{a}	0.08 ± 0.08^{b}	0.53 ± 0.20^{ab}
Faecalibacterium	0.003	4.20 ± 1.00^{ab}	5.87 ± 1.01^{a}	1.02 ± 0.52^{b}	2.69 ± 0.74^{b}

P-values are from Kruskal-Wallis test. Values represent means ± SEM. The genus-level groups with a relative abundance lower than 0.1% in both lines and phenotypes are not shown. Means within a row lacking a common superscript differ significantly ($P < 0.05$).

None of the quantitative environmental variables included in the RDA contributed to explaining the observed variation in gut microbiota composition at 10 or 30 weeks of age, except for serotonin and natural antibody IgG. Serotonin level showed a tendency for contributing to explaining the observed variation in mucosa-associated microbiota composition of the ileum at 30 weeks of age ($P = 0.068$). *Akkermansia*, *Bifidobacterium*, *Blautia*, *Dorea*, *Enterobacter*, *Faecalibacterium*, *Granulicatella*, *Klebsiella*, *Eubacterium*, *Lactococcus*, *Micrococcus*, *Parabacteroides*, *Prevotella*, *Rhodococcus*, *Roseburia*, *Serratia*, *Veillonella* and *Victivallis* and unclassified genus-level taxa within the *Enterobacteriaceae* and *Porphyromonadaceae* were positively correlated and *Helicobacter* was negatively correlated with serotonin level (Figure 2). Natural antibody IgG titer showed a tendency for contributing to explaining the observed variation in mucosa-associated microbiota composition of the caecum at 30 weeks of age ($P = 0.066$). *Alistipes*, *Butyricicoccus*, *Desulfovibrio* and an unclassified genus-level group within the *Rikenellaceae* were positively correlated to natural antibody IgG titer (Figure 3).

Richness, Diversity and Evenness Indices

FP genotype effects were found on all indices, where the HFP line had higher diversity ($\chi^2 = 4.85$, df = 1, $P = 0.028$) and evenness ($\chi^2 = 3.98$, df = 1, $P = 0.046$) indices for the mucosa-associated microbiota of the caecum compared to the LFP line at 30 weeks of age (see Table 5). Furthermore, the HFP line had higher richness

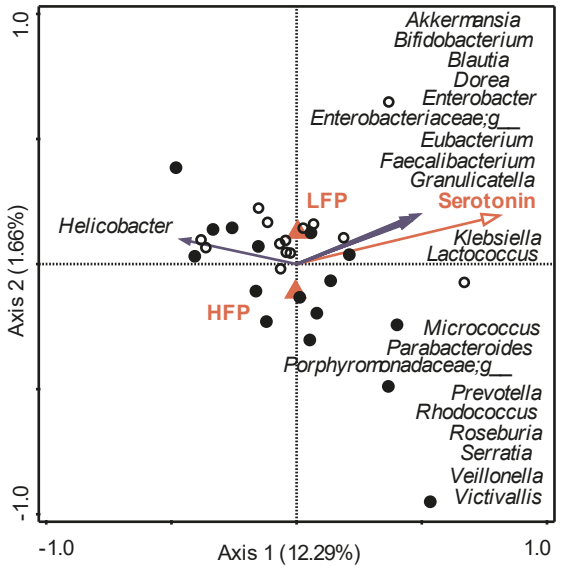

Figure 2. Triplot for RDA analysis of mucosa-associated microbiota composition of the ileum at 30 weeks of age. Nominal environmental variables high feather pecking (HFP) line and low feather pecking (LFP) line are represented by red triangles and quantitative environmental variable whole blood serotonin level is represented by the open red arrow. Samples are grouped by line: HFP (black filled circles) and LFP (black open circles). Microbial groups contributing at least 20% to the horizontal explanatory axis are represented as vectors (closed arrows). The horizontal axis explains 12.29% of the total variance in the dataset. Groups that could not be assigned to a specific genus are classified by the family name appended with ";g_".

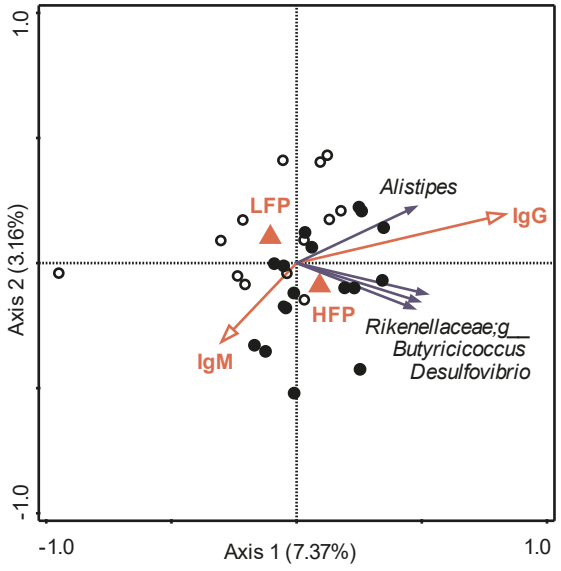

Figure 3. Triplot for RDA analysis of mucosa-associated microbiota composition of the caecum at 30 weeks of age. Nominal environmental variables high feather pecking (HFP) line and low feather pecking (LFP) line are represented by red triangles and quantitative environmental variables natural antibody (NAb) IgG and IgM titers are represented by open red arrows. Samples are grouped by line: HFP (black filled circles) and LFP (black open circles). Microbial groups contributing at least 20% to the horizontal explanatory axis are represented as vectors (closed arrows). The horizontal axis explains 7.37% of the total variance in the dataset. Groups that could not be assigned to a specific genus are classified by the family name appended with ";g_".

(χ^2 = 6.88, df = 1, P = 0.009), diversity (χ^2 = 10.43, df = 1, P = 0.001) and evenness (χ^2 = 10.43, df = 1, P = 0.001) indices for the luminal microbiota compared to the LFP line at 30 weeks of age. FP genotype * FP phenotype groups did not differ in richness, diversity or evenness indices, except for the diversity (χ^2 = 12.69, df = 3, P = 0.005) and evenness (χ^2 = 13.93, df = 3, P = 0.003) indices for the luminal microbiota (see Table 6). HFP neutrals had higher diversity and evenness indices compared to LFP feather peckers and neutrals (P < 0.05).

Table 5. Richness, diversity and evenness indices of mucosa-associated microbiota of the ileum and caecum, and luminal microbiota from the high feather pecking (HFP) and low feather pecking (LFP) lines at 10 and 30 weeks of age.

	HFP	**LFP**
Age (10 weeks)		
Ileum	**n=6**	**n=7**
Richness	31.8 ± 3.33	31.4 ± 4.92
Diversity	2.09 ± 0.19	2.10 ± 0.30
Evenness	0.60 ± 0.04	0.61 ± 0.08
Caecum	**n=10**	**n=8**
Richness	35.0 ± 1.83	36.5 ± 0.78
Diversity	2.73 ± 0.07	2.74 ± 0.06
Evenness	0.77 ± 0.01	0.76 ± 0.01
Age (30 weeks)		
Ileum	**n=16**	**n=14**
Richness	36.3 ± 3.26	37.7 ± 4.21
Diversity	1.93 ± 0.19	1.93 ± 0.19
Evenness	0.54 ± 0.05	0.53 ± 0.04
Caecum	**n=16**	**n=14**
Richness	59.9 ± 1.73	55.9 ± 2.68
Diversity	3.23 ± 0.09^a	3.02 ± 0.07^b
Evenness	0.79 ± 0.02^a	0.76 ± 0.02^b
Luminal	**n = 15**	**n = 14**
Richness	61.1 ± 1.84^a	50.8 ± 3.10^b
Diversity	3.22 ± 0.09^a	2.52 ± 0.18^b
Evenness	0.78 ± 0.02^a	0.64 ± 0.04^b

Values represent means ± SEM. Richness: the number of species in a sample (S). Diversity: the Shannon diversity index (H). Evenness: similarity of species abundance (H / log(S)). Means within a row lacking a common superscript differ significantly (P < 0.05).

Table 6. Richness, diversity and evenness indices of mucosa-associated microbiota of the ileum and caecum, and luminal microbiota from feather peckers (P) and neutrals (N) of the high feather pecking (HFP) and low feather pecking (LFP) lines at 10 and 30 weeks of age.

	HFP		LFP	
	P	**N**	**P**	**N**
Age (10 weeks)				
Ileum	**n=4**	**n=2**	**n=3**	**n=4**
Richness	31.3 ± 5.22	33.0 ± 1.00	37.3 ± 2.85	27.0 ± 8.09
Diversity	2.05 ± 0.29	2.16 ± 0.08	2.33 ± 0.13	1.93 ± 0.53
Evenness	0.60 ± 0.06	0.62 ± 0.02	0.64 ± 0.02	0.59 ± 0.14
Caecum	**n=5**	**n=5**	**n=4**	**n=4**
Richness	35.4 ± 1.69	34.6 ± 3.49	35.5 ± 1.32	37.5 ± 0.65
Diversity	2.72 ± 0.06	2.75 ± 0.13	2.71 ± 0.09	2.77 ± 0.07
Evenness	0.76 ± 0.01	0.78 ± 0.02	0.76 ± 0.02	0.77 ± 0.02
Age (30 weeks)				
Ileum	**n=6**	**n=10**	**n=4**	**n=10**
Richness	29.2 ± 5.22	40.5 ± 3.72	33.8 ± 7.81	39.3 ± 5.16
Diversity	1.62 ± 0.24	2.11 ± 0.25	1.65 ± 0.31	2.04 ± 0.24
Evenness	0.49 ± 0.07	0.57 ± 0.06	0.47 ± 0.07	0.56 ± 0.05
Caecum	**n=6**	**n=10**	**n=4**	**n=10**
Richness	58.8 ± 3.68	60.5 ± 1.84	56.5 ± 3.57	55.6 ± 3.57
Diversity	3.08 ± 0.21	3.32 ± 0.06	2.89 ± 0.10	3.07 ± 0.09
Evenness	0.75 ± 0.04	0.81 ± 0.01	0.72 ± 0.03	0.77 ± 0.02
Luminal	**n=5**	**n=10**	**n=4**	**n=10**
Richness	62.4 ± 3.37	60.5 ± 2.29	46.0 ± 4.22	52.7 ± 3.96
Diversity	3.08 ± 0.16^{ab}	3.29 ± 0.10^{a}	2.29 ± 0.18^{b}	2.61 ± 0.24^{b}
Evenness	0.75 ± 0.03^{ab}	0.80 ± 0.02^{a}	0.60 ± 0.03^{b}	0.66 ± 0.05^{b}

Values represent means ± SEM. Richness: the number of species in a sample (S). Diversity: the Shannon diversity index (H). Evenness: similarity of species abundance (H / log(S)). Means within a row lacking a common superscript differ significantly ($P < 0.05$).

Discussion

The aim of this study was to identify differences in gut microbiota composition in relation to feather pecking (FP) genotype and FP phenotype. We further determined whether or not behavioural or physiological parameters were related to gut microbiota composition. We identified mucosa-associated microbiota composition from the ileum and caecum of feather peckers and neutrals from the high FP (HFP)

and low FP (LFP) lines at young and adult age. We further identified luminal microbiota composition (combined content of the ileum, caeca and colon) at adult age.

The interaction between FP genotype and FP phenotype did not contribute to explaining the observed variation in mucosa-associated or luminal microbiota composition and we only found several differences in relative abundance of genus-level microbial groups. This suggests that FP phenotypes from the HFP and LFP lines did not differ in microbiota composition. Microbiota composition might thus not explain the behavioural and physiological differences previously found between feather peckers and neutrals (Daigle et al., 2015; Jensen et al., 2005; Kops et al., 2013) and between feather peckers and neutrals of the present study (**chapter 2** and **3**). Furthermore, HFP neutrals had higher diversity and evenness indices for the luminal microbiota compared to LFP phenotypes, suggesting that the microbiota in HFP neutrals was characterized by a more even distribution of relative abundances and higher diversity compared to LFP phenotypes. However, caution is needed when interpreting these results as we had relatively low sample sizes for FP phenotypes within FP genotypes. Furthermore, we observed FP behaviour for a limited amount of time which might have led to FP behaviour not being observed. However, continuous observation is impractical, and the strength of our study is that we identified phenotypes based on observations just prior to microbiota sampling.

FP genotype did not contribute to explaining the observed variation in mucosa-associated microbiota composition of the ileum or caecum at young or adult age, suggesting that divergent selection on FP did not affect mucosa-associated microbiota composition. Therefore, mucosa-associated microbiota composition might not explain the behavioural and physiological differences previously found in the FP selection lines (Kjaer, 2009; Kjaer and Guémené, 2009; Kops et al., 2017) and in HFP and LFP birds of the present study (**chapter 2** and **3**). However, the relative abundance of genus-level taxa within the Alteromonadales, Bacillales and Lactobacillales were higher and that of a genus-level group within the Erysipelotrichales was lower in the mucosa-associated microbiota of the ileum from HFP birds compared to LFP birds at adult age. For the mucosa-associated microbiota of the caeca HFP birds had a higher relative abundance of genus-level taxa within the Clostridiales compared to LFP birds at both young and adult age.

FP genotype did contribute to explaining the observed variation in luminal microbiota composition at adult age with genera of Clostridiales contributing more to the luminal microbiota of the HFP line, while *Lactobacillus* contributed more to that of the LFP line. However, it should be noted that FP genotype explained less

than 9% of the total variation in luminal microbiota composition. In addition, we identified luminal microbiota composition from combined content of the ileum, caeca and colon, while gut sections have been shown to differ in luminal microbiota composition in chickens (Awad et al., 2016). HFP birds also had a higher relative abundance of genera of the order Clostridiales, but lower relative abundance of *Staphylococcus* and *Lactobacillus* in the luminal microbiota compared to LFP birds. This supports our findings from the multivariate redundancy analysis and is similar to our findings from the mucosa-associated microbiota of the caecum. HFP birds further had higher diversity and evenness indices in the caecum and luminal content and a higher richness index in the luminal content compared to LFP birds at adult age, suggesting that the gut microbiota of HFP birds had a higher number of different species, a more even distribution of species relative abundances (meaning that less dominant species are present) and a higher diversity compared to the gut microbiota of LFP birds. Overall, our findings give first indications of differences in luminal microbiota composition and alpha diversity between the FP selection lines.

Behaviour (fearfulness) and physiology (serotonin, corticosterone and natural antibodies) did not contribute to explaining the observed variation in gut microbiota composition except for serotonin and natural antibody IgG. Serotonin tended to contribute to explaining the observed variation in mucosa-associated microbiota composition of the ileum and natural antibody IgG to that of the caecum at adult age. However, it should be noted that serotonin explained less than 13% and natural antibody IgG less than 8% of the total variation in mucosa-associated microbiota composition. Genera of the order Clostridiales were positively related to high serotonin levels. In general clostridia were found to modulate serotonin metabolism in the gut by for example, activating serotonin synthesis (Yano et al., 2015). Some of the genera, specifically *Faecalibacterium*, *Eubacterium* and *Roseburia*, are butyrate producers (Duncan et al., 2002a, 2002b; van den Abbeele et al., 2013) and butyrate can stimulate serotonin secretion (Fukumoto et al., 2003). Other genera that were positively related to serotonin level were *Klebsiella* and *Lactococcus*, which are serotonin producers (Özogul, 2004; Özogul et al., 2012), *Akkermansia*, which was shown to modulate tryptophan metabolism (i.e. serotonin precursor) in germ-free mice (Derrien et al., 2011) and *Bifidobacterium*, which increased tryptophan levels in the frontal cortex of rats (Desbonnet et al., 2008). Thus, serotonin levels might be higher due to the presence of these genera in the mucosa-associated microbiota of the ileum. *Alistipes* and unclassified members of the *Rikenellaceae* were positively related to natural antibody IgG titer. Species of the genera *Alistipes*, belonging to the family *Rikenellaceae*, are considered bacterial pathogens and were

found to induce intestinal inflammation in mice (Moschen et al., 2016). Thus, natural antibody IgG titers might be higher due to the presence of these genera in the mucosa-associated microbiota of the caecum.

Many genera within the order Clostridiales, which were higher or contributed more to the microbiota composition of HFP birds, are butyrate producers, such as *Anaerotruncus*, *Butyricicoccus*, *Eubacterium*, *Faecalibacterium*, *Flavonifractor* and *Subdoligranulum* (Duncan et al., 2002b; Eeckhaut et al., 2011; Li and Li, 2014). The relative abundance of *Butyricicoccus* and *Faecalibacterium* was also higher in luminal microbiota from HFP neutrals compared to LFP phenotypes. Butyrate has immunomodulatory potential (Vinolo et al., 2009; Wang et al., 2008) and can stimulate serotonin secretion, thereby activating the vagus nerve (Fukumoto et al., 2003). Many studies have identified effects of butyrate or butyrate producing bacteria on brain function and behaviour (Stilling et al., 2016). Although we did not measure butyrate concentrations, a previous study showed that HFP birds had higher n-butyrate, but lower i-butyrate in caecal digesta compared to LFP birds (Meyer et al., 2013). Thus, the behavioural and physiological differences seen between the FP selection lines might partly be caused by increased production of butyrate. Increased abundance of Clostridiales has further been related to autism spectrum disorders (Parracho et al., 2005; Williams et al., 2011) and major depressive disorder (Zheng et al., 2016) in humans. Patients with autism further had increased abundance of *Desulfovibrio* and *Sutterella* (Finegold, 2011; Williams et al., 2012), but reduced abundance of *Staphylococcus* (De Angelis et al., 2013). This is similar to what we found for the luminal microbiota composition of HFP birds compared to LFP birds, suggesting that specific gut microbiota compositions might be related to the development of these disorders and maladaptive behaviours, such as FP. Interestingly, HFP birds had higher richness, diversity and evenness indices compared to LFP birds. Autistic subjects were found to have higher faecal microbial richness and diversity compared to control subjects (De Angelis et al., 2013; Finegold et al., 2010), although the opposite or no relation has also been found (Kang et al., 2013; Kushak et al., 2017).

The relative abundance of *Lactobacillus* was lower in HFP birds and contributed more to the luminal microbiota composition of LFP birds. The relative abundance of *Lactobacillus* was also lower in luminal microbiota of HFP neutrals compared to LFP phenotypes. *Lactobacillus* can influence both the innate and adaptive immune systems in chickens (Brisbin et al., 2015, 2011) and behaviour in rodents, for example increasing locomotor activity or reducing anxiety and depression-related behaviour (Bravo et al., 2011; Liang et al., 2015; Liu et al., 2016a). This is potentially

done via stimulation of serotonin receptors (Horii et al., 2013) or by increasing serotonin and dopamine in the brain (Liu et al., 2016a). It is interesting to note that we previously found HFP birds to respond more actively to behavioural tests, suggesting they are less fearful compared to LFP birds (**chapter 2**), which is opposite to what would be expected based on the higher relative abundance of *Lactobacillus* in LFP birds. Thus, it remains to be determined whether differences in gut microbiota composition are causal to behavioural and physiological differences between the FP selection lines.

Gut microbiota composition is influenced by many factors, including endogenous and exogenous ones, such as genotype and diet (Spor et al., 2011). Thus, divergent selection on FP might cause the differences seen in gut microbiota composition. Our findings are supported by a previous study using the FP selection lines, where bacteria within the order Clostridiales showed increased relative abundance, but bacteria of the genus *Lactobacillus* showed reduced relative abundance in caecal droppings of HFP birds compared to LFP birds (Birkl et al., 2018). It is striking that these findings were consistent across studies, suggesting a strong influence of FP genotype on microbiota composition. It is further interesting to note that FP genotype contributed to explaining the observed variation in luminal microbiota composition, but not in mucosa-associated microbiota composition. Yet, luminal microbiota composition might be more determined by environmental effects such as diet, while mucosa-associated microbiota composition might be more determined by host genetics (van den Abbeele et al., 2011). A possible explanation for this discrepancy is that differences in luminal microbiota composition might arise because of feather eating in the HFP line. Previous studies have shown that HFP birds ingest more feathers compared to LFP birds (Harlander-Matauschek and Bessei, 2005; Harlander-Matauschek and Häusler, 2009). Furthermore, the FP selection lines differed in ileal and caecal microbial metabolites and HFP birds had a higher number of feather particles in their gizzards compared to LFP birds (Meyer et al., 2013). Moreover, birds fed feathers in their diet differed from control birds in microbial metabolites and microbial composition, with a higher number of Enterobacteria in the ileum and caecum, and a higher number of clostridia in the caecum (Meyer et al., 2012). Thus, feather eating might alter microbial composition. Interestingly, HFP birds had a higher relative abundance of *Nesterenkonia* in the ileum at adult age. *Nesterenkonia* species have been found to produce alkaline proteases which can hydrolyse keratinaceous substrates, such as feathers (Bakhtiar et al., 2005; Gessesse et al., 2003). Although we did not identify the level of feather eating or feather particles in the gut, HFP birds showed more FP compared to LFP birds throughout

the experiment (**chapter 2**). Further research is needed to identify whether differences between the FP selection lines in gut microbiota composition might be caused by feather eating.

In conclusion, FP phenotypes did not differ in luminal or mucosa-associated microbiota composition in the present study. However, birds from lines divergently selected for high respectively low FP differed in luminal, but not mucosa-associated, microbiota composition. Whether differences in microbiota composition are causal to FP or a consequence of FP (i.e. feather eating) remains to be elucidated.

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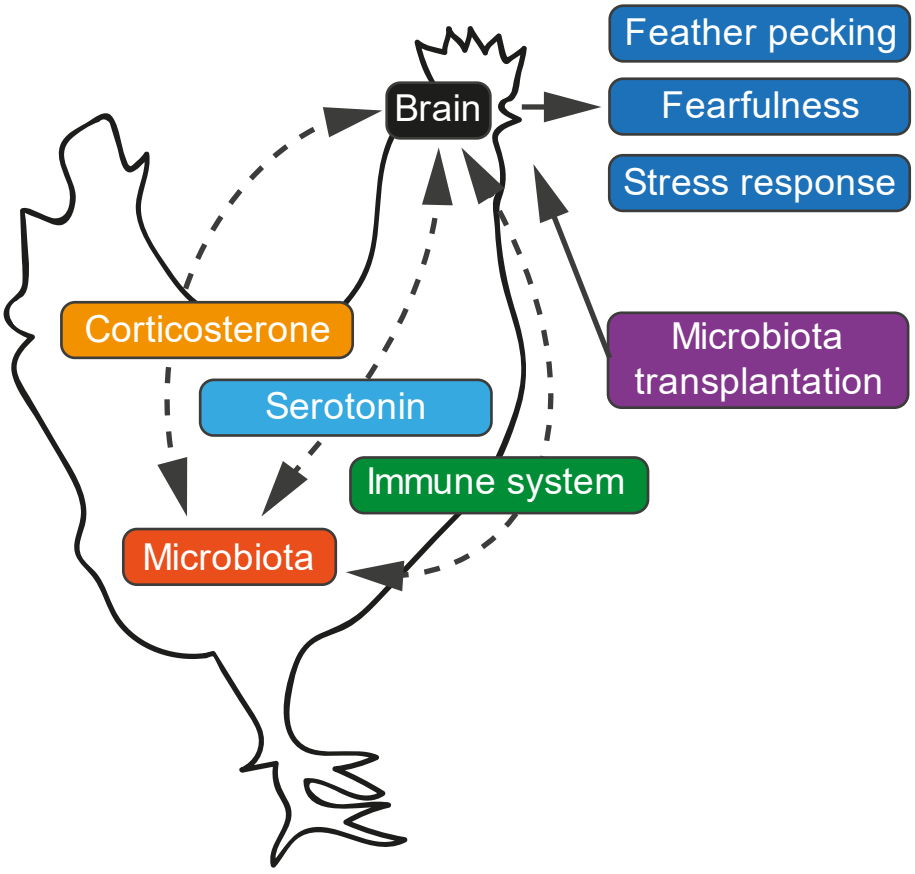
Chapter 6

Early-life microbiota transplantation affects behavioural responses, serotonin, and immune characteristics in chicken lines divergently selected on feather pecking



Jerine A.J. van der Eijk, T. Bas Rodenburg, Hugo de Vries, Joergen B. Kjaer, Hauke Smidt, Marc Naguib, Bas Kemp, Aart Lammers

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Abstract

Gut microbiota influences host behaviour and physiology, such as anxiety, stress, the serotonergic and immune systems. These behavioural and physiological characteristics have been related to feather pecking (FP), a damaging behaviour in chickens which reduces animal welfare and productivity. Moreover, high FP (HFP) and low FP (LFP) lines differed in microbiota composition. Yet, it is unknown whether microbiota can influence the development of FP. For the first time we identified effects of microbiota transplantation on FP and on behavioural and physiological characteristics related to FP.

HFP and LFP chicks received sterile saline (control), HFP or LFP microbiota transplantation during the first two weeks post hatch. Microbiota transplantation influenced behavioural responses of the HFP line during treatment and of the LFP line after treatment, immune characteristics (natural antibodies) in both lines and peripheral serotonin in the LFP line, but had limited effects on microbiota composition, stress response (corticosterone) and FP. Thus, early-life microbiota transplantation had immediate and long-term effects on behavioural responses, long-term effects on immune characteristics and peripheral serotonin, although effects were line dependent. Since early-life microbiota transplantation influenced behavioural and physiological characteristics that have been related to FP, it could thereby influence the development of FP later in life.

Introduction

Early-life is crucial for an animal's behavioural and physiological development and early-life factors can have a profound impact on this development (Pryce et al., 2005). An important moment early in life is the rapid microbial colonization of the gut, leading to the establishment of the gut microbiota. The gut microbiota has been shown to influence host behaviour and physiology (Cryan and Dinan, 2012; Foster et al., 2017; O'Mahony et al., 2015; Sommer and Bäckhed, 2013). Altering microbiota composition, via for example anti- or probiotic treatment, affected anxiety, stress and activity (Bravo et al., 2011; Ceylani et al., 2018; Hoban et al., 2016), but also the serotonergic and immune systems in rodents (Cao et al., 2018; Fröhlich et al., 2016; Ge et al., 2017; Yousefi et al., 2019). Moreover, germ-free mice colonized with microbiota from another mouse strain exhibited behaviour profiles of the donor strain (Bercik et al., 2011a). The gut microbiota seems to have similar effects in poultry where altering microbiota composition, via for example probiotic treatment, has been shown to affect fearfulness, memory, the serotonergic and immune systems (Cox and Dalloul, 2015; Parois et al., 2017; Yan et al., 2018). Moreover, microbiota transplantation to germ-free quails resulted in recipients adopting the fearful behaviour of donors early in life, although this was reversed later in life (Kraimi et al., 2019). These findings suggest that the gut microbiota influences behavioural and physiological characteristics in poultry and could therefore influence a bird's ability to cope with environmental and social challenges, such as those encountered in animal production systems.

Excessive damaging behaviours are indicative of an animal's inability to cope with a restrictive environment and are frequently seen in production animals. Feather pecking (FP) in chickens is such a damaging behaviour, which involves hens pecking and pulling at feathers of conspecifics, thereby reducing animal welfare and productivity (Brunberg et al., 2016). Feather pecking is multifactorial and has been linked to numerous behavioural characteristics, such as fearfulness, stress, activity, but also to physiological characteristics, such as the serotonergic, dopaminergic and immune systems (de Haas and van der Eijk, 2018; Parmentier et al., 2009; Rodenburg et al., 2013). Since similar behavioural and physiological systems related to FP are also affected by the gut microbiota, microbiota might play a role in the development of FP. Indeed, lines selected on high FP (HFP) and low FP (LFP) differed in behavioural responses, stress response, locomotor activity, central serotonergic and dopaminergic activity, peripheral serotonin, innate and adaptive immune characteristics (Buitenhuis et al., 2006; Kjaer, 2009; Kjaer and Guémené, 2009; Kops et al., 2017; **chapter 2, 3** and **4**). Moreover, the HFP and LFP lines

differed in intestinal microbial metabolites and in microbiota composition determined from caecal droppings and intestinal luminal samples (Birkl et al., 2018; Meyer et al., 2013; **chapter 5**). These findings point to a relationship between the gut microbiota and FP, yet it is unknown whether the gut microbiota could influence the development of FP.

Therefore, the aim of this study was to identify effects of early-life microbiota transplantation on FP and on behavioural and physiological characteristics related to FP in lines divergently selected on FP (HFP and LFP lines). Chicks received sterile saline (control), HFP or LFP microbiota transplantation daily during the first two weeks post hatch, a period where the brain is still developing (Atkinson et al., 2008). Fearfulness, stress response, serotonin and immune characteristics were measured as they are influenced by the gut microbiota (Cryan and Dinan, 2012; Foster et al., 2017; O'Mahony et al., 2015; Sommer and Bäckhed, 2013) and related to FP (de Haas and van der Eijk, 2018; Parmentier et al., 2009; Rodenburg et al., 2013). We further identified effects of microbiota transplantation on microbiota composition. We hypothesized that microbiota transplantation would result in recipients adopting a similar behavioural profile as that seen in the donor line. For example, LFP birds receiving HFP microbiota would show more FP and more active behavioural responses compared to LFP birds receiving LFP microbiota or control treatment.

Material and Methods

Animals and Housing

White leghorn birds from the 19th generation of lines selected on high (HFP) respectively low feather pecking (LFP) were used (see Kjaer et al., 2001 for the selection procedure). A total of 576 birds were produced in two batches of eggs. Eggs were placed in hatching baskets on embryonic day 18 and cardboard was placed on the bottom to prevent cross-contamination. From embryonic day 19 we collected hatched chicks (dry and wet chicks) and removed egg shells every 6 h. Chicks received a neck tag with an unique number, their first treatment and were weighed. Chicks were then placed in separate hatching baskets per line * treatment. On embryonic day 21 chicks were sexed and placed in pens per line * treatment with an approximate 50/50 male/female distribution. Non-beak-trimmed birds were used, which were housed in groups of 12 birds per pen. At 5 days, 2 weeks and 10 weeks of age group size was reduced for microbiota sampling (n = 10-11 birds per pen, n = 7-10 birds per pen and n = 6-9 birds per pen, respectively). Batches had the same housing conditions and experimental set-up with 4 pens per line * treatment (48 pens in total), but with 3 weeks between batches. At all times, water and feed were

provided *ad libitum.* Birds received a standard rearing diet 1 from hatch until 8 weeks of age and a standard rearing diet 2 from 8 until 16 weeks of age for laying hens. Each floor pen (h: 2 m, l: 1 m, w: 2 m) had wood shavings on the floor, two perches installed 45 cm above the floor and visual barriers of 1.5 m high to prevent birds in adjacent pens from seeing each other. Post hatch, temperature was kept around 33°C and gradually lowered to 24°C at 4 weeks of age. The light regime was 23L:1D post hatch, and was weekly, gradually reduced to 8L:16D at 4 weeks of age. Light intensity for each pen ranged between 45 and 81 LUX (average 62.6 LUX) as measured with a Voltcraft MS-1300 light meter (Conrad Electric Benelux). During hatching and for the first 5 weeks of age, extra hygienic measures were taken to prevent cross-contamination. Gloves were worn when handling birds and switched between treatments. Cover shoes were used when entering a pen and switched between pens. The experiment was approved by the Central Authority for Scientific Procedures on Animals according to Dutch law (no: AVD104002015150-1).

Treatment

Microbiota transplantation consisted of a mixed pool of luminal content of the ileum, caeca and colon from either HFP or LFP adult birds collected during a previous experiment (**chapter 5**). Pools were stored in -80 °C freezer until use. The number of viable aerobic and anaerobic microorganisms in pools was determined using plate cultures with blood agar medium. Plates were incubated overnight under aerobic or anaerobic conditions at 37 °C and colonies were counted. Before treatment, pools were defrosted in a 37 °C water bath for 5 min and then centrifuged at 5250 x g for 10 min. The microbial pellet was re-suspended in sterile saline (half of the original volume was added). The control treatment consisted of sterile saline. Treatments were kept on ice in between processing steps and during administration. The first treatment was given within 6 hrs post hatch and thereafter chicks received treatments daily during the first two weeks post hatch. Each chick orally received 100 µL of the treatment using a pipette (see Figure 1 for timeline of experiment).

Microbiota Sampling

At 5 days and 2 weeks of age 8 birds per line * treatment group (1 per pen) were randomly selected and sacrificed for the collection of gut microbiota. We collected luminal content from a ± 2 cm midsection of the ileum (between Meckel's diverticulum and caeca), one of the caeca and the colon. Samples were stored in cryovials at -80 °C until further analysis.

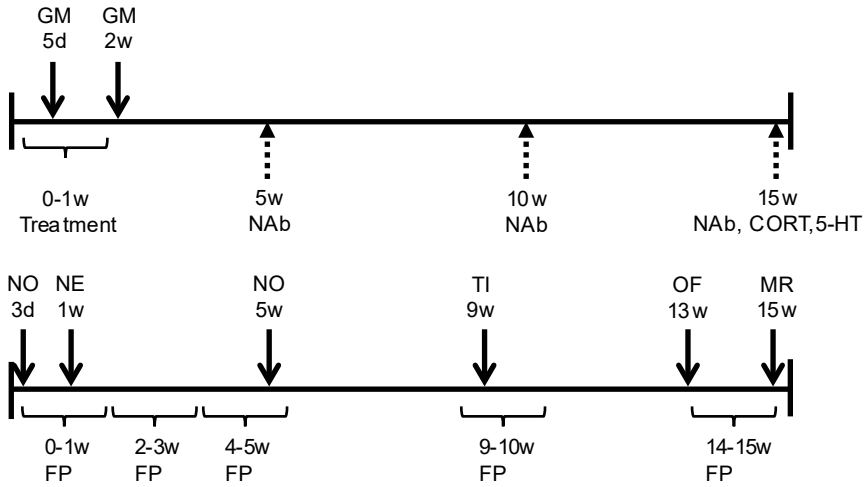

Figure 1. Timeline of experiment. Upper line indicates physiological measures: microbiota transplantation treatment and blood sampling (below line) and microbiota sampling (above line) performed at specific ages in days (d) or weeks (w). GM = gut microbiota, NAb = natural antibodies, CORT = corticosterone and 5-HT = serotonin. Lower line indicates behavioural measures: feather pecking observations (below line) and behavioural tests (above line). FP = feather pecking observations, NO = novel object test, NE = novel environment test, TI = tonic immobility test, OF = open field test and MR = manual restraint test.

Microbiota Analysis

Microbiota composition of pools and luminal content was determined via total DNA extraction, PCR amplification and sequencing as described previously (**chapter 5**). PCR amplification was carried out with primers directed to the V5-V6 region of the bacterial 16S rRNA gene, namely BSF784F (5'-RGGATTAGATACCC) and 1064R (5'-CGACRRCCATGCANCACCT). Data was processed and analysed using NG-Tax, an in-house bioinformatics pipeline, as described by Ramiro-Garcia et al., (2016), which resulted in a minimum of 29324 reads and a maximum of 607793 reads per sample.

Behavioural Observations and Tests

Feather pecking (FP) behaviour was observed between 0-1, 2-3, 4-5, 9-10 and 14-15 weeks of age. Birds were further subjected to five behavioural tests: novel object, novel environment, open field, tonic immobility and manual restraint. The order of testing was randomized at individual level, except for FP observations and novel

object tests which were randomized at pen level. The experimenters were blinded to the lines and treatments.

Feather Pecking Observations

FP behaviour was observed at pen level. Each observation lasted 30 min, either in the morning (8:30 h-12:30 h) or in the afternoon (12:30 h-16:30 h), after a 2.5 min habituation period. FP was categorized in gentle feather pecks (subdivided into exploratory FP and bouts of stereotyped FP) and severe FP as adapted from Newberry et al. (2007). Reliability between the two observers (inter-observer agreement) was high for all FP behaviours (Pearson correlations: exploratory FP (0.92), stereotyped FP (0.85) and severe FP (0.97)).

Novel Object Test

At 3 days and 5 weeks of age, the response to a novel object (NO) was tested at pen level. At 3 days of age (n = 48), the NO was a wooden block (h: 8 cm, l: 5 cm, w: 2.5 cm) wrapped with coloured tape (green, bright pink, light pink and yellow)(de Haas et al., 2014a). At 5 weeks of age (n = 48), the NO test was repeated with a plastic stick (l: 50 cm, d: 3.5 cm) wrapped with coloured tape (red, white, green, black, and yellow)(Welfare Quality®, 2009). The test started 10 sec after the experimenter had placed the NO on the floor in the centre of the home pen. The latency for three birds to approach the NO at a distance of < 25 cm and the number of birds that were within < 25 cm of the NO was recorded every 10 sec for the 2 min test duration. Two experimenters tested all pens at 3 days and 5 weeks of age.

Novel Environment Test

At 1 week of age, the response to a novel environment (de Haas et al., 2014a) was tested for a duration of 1 min (n = 520). All birds from a pen were taken and put in a cardboard box in front of the home pen. The average time difference between the first and last bird to be tested was 9 min. Birds were then individually taken to one of three test locations, where birds were placed inside a white bucket (h: 57 cm, l: 32 cm, w: 22 cm) at the start of the test. The latency to vocalize, the number of vocalizations and flight attempts were recorded. Together, three experimenters tested all birds where each experimenter tested approximately one third of the birds.

Tonic Immobility Test

At 9 weeks of age, birds were tested in a tonic immobility (TI) test (Jones and Faure, 1981) for a maximum duration of 5 min (n = 458). Half of the birds in a pen were

taken and transported in a cardboard box to a room near the testing rooms. The average time difference between the first and last bird to be tested was 8 min. Birds were individually taken to one of two test rooms, where they were placed in a supine position in a metal cradle with their head suspended from the side of the cradle. Each bird was restrained for 10 sec and when the bird remained in this position after releasing, TI duration was recorded until the bird returned to upright position. If this happened within 10 sec after release TI was induced again, with a maximum of three attempts at inducing TI. Together, two experimenters tested all birds where each experimenter tested approximately half of the birds.

Open Field Test

At 13 weeks of age, birds were tested in an open field (OF) test (Rodenburg et al., 2009) for a duration of 5 min (n = 409). Birds were individually taken and transported to the test room in a cardboard box. The OF was a square wooden enclosure (h: 1.22 m, l: 1.15 m, w: 1 m) with a video-camera positioned above it. A bird was placed in the centre of the OF and the test started when the lights were switched on. One experimenter recorded the latency to vocalize and number of vocalizations. A second experimenter recorded the latency to step and number of steps from a monitor in an adjacent room. Three experimenters tested all birds where each experimenter tested approximately one third of the birds for vocalizations or steps.

Manual Restraint Test

At 15 weeks of age, birds were tested in a manual restraint (MR) test (Bolhuis et al., 2009) for a duration of 5 min (n = 247). Birds were individually taken and transported to one of two test rooms in a cardboard box. Birds were placed on their right side on a table, with the right hand of the experimenter covering the bird's back and the left hand gently stretching the bird's legs. The latencies to vocalize and to struggle and the number of vocalizations and struggles were recorded. Together, four experimenters tested the birds, where each experimenter tested approximately one fourth of the birds. Fifteen min after the start of MR, blood samples were drawn from the wing vein for assessment of the peak in plasma corticosterone level (Fraisse and Cockrem, 2006).

Blood Collection and Analyses

Blood was collected from all birds at 5, 10, and 15 weeks of age. Blood was taken from the wing vein using a heparinized syringe and kept on ice after blood sampling.

Blood samples for corticosterone concentrations and natural antibody titers were centrifuged at 5250 x g for 10 min at room temperature and the obtained plasma was stored at -20 °C until further analysis. Whole blood samples (1 mL) for determination of serotonin were stored at -20 °C until further analysis.

Plasma Corticosterone

Samples from week 15 were used for determination of plasma corticosterone concentrations via a radioimmunoassay kit (MP Biomedicals) as described previously (Buyse et al., 1987).

Whole Blood Serotonin

Samples from week 15 were used for determination of whole blood serotonin concentration (nmol/mL) via a fluorescence assay as described previously (Bolhuis et al., 2009). The centrifugation steps were performed at 931 x g and fluorescence was determined in a Perkin-Elmer 2000 Fluorescence spectrophotometer at 295 and 540 nm.

Plasma IgM and IgG Natural Antibody Titers

Samples from all weeks were used for determination of IgM and IgG natural antibody titers against keyhole limpet hemocyanin via an indirect enzyme-linked immunosorbent assay as described previously (Berghof et al., 2015) with the following modifications. Serial dilutions of plasma were made in four steps starting at dilution 1:40,000 in phosphate buffer saline containing 0.05% Tween 20 and 1% horse plasma (100 µL in each well). Peroxidase conjugated goat-anti-chicken IgM (A30-102P, Bethyl; dilution 1:20,000) or goat-anti-chicken IgG (A30-104P, Bethyl; dilution 1:20,000) was used as secondary antibody (100 µL in each well).

Statistical Analysis

In order to relate changes in microbial composition to explanatory variables, redundancy analysis was used as implemented in the CANOCO 5 software package (Biometris). Line * treatment interaction was introduced as nominal variable and we further tested treatment and treatment effects within lines separately. The relative contribution of 259 genus-level phylogenetic groups identified by 16S rRNA gene sequencing were used as response variables. Analyses were performed for each age (5 days and 2 weeks of age) and gut section (ileum, caecum or colon) separately. The Monte Carlo Permutation test (number of permutations 499) was applied to test for significance of the effect of line * treatment or treatment within lines on microbiota

composition. Batch and sex were included as covariates. P-values were corrected using Bonferroni correction.

SAS Software version 9.4 was used for statistical analysis (SAS Institute). Linear mixed models for line * treatment effects were tested for each age separately and consisted of fixed effects of line * treatment, line, treatment, sex and batch and the random effect of pen within line and treatment. Linear mixed models for treatment effects within lines consisted of fixed effects of treatment, sex and batch and the random effect of pen within treatment. Linear mixed models for FP behaviours and behavioural responses to NO did not include the fixed effect of sex or a random effect as they were tested at pen level. Test time (morning 8:00 h-12:30 h or afternoon 12:30 h-18:00 h) was added as fixed effect for the TI, OF and MR tests. Experimenter was added as fixed effect for the NE, TI, OF and MR tests. The model residuals were visually examined for normality. Variables were square root transformed (i.e. latency to vocalize and frequency of vocalizations in the NE test; TI duration; latency to vocalize and step, step and vocalization frequency in the OF test; latency to struggle and vocalize in the MR test; corticosterone and serotonin levels) to obtain normality of model residuals. Generalized linear mixed models with a Binary distribution were used to test effects of line*treatment, line and treatment or treatment effects within lines for flight attempts in the NE test, for number of inductions needed to reach TI and for struggle and vocalization frequency in the MR test. A backward regression procedure was used when fixed effects (i.e. line * treatment, test time or experimenter) had $P > 0.1$. Post hoc pairwise comparisons were corrected by Tukey–Kramer adjustment. Kruskal Wallis tests were used to analyse line * treatment, treatment and treatment effects within lines for individual microbial groups, stereotyped FP, severe FP and behavioural responses to the NO test and post hoc comparisons were made with Dwass, Steel, Critchlow-Fligner method. All data is presented as (untransformed) mean ± standard error (SE).

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

<u>High and low feather pecking transplantation pools had distinct microbiota composition</u>

Gut microbiota was collected from adult chickens of the high (HFP) and low feather pecking (LFP) lines (**chapter 5**). Transplantation pools were made per line and could be distinguished from each other in terms of microbiota composition using a principal component analysis (Figure 2A). The orders of Clostridiales and

Lactobacillales had the highest relative abundance in both pools, where the HFP pool had a higher relative abundance of Clostridiales and the LFP pool had a higher relative abundance of Lactobacillales (Figure 2B). The number of viable microorganisms in the pools was analysed using plate cultures and both pools contained on average 4.75 x 10^6 viable aerobic colony forming units / mL and 5.1 x 10^6 viable anaerobic colony forming units / mL.

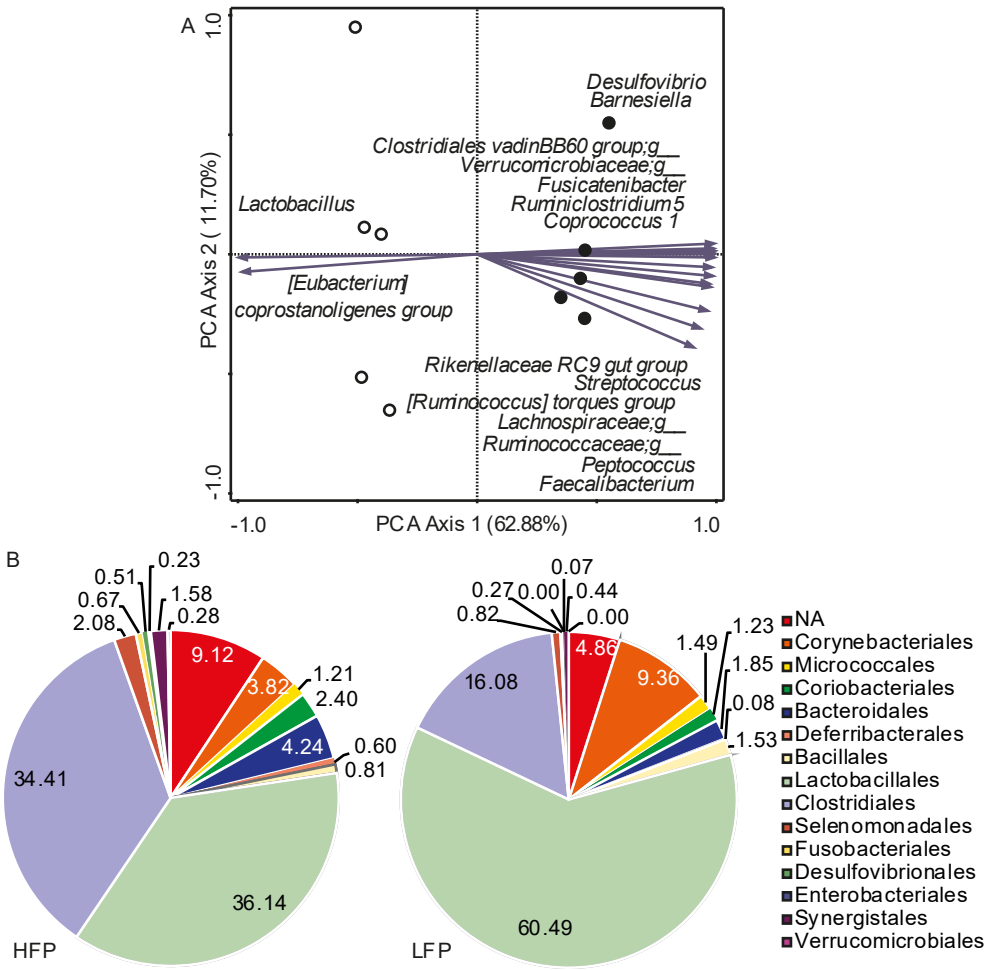

Figure 2. A) Biplot for principal component analysis (PCA) of transplantation pools' microbiota composition. Samples are grouped by line: HFP (closed circles) and LFP (open circles). Microbial groups for which the variation in relative abundance in the data is explained for at least 95% by the axes are represented as vectors. Groups that could not be assigned to a specific genus are classified by the family name appended with ";g_". B) Pie charts with average relative abundances of orders present in the HFP (left) and LFP (right) transplantation pools.

Early-life microbiota transplantation had limited effects on microbiota composition

Newly hatched chicks received a control treatment, HFP or LFP microbiota transplantation within 6 h post hatch to influence bacterial colonization (Ballou et al., 2016) and every day during the first 2 weeks post hatch, a period where synapses are still being formed (Atkinson et al., 2008). Microbiota of chicks was sampled from luminal content of the ileum, caecum and colon at 5 days and 2 weeks of age to assess effects of microbiota transplantation on microbiota composition using 16S rRNA gene sequencing. Multivariate redundancy analysis (RDA) of 16S rRNA gene sequencing profiles at the approximate genus-level showed a high overlap of line * treatment groups (Sup. Figure 1), treatments (Sup. Figure 2) and treatments within lines (Sup. Figures 3-5). Except for birds receiving the control treatment which could be distinguished from birds receiving microbiota transplantation for the caecal microbial composition at 5 days and 2 weeks of age, where the control treatment contributed to explaining 5,5% and 6.3%, respectively of the observed variation in microbiota composition ($P = 0.032$ and $P = 0.004$, respectively). Furthermore, within the HFP line, HFP birds receiving control treatment could be distinguished from other groups for caecal microbial composition at 2 weeks of age, where the control treatment contributed to explaining 11.2% of the observed variation in microbiota composition ($P = 0.036$; Figure 3). This suggests microbiota composition of HFP birds receiving control treatment was distinct from that of HFP birds receiving HFP or LFP microbiota. To further analyse differences in microbiota composition we determined whether line * treatment groups, treatments or treatments within lines differed in relative abundances of microbial groups. However, only few microbial groups differed between line * treatment groups, treatments or treatments within lines and most microbial groups that differed had on average low relative abundances (< 1%) (Sup. Tables 1 and 2). Overall, these results suggest immediate effects of early-life microbiota transplantation on microbiota composition.

Early-life microbiota transplantation influenced behavioural responses

Several behavioural tests were performed to assess fearfulness and stress response (Forkman et al., 2007; Korte et al., 1997). During treatment, birds were tested in a novel object test at 3 days of age and a novel environment test at 1 week of age. After treatment, birds were tested in a second novel object test at 5 weeks of age, a tonic immobility test at 9 weeks of age, an open field test at 13 weeks of age and a manual restraint test at 15 weeks of age. Fearfulness and stress response were measured, as many studies show that anxiety-like behaviour and stress were influenced by gut microbiota (Cryan and Dinan, 2012; Foster et al., 2017), and fearfulness and stress

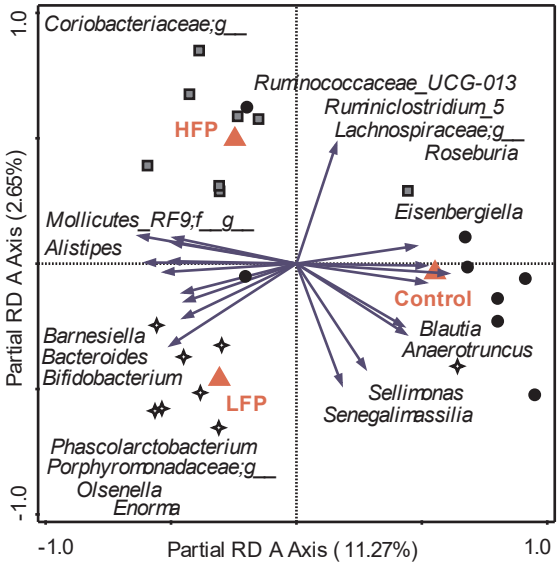

Figure 3. Triplot for partial redundancy analysis (RDA) of caecum microbiota composition of the high feather pecking (HFP) line at 2 weeks of age. Microbial groups for which the variation in relative abundance in the data is explained for at least 20% by the axes are represented as vectors. Nominal environmental treatment variables are represented by red triangles. Samples are grouped by treatment: HFP microbiota (grey squares), Control (black circles) and low feather pecking (LFP) microbiota (white stars). Groups that could not be assigned to a specific genus are classified by the family name appended with ";g_".

sensitivity have been related to the development of feather pecking (FP) (Rodenburg et al., 2013). Here, tendencies and significant findings were reported with regard to effects of line * treatment interactions, treatment and treatment within lines, but a complete overview is given in Sup. Table 3.

During treatment, significant line * treatment interactions were found on latency to approach the novel object (χ^2 = 16.32, df = 5, P = 0.006), on the percentage of birds approaching the novel object (χ^2 = 22.69, df = 5, P < 0.001) (Sup. Figure 6) and on flight attempts during the novel environment test ($F_{2,41}$ = 3.27, P = 0.048) (Sup. Figure 7). However, no treatment effects were found. We further analysed effects of treatment within lines, as we were interested whether treatments within lines differed from each other. Interestingly, during the treatment period we only found treatment effects within the HFP line, where it tended to affect the latency to approach the novel object (χ^2 = 5.71, df = 2, P = 0.058; Figure 4A), percentage of birds approaching the novel object (χ^2 = 5.17, df = 2, P = 0.075; Figure 4B) and the latency to vocalize in the novel environment test ($F_{2,20}$ = 2.63, P = 0.097; Figure 4C). HFP chicks receiving HFP microbiota tended to approach the novel object sooner (P = 0.064) and more birds tended to approach it (P = 0.091) compared to HFP chicks receiving LFP microbiota. Furthermore, HFP chicks receiving HFP microbiota

tended to vocalize sooner compared to HFP chicks receiving control treatment (P = 0.091). These results suggest that during treatment HFP chicks receiving HFP microbiota showed more active behavioural responses compared to HFP chicks receiving LFP microbiota or control treatment.

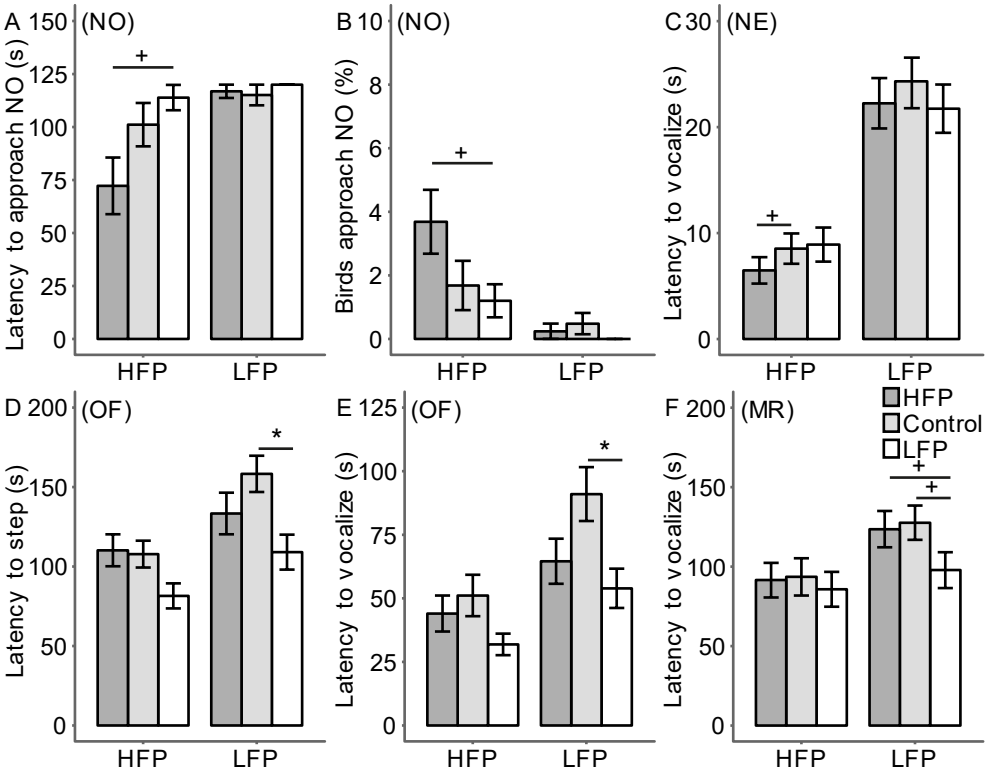

Figure 4. A) Mean latency (± SE) for three birds to approach the novel object (NO) and B) mean percentage (± SE) of birds approaching the NO at 3 days of age; C) mean latency to vocalize (± SE) in the novel environment (NE) test at 1 week of age; D) mean latency to step (± SE) and E) mean latency to vocalize (± SE) in the open field (OF) test at 13 weeks of age; F) mean latency to vocalize (± SE) in the manual restraint (MR) test at 15 weeks of age for the high (HFP) and low feather pecking (LFP) lines treated with HFP microbiota, control or LFP microbiota. $^{+}$ show tendencies ($P < 0.1$) and * show significant differences ($P < 0.05$) between treatments within lines.

After treatment, significant line * treatment interactions were found on latency to approach the novel object ($\chi^2 = 20.38$, df = 5, P = 0.001) and percentage of birds approaching the novel object ($\chi^2 = 19.35$, df = 5, P = 0.002) (Sup. Figure 6). Treatment effects were found on number of inductions needed to reach tonic immobility ($F_{2,43} = 3.39$, P = 0.043), and on latency to step ($F_{2,43} = 7.42$, P = 0.002)

and vocalize ($F_{2,43}$ = 5.66, P = 0.007) in the open field test. Birds receiving LFP microbiota needed fewer inductions to reach tonic immobility compared to birds receiving HFP microbiota (P = 0.043) (Sup. Figure 8A). In the open field test, birds receiving LFP microbiota stepped and vocalized sooner compared to birds receiving control treatment (P = 0.001 and P = 0.005, respectively) and tended to step sooner compared to birds receiving HFP microbiota (P = 0.059) (Sup. Figures 8B and 8C). Furthermore, a tendency for a treatment effect was found on latency to struggle during restraint ($F_{2,43}$ = 2.48, P = 0.096), but no differences were found after correction for multiple comparisons. To explore these treatment effects in more detail we analysed treatment effects within lines. Interestingly, after the treatment period we only found treatment effects within the LFP line, where it affected latency to step ($F_{2,20}$ = 5.77, P = 0.011; Figure 4D) and vocalize ($F_{2,20}$ = 5.13, P = 0.016; Figure 4E) in the open field test and latency to vocalize during restraint ($F_{2,20}$ = 3.79, P = 0.04; Figure 4F). In the open field test, LFP birds receiving LFP microbiota stepped (P = 0.008) and vocalized (P = 0.013) sooner compared to LFP birds receiving control treatment. In the manual restraint test, LFP birds receiving LFP microbiota tended to vocalize sooner compared to LFP birds receiving HFP microbiota or control treatment (P = 0.096 and P = 0.051, respectively). These results suggest that after treatment LFP birds receiving LFP microbiota showed more active behavioural responses compared to LFP birds receiving HFP microbiota or control treatment.

In summary, these results indicate that behavioural responses were influenced by early-life microbiota transplantation, where effects were found during treatment in the HFP line and after treatment in the LFP line. Furthermore, birds receiving homologous microbiota transplantation (i.e. receiving microbiota from their own line) showed more active responses in both lines.

<u>Early-life microbiota transplantation influenced natural antibodies and peripheral serotonin, but not corticosterone</u>

Several physiological characteristics were measured after the treatment period. Natural antibody (NAb, antibody that binds antigen without prior intentional exposure to that antigen (Baumgarth et al., 2005)) titers were measured at 5, 10 and 15 weeks of age as they play an essential role in both innate and adaptive immunity (Lutz et al., 2009; Ochsenbein and Zinkernagel, 2000; Panda and Ding, 2015) and were therefore used as a general immune characteristic. At 15 weeks of age, corticosterone level after manual restraint was used as indicator of the physiological stress response (Kjaer and Guémené, 2009) and whole blood serotonin level as

indicator of central serotonin levels (Uitdehaag et al., 2011). These physiological characteristics were measured as many studies show that the immune, stress and serotonergic systems are influenced by gut microbiota (Foster et al., 2017; O'Mahony et al., 2015; Sommer and Bäckhed, 2013) and these systems have all been related to FP (de Haas and van der Eijk, 2018; Parmentier et al., 2009; Rodenburg et al., 2013). Here, tendencies and significant findings were reported with regard to effects of line * treatment interactions, treatment and treatment within lines, but a complete overview is given in Sup. Table 4.

Treatment effects were found on IgM NAb titers at 5 ($F_{2,43}$ = 7.87, P = 0.001) and 10 weeks of age ($F_{2,43}$ = 7.94, P = 0.031). Birds receiving control treatment had lower IgM titers compared to birds receiving HFP (P = 0.018) or LFP microbiota (P = 0.001) at 5 weeks of age, but at 10 weeks of age they had higher IgM titers compared to birds receiving HFP microbiota (P = 0.026) (Sup. Figure 9). To explore these treatment effects in more detail we analysed treatment effects within lines. In the HFP line, treatment tended to influence IgM NAb at 5 weeks of age ($F_{2,20}$ = 3.18, P = 0.063) and significantly influenced IgM NAb at 10 weeks of age ($F_{2,20}$ = 4.03, P = 0.034) (Figure 5A). HFP birds receiving LFP microbiota tended to have higher IgM titers compared to HFP birds receiving control treatment at 5 weeks of age (P = 0.064) and further had higher IgM titers compared to HFP birds receiving HFP microbiota at 10 weeks of age (P = 0.031). In the LFP line, we found a treatment effect on IgM NAb at 5 weeks of age ($F_{2,20}$ = 4.41, P = 0.026) and a tendency for a treatment effect at 10 weeks of age ($F_{2,20}$ = 2.87, P = 0.08) (Figure 5A). LFP birds receiving LFP microbiota had higher IgM titers compared to LFP birds receiving control treatment at 5 weeks of age (P = 0.025). Furthermore, treatment tended to influence peripheral serotonin within the LFP line ($F_{2,20}$ = 2.62, P = 0.098; Figure 5B), where LFP birds receiving HFP microbiota tended to have lower serotonin levels compared to LFP receiving control treatment (P = 0.084).

Overall, these results indicate that physiological characteristics were influenced by early-life microbiota transplantation, where effects were seen on IgM NAbs in both lines and on peripheral serotonin in the LFP line after the treatment period, whereas no effects were seen on IgG NAbs or corticosterone.

Early-life microbiota transplantation had limited effects on feather pecking

Feather pecking was observed between 0-1, 2-3, 4-5, 9-10 and 14-15 weeks of age at pen level and was categorized into gentle FP (subdivided into exploratory and stereotyped FP) and severe FP, where gentle FP usually does not result in damage

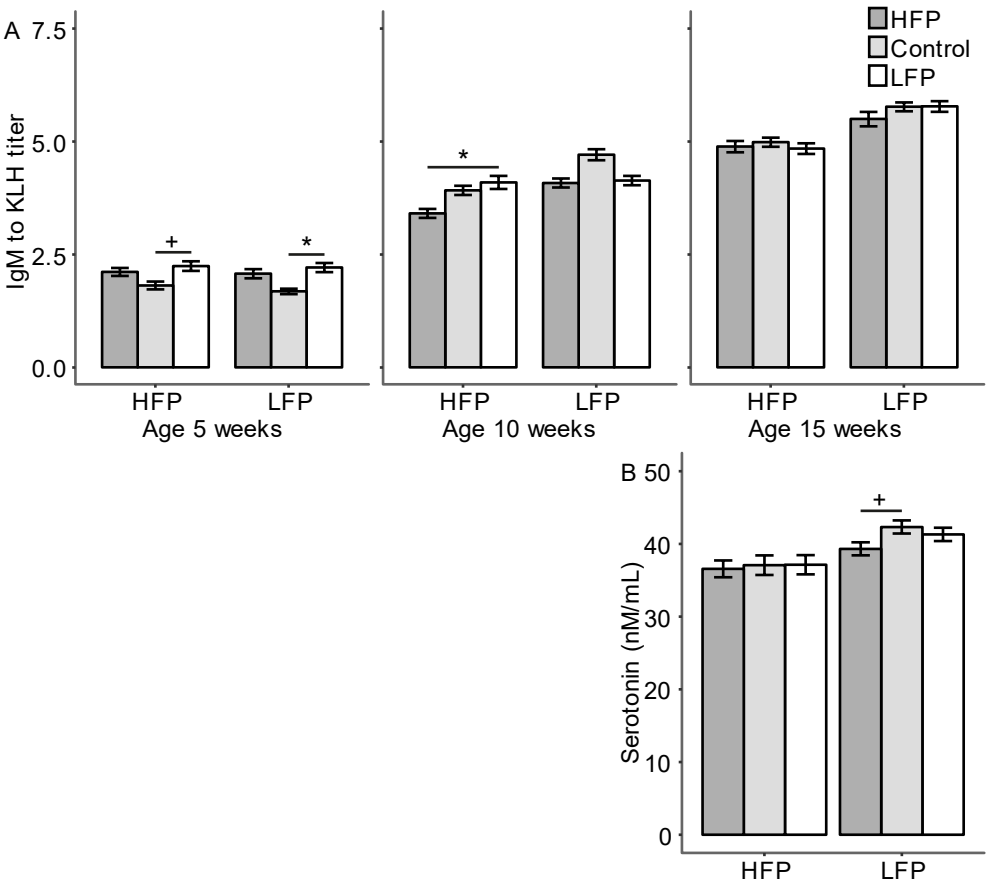

Figure 5. A) Mean IgM natural antibody titers to keyhole limpet hemocyanin (KLH) (± SE) at 5, 10 and 15 weeks of age and B) mean serotonin level (± SE) for the high (HFP) and low feather pecking (LFP) lines treated with HFP microbiota, control or LFP microbiota. $^{+}$ show tendencies ($P < 0.1$) and * show significant differences ($P < 0.05$) between treatments within lines.

and severe FP is the problematic behaviour in terms of damage to the recipient in animal husbandry (Rodenburg et al., 2013). During and after the treatment period, significant line * treatment interactions were found on stereotyped FP and severe FP, but not on exploratory FP (for a complete overview see Sup. Table 5 and Sup. Figures 10A-C). After treatment, a tendency for a treatment effect on exploratory FP was found in week 2-3 ($F_{2,43} = 2.34$, $P = 0.083$), where birds receiving HFP microbiota tended to show less exploratory FP compared to birds receiving LFP microbiota ($P = 0.085$) (Sup. Table 5). We explored treatment effects in more detail by analysing treatment effects within lines. In the HFP line, a tendency for a treatment effect on exploratory FP was found in week 2-3 ($\chi^2 = 4.81$, df = 2, P =

0.091). In the LFP line, tendencies for treatment effects were found on exploratory FP in week 4-5 ($\chi^2 = 5.16$, df = 2, P = 0.076) and on severe FP in week 14-15 ($\chi^2 = 5.35$, df = 2, P = 0.069). LFP birds receiving HFP microbiota tended to show more exploratory FP compared to LFP birds receiving control (P = 0.09) at 4-5 weeks of age. However, for other treatment effects within lines no differences were found after correction for multiple comparisons. In summary, these results indicate that FP was not clearly influenced by early-life microbiota transplantation in the present study.

Discussion

The aim of this study was to identify effects of early-life microbiota transplantation on feather pecking (FP) and on behavioural and physiological characteristics related to FP in high FP (HFP) and low feather pecking (LFP) selection lines. We hypothesized that microbiota transplantation would result in recipients adopting a similar behavioural profile as that seen in the donor line. To summarize, behavioural responses were influenced by early-life microbiota transplantation, where effects of homologous transplantation were seen during treatment in the HFP line and after treatment in the LFP line. With regard to physiological characteristics, we found effects on IgM natural antibodies in both lines and on peripheral serotonin in the LFP line, but not on IgG natural antibodies or corticosterone. Furthermore, early-life microbiota transplantation had limited effects on microbiota composition and FP.

Microbiota composition was not clearly influenced by early-life microbiota transplantation, which could be explained by transplantation pools consisting of adult microbiota. Adult microbiota might not be able to settle and remain within the gut of newly hatched chicks, since the gut microbiota is still developing and undergoes a rapid succession until it is fully developed and stable around seven weeks of age (Ballou et al., 2016; Hill et al., 2011). Furthermore, we sampled luminal content instead of mucosal scrapings. Mucosa-associated microbiota composition might be more involved in communication to the host because of its close proximity (Ouwerkerk et al., 2013) and was further shown to differ from luminal microbiota composition (Awad et al., 2016; Olsen et al., 2008). Mucosa-associated microbiota composition might be altered by our microbiota transplantation, but previously the FP selection lines did not differ in mucosa-associated microbiota composition (**chapter 5**). Previous studies did find effects of microbiota transplantation on microbiota composition in rats and quails (Kraimi et al., 2019; Yang et al., 2019). However, these studies used pseudo germ-free (i.e. received antibiotic treatment prior to transplantation) or germ-free animals and most studies to date that identified effects of microbiota transplantation on behaviour used (pseudo) germ-free animals

(Bercik et al., 2011a; Kelly et al., 2016; Sharon et al., 2019; Zheng et al., 2016). Yet, (pseudo) germ-free animal models are rather extreme, making it difficult to translate findings to 'normally' occurring situations, especially when animals are housed in sterile isolators.

It is striking that without using (pseudo) germ-free chicks or housing them in sterile isolators, we did find effects on behavioural responses, natural antibodies and peripheral serotonin. Microbiota transplantation may have influenced brain, immune and serotonergic system functioning. Microbiota transplantation was given during the first two weeks post hatch, when synapses are still being formed (Atkinson et al., 2008). Furthermore, the immune system is still under development, as secondary immune organs are being formed (Fellah et al., 2014) and the early-life period is of major immunological importance, since the chick becomes exposed to a wide variety of environmental antigens (Bar-Shira et al., 2003), which activate and educate the immune system (Cebra, 1999). There is extensive evidence that the gut microbiota affects brain, central serotonergic and immune system functioning (Burokas et al., 2017; O'Mahony et al., 2015; Sommer and Bäckhed, 2013) and it seems to influence peripheral serotonergic system functioning as well. For example, germ-free mice had decreased mRNA levels of tryptophan hydroxylase (enzyme for serotonin synthesis), and increased mRNA levels of the serotonin transporter in intestinal cells (Sjögren et al., 2012). In the transplantation pools the most abundant orders were Lactobacillales and Clostridiales. These orders and genera that were associated with the transplantation pools have been shown to influence behaviour, natural antibodies and peripheral serotonin. For example, *Lactobacillus* reduced anxiety-like behaviour (Bravo et al., 2011) and intestinal mRNA levels of the serotonin transporter in rodents (Cao et al., 2018). Furthermore, the orders of Clostridiales and Lactobacillales were associated with the development of natural antibody repertoire in GalT-KO mice (Bello-Gil et al., 2019) and in general clostridia were found to modulate serotonin metabolism in the gut, for example by activating serotonin synthesis (Yano et al., 2015). However, it should be noted that we cannot exclude that other factors in the transplantation pools might have contributed to altered behaviour, natural antibodies and peripheral serotonin, for example viruses or fungi (Cadwell, 2015; Enaud et al., 2018). We suggest that microbiota transplantation influenced brain, serotonergic and immune system functioning, which (in)directly resulted in differences in behavioural responses, natural antibodies and serotonin.

During treatment, microbiota transplantation influenced behavioural responses in the HFP line, while after treatment it influenced behavioural responses in the LFP line. A potential explanation for this could be that the HFP line seems to have a more

responsive immune system, which reacts more strongly to the environment (Buitenhuis et al., 2006; **chapter 3** and **4**). However, it should be noted that after the treatment period both lines responded similarly to microbiota transplantation with regard to IgM natural antibodies and no differences were found for IgG natural antibodies. Still, having a more responsive immune system might result in HFP birds responding stronger to microbiota transplantation with the synthesis and release of pro-inflammatory cytokines. Peripherally produced pro-inflammatory cytokines can act on the brain (Dantzer et al., 2008) where they reduce serotonergic and dopaminergic neurotransmission (Miller et al., 2013), which have been indicated to play a role in the development of FP (de Haas and van der Eijk, 2018). Thus, having a more responsive immune system might result in the HFP line responding more strongly to microbiota transplantation with the production of pro-inflammatory cytokines. In turn, these cytokines act on the brain and alter neurotransmission, thereby potentially influencing behavioural responses and the development of FP. Direct-fed microbials were shown to alter intestinal mRNA levels of pro-inflammatory cytokines (Lee et al., 2015) and probiotic treatment altered serotonergic and dopaminergic neurotransmission (Yan et al., 2018) in broilers, indicating microbiota could influence cytokine levels, serotonergic and dopaminergic neurotransmission in poultry. However, we did not identify pro-inflammatory cytokine levels or brain neurotransmission in the present study. Therefore, further research is needed to identify effects of microbiota transplantation on cytokine levels and brain neurotransmission in poultry.

After treatment, microbiota transplantation influenced behavioural responses in the LFP line. These effects do not seem to be explained by differences in peripheral serotonin, as LFP birds receiving HFP microbiota tended to have lower peripheral serotonin levels compared to LFP birds receiving control treatment, while behavioural differences were seen between LFP birds receiving LFP microbiota and LFP birds receiving HFP microbiota or control treatment. Moreover, it should be noted that serotonin cannot cross the blood-brain barrier (Pietraszek et al., 1992), thus caution is needed when using peripheral serotonin levels as indicator for central serotonin levels. Still, it is interesting that LFP birds receiving HFP microbiota had lower peripheral serotonin, as we previously found that HFP birds had lower peripheral serotonin compared to LFP birds (**chapter 3**). This might point to an increased risk for developing FP in LFP birds receiving HFP microbiota, which is supported by findings in commercial lines where low peripheral serotonin levels were related to high FP (de Haas et al., 2014a, 2013; Uitdehaag et al., 2011). However, treatment effects on peripheral serotonin levels do not seem to be

explained by the differences in microbiota composition of the HFP pool compared to the LFP pool, as genera of the order Clostridiales were positively related to high peripheral serotonin levels (**chapter 5**) and in general clostridia were found to activate serotonin synthesis in the gut (Yano et al., 2015). Thus, it remains unclear through which potential pathway microbiota transplantation influences behavioural responses in LFP birds.

Previously, the HFP line showed more active responses compared to the LFP line in the same behavioural tests that were also performed in the present study (Kops et al., 2017; **chapter 2** and **3**) and in other behavioural tests (de Haas et al., 2010). Therefore, during the treatment period behavioural responses seem to be adopted from donors in the HFP line, as HFP chicks receiving HFP microbiota tended to show more active responses (i.e. approached novel object sooner and more birds approached it, vocalized sooner) compared to HFP chicks receiving LFP microbiota or control treatment. In contrast, after the treatment period behavioural responses do not seem to be adopted from donors in the LFP line, as LFP birds receiving LFP microbiota showed more active responses (i.e. stepped and vocalized sooner) compared to LFP birds receiving HFP microbiota or control treatment. Similarly, a recent study showed that microbiota transplantation early in life had long-term effects on behaviour in quails, where quails adopted the behaviour of donor birds early in life, but these effects were reversed later in life (Kraimi et al., 2019). Thus, early-life microbiota transplantation can have long-term effects on behaviour in poultry and effects seem to depend on host genotype and age of testing.

It is interesting that homologous transplantation (i.e. receiving microbiota from their own line) resulted in birds showing more active responses, which suggests reduced fearfulness, as silence and inactivity have been related to high fearfulness (Forkman et al., 2007; Jones, 1996). Therefore, homologous microbiota transplantation could be a potential approach to reduce fearfulness in chickens. Many studies show that FP is related to high fearfulness (de Haas et al., 2014a; Rodenburg et al., 2004a; Uitdehaag et al., 2008a), indicating that receiving microbiota from their own line might reduce the risk of birds developing FP. However, it should be noted that no treatment effects within lines were found on severe FP or on tonic immobility duration, the measure for innate fearfulness in chickens (Forkman et al., 2007). Previously, HFP birds had shorter tonic immobility duration compared to LFP birds (**chapter 2**), suggesting FP is related to reduced fearfulness in the FP selection lines. However, another study reported no difference in tonic immobility duration between the FP selection lines (Rodenburg et al., 2010). Thus, further research is needed to

identify whether homologous transplantation can be used to reduce fearfulness in poultry, thereby potentially reducing the development of FP.

FP was not clearly influenced by early-life microbiota transplantation. An explanation for this finding might be that FP usually increases from the egg laying period onwards (around 20 weeks of age) (Bright, 2009; Newberry et al., 2007) and we observed FP till 15 weeks of age. However, in this study as well as previous studies, the FP selection lines clearly differed in all types of FP already around five weeks of age (Kops et al., 2017; **chapter 2**), although one study reported no differences in FP around five weeks of age (Kjaer, 2009). FP around the five week period has been indicated as a critical time point for the development of severe FP later in life (de Haas, 2014; Huber-Eicher and Sebö, 2001). Interestingly, we found a peak in severe FP between two and five weeks of age in the HFP line (Sup. Figure 10C). Furthermore, it should be noted that on average LFP chicks receiving HFP microbiota showed more severe FP compared to LFP chicks receiving LFP microbiota or control treatment and for this group we also found a peak between two and five weeks of age (Sup. Table 5 and Sup. Figure 10C). These findings might point to potential differences in severe FP at adult age (> 17 weeks of age). It should further be noted that effects on FP might be missed, as there was quite some variation in severe FP between pens, which could be caused by severe FP only being performed by few individuals (Newberry et al., 2007). Further research is needed to identify potential effects of microbiota transplantation on severe FP at adult age.

This is the first study that investigated effects of early-life microbiota transplantation on FP and on behavioural and physiological characteristics related to FP. In conclusion, early-life microbiota transplantation influenced behavioural responses that have been related to FP, where effects were seen during treatment in the HFP line and after treatment in the LFP line. Early-life microbiota transplantation further influenced physiology, including immune characteristics (natural antibodies) in both lines and peripheral serotonin in the LFP line after treatment, but had limited effects on microbiota composition, the physiological stress response (corticosterone) and FP. Thus, early-life microbiota transplantation had immediate and long-term effects on behavioural responses, long-term effects on immune characteristics and peripheral serotonin, although effects were dependent on host genotype. Since microbiota transplantation influenced behavioural and physiological characteristics that have been related to FP, it could thereby influence the development of FP. However, more research is needed to identify whether the gut microbiota plays a role in the development of FP.

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Table 1. Relative abundance (%) of microbial groups of the high (HFP) and low feather pecking (LFP) lines receiving HFP, control or LFP microbiota treatment as determined from luminal content of the ileum, caecum or colon at 5 days of age.

Line	**HFP**			**LFP**			**P-value**				
Treatment	**HFP**	**Control**	**LFP**	**HFP**	**Control**	**LFP**	**L * T**	**L**	**T**	**T within L**	
										HFP	**LFP**
Ileum											
Candidatus Arthromitus	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.04	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.18	< 0.01	ns	< 0.01	ns	< 0.01
Enterococcus	0.3 ± 0.10	3.9 ± 2.4	0.8 ± 0.38	0.6 ± 0.58	0.1 ± 0.05	0.3 ± 0.21	ns	< 0.01	ns	ns	ns
Caecum											
Anaerofilum	0.2 ± 0.09	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.09	0.2 ± 0.12	0.0 ± 0.0	ns	ns	< 0.05	< 0.05	ns
Anaerotruncus	0.4 ± 0.14	0.1 ± 0.04	0.7 ± 0.57	0.5 ± 0.25	0.4 ± 0.28	0.7 ± 0.40	ns	ns	< 0.05	ns	ns
Coriobaceriaceae;g__	0.3 ± 0.35	0.0 ± 0.0	0.0 ± 0.02	0.3 ± 0.16	0.1 ± 0.08	0.0 ± 0.03	< 0.05	ns	< 0.01	< 0.05	ns
Clostridiales	0.7 ± 0.43	0.3 ± 0.22	2.0 ± 1.0	0.7 ± 0.20	0.5 ± 0.34	0.8 ± 0.22	ns	ns	< 0.05	ns	ns
Lachnoclostridium	1.7 ± 0.38	0.2 ± 0.10	1.5 ± 0.61	1.3 ± 0.76	0.5 ± 0.28	1.0 ± 0.32	< 0.05	ns	< 0.05	< 0.05	ns
Tyzzerella	0.3 ± 0.10	0.0 ± 0.01	0.1 ± 0.06	0.1 ± 0.07	0.1 ± 0.07	0.0 ± 0.01	ns	ns	ns	< 0.05	ns
Ruminococcus	0.5 ± 0.30	0.0 ± 0.0	0.0 ± 0.01	0.0 ± 0.04	0.0 ± 0.02	0.0 ± 0.0	< 0.05	ns	ns	< 0.05	ns
Ruminococcaceae;g__	0.7 ± 0.17	0.1 ± 0.06	0.9 ± 0.22	1.3 ± 0.43	0.5 ± 0.27	1.0 ± 0.38	< 0.05	ns	< 0.01	< 0.05	ns
Ruminiclostridium 9	0.3 ± 0.08	0.2 ± 0.07	1.0 ± 0.22	1.0 ± 0.58	0.9 ± 0.19	0.6 ± 0.16	< 0.01	< 0.05	ns	< 0.01	ns
Ruminococcaceae UCG	0.0 ± 0.04	0.0 ± 0.02	0.2 ± 0.07	0.2 ± 0.16	0.0 ± 0.03	0.1 ± 0.08	ns	ns	ns	< 0.05	ns
Lactobacillus	9.2 ± 1.90	13.9 ± 3.72	9.5 ± 3.06	18.9 ± 4.33	8.0 ± 1.47	6.2 ± 1.31	ns	ns	ns	ns	< 0.05
Lachnospiraceae FCS020	0.1 ± 0.08	0.0 ± 0.02	0.0 ± 0.05	0.0 ± 0.03	0.0 ± 0.0	0.2 ± 0.09	< 0.05	ns	< 0.05	ns	< 0.01
Klebsiella	0.0 ± 0.0	0.7 ± 0.54	0.2 ± 0.13	0.9 ± 0.94	1.9 ± 1.58	0.0 ± 0.0	ns	ns	< 0.05	ns	ns
Intestinimonas	0.1 ± 0.05	0.1 ± 0.07	0.3 ± 0.11	0.1 ± 0.06	0.1 ± 0.07	0.3 ± 0.11	ns	ns	< 0.05	ns	< 0.05
Colon											
Anaerofilum	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.2	0.2 ± 0.16	0.0 ± 0.02	ns	< 0.05	ns	ns	ns
Candidatus Arthromitus	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.07	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.32	< 0.01	ns	< 0.01	ns	< 0.01
Erysipelotrichaceae;g__	0.1 ± 0.06	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.47	ns	< 0.05	ns	ns	ns

L: Line and T: Treatment; Values represent means ± SE; The genus-level groups with a relative abundance lower than 0.1% in all treatment groups are not shown; Microbial groups that could not be assigned to a specific genus are classified by the family name appended with ";g_".

Table 2. Relative abundance (%) of microbial groups of the high (HFP) and low feather pecking (LFP) lines receiving HFP, control or LFP microbiota treatment as determined from luminal content of the ileum, caecum or colon at 2 weeks of age.

Line	**HFP**			**LFP**			**P-value**				
Treatment	**HFP**	**Control**	**LFP**	**HFP**	**Control**	**LFP**	**L * T**	**L**	**T**	**T within L**	
										HFP	**LFP**
Ileum											
Peptostreptococcaceae;g__	1.7 ± 0.63	0.2 ± 0.13	2.1 ± 1.52	0.6 ± 0.20	0.1 ± 0.08	0.5 ± 0.34	< 0.05	ns	< 0.01	ns	< 0.05
Aeriscardovia	0.0 ± 0.03	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.23	0.0 ± 0.02	0.5 ± 0.47	ns	< 0.05	ns	ns	ns
Streptococcus	0.2 ± 0.15	0.3 ± 0.31	0.4 ± 0.35	0.2 ± 0.18	0.0 ± 0.0	0.0 ± 0.02	ns	< 0.05	ns	ns	ns
Caecum											
Bifidobacterium	0.5 ± 0.13	0.1 ± 0.06	1.9 ± 0.74	1.8 ± 1.23	1.8 ± 1.25	0.5 ± 0.21	ns	ns	ns	< 0.05	ns
Enorma	0.2 ± 0.14	0.0 ± 0.0	0.2 ± 0.08	0.4 ± 0.24	0.0 ± 0.03	0.4 ± 0.22	< 0.01	ns	< 0.01	< 0.05	< 0.05
Coriobacteriaceae;g__	0.1 ± 0.04	0.6 ± 0.39	0.2 ± 0.12	0.9 ± 0.60	0.3 ± 0.12	0.3 ± 0.19	ns	ns	ns	< 0.05	ns
Porphyromonadaceae;g__	0.3 ± 0.16	0.0 ± 0.02	0.3 ± 0.08	0.2 ± 0.15	0.1 ± 0.05	0.3 ± 0.15	ns	ns	< 0.05	< 0.05	ns
Eisenbergiella	0.5 ± 0.11	2.4 ± 0.83	0.7 ± 0.20	0.6 ± 0.18	1.2 ± 0.29	1.1 ± 0.29	< 0.05	ns	< 0.01	< 0.05	ns
Barnesiella	0.2 ± 0.09	0.0 ± 0.02	0.3 ± 0.14	0.1 ± 0.06	0.0 ± 0.02	0.2 ± 0.10	ns	ns	< 0.05	ns	ns
Alistipes	2.3 ± 0.63	1.5 ± 1.23	2.0 ± 0.55	1.8 ± 0.93	0.7 ± 0.42	2.5 ± 0.75	ns	ns	< 0.05	ns	ns
Hydrogenoanaerobacteriu	0.0 ± 0.0	0.1 ± 0.04	0.0 ± 0.01	0.0 ± 0.0	0.1 ± 0.05	0.0 ± 0.02	ns	ns	< 0.05	ns	ns
Roseburia	0.0 ± 0.0	0.3 ± 0.18	0.0 ± 0.0	0.1 ± 0.07	0.0 ± 0.0	0.0 ± 0.04	< 0.05	ns	ns	< 0.05	ns
Collinsella	0.0 ± 0.02	0.0 ± 0.0	0.0 ± 0.02	0.1 ± 0.03	0.0 ± 0.0	0.0 ± 0.01	ns	ns	< 0.05	ns	< 0.05
Slackia	0.2 ± 0.12	0.0 ± 0.03	0.3 ± 0.20	0.1 ± 0.04	0.0 ± 0.03	0.4 ± 0.18	ns	ns	< 0.05	ns	< 0.05
Christensenellaceae R 7	0.8 ± 0.33	0.4 ± 0.22	0.6 ± 0.20	0.1 ± 0.07	1.3 ± 1.11	0.7 ± 0.19	ns	ns	ns	ns	< 0.05
Faecalitalea	0.0 ± 0.0	0.0 ± 0.02	0.0 ± 0.0	0.5 ± 0.31	0.0 ± 0.0	0.0 ± 0.0	< 0.05	ns	ns	ns	< 0.05
Phascolarctobacterium	0.6 ± 0.26	0.5 ± 0.39	0.9 ± 0.30	1.3 ± 0.57	0.3 ± 0.19	1.0 ± 0.36	ns	ns	< 0.05	ns	ns
Clostridiales	0.1 ± 0.11	0.6 ± 0.27	0.1 ± 0.03	0.2 ± 0.08	0.7 ± 0.25	0.2 ± 0.10	ns	ns	< 0.05	ns	ns
Parabacteroides	0.2 ± 0.11	0.0 ± 0.0	0.1 ± 0.04	0.4 ± 0.21	0.1 ± 0.08	0.4 ± 0.13	ns	< 0.05	ns	ns	ns
Tyzzerella 3	0.0 ± 0.0	0.0 ± 0.02	0.0 ± 0.0	0.1 ± 0.08	0.2 ± 0.10	0.1 ± 0.11	ns	< 0.05	ns	ns	ns
Oscillospira	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.02	0.0 ± 0.02	0.1 ± 0.03	ns	< 0.01	ns	ns	ns
Subdoligranulum	3.3 ± 0.65	2.0 ± 0.52	4.3 ± 1.89	2.0 ± 0.84	2.3 ± 0.63	1.6 ± 0.35	ns	< 0.05	ns	ns	ns
Colon											
Bifidobacterium	1.1 ± 1.06	0.0 ± 0.0	0.4 ± 0.17	0.1 ± 0.06	0.2 ± 0.13	0.1 ± 0.03	ns	ns	ns	< 0.05	ns
Lactobacillus	52.2 ± 9.88	56.2 ± 13.12	58.6 ± 12.55	34.6 ± 10.65	82.2 ± 4.40	36.4 ± 9.93	ns	ns	ns	ns	< 0.05
Peptostreptococcaceae;g	34.3 ± 8.26	11.5 ± 1.73	10.8 ± 4.80	30.5 ± 9.28	4.3 ± 1.70	20.1 ± 6.37	< 0.05	ns	< 0.05	ns	< 0.05

L: Line and T: Treatment; Values represent means ± SE; The genus-level groups with a relative abundance lower than 0.1% in all treatment groups are not shown; Microbial groups that could not be assigned to a specific genus are classified by the family name appended with ";g_".

Table 3. Responses to behavioural tests at different ages of the high (HFP) and low feather pecking (LFP) lines receiving HFP, control or LFP microbiota treatment.

Line	**HFP**			**LFP**			**P-value**				
Treatment	**HFP**	**Control**	**LFP**	**HFP**	**Control**	**LFP**	**L * T**	**L**	**T**	**T within L**	
										HFP	**LFP**
Novel object											
Lat. to approach	72.3 ± 13.37	101.1 ± 10.23	113.9 ± 5.98	116.9 ± 3.1	115.1 ± 4.88	120.0 ± 0.0	< 0.01	< 0.01	= 0.1	< 0.1	ns
# birds approach	3.7 ± 1.00	1.7 ± 0.78	1.2 ± 0.52	0.2 ± 0.24	0.5 ± 0.34	0.0 ± 0.0	< 0.01	< 0.01	ns	< 0.1	ns
Novel environment											
Lat. to vocalize	6.5 ± 1.25	8.5 ± 1.43	9.0 ± 1.59	22.2 ± 2.38	24.3 ± 2.39	21.7 ± 2.28	ns	< 0.01	ns	< 0.1	ns
Vocalization freq.	1.2 ± 0.06	1.1 ± 0.06	1.1 ± 0.06	0.4 ± 0.04	0.3 ± 0.04	0.3 ± 0.04	ns	< 0.01	ns	ns	ns
Flight attempts	0.4 ± 0.10	0.6 ± 0.13	0.6 ± 0.11	0.3 ± 0.08	0.1 ± 0.05	0.3 ± 0.07	< 0.05	< 0.01	ns	ns	ns
Novel object											
Lat. to approach	84.6 ± 12.08	87.6 ± 9.69	78.1 ± 13.35	120.0 ± 0.0	112.1 ± 7.88	120.0 ± 0.0	< 0.01	< 0.01	ns	ns	ns
# birds approach	4.5 ± 1.29	2.1 ± 0.66	5.7 ± 1.64	0.5 ± 0.55	1.2 ± 0.87	0.5 ± 0.21	< 0.01	< 0.01	ns	ns	ns
Tonic immobility											

L: Line and T: Treatment; Lat: latency in seconds; #: number; Freq: frequency in number/min; TI duration: tonic immobility duration in seconds; Values represent means ± SE .

Table 4. Physiological characteristics at different ages of the high (HFP) and low feather pecking (LFP) lines receiving HFP, control or LFP microbiota treatment.

Line	**HFP**			**LFP**			**P-value**				
Treatment	**HFP**	**Control**	**LFP**	**HFP**	**Control**	**LFP**	**L * T**	**L**	**T**	**T within L**	
										HFP	**LFP**
Natural antibody titers											
IgM	2.1 ± 0.09	1.8 ± 0.09	2.2 ± 0.11	2.1 ± 0.10	1.7 ± 0.06	2.2 ± 0.10	ns	ns	< 0.01	< 0.1	< 0.05
IgG	1.6 ± 0.09	1.5 ± 0.07	1.6 ± 0.08	1.4 ± 0.10	1.5 ± 0.09	1.5 ± 0.10	ns	ns	ns	ns	ns
Natural antibody titers											
IgM	3.4 ± 0.10	3.9 ± 0.10	4.1 ± 0.14	4.1 ± 0.10	4.7 ± 0.12	4.1 ± 0.10	ns	< 0.01	< 0.05	< 0.05	< 0.1
IgG	3.3 ± 0.20	3.0 ± 0.13	3.2 ± 0.22	2.8 ± 0.17	2.9 ± 0.15	2.6 ± 0.12	ns	< 0.05	ns	ns	ns
Natural antibody titers											
IgM	4.9 ± 0.13	5.0 ± 0.10	4.8 ± 0.12	5.5 ± 0.16	5.8 ± 0.10	5.8 ± 0.12	ns	< 0.01	ns	ns	ns
IgG	4.5 ± 0.21	4.5 ± 0.21	4.3 ± 0.21	3.6 ± 0.19	4.0 ± 0.20	3.9 ± 0.20	ns	< 0.01	ns	ns	ns
Corticosterone (ng/mL)	4.9 ± 0.37	6.0 ± 0.51	6.9 ± 0.79	5.7 ± 0.36	6.2 ± 0.49	6.3 ± 0.64	ns	ns	ns	ns	ns
Serotonin (nM/mL)	36.6 ± 1.15	37.1 ± 1.35	37.1 ± 1.32	39.3 ± 0.90	42.3 ± 0.90	41.3 ± 0.92	ns	< 0.01	ns	ns	< 0.1

L: Line and T: Treatment; Values represent means ± SE .

Table 5. Feather pecking (FP) behaviour (exploratory FP, stereotyped FP (bouts) and severe FP) at different ages of the high (HFP) and low feather pecking (LFP) lines receiving HFP, control or LFP microbiota treatment.

Line	**HFP**			**LFP**			**P-value**				
Treatment	**HFP**	**Control**	**LFP**	**HFP**	**Control**	**LFP**	**L * T**	**L**	**T**	**T within L**	
										HFP	**LFP**
Age 0-1 weeks											
Exploratory FP	5.3 ± 0.49	5.8 ± 0.34	4.6 ± 0.58	2.4 ± 0.15	2.1 ± 0.22	2.3 ± 0.17	ns	< 0.01	ns	ns	ns
Stereotyped FP	0.4 ± 0.10	0.9 ± 0.37	0.6 ± 0.20	0.2 ± 0.07	0.1 ± 0.05	0.1 ± 0.04	< 0.05	< 0.01	ns	ns	ns
Severe FP	4.5 ± 3.28	4.1 ± 1.96	1.4 ± 0.33	1.4 ± 0.96	0.3 ± 0.07	0.5 ± 0.16	< 0.01	< 0.01	ns	ns	ns
Age 2-3 weeks											
Exploratory FP	3.3 ± 0.53	4.8 ± 0.57	5.5 ± 0.83	2.5 ± 0.35	2.9 ± 0.61	2.8 ± 0.42	ns	< 0.01	< 0.1	< 0.1	ns
Stereotyped FP	0.7 ± 0.28	1.5 ± 0.38	1.2 ± 0.44	0.3 ± 0.14	0.4 ± 0.13	0.2 ± 0.11	< 0.05	< 0.01	ns	ns	ns
Severe FP	13.4 ± 7.21	31.8 ± 13.82	12.2 ± 4.13	3.0 ± 1.79	1.1 ± 0.82	2.2 ± 1.43	= 0.01	< 0.01	ns	ns	ns
Age 4-5 weeks											
Exploratory FP	2.5 ± 0.41	3.3 ± 0.86	3.8 ± 0.56	3.3 ± 0.66	1.8 ± 0.47	2.7 ± 0.53	ns	ns	ns	ns	< 0.1
Stereotyped FP	1.2 ± 0.66	2.5 ± 1.53	1.2 ± 0.59	0.6 ± 0.29	0.2 ± 0.08	0.7 ± 0.38	ns	< 0.05	ns	ns	ns
Severe FP	6.3 ± 4.81	15.8 ± 6.64	25.5 ± 12.3	6.5 ± 4.60	0.7 ± 0.39	1.8 ± 0.88	< 0.05	< 0.01	ns	ns	ns
Age 9-10 weeks											
Exploratory FP	3.1 ± 0.77	3.0 ± 0.86	2.0 ± 0.64	1.7 ± 0.26	1.4 ± 0.23	1.4 ± 0.11	ns	< 0.05	ns	ns	ns
Stereotyped FP	0.3 ± 0.09	0.5 ± 0.26	0.2 ± 0.06	0.2 ± 0.07	0.1 ± 0.03	0.1 ± 0.03	ns	< 0.05	ns	ns	ns
Severe FP	1.1 ± 0.52	6.6 ± 4.44	2.9 ± 2.29	1.0 ± 0.41	0.6 ± 0.16	0.5 ± 0.12	ns	ns	ns	ns	ns
Age 14-15 weeks											
Exploratory FP	3.6 ± 0.47	2.5 ± 0.71	2.9 ± 0.74	1.6 ± 0.39	1.9 ± 0.41	1.1 ± 0.25	ns	< 0.01	ns	ns	ns
Stereotyped FP	0.6 ± 0.11	0.3 ± 0.13	0.8 ± 0.30	0.2 ± 0.06	0.2 ± 0.06	0.2 ± 0.08	< 0.1	< 0.05	ns	ns	ns
Severe FP	4.5 ± 0.63	6.4 ± 4.30	6.3 ± 2.11	1.2 ± 0.28	1.0 ± 0.34	0.5 ± 0.21	< 0.01	< 0.01	ns	ns	< 0.1

L: Line and T: Treatment; Average number of pecks or bouts per bird per hour (number of pecks or bouts per pen per hour divided by the number of birds in that pen); Values represent means ± SE.

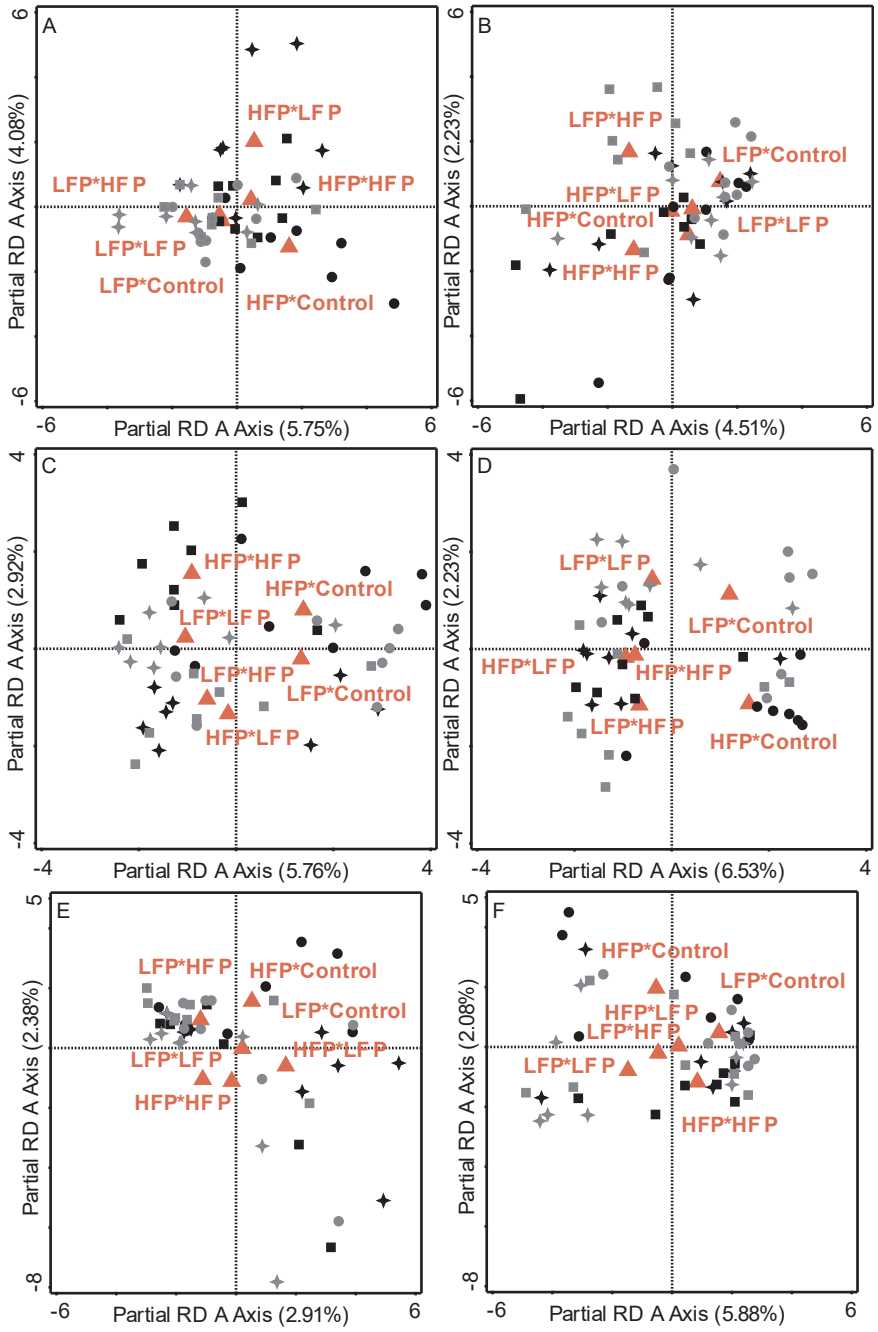

Figure 1. Biplot for partial RDA analysis of ileum (A and B), caecum (C and D), and colon microbiota composition (E and F) at 5 days and 2 weeks of age, respectively. Nominal environmental interaction variables are represented by red triangles. Samples are grouped by line: high feather pecking (HFP; black) and low feather pecking (LFP; grey), and by treatment: HFP (squares), Control (circles) and LFP (stars).

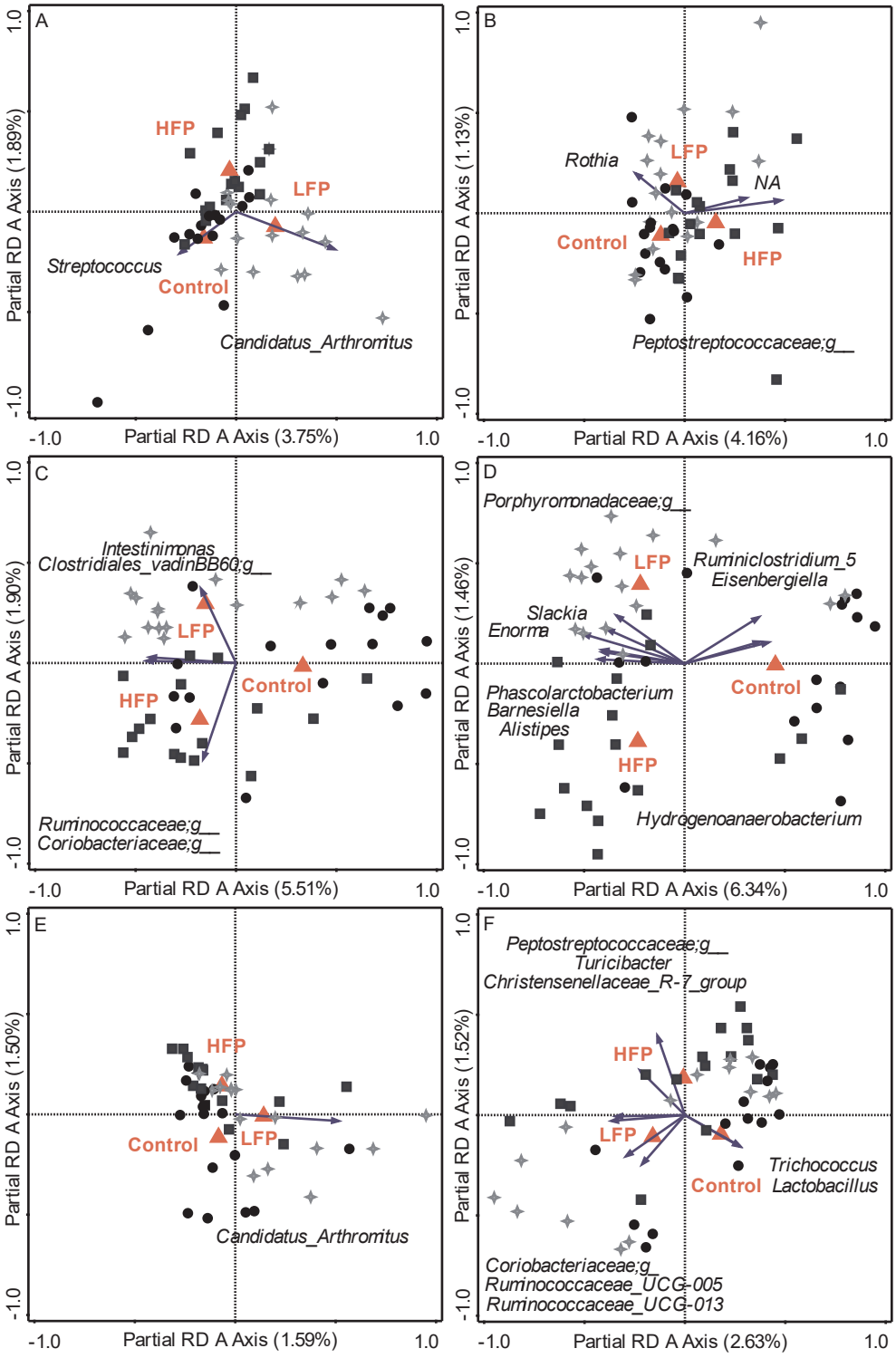
A
HFP
LFP
Streptococcus
Control
Candidatus_Arthromitus
Partial RD A Axis (1.89%)
Partial RD A Axis (3.75%)
B
Rothia
LFP
NA
Control
HFP
Peptostreptococcaceae;g__
Partial RD A Axis (1.13%)
Partial RD A Axis (4.16%)
C
Intestinimonas
Clostridiales_vadinBB60;g__
LFP
HFP
Control
Ruminococcaceae;g__
Coriobacteriaceae;g__
Partial RD A Axis (1.90%)
Partial RD A Axis (5.51%)
D
Porphyromonadaceae;g__
LFP
Ruminiclostridium_5
Eisenbergiella
Slackia
Enorma
Phascolarctobacterium
Barnesiella
Alistipes
Control
HFP
Hydrogenoanaerobacterium
Partial RD A Axis (1.46%)
Partial RD A Axis (6.34%)
E
HFP
Control
LFP
Candidatus_Arthromitus
Partial RD A Axis (1.50%)
Partial RD A Axis (1.59%)
F
Peptostreptococcaceae;g__
Turicibacter
Christensenellaceae_R-7_group
HFP
LFP
Control
Trichococcus
Lactobacillus
Coriobacteriaceae;g_
Ruminococcaceae_UCG-005
Ruminococcaceae_UCG-013
Partial RD A Axis (1.52%)
Partial RD A Axis (2.63%)
1.0
-1.0

Figure 2. Triplot for partial RDA analysis of ileum (A and B), caecum (C and D), and colon microbiota composition (E and F) at 5 and 2 weeks of age, respectively. Nominal environmental treatment variables are represented by red triangles. Samples are grouped by treatment: HFP (grey squares), Control (black circles) and LFP (light grey stars). Microbial groups for which the variation in relative abundance in the data is explained for at least 15% for the caecum and 10% for the ileum and colon by the axes are represented as vectors. Groups that could not be assigned to a specific genus are classified by the family name appended with ";g_".

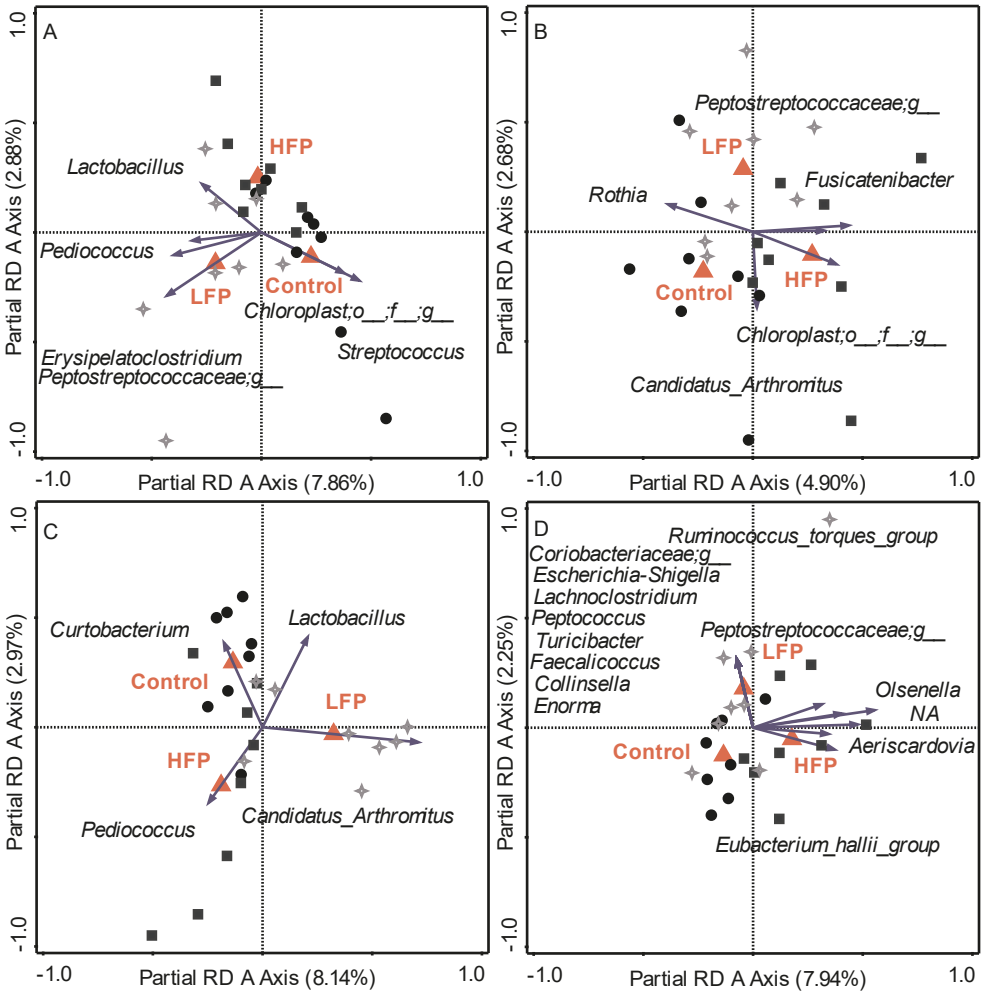

Figure 3. Triplot for partial RDA analysis of ileum microbiota composition of the high feather pecking (HFP; A and B) line and the low feather pecking (LFP; C and D) line at 5 and 14 days of age. Nominal environmental treatment variables are represented by red triangles. Samples are grouped by treatment: HFP (grey squares), Control (black circles) and LFP (light grey stars). Microbial groups for which the variation in relative abundance in the data is

explained for at least 10% by the axes are represented as vectors. Groups that could not be assigned to a specific genus are classified by the family name appended with ";g_".

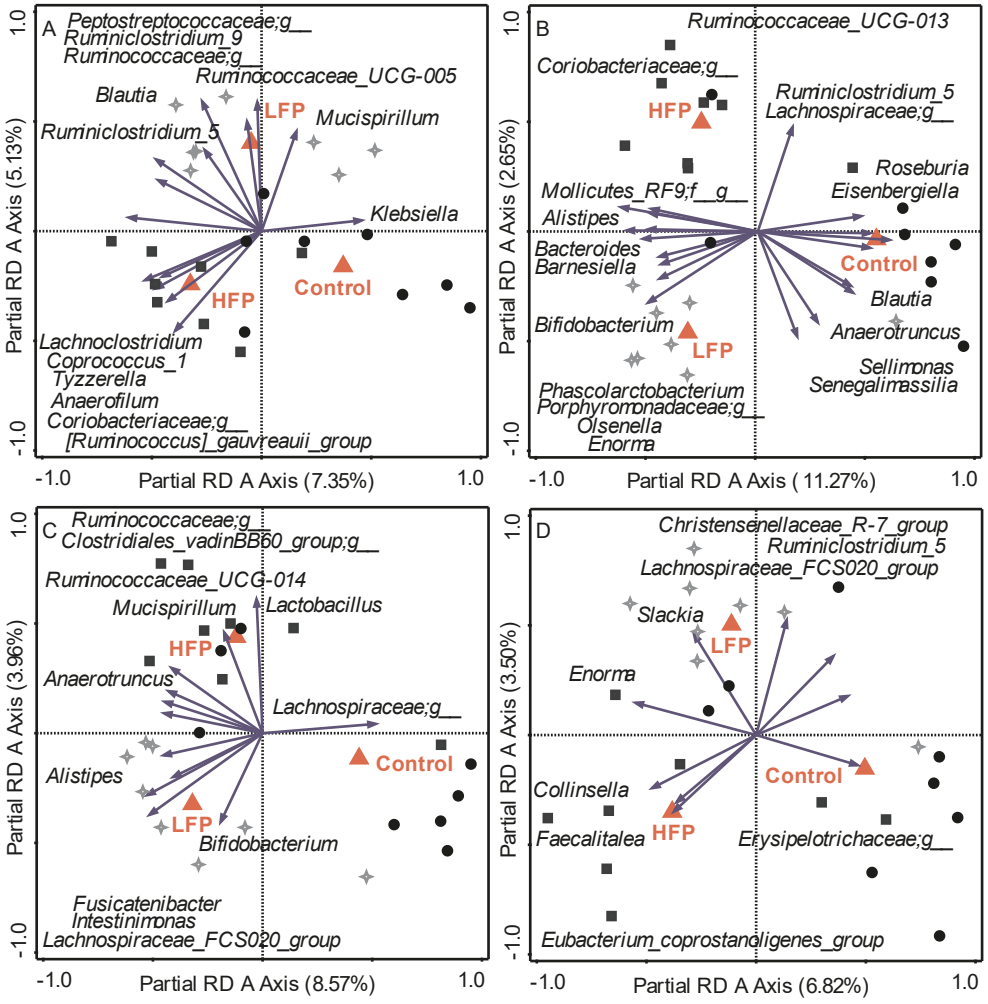

Figure 4. Triplot for partial RDA analysis of caecum microbiota composition of the high feather pecking (HFP; A and B) line and the low feather pecking (LFP; C and D) line at 5 and 14 days of age. Nominal environmental treatment variables are represented by red triangles. Samples are grouped by treatment: HFP (grey squares), Control (black circles) and LFP (light grey stars). Microbial groups for which the variation in relative abundance in the data is explained for at least 20% by the axes are represented as vectors. Groups that could not be assigned to a specific genus are classified by the family name appended with ";g_".

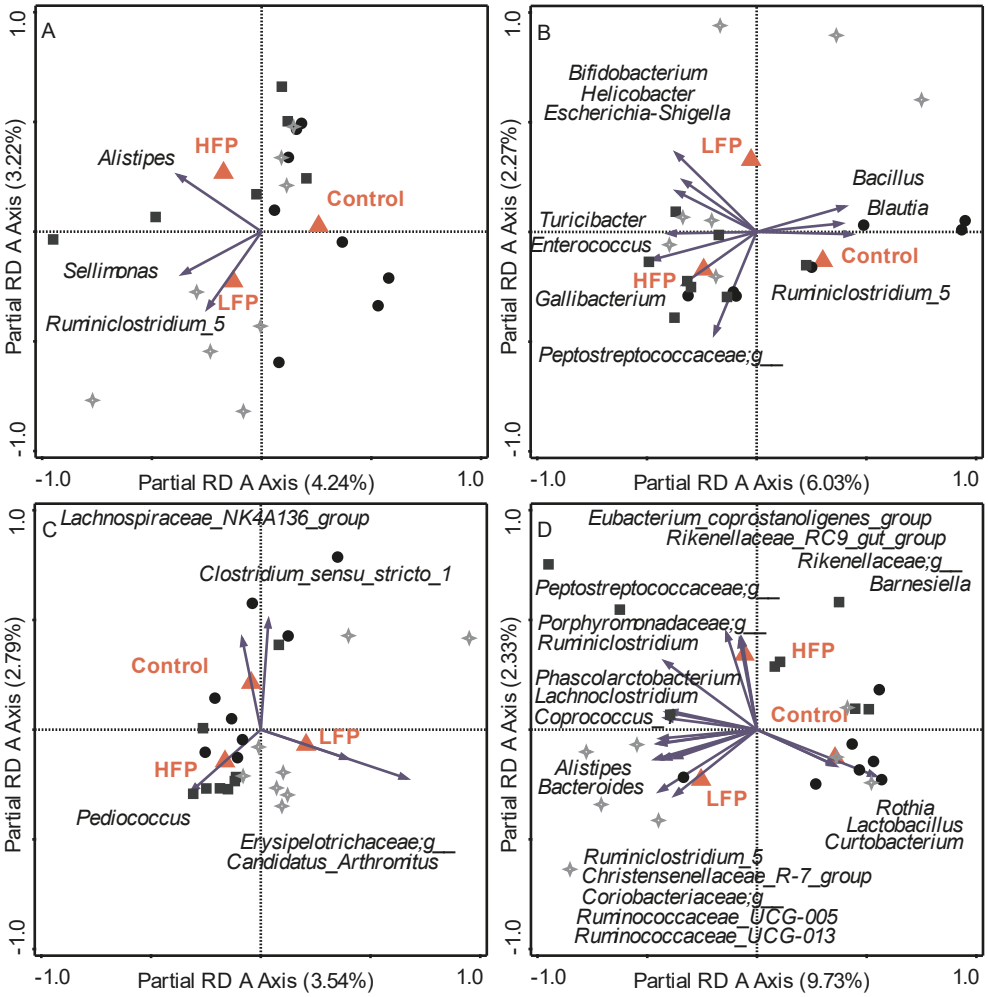

Figure 5. Triplot for partial RDA analysis of colon microbiota composition of the high feather pecking (HFP; A and B) line and the low feather pecking (LFP; C and D) line at 5 and 14 days of age. Nominal environmental treatment variables are represented by red triangles. Samples are grouped by treatment: HFP (grey squares), Control (black circles) and LFP (white stars). Microbial groups for which the variation in relative abundance in the data is explained for at least 15% by the axes are represented as vectors. Groups that could not be assigned to a specific genus are classified by the family name appended with ";g_".

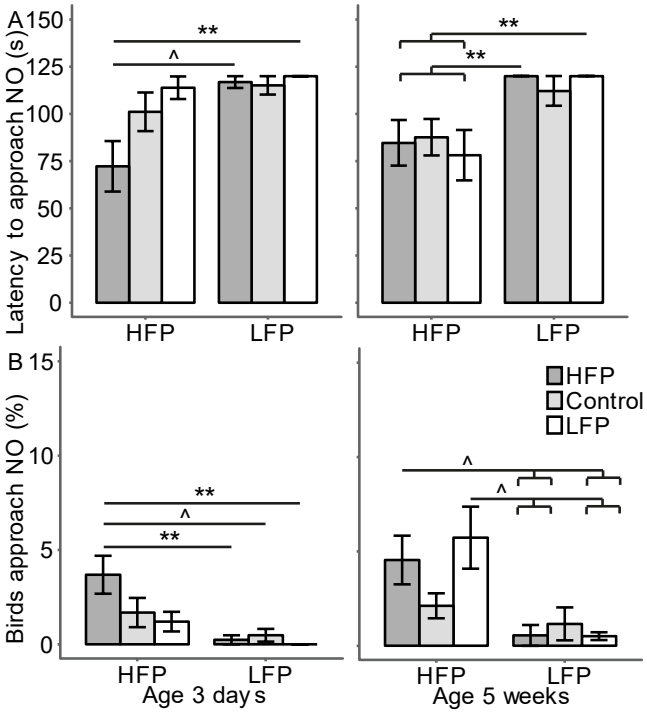

Figure 6. A) Mean latency (± SE) for three birds to approach the novel object (NO) and B) mean percentage (± SE) of birds approaching the NO at 3 days and 5 weeks of age for the high (HFP) and low feather pecking (LFP) lines treated with HFP microbiota, control or LFP microbiota. ^ show tendencies ($P < 0.1$) and ** show significant differences ($P < 0.05$) between line * treatment interactions.

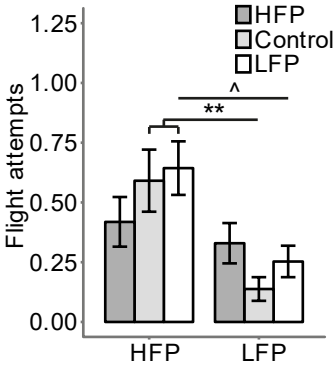

Figure 7. Mean number of flight attempts (± SE) in the novel environment test for the high (HFP) and low feather pecking (LFP) lines treated with HFP microbiota, control or LFP microbiota. ^ show tendencies ($P < 0.1$) and ** show significant differences ($P < 0.05$) between line * treatment interactions.

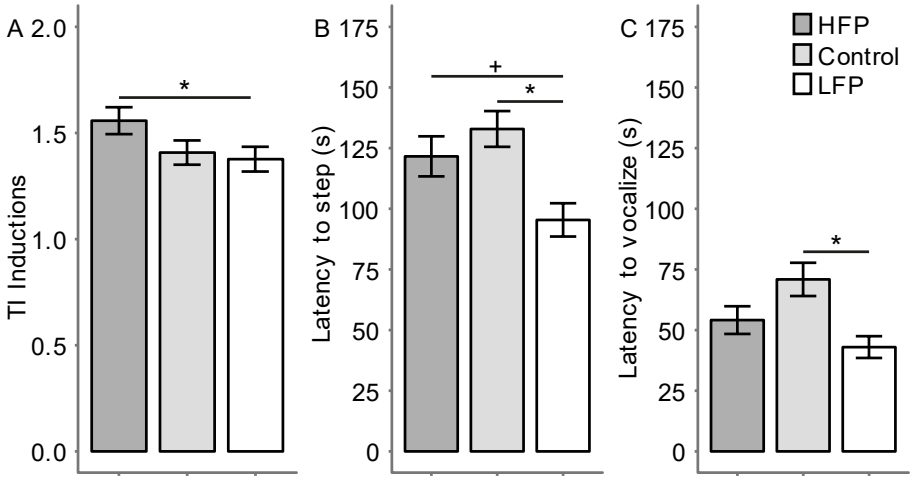

Figure 8. A) Mean number of inductions needed to reach tonic immobility (TI) (± SE) and mean latency (± SE) to B) step and C) vocalize in the open field test for birds treated with high feather pecking (HFP) microbiota, control or low feather pecking (LFP) microbiota. $^{+}$ show tendencies ($P < 0.1$) and * show significant differences ($P < 0.05$) between treatments.

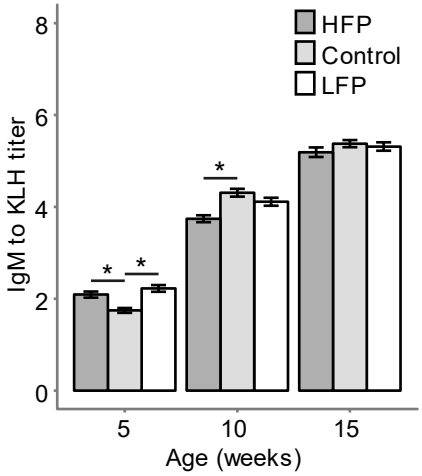

Figure 9. Mean IgM natural antibody titers to keyhole limpet hemocyanin (KLH) (± SE) at 5, 10 and 15 weeks of age for birds treated with high feather pecking (HFP) microbiota, control or low feather pecking (LFP) microbiota. * show significant differences ($P < 0.05$) between treatments.

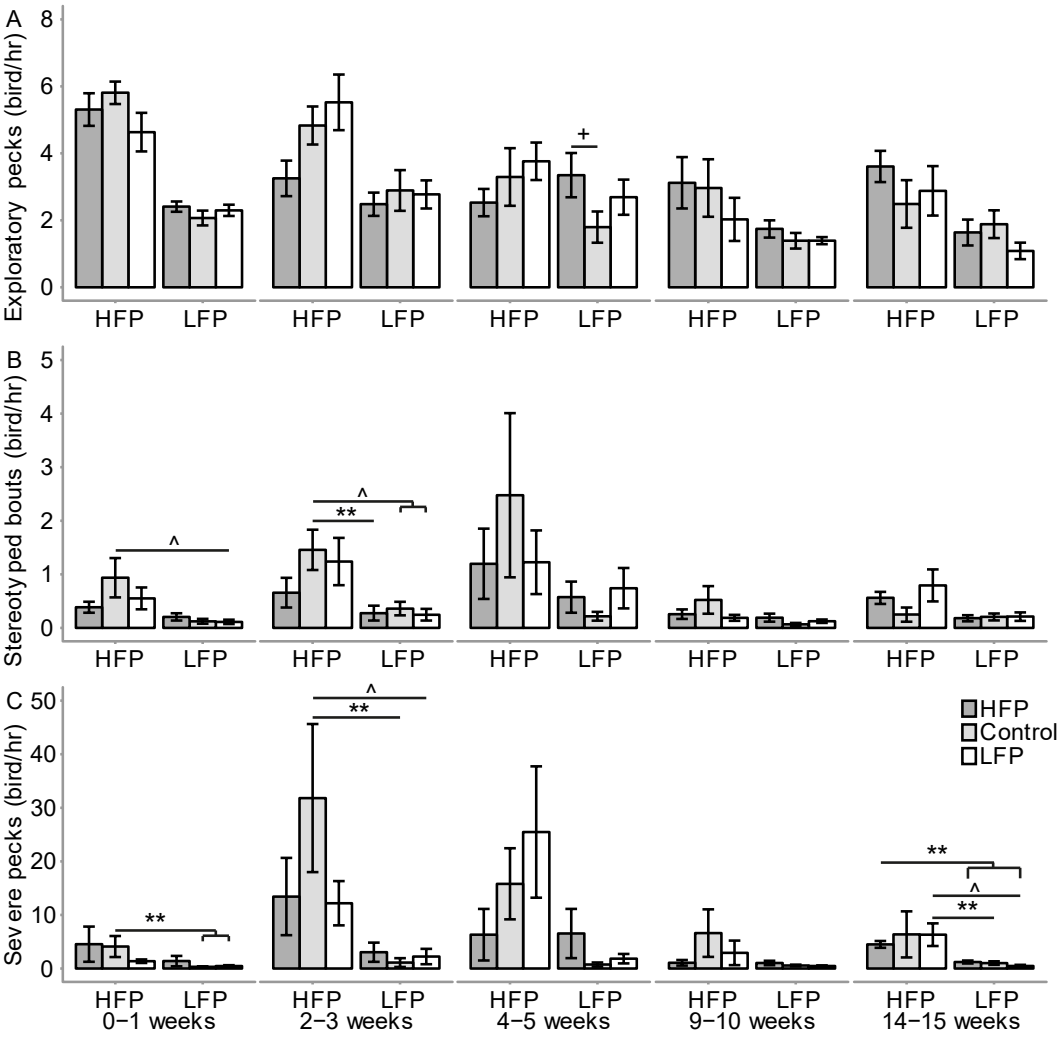

Figure 10. A) Mean exploratory pecks (± SE), B) mean stereotyped bouts (± SE) and C) mean severe pecks (± SE) per bird per hour at 0-1, 2-3, 4-5, 9-10 and 14-15 weeks of age for the high (HFP) and low feather pecking (LFP) lines treated with HFP microbiota, control or LFP microbiota. $^{+}$ show tendencies ($P < 0.1$) between treatments within lines; $^{\wedge}$ show tendencies ($P < 0.1$) and ** show significant differences ($P < 0.05$) between line * treatment interactions.

Chapter 7

General discussion

Understanding the role of gut microbiota in host behaviour and physiology is a rapidly developing field. Most studies to date have focussed on gut microbiota effects in rodents, yet only a limited number of studies focussed on gut microbiota effects in poultry. Moreover, it is unknown whether gut microbiota influences the development of feather pecking (FP), a damaging behaviour in chickens which reduces animal welfare and productivity. Therefore, with this thesis I aimed to identify gut microbiota effects on the development of FP. The first objective was to identify behavioural and physiological characteristics of FP genotypes (i.e. high (HFP) and low (LFP) FP selection lines) and of FP phenotypes (i.e. feather pecker, victim, feather pecker-victim and neutral), where I focused on characteristics that were shown to be influenced by the gut microbiota and to be related to FP. I addressed this objective in **chapters 2, 3, 4** and **5**. The second objective was to identify whether gut microbiota influences the development of FP, where I focused on the same behavioural and physiological characteristics. I addressed this objective in **chapter 6**. In this final chapter I synthesize and discuss my main findings. First, I will discuss behavioural and physiological characteristics of FP genotypes and phenotypes, where I focus on fearfulness, the stress response, the serotonergic system, the immune system and gut microbiota. Second, I will discuss microbiota transplantation effects on the development of FP, where I focus on gut microbiota, fearfulness, the stress response, the serotonergic system, the immune system and FP. In each section I will discuss my findings, compare them with the current knowledge, identify research gaps and suggest further research.

Behavioural and physiological characteristics of feather pecking genotypes and phenotypes

Feather pecking genotypes

FP genotypes clearly differed in behavioural responses, where HFP birds had more active behavioural responses (i.e. approached a novel object sooner and more birds approached it, vocalized sooner and more, showed more flight attempts, stepped sooner and more, and had shorter tonic immobility duration) compared to LFP birds (**chapter 2, 3** and **6**). These findings are supported by previous studies using the FP selection lines (Bögelein et al., 2014; de Haas et al., 2010; Kops et al., 2017). The active behavioural responses suggest lower fearfulness in HFP birds compared to LFP birds, as silence, inactivity and long tonic immobility duration have been related to high fearfulness (Forkman et al., 2007; Jones, 1996). Furthermore, they suggest higher social and exploration motivation in HFP birds compared to LFP birds, as

vocalizing sooner and more has been related to high social motivation, and approaching a novel object sooner and more or stepping sooner and more have been related to high exploration motivation (Forkman et al., 2007; Gallup and Suarez, 1980; Suarez and Gallup, 1983). Moreover, more active behavioural responses might be caused by differences in activity, as previous studies show HFP birds had higher locomotor activity in their home-pen and in a novel environment compared to LFP birds (de Haas et al., 2010; Kjaer, 2009). Overall, differences between FP genotypes in fearfulness, social motivation, exploration motivation and activity are not necessarily mutually exclusive.

For the stress response, HFP birds struggled later and less, but vocalized sooner and more compared to LFP birds during restraint (**chapter 3** and **6**), which is supported by Kops et al. (2017). However, FP genotypes did not differ in corticosterone (CORT) level after restraint, providing no physiological support for the behavioural differences observed (**chapter 3** and **6**). Previously, HFP birds had higher CORT levels after restraint compared to LFP birds (Kjaer and Guémené, 2009). However, for both the behavioural and physiological stress responses there is no consistent difference between FP genotypes in coping style. Coping style is defined as a coherent set of behavioural and physiological stress responses which is consistent over time and situations (proactive vs. reactive, Koolhaas et al., 1999). The struggles and high CORT level after restraint suggest HFP birds were reactive, while the vocalizations suggest they were proactive compared to LFP birds and no difference in CORT level after restraint was found (**chapter 3** and **6**). Moreover, HFP birds had higher heart rate and lower heart rate variability (Kjaer and Jørgensen, 2011), suggesting they were proactive compared to LFP birds. Thus, FP genotypes cannot clearly be categorized into specific coping styles.

With regard to the serotonergic system, whole blood serotonin (5-Hydroxytryptamine or 5-HT) was used as an indicator for central 5-HT (Uitdehaag et al., 2011). HFP birds had lower whole blood 5-HT levels compared to LFP birds (**chapter 3** and **6**). Previously, HFP birds had higher plasma 5-HT levels compared to LFP birds (Buitenhuis et al., 2006), but this discrepancy might be explained by the method used, as whole blood 5-HT more likely reflects storage concentration of 5-HT, while plasma 5-HT reflects unbound 5-HT (Shajib and Khan, 2015). The central and peripheral serotonergic system show similar characteristics in their transporters and receptors (Yubero-Lahoz et al., 2013). Previously, HFP chicks had lower central serotonergic activity compared to LFP chicks in several brain regions (Kops et al., 2017). Yet, at adult age the differences in central serotonergic activity had disappeared or were opposite to what was found at young age (Kops et al., 2017).

Thus, HFP birds seem to have lower central serotonergic activity at young age and lower whole blood 5-HT levels at adult age compared to LFP birds.

For the immune system, nitric oxide production by monocytes was measured as indicator for innate pro-inflammatory immune functioning (Murray and Wynn, 2011; Uehara et al., 2015) and specific antibody level was measured as part of the adaptive immune system. Furthermore, natural (auto)antibody level was measured, as natural antibodies play an essential role in both innate and adaptive immunity (Ochsenbein and Zinkernagel, 2000; Panda and Ding, 2015). Natural antibodies are antibodies that can bind antigen without intentional prior exposure to that antigen, where natural antibodies bind to non-self-antigen and natural autoantibodies bind to self-antigen (Avrameas, 1991; Baumgarth et al., 2005). In addition, immune cell subsets were included to identify whether potential differences in immune characteristics were reflected by differences in immune cell subsets. HFP birds had lower IgM and higher IgG natural (auto)antibody levels, higher nitric oxide production by monocytes and a tendency for higher IgM and IgG specific antibody levels compared to LFP birds, but did not differ in relative abundances of immune cell subsets (**chapter 3, 4** and **6**). Supporting these findings, HFP birds had higher IgG specific antibody levels, although LFP birds were found to have a higher number of white blood cells and higher expression of MHC class I molecules on T and B cells (Buitenhuis et al., 2006). Thus, HFP birds seem to have a more responsive immune system compared to LFP birds.

FP genotypes had distinct luminal microbiota composition, where HFP birds had a higher relative abundance of genera of the order Clostridiales, but lower relative abundance of *Lactobacillus* compared to LFP birds at adult age (**chapter 5**), which is supported by Birkl et al. (2018). It is striking that findings were consistent across studies, suggesting a strong influence of genotype on microbiota composition. However, no clear differences were found in mucosa-associated microbiota composition (**chapter 5**). Luminal microbiota composition might be more determined by environmental effects such as diet, while mucosa-associated microbiota composition might be more determined by host genetics (van den Abbeele et al., 2011). A possible explanation for this discrepancy is that differences in luminal microbiota composition at adult age might arise because of feather eating in the HFP line. Previously, HFP birds ingested more feathers (Harlander-Matauschek and Bessei, 2005; Harlander-Matauschek and Häusler, 2009), differed in ileal and caecal microbial metabolites and had a higher number of feather particles in their gizzards compared to LFP birds (Meyer et al., 2013). Moreover, birds fed feathers in their diet differed from control birds in microbial metabolites and

composition, with a higher number of enterobacteria in the ileum and caecum, and a higher number of clostridia in the caecum (Meyer et al., 2012). Thus, feather eating might alter microbiota composition. Therefore, differences between FP genotypes in microbiota composition could be a consequence of FP.

In summary, these findings suggest that divergent selection on FP not only affects FP but also behavioural responses, peripheral 5-HT, immune responsiveness and microbiota composition, but did not affect CORT level (see Figure 1). However, cause and consequence cannot be disentangled from each other here. Especially since bidirectional communication exists between the gut microbiota and the brain (Grenham et al., 2011), potentially through the stress, serotonergic or immune systems (de Weerth, 2017; O'Mahony et al., 2015; Powell et al., 2017). In turn, these systems can also influence each other (Leonard, 2007; Mössner and Lesch, 1998; O'Mahony et al., 2015). Furthermore, certain (peripheral) indicators of the stress, serotonergic and immune systems were used, but these systems are of course more complex and no central measurements were included. Thus, the brain remains a 'black box', where behaviour was used as a type of output measurement of the brain.

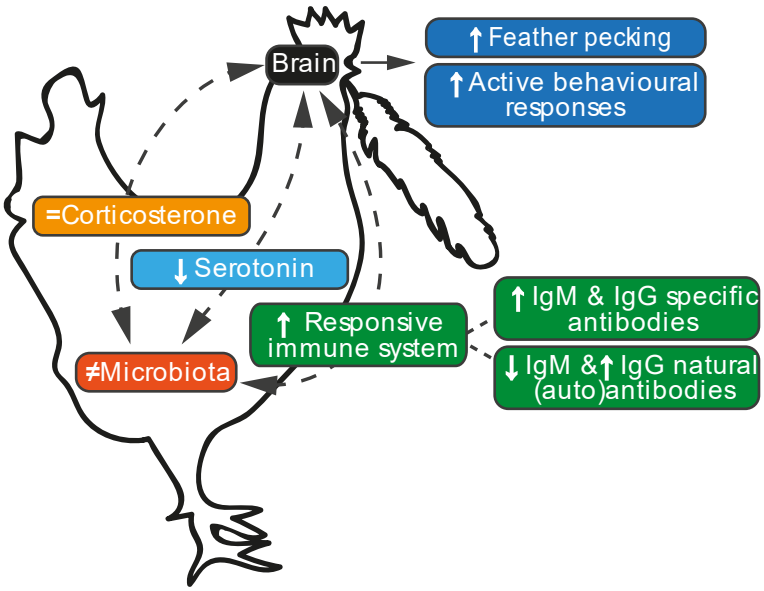

Figure 1. Comparison between HFP vs. LFP birds for behavioural and physiological characteristics as determined in **chapter 2, 3, 4** and **5**.

Many behavioural disorders in humans have been related to altered gut microbiota composition, for example depression, anxiety disorder, autism and attention deficit hyperactivity disorder (ADHD) (Cheung et al., 2019; Felice and O'Mahony, 2017;

Liu et al., 2019), which points to interesting relations between microbiota composition and behavioural abnormalities in mammals. Moreover, these behavioural disorders have also been linked to altered functioning of the stress, serotonergic and immune systems (Dean and Keshavan, 2017; Gold, 2015; Hughes et al., 2018; Kamradt et al., 2018; Muller et al., 2016; Taylor and Corbett, 2014). Although findings with regard to altered functioning of these systems are not always in the same direction for the different behavioural disorders, it still indicates that intricate relationships exist between these systems, the gut microbiota and behavioural abnormalities.

Interestingly, Kjaer (2009) proposed that FP in the HFP line could be seen as a hyperactivity disorder model and there seem to be similarities between ADHD patients and HFP birds. Apart from HFP birds having higher locomotor activity level (Kjaer, 2009) and more active behavioural responses (Bögelein et al., 2014; de Haas et al., 2010; Kops et al., 2017; **chapter 2, 3** and **6**), there are also similarities between characteristics of ADHD patients and HFP birds with regard to the stress, serotonergic and immune systems. For the stress response, ADHD patients generally had higher stress-induced cortisol levels compared to controls (Palma et al., 2012; Raz and Leykin, 2015). Although no difference in CORT level after restraint was found (**chapter 3** and **6**), previously HFP birds had higher stress-induced CORT levels compared to LFP birds (Kjaer and Guémené, 2009). The serotonergic system has been suggested to be involved in both ADHD and FP (Banerjee and Nandagopal, 2015; de Haas and van der Eijk, 2018). ADHD patients generally have lower peripheral 5-HT levels compared to controls (Coleman, 1971; Spivak et al., 1999; Wang et al., 2018), similar to HFP birds compared to LFP birds (**chapter 3** and **6**). For the immune system, many studies find positive associations between ADHD and inflammatory or autoimmune disorders, suggesting an altered immune response in ADHD patients (Leffa et al., 2018). Furthermore, ADHD patients had higher total IgE and IgG levels and tended to have lower levels of the cytokine interleukin-5 (IL-5) compared to controls, although no differences were found for other cytokines (i.e. IL-1β, IL-6, IL-8, IL-10 or tumour necrosis factor) (Verlaet et al., 2019). The higher IgG levels are similar to findings for HFP birds compared to LFP birds (Buitenhuis et al., 2006; **chapter 3, 4** and **6**). Thus, similarities seem to exist between characteristics of ADHD patients and of HFP birds. However, it remains unknown whether these similarities are caused by similar underlying mechanisms or might be a consequence of differences in activity.

Feather pecking phenotypes

FP phenotypes differed in behavioural responses, where feather peckers tended to have more active behavioural responses (i.e. more flight attempts and stepped more) compared to victims and neutrals at young age (**chapter 2**), which is supported by previous studies (Bögelein et al., 2014; Jensen et al., 2005). More active behavioural responses suggest lower fearfulness and higher exploration motivation in feather peckers. Furthermore, victims had more active responses (i.e. more flight attempts) compared to neutrals at young age (**chapter 2**), suggesting lower fearfulness in victims. However, previously the performance of FP was positively related to fearfulness (Vestergaard et al., 1993) and receiving FP showed no clear relation with fearfulness (Bögelein et al., 2014). These discrepancies might be caused by the fact that these studies identified correlations between FP and fearfulness, instead of identifying differences between FP phenotypes (**chapter 2**). Moreover, more active behavioural responses of feather peckers and victims might be caused by differences in activity. Yet, differences between FP phenotypes in fearfulness, exploration motivation and activity are not necessarily mutually exclusive.

For the stress response, feather peckers tended to vocalize less compared to neutrals, while victims struggled sooner, vocalized sooner and more compared to other phenotypes (**chapter 3**). These findings suggest feather peckers were reactive, while victims were proactive. Yet, previously feather peckers showed more jumps during restraint compared to non-feather peckers (Jensen et al., 2005), suggesting feather peckers were proactive. Furthermore, FP phenotypes did not differ in CORT level after restraint (**chapter 3**), which is supported by previous studies (Daigle et al., 2015; Jensen et al., 2005). However, poor plumage condition was related to low CORT level after restraint (Jensen et al., 2005), suggesting victims were proactive. This discrepancy might be caused by birds with poor plumage condition being either a victim or a feather pecker-victim, making it difficult to disentangle between effects being caused by receiving or performing FP. Thus, it seems that victims are proactive, but other phenotypes cannot clearly be categorized into specific coping styles.

With regard to the serotonergic system, feather peckers had higher whole blood 5-HT levels compared to neutrals at adult age (**chapter 3**). Previously, neutrals had higher whole blood 5-HT levels compared to victims and feather pecker-victims (Daigle et al., 2015) and feather peckers had higher central serotonergic activity compared to neutrals at adult age (Kops et al., 2013). Thus, feather peckers seem to have higher whole blood 5-HT levels, which might be explained by feather peckers

eating feathers causing the gut to release 5-HT, as I will explain in more detail in the next section.

For the immune system, FP phenotypes did not differ in IgM or IgG natural antibody level (**chapter 3**). Previously, feather peckers showed upregulation or downregulation of genes involved in immune defence compared to neutrals (Brunberg et al., 2011), indicating that FP phenotypes might differ with regard to immune system functioning. However, further research is needed to identify whether FP phenotypes might differ in other immune characteristics.

With regard to microbiota composition, I focused on feather peckers and neutrals in order to identify microbiota composition in relation to performing FP and to neither performing nor receiving FP. However, feather peckers and neutrals did not differ in mucosa-associated or luminal microbiota composition, which might be caused by the relatively low sample sizes for FP phenotypes (**chapter 5**). These findings suggest that the actual performance of FP might not be related to microbiota composition.

In summary, these findings suggest that both performing and receiving FP is related to more active behavioural responses. Furthermore, performing FP is related to high whole blood 5-HT level, but FP phenotypes did not differ in CORT level after restraint, natural antibody level or microbiota composition (see Figure 2). Similar to findings for FP genotypes, cause and consequence cannot be disentangled from each other here and certain (peripheral) indicators were measured for the stress, serotonergic and immune systems, but these systems are more complex. Therefore, further research is needed to identify these systems in more detail in relation to FP phenotypes, especially the serotonergic system.

Feather pecking genotypes vs. phenotypes

When comparing findings from FP genotypes to those from FP phenotypes, there is a similar relation between high FP and behavioural responses. HFP birds had more active responses to behavioural tests (Bögelein et al., 2014; de Haas et al., 2010; Kops et al., 2017; **chapter 2, 3** and **6**) and were further shown to have higher locomotor activity in their home pen and in a novel environment compared to LFP birds (de Haas et al., 2017a; Kjaer, 2009). Kjaer (2009) suggested that FP in the HFP line might be linked to changes in intrinsic motivation, which either directly or indirectly leads to higher locomotor activity. When HFP birds are indeed more active in general because of altered intrinsic motivation, this might result in more active responses to any type of behavioural test. Thereby, activity level could be used as a potential indicator for FP at group level.

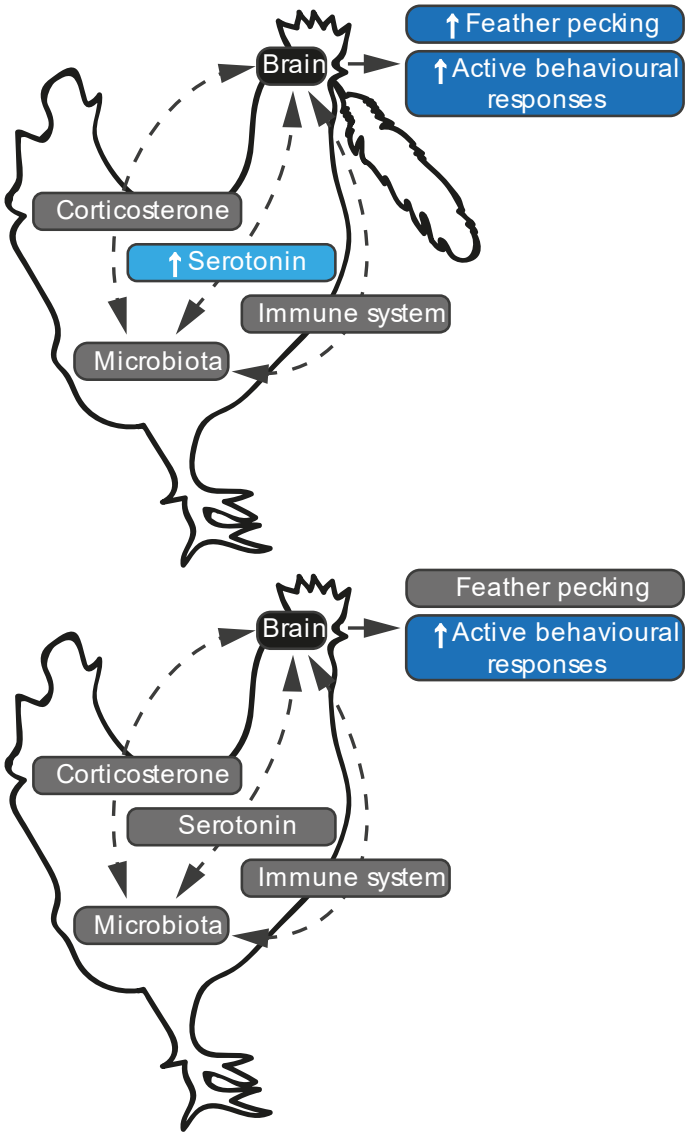

Figure 2. Top) comparison between feather peckers vs. neutrals, bottom) comparison between victims vs. other phenotypes for behavioural and physiological characteristics as determined in **chapter 2, 3** and **5**.

Indeed, several studies have indicated relations between activity level and FP, although not all studies report the same direction. Using structural equation models, Lutz et al. (2016) indicated that an increase in locomotor activity resulted in higher FP. Furthermore, a positive correlation was found between foraging behaviour at young age and FP at adult age (Newberry et al., 2007), suggesting high activity level at young age was related to high FP. However, low correlations between FP and locomotor activity or foraging activity have also been found (Bessei et al., 2018; Grams et al., 2015; Lutz et al., 2016), suggesting no relation between activity level

and FP. Thus, further research is needed to identify whether activity levels could be used to identify FP in a group, especially in commercial lines and settings.

Interestingly, activity level has been related to tail biting in pigs, a damaging behaviour which involves chewing on and biting tails of conspecifics and which was suggested to have similar underlying principles and mechanisms as FP in laying hens (Brunberg et al., 2016). High activity was observed in pens with high levels of tail biting (Ursinus et al., 2014a) and 5 days before tail damage occurred, tail damage pens had higher activity levels compared to control pens (Larsen et al., 2019), suggesting increased activity is related to tail biting and might be a potential predictor of tail biting in a group.

Similar to HFP birds having more active responses compared to LFP birds, feather peckers had more active responses compared to neutrals (**chapter 2**), non-feather peckers (Jensen et al., 2005) and a high number of feather pecks given tended to be related to more active responses in the HFP line (Bögelein et al., 2014). Furthermore, victims had more active responses compared to other phenotypes (**chapter 2** and **3**). Thus, feather peckers and victims had more active behavioural responses. Thereby, activity level might be used as indicator for individuals that perform or receive FP when measured at individual level. Since feather peckers had more active responses at young age (**chapter 2**), it would further be interesting to identify whether activity level at a young age could be used to predict which individuals will become feather peckers. Furthermore, Newberry et al. (2007) showed that birds that performed more foraging behaviour when young were more likely to become feather peckers as adults, indicating that high activity at young age is related to high FP at adult age. Further research is needed to identify whether activity levels could be used to identify feather peckers and victims within a group and to identify whether activity level at young age can be used as predictor for FP at adult age. Interestingly, preliminary findings indeed show that FP phenotypes differed in activity level, where adult feather peckers walked a longer distance compared to victims in a novel environment test (de Haas et al., 2017b).

There is an opposite relation between high FP and whole blood 5-HT level, where HFP birds had lower whole blood 5-HT levels compared to LFP birds (**chapter 3** and **6**), while feather peckers had higher whole blood 5-HT levels compared to neutrals (**chapter 3**). Previously, HFP chicks had lower central serotonergic activity compared to LFP chicks in several brain regions (Kops et al., 2017). However, at adult age the differences between FP genotypes in central serotonergic activity had disappeared or were opposite to what was found at young age (Kops et al., 2017). Interestingly, feather peckers also had higher central serotonergic activity compared

to neutrals (Kops et al., 2013). Thus, the actual performance of FP might increase peripheral 5-HT level in feather peckers, possibly due to feather eating. HFP birds were more prone to eat feathers compared to LFP birds (Harlander-Matauschek and Bessei, 2005; Harlander-Matauschek and Häusler, 2009) and feather peckers showed more feather eating compared to non-peckers (McKeegan and Savory, 2001). Ingestion of feathers may increase peripheral 5-HT level by providing structural components, as the gut releases 5-HT in reaction to sensory perception of the mucosal layer (Coates et al., 2017). However, this relation between feather eating and increased peripheral 5-HT level remains speculative and needs further investigation. Since birds already started to feather peck at a young age (**chapter 3**), I cannot distinguish between cause or consequence of FP in relation to peripheral 5-HT level here. Therefore, it would be interesting to identify whole blood 5-HT level in birds prior to and after the development of FP.

Similar to findings for FP genotypes, FP phenotypes did not differ in CORT level after restraint, indicating that the stress response might not be related to FP in FP genotypes and phenotypes. Furthermore, although differences between FP genotypes were found for the immune system and microbiota composition, no such differences were identified for FP phenotypes. This might indicate that differences in immune characteristics and microbiota composition are more related to genotype than to actual FP behaviour. However, there are limitations to the categorization of FP phenotypes, as described in the next paragraph, which might have caused differences to be less pronounced between FP phenotypes.

Limitations of feather pecking observations and feather pecking phenotypes

A limitation in all my experiments was that FP was observed for a limited amount of time (ranging between 30 - 60 min) at various ages, which might have led to FP behaviour not being observed. Of course continuous observation is impractical, but what is clearly missing in literature is a study that identifies the observation time and moment of observation during the day (i.e. morning or afternoon) needed to get an indication of the level of FP in a group or of an individual. Most studies to date identified FP based on feather damage or on observations ranging between 10 – 420 min at group level and 5 – 30 min at individual level, with observations at one age and sometimes including both morning and afternoon observations. Creating consistency between FP observation methods will probably improve our understanding of factors related to FP and will further improve identification of potential causal factors. Furthermore, as FP is performed by only a few individuals (Newberry et al., 2007) it is essential to identify factors in individuals, because they

can become a feather pecker, feather pecker-victim, victim or neutral (i.e. FP phenotypes) (Daigle et al., 2015). This is especially important since FP phenotypes differed in behavioural responses and whole blood 5-HT level (**chapter 2** and **3**). Furthermore, relations between high FP and behavioural or physiological characteristics were not always in the same direction for comparisons between HFP and LFP birds or between feather peckers and victims or neutrals. However, it should be noted that FP phenotypes can switch and are therefore not consistent over time (Daigle et al., 2015), indicating the importance of FP observations being performed at various ages. Furthermore, for FP phenotypes there is also the need for consistent identification and categorization methods in order to identify potential causal factors. An objective measure to identify individuals performing FP would be ideal, as it eliminates any inter and intra observer issues, for example activity level. Whether activity level can be used to identify FP in a group or even at individual level is currently being studied.

Divergent selection on feather pecking vs. no selection on feather pecking

When comparing selection for (i.e. HFP) and against FP (i.e. LFP) to no selection (i.e. the unselected control line, CON), CON birds were similar to LFP birds in their behavioural responses and FP, especially at young ages, but CON and LFP birds differed from HFP birds (**chapter 2** and **3**). CON birds were also similar to LFP birds with regard to whole blood 5-HT and IgM natural antibody levels, but CON and LFP birds differed from HFP birds. However, CON birds had higher IgG natural antibody levels compared to LFP birds, but did not differ from HFP birds (**chapter 3**). Thus, selection for FP seems to be more effective in altering behavioural and physiological characteristics compared to selection against FP. This might be explained by the change in phenotypic variability seen after some generations of selection when the mean level of FP becomes low (Kjaer et al., 2001). FP is a threshold trait (Kjaer and Jørgensen, 2011) and when the general level of FP is low, most birds will not show any FP even if they differ in their genetic propensity to perform FP. This makes it impossible to distinguish feather peckers from neutrals for selection and the selection for less FP is no longer effective. Thereby, selection for FP might cause stronger alterations to the genotype, ultimately resulting in stronger differences in behavioural and physiological characteristics compared to selection against FP.

Microbiota and the development of feather pecking

Since FP genotypes had distinct gut microbiota composition, but FP phenotypes did not, for the second objective I focussed on FP genotypes. The difference between FP genotypes in microbiota composition was used to create a HFP and LFP microbiota pool. In **chapter 6** I identified effects of early-life microbiota transplantation on FP and on behavioural and physiological characteristics related to FP. Newly hatched HFP and LFP chicks received a control treatment, HFP or LFP microbiota daily during the first two weeks post hatch.

Microbiota

Early-life microbiota transplantation had limited effects on microbiota composition. However, previous studies that identified effects of microbiota transplantation on behaviour did find effects on microbiota composition (Kraimi et al., 2019; Yang et al., 2019). In pseudo germ-free rats (i.e. received antibiotic treatment prior to transplantation), microbiota transplantation altered diversity, composition and relative abundances of class, order, family and genus level microbial groups (Yang et al., 2019). Furthermore, in germ-free quails, microbiota transplantation altered diversity, evenness and relative abundances of phylum and family level microbial groups (Kraimi et al., 2019). However, both studies used (pseudo) germ-free animals and identified microbiota composition from faecal samples. Using (pseudo) germ-free animals allows for stronger potential effects of microbiota transplantation on microbiota composition, as these animals are sterile or depleted of microbiota, but are rather extreme. Furthermore, identifying microbiota composition from different gut sections (i.e. ileum, caecum or colon) is crucial, as microbiota composition of faecal samples is variable because faeces can originate from different gut sections (Sekelja et al., 2012), which have been shown to differ in microbiota composition (Awad et al., 2016; Lu et al., 2003). Thus, differences found might be caused by faecal samples originating from different gut sections. Redundancy analysis was used to identify differences in microbiota composition (**chapter 5** and **6**), as one can question whether differences in relative abundances of genus level microbial groups are biologically relevant, since species within a genus or even strains within a species can have opposite effects on the host. For example, *Escherichia coli* species include probiotic strains that benefit host health (Wassenaar, 2016), but also pathogenic strains which are disadvantageous to host health (Hu and Torres, 2015). Thus, it is more important to focus on overall microbiota composition, than on relative abundances of specific family or genus level microbial groups.

A potential explanation for why limited effects on microbiota composition were found could be that transplantation pools consisted of adult microbiota composition. Adult microbiota composition might not be able to settle and remain within the gut of newly hatched chicks, since the gut microbiota is still developing and undergoes a rapid succession until it is fully developed and stable around 7 weeks of age (Ballou et al., 2016; Hill et al., 2011). Furthermore, luminal content was sampled instead of mucosal scrapings. Mucosa-associated microbiota composition might be more involved in communication to the host because of its close proximity (Ouwerkerk et al., 2013) and was further shown to differ from luminal microbiota composition (Awad et al., 2016; Olsen et al., 2008). Mucosa-associated microbiota composition might be altered by microbiota transplantation, although FP genotypes did not differ in mucosa-associated microbiota composition (**chapter 5**) and luminal microbiota was used for transplantation (**chapter 6**).

Although limited effects of early-life microbiota transplantation on microbiota composition were found, microbiota transplantation did affect behavioural responses, natural antibody level and whole blood 5-HT level. Microbiota transplantation may have influenced brain, immune and serotonergic system functioning. Microbiota transplantation was given during the first two weeks post hatch, as both the brain and immune system are still developing (Atkinson et al., 2008; Fellah et al., 2014). Extensive evidence suggests microbiota affects functioning of the brain, and the central serotonergic and immune systems (Burokas et al., 2017; O'Mahony et al., 2015; Sommer and Bäckhed, 2013) and first indications show that microbiota influences peripheral serotonergic system functioning as well (Sjögren et al., 2012). However, it should be noted that other factors in the transplantation pools might have contributed to altered behaviour, natural antibody level and whole blood 5-HT level, for example viruses or fungi (Cadwell, 2015; Enaud et al., 2018). Still, it seems that microbiota transplantation influenced functioning of the brain, and the serotonergic and immune systems, which (in)directly resulted in differences in behavioural responses, natural antibody level and whole blood 5-HT level.

Fearfulness

During treatment, HFP chicks receiving HFP microbiota tended to show more active behavioural responses (i.e. approached novel object sooner and more birds approached it, vocalized sooner) compared to HFP chicks receiving LFP microbiota or control treatment. Similarly, after treatment LFP birds receiving LFP microbiota showed more active behavioural responses (i.e. stepped and vocalized sooner)

compared to LFP birds receiving HFP microbiota or control treatment. These active behavioural responses suggest low fearfulness in birds receiving microbiota from their own line (i.e. homologous transplantation), as silence and inactivity have been related to high fearfulness (Forkman et al., 2007; Jones, 1996; Suarez and Gallup, 1983). However, no effects were found on tonic immobility duration, the measure for innate fearfulness in chickens (Forkman et al., 2007).

Manipulating microbiota composition via anti-, pro-, pre- or synbiotics was shown to alter anxiety-like behaviour in rodents. Antibiotic treatment increased (Ceylani et al., 2018), reduced (Desbonnet et al., 2015) or did not alter anxiety-like behaviour in rodents (Hoban et al., 2016; O'Mahony et al., 2014). Pro-, pre- and synbiotic treatment generally reduced anxiety-like behaviour in rodents (Barrera-Bugueño et al., 2017; Bravo et al., 2011; Burokas et al., 2017; Liu et al., 2016a; Liu et al., 2016b; Savignac et al., 2014) and fearfulness in quails (Parois et al., 2017). Thus, similar as homologous transplantation, manipulating microbiota composition via pro-, pre- or synbiotic treatment reduced anxiety-like behaviour, although findings with regard to antibiotic treatment were less consistent.

Early-life microbiota transplantation was shown to affect fearfulness in quails (Kraimi et al., 2019). Microbiota from lines divergently selected on tonic immobility duration was given to germ-free quails of the line selected on long tonic immobility duration. Opposite to findings at young age (**chapter 6**), birds receiving homologous transplantation showed increased fearfulness early in life (Kraimi et al., 2019). Yet, this effect was reversed later in life, with birds receiving homologous transplantation showing reduced fearfulness (Kraimi et al., 2019), which is similar to findings at adolescent age (**chapter 6**). However, it should be noted that these lines were selected on tonic immobility duration and included no control treatment to account for potential effects of handling during treatment. Overall, microbiota transplantation affects fearfulness in poultry, but effects are dependent on age and genotype.

Stress response

LFP birds receiving LFP microbiota tended to have more active stress responses (i.e. vocalized sooner) compared to LFP birds receiving HFP microbiota or control treatment. However, microbiota transplantation did not influence CORT levels after restraint, suggesting differences in behavioural responses might not be related to stress. Similarly, manipulating microbiota composition via anti-, pro- or prebiotic treatment did not affect stress-induced CORT levels in rodents (Barrera-Bugueño et al., 2017; Ceylani et al., 2018; Desbonnet et al., 2015; Hoban et al., 2016; Liu et al.,

2016a; Savignac et al., 2014; Sudo et al., 2004; Tarr et al., 2015), except for two studies using probiotic treatment and one study using prebiotic treatment where stress-induced CORT level was reduced (Ait-Belgnaoui et al., 2014; Bravo et al., 2011; Burokas et al., 2017). Inconsistency between results might be explained by different treatments used, still the majority of studies report no effect on stress-induced CORT level. Furthermore, microbiota transplantation had no effect on stress-induced CORT level in mice (Kelly et al., 2016). Thus, microbiota transplantation does not seem to affect the physiological stress response and had limited effects on the behavioural stress response.

Other interpretations of behavioural responses

Several other factors could have influenced bird's responses to behavioural tests, such as 1) coping style, 2) social motivation, 3) exploration and 4) activity.

1) The shorter latency to vocalize during restraint suggests LFP birds receiving LFP microbiota were more proactive compared to LFP birds receiving HFP microbiota or control treatment. However, there was no effect of microbiota transplantation on CORT levels after restraint. In addition, the manual restraint test was only performed once (**chapter 6**), therefore consistency of behavioural and physiological stress responses cannot be assessed. Thus, further research is needed to identify whether microbiota transplantation influences coping style.

2) Birds receiving homologous transplantation vocalized sooner, suggesting higher social motivation, compared to birds receiving microbiota from the other line or control treatment. Indeed, gut microbiota seems to influence social motivation, as germ-free mice were found to have reduced (Desbonnet et al., 2014) or increased social motivation (Arentsen et al., 2015) compared to conventional or specific pathogen free mice. However, germ-free quails did not differ from colonized quails in time spent near conspecifics in a social separation test (Kraimi et al., 2018). Oxytocin and vasopressin play a role in social motivation (Gordon et al., 2011; Insel, 2010) and antibiotic treatment reduced hypothalamic mRNA levels of these neuropeptides in mice (Desbonnet et al., 2015), suggesting that depletion of microbiota alters their activity and potentially alters host social motivation. Microbiota may alter host social behaviour to maximize their own fitness, for example by increasing their ability to access new hosts or environments (Archie and Tung, 2015). Further research is needed to identify effects of manipulating microbiota or microbiota transplantation on social motivation.

3) Birds receiving homologous transplantation approached a novel object sooner and more birds approached it, and stepped sooner in an open field test, suggesting higher

exploration motivation compared to birds receiving microbiota from the other line or control treatment. Indeed, gut microbiota influences exploratory behaviour, as germ-free mice travelled for longer distances and spent more time in the centre of a novel environment (Arentsen et al., 2015) and germ-free quails spent more time near a novel object (Kraimi et al., 2018). Similarly, antibiotic treatment increased exploratory behaviour in mice (Bercik et al., 2011a) and probiotic treatment reduced latency to the centre of a novel environment (Savignac et al., 2014), although this was dependent on the probiotic strain used and probiotic treatment did not affect distance travelled. Thus, manipulating microbiota composition via anti- or probiotic treatment seems to increase exploratory behaviour. Moreover, germ-free mice receiving microbiota from another strain showed exploratory behaviour similar to that of the donor strain (Bercik et al., 2011a) and germ-free quails receiving microbiota from another line showed more exploratory behaviour early in life compared to quails that received homologous transplantation, although this was reversed later in life (Kraimi et al., 2019). Thus, microbiota transplantation affects exploratory behaviour, but effects are dependent on age and genotype. Similar to the effects on host social behaviour, microbiota may manipulate host exploratory behaviour to maximize their fitness for example, by increasing their ability to access new environments.

4) Birds receiving homologous transplantation had more active responses (i.e. shorter latencies to vocalize, step and approach a novel object, and more birds approached the novel object), suggesting increased activity, compared to birds receiving microbiota from the other line or control treatment. Indeed, gut microbiota affects activity, as germ-free mice were more active compared to conventionalized germ-free mice or specific pathogen free mice (Arentsen et al., 2015; Nishino et al., 2013), but germ-free quails were less active compared to colonized quails (Kraimi et al., 2018). Similarly, antibiotic treatment reduced (Ceylani et al., 2018) or did not alter activity in mice (Bercik et al., 2011a; O'Mahony et al., 2014). Probiotic treatment reduced (Nishino et al., 2013; Savignac et al., 2015) or increased activity in mice (Liu et al., 2016a; Liu et al., 2016b), but did not alter activity in quails (Parois et al., 2017). Furthermore, prebiotic treatment did not alter activity in mice (Burokas et al., 2017). Discrepancies between findings might be explained by different anti- or probiotics being used. Thus, manipulating microbiota composition can influence activity, but effects are inconsistent even within treatments used. However, microbiota transplantation did not affect activity in quails (Kraimi et al., 2019). Thus, findings with regard to microbiota transplantation affecting activity remain

inconsistent and further research is needed to identify whether microbiota transplantation affects activity.

Differences in fearfulness, social motivation, exploration or activity are not necessarily mutually exclusive. Overall, microbiota transplantation seems to influence fearfulness, social motivation, exploration and activity.

Are recipients adopting behaviour of donors?

During treatment, behavioural responses seem to be adopted from donors in the HFP line, as HFP chicks receiving HFP microbiota tended to show more active responses compared to HFP chicks receiving LFP microbiota or control treatment, and HFP birds had more active responses compared to LFP birds in various behavioural tests (de Haas et al., 2010; Kops et al., 2017; **chapter 2, 3** and **6**). In contrast, after treatment, behavioural responses do not seem to be adopted from donors in the LFP line, as LFP birds receiving LFP microbiota showed more active responses compared to LFP birds receiving HFP microbiota or control treatment. Rodent studies show that behavioural profiles of donors are adopted by recipients via microbiota transplantation (Bercik et al., 2011a; Collins et al., 2013; Kelly et al., 2016; Yang et al., 2019). Similar to findings from **chapter 6**, quails adopted the behavioural profile of donors early in life, but this was reversed later in life (Kraimi et al., 2019), suggesting that microbiota transplantation effects depend on age in poultry. LFP birds receiving LFP microbiota might not respond like donors because the LFP pool had a higher relative abundance of the order Lactobacillales and particularly of *Lactobacillus* compared to the HFP pool. In rodents, *Lactobacillus* increased locomotor activity and reduced anxiety-like behaviour (Bravo et al., 2011; Liu et al., 2016a; Miyazaki et al., 2014; Wang et al., 2015). However, microbiota composition did not differ between treatments within the LFP line (**chapter 6**). Thus, it is unlikely that behavioural responses were influenced by *Lactobacillus*. Overall, it seems that behavioural profiles are adopted via microbiota transplantation, but these effects depend on genotype and age.

Serotonergic system

LFP birds receiving HFP microbiota tended to have lower whole blood 5-HT levels compared to LFP birds receiving control treatment. Previously, manipulating microbiota composition via anti- or probiotics altered central and peripheral 5-HT levels. Antibiotic treatment reduced colonic and hippocampal 5-HT level (Ge et al., 2017; Hoban et al., 2016), but no effects on small intestinal or colonic 5-HT level has also been reported in rodents (Bercik et al., 2011a). Heat killed probiotic

treatment increased colonic 5-HT level in mice (Hara et al., 2018) and probiotic treatment increased 5-HT level in the striatum and prefrontal cortex, but no effects on 5-HT level in several brain regions has also been found in mice (Liu et al., 2016a; Liu et al., 2016b). Furthermore, probiotic treatment increased 5-HT level in raphe nuclei, but did not affect plasma 5-HT level in broilers (Yan et al., 2018). Thus, overall antibiotic treatment seems to reduce peripheral and central 5-HT levels, while probiotic treatment seems to increase these levels. However, microbiota transplantation did not influence small intestinal or colonic 5-HT levels in mice (Bercik et al., 2011a). Thus, there is inconsistency in findings with regard to microbiota transplantation affecting peripheral 5-HT level.

It is interesting that LFP birds receiving HFP microbiota had lower whole blood 5-HT levels, as HFP birds had lower whole blood 5-HT levels compared to LFP birds (**chapter 3** and **6**). However, these effects do not seem to be explained by the higher relative abundance of Clostridiales in the HFP pool, as genera of the order Clostridiales were positively related to high whole blood 5-HT level (**chapter 5**) and in general Clostridia were found to modulate 5-HT metabolism in the gut for example, by activating 5-HT synthesis (Yano et al., 2015). Effects might be explained by the lower relative abundance of *Lactobacillus* in the HFP pool, as *Lactobacillus* increased central 5-HT level in rodents, although also no effects on central 5-HT level was found (Liu et al., 2016a; Liu et al., 2016b). Thus, there is inconsistency in findings with regard to effects of *Lactobacillus* on central 5-HT level and no studies to date have identified effects of *Lactobacillus* on peripheral 5-HT level. Overall, microbiota transplantation effects on peripheral 5-HT might not be explained by differences in HFP and LFP pools' microbiota composition. Especially since microbiota composition did not differ between treatments within the LFP line. It remains unknown whether microbiota transplantation influences brain neurotransmission in poultry, for example serotonergic and dopaminergic neurotransmission. Further research should identify effects of microbiota transplantation on these systems, especially in relation to FP as they were suggested to be involved in the development of FP (de Haas and van der Eijk, 2018).

Immune system

Manipulating microbiota composition via pro- or prebiotic treatment alters immune system functioning in mammals (Peters et al., 2019; Yousefi et al., 2019) and increasing evidence suggests the same in poultry (Adhikari and Kim, 2017). Chicks receiving LFP microbiota had higher IgM natural antibody levels compared to chicks receiving control treatment. Similarly, manipulating microbiota composition via

probiotic treatment increased IgM, but also IgG natural antibody level in broilers (Haghighi et al., 2006). Yet, anti- or prebiotic treatment did not alter IgG natural antibody level in broilers (Shang et al., 2015). Thus, manipulating microbiota composition seems to increase IgM natural antibody level, but findings are inconsistent for IgG natural antibody level. Interestingly, IgG natural antibodies were suggested to be dependent upon exogenous antigen stimulation, while IgM natural antibodies were not (Holodick et al., 2017). Thus, IgM natural antibody level may be more under genetic influence, while IgG natural antibody level may reflect immunomodulating environmental influences. Based on this one would expect effects of microbiota transplantation or manipulation on IgG natural antibody level. LFP birds had higher IgM, but lower IgG natural antibody levels compared to HFP birds (**chapter 3, 4** and **6**), suggesting birds receiving LFP microbiota might somehow adopt the higher IgM natural antibody level of the LFP line. However, chicks receiving LFP or HFP microbiota had higher IgM natural antibody levels compared to chicks receiving control treatment (**chapter 6**). Thus, being exposed to adult microbiota composition might be sufficient to increase IgM natural antibody level. Previous studies show that microbiota transplantation can affect immune characteristics in mice (Ekmekciu et al., 2017) and pigs (Cheng et al., 2019). Yet, further research is needed to identify whether microbiota transplantation could influence other immune characteristics in poultry, such as innate and adaptive immune characteristics, giving a more complete overview of effects on different arms of the immune system.

Feather pecking

For the first time effects of microbiota transplantation on FP were investigated. However, early-life microbiota transplantation had limited effects on FP. An explanation for this finding might be that FP usually increases from the egg laying period onwards (around 20 weeks of age) (Bright, 2009; Newberry et al., 2007), while FP was observed till 15 weeks of age (**chapter 6**). However, previously FP genotypes clearly differed in all types of FP already around 5 weeks of age (Kops et al., 2017; **chapter 2, 3, 4** and **6**), although one study reported no differences in FP around 5 weeks of age (Kjaer, 2009). It should be noted that on average LFP chicks receiving HFP microbiota showed more FP compared to LFP chicks receiving LFP microbiota or control treatment and for this group a peak can be seen between 2 and 5 weeks of age. These findings might point to potential differences in FP at adult age (> 17 weeks of age), as FP around the 5 week period has been indicated as a critical time point for the development of FP later in life (de Haas, 2014; Huber-Eicher and

Sebö, 2001). Furthermore, effects on FP might be missed, as there was quite some variation in FP between pens, which could be caused by FP only being performed by few individuals (Newberry et al., 2007). Further research is needed to identify potential effects of microbiota transplantation on FP at adult age.

Microbiota transplantation to (pseudo) germ-free animals vs. newly hatched chicks

All rodent and poultry studies to date that identified effects of microbiota transplantation on behaviour used germ-free (Bercik et al., 2011a; Kraimi et al., 2019; Sharon et al., 2019; Zheng et al., 2016) or pseudo germ-free animals (Kelly et al., 2016; Yang et al., 2019). Even in humans antibiotic treatment was given prior to microbiota transplantation for identifying effects on autism symptoms (Kang et al., 2019, 2017). However, (pseudo) germ-free animal models are rather extreme, making it difficult to translate findings to 'normally' occurring situations. Especially since germ-free animals were shown to have increased permeability of the blood-brain barrier (Braniste et al., 2014) and certain antibiotics can penetrate the blood-brain barrier (Nau et al., 2010). Furthermore, antibiotic treatment influences an animal's behaviour and physiology. Thus, it is difficult to disentangle effects of microbiota transplantation after antibiotic treatment, as these effects on behaviour and physiology could be the result of the antibiotic treatment. Furthermore, when animals are kept in sterile isolators, like for (Bercik et al., 2011a; Kraimi et al., 2019; Sharon et al., 2019; Zheng et al., 2016), effects of microbiota transplantation on the host are likely more extreme then when they would have been housed in a non-sterile environment. Thereby, findings from these studies should be interpreted with caution, especially when transferring them to humans or production animals which are in a non-sterile environment.

For the first time, microbiota transplantation was given to newly hatched chicks, which were not (pseudo) germ-free or housed in sterile isolators, and still microbiota transplantation influenced behavioural responses, natural antibody level and whole blood 5-HT level (**chapter 6**). Thereby, this thesis provides first indications that microbiota transplantation is able to alter host behaviour and physiology in more 'normal' situations. Similarly, early-life microbiota transplantation, without using (pseudo) germ-free pigs or housing them in sterile isolators, influenced immune characteristics (Cheng et al., 2019). It is important to identify whether gut microbiota actually affects host behaviour and physiology in more 'normally' occurring situations, as only then can we get an overview of what kind of treatments would be

beneficial, for example for patients with behavioural disorders or for production animals that show damaging behaviours.

Effects of microbiota transplantation depend on genotype

During treatment, microbiota transplantation influenced behavioural responses in the HFP line, while after treatment it influenced behavioural responses in the LFP line (see Figure 3). A potential explanation for this could be that the HFP line has a more responsive immune system, which responds more strongly to the environment (Buitenhuis et al., 2006; **chapter 3, 4** and **6**). Although it should be noted both lines responded similarly to microbiota transplantation with regard to IgM natural antibody level and microbiota transplantation did not affect IgG natural antibody level. Still, having a more responsive immune system might result in HFP birds responding stronger to microbiota transplantation with the synthesis and release of pro-inflammatory cytokines. Peripherally produced pro-inflammatory cytokines can act on the brain (Dantzer et al., 2008) where they reduce serotonergic and dopaminergic neurotransmission (Miller et al., 2013), thereby potentially influencing behavioural responses. Indeed, direct-fed microbials were shown to alter intestinal mRNA levels of pro-inflammatory cytokines (Lee et al., 2015) and probiotic treatment altered serotonergic and dopaminergic neurotransmission in broilers (Yan et al., 2018), indicating microbiota could influence cytokine levels, and the serotonergic and dopaminergic systems in poultry. However, behavioural responses in HFP chicks were mainly altered by receiving HFP microbiota in comparison to LFP microbiota or control treatment, indicating that receiving any type of adult microbiota composition was not sufficient to alter behavioural responses in the HFP line. It might be that HFP microbiota had specific effects on other immune characteristics, although this needs further investigation. In addition, further research is needed to identify effects of microbiota transplantation on cytokine levels and brain neurotransmission in relation to FP.

After treatment, microbiota transplantation influenced behavioural responses in the LFP line. These effects do not seem to be explained by the difference in peripheral 5-HT level, as LFP birds receiving HFP microbiota tended to have lower whole blood 5-HT levels compared to LFP birds receiving control treatment. At the same time, behavioural differences were seen in LFP birds receiving LFP microbiota compared to LFP birds receiving HFP microbiota or control treatment. Still, it is interesting that LFP birds receiving HFP microbiota had lower peripheral 5-HT levels, as HFP birds had lower whole blood 5-HT levels compared to LFP birds (**chapter 3** and **6**). This might point to an increased risk for developing FP in LFP

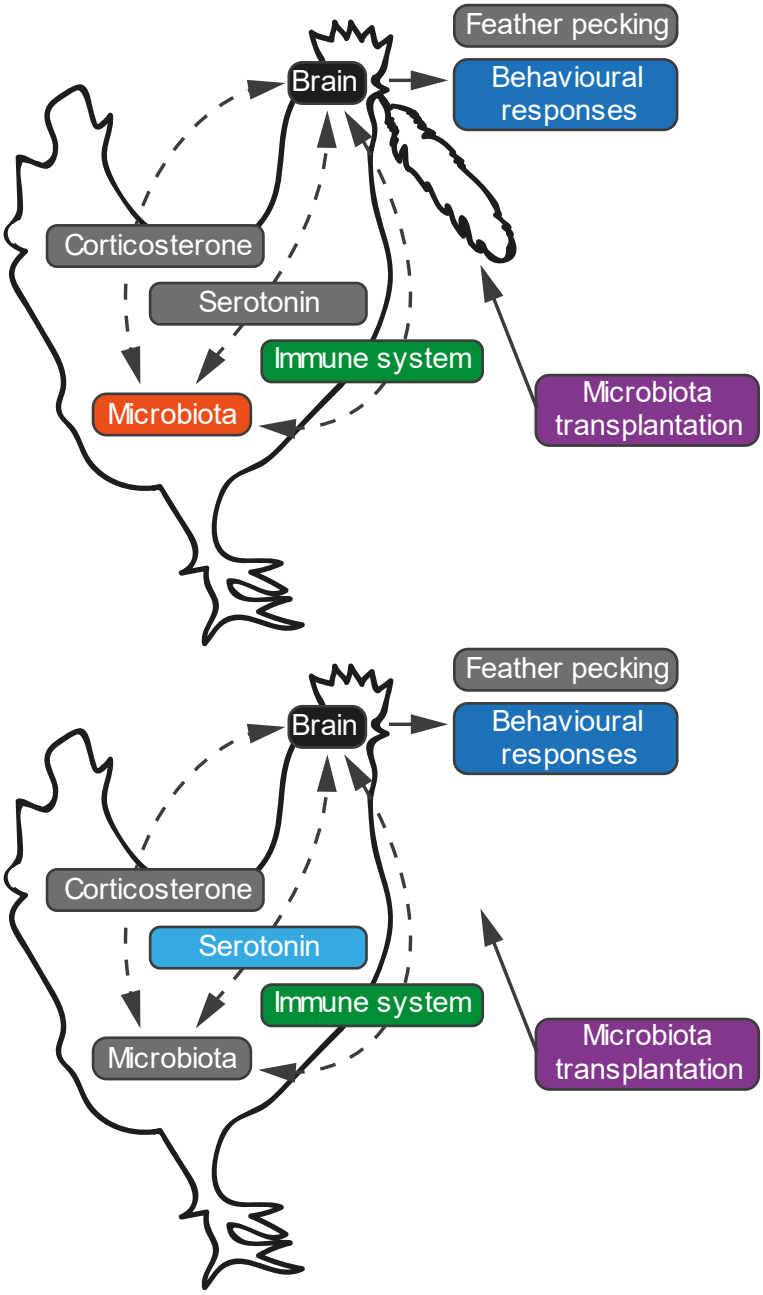

Figure 3. Microbiota transplantation effects on behavioural and physiological characteristics as determined in **chapter 6** for the top) HFP line and bottom) LFP line.

birds receiving HFP microbiota, which is further supported by high FP being related to low peripheral 5-HT level in commercial lines (de Haas et al., 2014a, 2013; Uitdehaag et al., 2011). Furthermore, on average LFP chicks receiving HFP microbiota showed more FP compared to LFP chicks receiving LFP microbiota or control treatment and showed a peak in FP between 2-5 weeks of age (**chapter 6**),

pointing to potential differences in FP at adult age (> 17 weeks of age). However, it remains unclear through which pathway microbiota transplantation influences behavioural responses in LFP birds.

Homologous transplantation

Homologous transplantation (i.e. receiving microbiota from their own line) resulted in birds showing more active responses (see Figure 4), suggesting reduced fearfulness. Therefore, homologous transplantation could be a potential approach to reduce fearfulness in chickens. FP is usually related to high fearfulness (de Haas et al., 2014a; Rodenburg et al., 2004a; Uitdehaag et al., 2008a), indicating that receiving homologous transplantation might further reduce FP. However, no effects were found on FP or on tonic immobility duration (**chapter 6**), the measure for innate fearfulness in chickens (Forkman et al., 2007). Furthermore, HFP birds had shorter tonic immobility duration compared to LFP birds (Bögelein et al., 2014; **chapter 2**), suggesting FP is related to reduced fearfulness in the FP selection lines, although no difference in tonic immobility duration has also been reported (Rodenburg et al., 2010; **chapter 6**). What is further interesting to note is that germ-free rodents generally show reduced anxiety-like behaviour compared to specific pathogen free or conventional rodents (Arentsen et al., 2015; Clarke et al., 2013; De Palma et al., 2015; Heijtz et al., 2011; Huo et al., 2017; Neufeld et al., 2011), suggesting that gut microbiota increases anxiety-like behaviour. However, homologous transplantation reduced fearfulness as opposed to receiving microbiota from the other line or the control treatment. Homologous transplantation might result in reduced fearfulness because of a match between transplanted microbiota and host genotype as opposed to a mismatch or control treatment.

Microbiota transplantation might be seen as a type of vertical transmission, where microbiota is transferred from mother hens to chicks. This could potentially occur when chicks peck at faeces from the mother hen, as chicks spend considerable time on ground pecking or pecking at objects (Hogan, 1971). Interestingly, chicks reared with a broody hen were less fearful, showed more active behavioural responses, had higher social motivation and showed less FP compared to chicks reared without a broody hen (Perré et al., 2002; Riber et al., 2007; Roden and Wechsler, 1998; Rodenburg et al., 2009), although no differences in feather damage or FP were also observed (Roden and Wechsler, 1998; Rodenburg et al., 2009). These findings seem to be similar to findings for homologous transplantation, suggesting effects of broody hens on chicks might be caused by vertical transmission of microbiota.

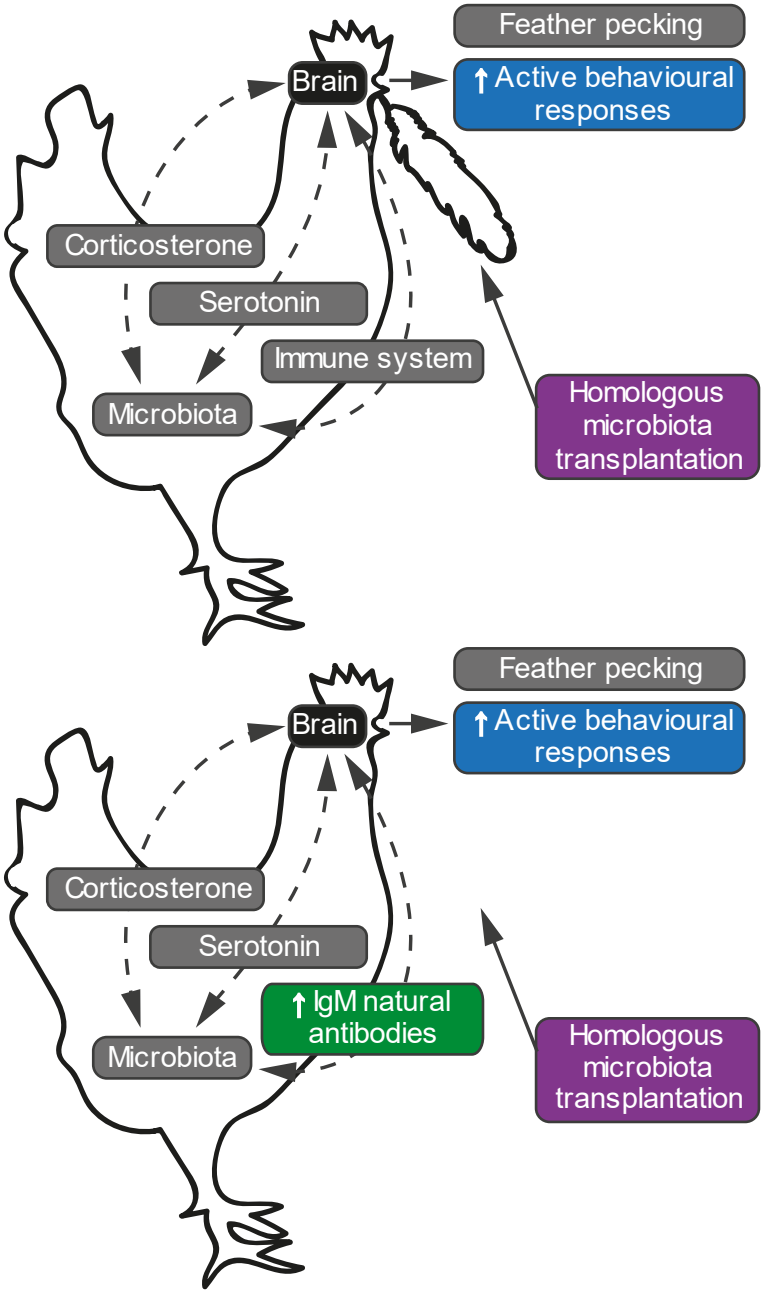

Figure 4. Homologous transplantation effects on behavioural and physiological characteristics as determined in **chapter 6** for the top) HFP line and bottom) LFP line.

Further supporting this, chicks reared with a broody hen spent more time exploring the environment and performed more ground pecking compared to chicks reared without a broody hen (Riber et al., 2007; Wauters et al., 2002a, 2002b), suggesting they are more likely to encounter and peck at faeces of mother hens. However, it should be noted that the broody hens used in these studies are often from different

strains than the chicks. Still, it is interesting that similarities exist between findings for chicks receiving homologous transplantation and chicks reared with a broody hen.

Vertical transmission might play an important role in initiating a host-specific gut microbiota, which could improve host immune system and brain development. This is supported by a study where germ-free mice colonized with human microbiota showed impaired immune system development compared to mice colonized with mouse microbiota (Chung et al., 2012), suggesting host-specific microbiota is required for immune system maturation. Although this is of course comparing inter vs. intra species microbiota transplantation, it could still point to improved immune system development through homologous transplantation. Moreover, germ-free mice receiving homologous transplantation had higher brain-derived neurotrophic factor (BDNF) levels in the hippocampus, but not in the amygdala, compared to germ-free mice receiving microbiota from the other strain (Bercik et al., 2011a). This suggests that receiving homologous transplantation might improve brain development, since BDNF is involved in synaptic plasticity, thereby potentially influencing behaviour as well. Thus, receiving homologous transplantation could improve immune system and brain development, ultimately altering behavioural responses. Furthermore, fearfulness was shown to decrease with age in chickens (Albentosa et al., 2003; Hocking et al., 2001), suggesting that receiving homologous transplantation might result in a faster behavioural development. Further research is needed to identify whether homologous transplantation improves immune system and brain development in poultry. Furthermore, it would be interesting to identify whether homologous transplantation, for example by providing faeces from mother hens to chicks, can be used to reduce fearfulness in poultry and FP in laying hens.

Addressing a multifactorial behaviour using a multifactorial approach

Both FP and the gut microbiota can be influenced by many internal and external factors, i.e. they are both multifactorial. This makes it extremely difficult to distinguish between cause and consequence. Furthermore, one factor cannot be the solution to control or prevent FP, especially since despite over four decades of research it remains difficult to find solutions to control or prevent FP. Yet, as the gut microbiota influences many aspects of host behaviour and physiology that have been related to FP, it could be a potential candidate to address FP. Yet, findings with regard to the gut microbiota remain complex. Especially since it relatively recently became a topic of interest and we are not yet completely aware of all the factors that shape the gut microbiota and the effects it has on its host. Still, an interdisciplinary

approach is key in behavioural research, since behavioural and physiological systems interact. Unfortunately, there are limitations in the number of characteristics one can measure, mostly due to time, financial, or practical constraints. It would have been interesting to look more in detail into the different physiological systems, such as central stress, serotonergic and immune system measurements or peripheral tryptophan and cytokine levels. However, integrating all these physiological systems is also complex and therefore peripheral indicators of each system were used in this thesis. Furthermore, identifying gut microbial metabolites might have provided further insight into the pathway through which microbiota transplantation altered behavioural responses.

Role for the immune system in feather pecking?

There is increasing evidence for a role of the immune system in FP. To summarize, an individual's feather damage was associated with high natural antibody level and mutations in cytokine genes of cage mates (Biscarini et al., 2010; Sun et al., 2014); a genetic correlation was found between FP and the specific antibody response (Buitenhuis et al., 2004); a line with high FP tendency showed upregulation of genes related to immune system processes in the brain compared to a line with low FP tendency (Habig et al., 2014, 2012); HFP birds had higher specific antibody levels, IgG natural (auto)antibody levels and nitric oxide production by monocytes compared to LFP birds (Buitenhuis et al., 2006; **chapter 3, 4** and **6**); and activation of the specific immune response at young age increased feather damage at adult age (Parmentier et al., 2009). These findings suggest that FP is related to activation of or a more responsive immune system. Although it should be noted that no differences were found in natural antibody level for FP phenotypes (**chapter 3**). Interestingly, the immune system could be a potential route through which microbiota transplantation affects behavioural responses, especially in HFP birds as they seem to have a more responsive immune system (**chapter 3, 4** and **6**) and microbiota transplantation had immediate but no long-term effects on behavioural responses in HFP birds (**chapter 6**). However, it remains unknown whether and how the immune system affects the development of FP, providing an interesting avenue for further research. For example, one could identify how individual differences in FP relate to innate and adaptive immune characteristics, especially cytokines, as they are the key signalling molecules of the immune system and can act on the brain and alter neurotransmission (Dantzer et al., 2008). Further relating these differences in FP and immune characteristics to the serotonergic and dopaminergic systems in the brain would be of added value since these systems seem to be involved in the development

of FP (de Haas and van der Eijk, 2018). Furthermore, differences in hygienic conditions, as an immune activation model, could be used to identify whether individuals with certain immune characteristics are more likely to develop FP in immune activating conditions.

Feather pecking and tail biting

Both chickens and pigs are omnivores and have a highly explorative nature. Interestingly, FP in chickens and tail biting in pigs, both injurious behaviours directed toward conspecifics, were suggested to have similar underlying principles and mechanisms (Brunberg et al., 2016). Indeed, there are striking similarities between findings for FP and tail biting.

As mentioned previously tail biting seems to be related to increased activity (Larsen et al., 2019; Ursinus et al., 2014a), but also to fearfulness and the stress response. Tail biters were suggested to be more fearful compared to other phenotypes (Ursinus et al., 2014b), although the opposite has also been found (Zupan et al., 2012). The latter is in agreement with findings for FP genotypes (Bögelein et al., 2014; de Haas et al., 2010; Kops et al., 2017; **chapter 2** and **6**) and FP phenotypes (**chapter 2** and **3**), while the first is in agreement with previous studies in other experimental and commercial lines that showed high FP is related to high fearfulness (de Haas et al., 2014; Jones et al., 1995; Rodenburg et al., 2004; Uitdehaag et al., 2008). With regard to the stress response, victims had lower stress-induced cortisol levels compared to control pigs (Valros et al., 2013). Although this is not in agreement with findings for FP phenotypes (Daigle et al., 2015; Jensen et al., 2005; **chapter 3**), tail biters were shown to have lower heart rate variability compared to victims, similar to findings for FP genotypes (Kjaer and Jørgensen, 2011) and other experimental lines (Korte et al., 1997). Yet, tail biters did not differ from controls in heart rate variability (Zupan et al., 2012).

Tail biting has further been related to altered central and peripheral serotonergic systems. High levels of tryptophan in the diet reduced tail biting compared to low levels of tryptophan (Martínez-Trejo et al., 2009) and tail biters had higher platelet 5-HT uptake, but lower whole blood and platelet 5-HT levels compared to other phenotypes (Ursinus et al., 2014b). Similarly, supplementing tryptophan in the diet reduced FP (van Hierden et al., 2004b) and high FP is generally related to low whole blood 5-HT level (de Haas et al., 2014, 2013; Uitdehaag et al., 2011; **chapter 3** and **6**), although feather peckers had higher whole blood 5-HT levels compared to neutrals (**chapter 3**). For the central serotonergic system, tail biters had higher 5-HIAA (5-HT metabolite) in the prefrontal cortex compared to victims and controls,

while victims had higher 5-HIAA and 5-HT turnover (5-HIAA/5-HT) compared to tail biters and controls in several brain regions (Valros et al., 2015). For FP phenotypes, feather peckers and victims had higher 5-HIAA and 5-HT turnover in the dorsal thalamus compared to non-peckers, while victims had lower 5-HIAA in the medial striatum compared to feather peckers and non-peckers (Kops et al., 2013). Although these findings are not always in agreement, it still points to a potential role for the serotonergic system in both damaging behaviours.

Similar as FP, tail biting seems to be related to the immune system. Altered cytokine levels were associated with tail biting (Munsterhjelm et al., 2017), in low hygienic conditions (i.e. immune activating conditions) more tail biting occurred compared to high hygienic conditions (van der Meer et al., 2017), and an immune challenge increased tail biting (Munsterhjelm et al., 2019), potentially through increased central levels of the pro-inflammatory cytokine interferon γ (Nordgreen et al., 2018). These findings suggest that tail biting is related to immune activation, providing an interesting research avenue to identify whether the immune system plays a role in tail biting and FP.

To date, no studies have identified microbiota composition in relation to tail biting, although it was suggested that the microbiota-gut-brain axis could play a role in the development of tail biting (Brunberg et al., 2016). A first indication for a relation between gut microbiota and tail biting was shown, as antibiotic treated groups were more likely to have tail lesions compared to non-treated groups, although there was no difference in actual tail biting behaviour (Diana et al., 2017). Since there are striking similarities between FP and tail biting, it would be interesting to see whether tail biting is related to gut microbiota composition and whether gut microbiota might play a role in the development of tail biting.

Feather pecking selection lines as model system

The HFP and LFP lines were used throughout this thesis as a model system to identify effects of gut microbiota on FP. As these lines were specifically selected on high and low FP (Kjaer et al., 2001), findings with regard to FP should be interpreted with caution when transferring them to other experimental or commercial lines. Overall, high FP was related to low fearfulness, low whole blood 5-HT level and a more responsive immune system in the FP selection lines (**chapter 2, 3, 4** and **6**). Is this in agreement with previous findings in other experimental or commercial lines? In experimental lines selected on egg production and commercial lines high FP was related to high fearfulness (Dekalb White vs. ISA Brown: de Haas et al., 2014a; HP vs. LP line: Jones et al., 1995; Rodenburg et al., 2004a; White Leghorn vs. Rhode

Island Red: Uitdehaag et al., 2008a) and low whole blood 5-HT level (Dekalb White vs. ISA Brown: de Haas et al., 2013; White leghorn vs. Rhode Island Red: Uitdehaag et al., 2011). Furthermore, when conspecifics within a cage had higher IgG natural antibody level, the individual had more feather damage (White Leghorn: Sun et al., 2014) and activation of the specific immune response resulted in more feather damage (White Leghorn: Parmentier et al., 2009), indicating that a more responsive immune system or activation of the immune system is related to high FP. Findings with regard to CORT level after restraint are less consistent, with high FP being related to low CORT level after restraint (HP vs. LP line: Korte et al., 1997; van Hierden et al., 2002) or not (White Leghorn vs. Rhode Island Red: Uitdehaag et al., 2011). Thus, the FP selection lines seem to show similar relations between high FP, whole blood 5-HT level and immune responsiveness as commercial lines, but an opposite relation between high FP and fearfulness as other experimental and commercial lines. Furthermore, there is inconsistency with regard to the relation between high FP and the stress response. Thus, findings from the FP selection lines should be used with caution when developing control or preventive methods that are to be applied in commercial settings. Still, this thesis provides new interesting insights into the relation between FP, behavioural and physiological characteristics related to FP and the gut microbiota. Further research is needed to identify whether microbiota can affect FP or behavioural and physiological characteristic related to FP in commercial lines, especially since effects of microbiota transplantation are dependent on genotype. It would be interesting to identify whether homologous transplantation, for example by providing faeces from mother hens to chicks, can be used to reduce fearfulness in commercial lines, thereby potentially reducing the risk of birds developing FP.

Conclusion

Divergent selection on FP affects fearfulness, activity, peripheral serotonin, immune characteristics and gut microbiota composition, but not the physiological stress response (i.e. corticosterone). FP phenotypes differ in fearfulness, activity and peripheral serotonin, but not in the physiological stress response, immune characteristics or gut microbiota composition. Yet, relations between high FP and behavioural or physiological characteristics are not always similar for FP genotypes and phenotypes, indicating the importance of taking FP genotype and phenotype into account when studying FP.

Gut microbiota could influence the development of FP, as early-life microbiota transplantation affects fearfulness, activity, peripheral serotonin and immune characteristics, with effects being either immediate or long-term. However, effects depend on age, donor's and recipient's genotype, indicating the importance of taking donor's and recipient's genotype into account when studying microbiota transplantation effects on behaviour. Overall, this thesis provides new interesting insights into the relationship between gut microbiota, host behaviour and physiology in poultry, which could further be of interest for other species.

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Summary

Early-life factors can have a profound impact on an animal's behavioural development. An important moment early in life is the rapid microbial colonization of the gut, leading to the establishment of the gut microbiota. From rodent studies it is clear that the gut microbiota influences host behaviour and physiology, such as anxiety, stress, and the serotonergic and immune systems. First indications show that microbiota affects similar behavioural and physiological characteristics in poultry. Through these effects microbiota could alter an animal's ability to cope with environmental and social challenges, such as those encountered in animal production systems, and could thereby affect the development of damaging behaviours in production animals.

Fearfulness, stress, and the serotonergic and immune systems have been related to severe feather pecking (FP), a damaging behaviour in chickens which involves pecking and pulling out feathers of conspecifics, negatively affecting animal welfare and productivity. Furthermore, high FP (HFP) and low FP (LFP) selection lines were shown to differ in gut microbial metabolites and microbiota composition determined from caecal droppings. These findings suggest a link between the gut microbiota and FP. Yet, it is unknown whether gut microbiota influences the development of FP. Therefore, the aim of this thesis was to identify effects of gut microbiota on the development of FP. First, I identified behavioural and physiological characteristics in FP genotypes (i.e. HFP and LFP lines) and FP phenotypes (i.e. feather pecker, victim, feather pecker-victim and neutral) that were related to FP and shown to be influenced by microbiota. Second, I identified whether microbiota influences FP, and behavioural and physiological characteristics related to FP.

Feather pecking genotypes

FP genotypes differed in behavioural responses, where HFP birds had more active behavioural responses compared to LFP birds (**chapter 2, 3** and **6**), especially at young age. The active behavioural responses suggest lower fearfulness, higher social and exploration motivation, or higher activity in HFP birds compared to LFP birds. For the stress response, HFP birds struggled later and less, but vocalized sooner and more compared to LFP birds during restraint (**chapter 3** and **6**). However, FP genotypes did not differ in corticosterone (CORT, the major stress hormone) level after restraint (**chapter 3** and **6**), suggesting differences in behavioural findings might not be related to stress. With regard to the serotonergic system, whole blood

serotonin (5-Hydroxytryptamine or 5-HT) level was measured as indicator for central 5-HT and HFP birds had lower whole blood 5-HT levels compared to LFP birds (**chapter 3** and **6**). For the immune system, nitric oxide production by monocytes was measured as indicator for the innate immune system, specific antibody level was measured as part of the adaptive immune system, and natural (auto)antibody level was measured, as natural antibodies play an essential role in both innate and adaptive immunity. HFP birds had lower IgM and higher IgG natural (auto)antibody levels, higher nitric oxide production by monocytes, and a tendency for higher IgM and IgG specific antibody levels compared to LFP birds, but did not differ in relative abundances of immune cell subsets (**chapter 3, 4** and **6**). Moreover, FP genotypes had distinct luminal microbiota composition, where HFP birds had a higher relative abundance of genera of the order Clostridiales, but lower relative abundance of *Lactobacillus* compared to LFP birds (**chapter 5**). Yet, FP genotypes did not differ in mucosa-associated microbiota composition. In summary, these findings indicate that divergent selection on FP not only affects FP but also (in)directly affects behavioural responses, peripheral 5-HT level, different arms of the immune system and microbiota composition, but did not affect CORT level.

Feather pecking phenotypes

FP phenotypes differed in behavioural responses, where feather peckers tended to have more active behavioural responses compared to victims and neutrals at young age (**chapter 2**), which suggests lower fearfulness, higher exploration motivation or activity in feather peckers. Furthermore, victims had more active responses compared to neutrals at young age (**chapter 2**), which suggests lower fearfulness or higher activity in victims. For the stress response, feather peckers tended to have less active behavioural responses compared to neutrals, while victims had more active behavioural responses compared to other phenotypes during restraint (**chapter 3**). However, FP phenotypes did not differ in CORT level after restraint (**chapter 3**), suggesting differences in behavioural findings might not be related to stress. With regard to the serotonergic system, feather peckers had higher whole blood 5-HT levels compared to neutrals at adult age (**chapter 3**). However, FP phenotypes did not differ in natural antibody level (**chapter 3**) or gut microbiota composition (**chapter 5**). In summary, these findings indicate that performing and receiving FP is related to more active behavioural responses and that performing FP is further related to high peripheral 5-HT level.

Feather pecking genotypes vs. phenotypes

When comparing findings from FP genotypes to those from FP phenotypes, there is a similar relation between high FP and behavioural responses. HFP birds had more active responses compared to LFP birds (**chapter 2, 3** and **6**) and similarly feather peckers tended to have more active responses compared to victims and neutrals (**chapter 2**), especially at young age. Furthermore, victims had more active responses compared to other phenotypes at adult age (**chapter 3**). Thus, activity level might be used as potential indicator for FP at group level or even as indicator for individuals that perform or receive FP. Since feather peckers seem to have more active responses at young age (**chapter 2**), it would be interesting to identify whether activity level at young age could be used to predict which individuals will become feather peckers.

There is an opposite relation between high FP and whole blood 5-HT level, where HFP birds had lower whole blood 5-HT levels compared to LFP birds (**chapter 3** and **6**), while feather peckers had higher whole blood 5-HT levels compared to neutrals (**chapter 3**). The actual performance of FP might increase peripheral 5-HT level in feather peckers, possibly due to feather eating. Feather peckers often ingest feathers, which may increase peripheral 5-HT level by providing structural components, as the gut releases 5-HT in reaction to these structural components. However, this relation between feather eating and increased peripheral 5-HT level needs further investigation.

Similar to findings for FP genotypes, FP phenotypes did not differ in CORT level after restraint, indicating that the stress response might not be related to FP in FP genotypes and phenotypes. Furthermore, although differences between FP genotypes were found for the immune system and gut microbiota composition, no such differences were identified for FP phenotypes. This might indicate that differences in immune characteristics and gut microbiota composition are more related to genotype than to actual FP behaviour. Yet, cause and consequence cannot be disentangled from each other based on these findings. Therefore, microbiota transplantation was used to identify gut microbiota effects on the development of FP.

Microbiota and the development of feather pecking

Since FP genotypes differed in gut microbiota composition, but FP phenotypes did not, I focussed on FP genotypes for the second objective. The difference in microbiota composition was used to create a HFP and LFP microbiota pool. I identified effects of early-life microbiota transplantation on FP and on the same

behavioural and physiological characteristics that were identified in **chapter 2, 3** and **5**. Newly hatched HFP and LFP chicks received a control treatment, HFP or LFP microbiota daily during the first two weeks post hatch.

Although limited effects of early-life microbiota transplantation on microbiota composition were found, microbiota transplantation did affect behavioural responses, natural antibody level and whole blood 5-HT level. Thus, microbiota transplantation may have influenced brain, immune and serotonergic system functioning, which (in)directly resulted in differences in behavioural responses, natural antibody level and whole blood 5-HT level.

With regard to behavioural responses, birds receiving microbiota from their own line (i.e. homologous transplantation) had more active behavioural responses compared to birds receiving microbiota from the other line or control treatment. These active behavioural responses suggest low fearfulness, high exploration and social motivation or activity in birds receiving homologous transplantation. For the stress response, LFP birds receiving homologous transplantation had more active stress responses compared to LFP birds that received HFP microbiota or control treatment. However, microbiota transplantation did not influence CORT level after restraint, suggesting these behavioural findings might not be related to stress.

With regard to the serotonergic system, LFP birds receiving HFP microbiota tended to have lower whole blood 5-HT level compared to LFP birds receiving control treatment. Yet, microbiota transplantation effects on whole blood 5-HT level do not seem to be explained by the HFP pools' microbiota composition. Especially since microbiota composition did not differ between treatments within the LFP line.

For the immune system, birds receiving LFP microbiota had higher IgM natural antibody level compared to birds receiving control treatment, but microbiota transplantation did not affect IgG natural antibody level. Thus, being exposed to an adult microbiota composition might be sufficient to increase IgM natural antibody level. Further research is needed to identify whether microbiota transplantation could influence other immune characteristics in poultry, such as innate and adaptive immune characteristics.

For the first time, effects of early-life microbiota transplantation on FP were investigated. However, early-life microbiota transplantation had limited effects on FP at young age (till 15 weeks of age) (**chapter 6**), which might be explained by FP usually increasing from the egg laying period onwards (around 20 weeks of age). Thus, further research is needed to identify effects of microbiota transplantation on FP at adult age.

Effects of microbiota transplantation depend on genotype

During treatment, microbiota transplantation influenced behavioural responses in the HFP line, while after treatment it influenced behavioural responses in the LFP line. A potential explanation for this could be that the HFP line has a more responsive immune system (**chapter 3, 4** and **6**), which responds more strongly to the environment or in this case to microbiota transplantation, with the synthesis and release of pro-inflammatory cytokines. These cytokines in turn act on the brain and alter neurotransmission, thereby potentially influencing behavioural responses. After treatment, microbiota transplantation influenced behavioural responses in the LFP line. These effects do not seem to be explained by the difference in whole blood 5-HT level. Still, it is interesting that LFP birds receiving HFP microbiota had lower whole blood 5-HT levels, as HFP birds had lower whole blood 5-HT levels compared to LFP birds (**chapter 3** and **6**). This might increase the risk for developing FP in LFP birds receiving HFP microbiota, as high FP is usually related to low whole blood 5-HT level. However, it remains unknown through which pathway microbiota transplantation influences behavioural responses in the LFP line.

Homologous transplantation

It is interesting to note that homologous transplantation resulted in birds having more active responses, suggesting reduced fearfulness. Therefore, homologous transplantation could be a potential approach to reduce fearfulness in chickens. High FP is usually related to high fearfulness, indicating that receiving homologous transplantation might reduce FP. Homologous transplantation might result in reduced fearfulness because of a match between transplanted microbiota composition and host genotype as opposed to a mismatch or control treatment. Homologous transplantation could be seen as a type of vertical transmission, where microbiota is transferred from mother hens to chicks. Vertical transmission might play an important role in initiating a host-specific gut microbiota, which might improve host immune system and brain development. Thus, homologous transplantation might have improved immune system and brain development, thereby altering behavioural responses. It would be interesting to identify whether homologous transplantation can be used to reduce fearfulness in poultry and FP in laying hens.

Role for the immune system in feather pecking?

There is increasing evidence for a role of the immune system in FP. For FP genotypes, HFP birds had higher specific antibody levels, IgG natural (auto)antibody

levels and nitric oxide production by monocytes (**chapter 3, 4** and **6**), suggesting that high FP is related to a more responsive innate and adaptive immune system. Although it should be noted that FP phenotypes did not differ in natural antibody level (**chapter 3**). The immune system could be a potential route through which microbiota transplantation affects behavioural responses, especially in HFP birds as they seem to have a more responsive immune system (**chapter 3, 4** and **6**) and microbiota transplantation had immediate but no long-term effects on behavioural responses in HFP birds (**chapter 6**). Yet, the exact mechanisms through which the immune system affects the development of FP remain unknown, providing an interesting avenue for further research.

Feather pecking selection lines as model system

The HFP and LFP lines were used throughout this thesis as a model system to identify effects of gut microbiota on FP. As these lines are specifically selected on high and low FP, findings with regard to FP should be interpreted with caution when transferring them to other experimental or commercial lines. Overall, high FP was related to low fearfulness, low whole blood 5-HT level and a more responsive immune system in the FP selection lines (**chapter 2-6**). Previous studies in other experimental and commercial lines show that high FP is related to high fearfulness, low whole blood 5-HT level and a more responsive immune system. Findings with regard to CORT level after restraint are less consistent, with high FP being related to low CORT level after restraint or not. Thus, the FP selection lines seem to show similar relations between high FP, whole blood 5-HT level and immune responsiveness as commercial lines, but an opposite relation between high FP and fearfulness as other experimental and commercial lines. Furthermore, there is inconsistency with regard to the relation between high FP and the stress response. Therefore, findings from the FP selection lines should be used with caution when developing control or preventive methods that are to be applied in production systems. Still, this thesis provides new interesting insights into the relation between FP, behavioural and physiological characteristics related to FP and the gut microbiota.

Conclusion

Divergent selection on FP affects fearfulness, activity, peripheral serotonin, immune characteristics and gut microbiota composition, but not the physiological stress response (i.e. corticosterone). FP phenotypes differ in fearfulness, activity and peripheral serotonin, but not in the physiological stress response, immune

characteristics or gut microbiota composition. Yet, relations between high FP and behavioural or physiological characteristics are not always similar for FP genotypes and phenotypes, indicating the importance of taking FP genotype and phenotype into account when studying FP.

Gut microbiota could influence the development of FP, as early-life microbiota transplantation affects fearfulness, activity, peripheral serotonin and immune characteristics, with effects being either immediate or long-term. However, effects depend on age, donor's and recipient's genotype, indicating the importance of taking donor's and recipient's genotype into account when studying microbiota transplantation effects on behaviour. Overall, this thesis provides new interesting insights into the relationship between gut microbiota, host behaviour and physiology in poultry, which could further be of interest for other species.

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About the author

I, Jerine Alexandra Johanna van der Eijk, was born on 22 October 1989 in Voorburg, the Netherlands. From an early age I have been fascinated with nature, in particular with animals. That's why I enrolled into the B.Sc. programme Biology at Utrecht University. During my bachelor I learned that I was especially enthusiastic about animal behaviour and specialised in Behavioural Sciences and even attended some courses related to this topic at Wageningen University. I enrolled into the M.Sc. programme Animal Sciences at Wageningen University with a specialisation in Animal Health and Behaviour and started with my minor thesis entitled 'Effects of restricting microbiota development early in life on fearfulness in laying hens'. My major thesis was on the effects of neighbours on dawn singing behaviour in Great tits (*Parus major*). At the end of my master, I participated in the Research Master Cluster course and wrote a research proposal entitled 'Effects of early life gut microbiota on social behaviour, with a focus on feather pecking in laying hens'. For this proposal I received the Tjeerd de Jong award. The best proposals continued to compete in the Wageningen Institute for Animal Sciences (WIAS) Graduate Programme. I received one of four grants funded by the Netherlands Organisation for Scientific Research (NWO), which allowed me to do my Ph.D. at the Behavioural Ecology (BHE) and Adaptation Physiology (ADP) groups of Wageningen University. Before actually starting, I was a research assistant at ADP and worked on a literature study focussing on the effects of early feeding on the behavioural and physiological development of chickens. My Ph.D. focused on gut microbiota and feather pecking where I investigated whether early-life gut microbiota influences behavioural and physiological characteristics related to feather pecking in chicken lines divergently selected on feather pecking behaviour. Currently, I'm appointed as Postdoctoral researcher at ADP to write a research proposal looking more into the relationship between the immune system and behaviour in chickens. Ultimately, my goal is to contribute to the knowledge we have on animal behaviour and physiology, and to see how this knowledge can be incorporated into procedures and management measures designed to ensure and improve animal health and welfare.

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Other publications related to this thesis

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WIAS Education & training certificate

Description	Year
The Basic Package (3.0 ECTS[1])	
WGS course Research integrity and ethics in animal sciences, Wageningen, the Netherlands	2017
WIAS course Essential skills, Wageningen, the Netherlands	2017
WIAS course Introduction day, Wageningen, the Netherlands	2015
In Depth Studies (8.25 ECTS)	
GroupHouseNet Training school, Genetics, epigenetics and neurobiology of damaging behaviour, Bratislava, Slovakia	2018
GroupHouseNet Training school, Prenatal and early life influences on damaging behaviour in pigs and poultry, Bilbao, Spain	2017
ISAE Satellite meeting From beak to tail, Aarhus, Denmark	2017
WIAS course Statistics for the life sciences, Wageningen, the Netherlands	2016
GroupHouseNet Training school, Damaging behaviour and health, Belgrade, Serbia	2016
Professional Skills Support Courses (7.25 ECTS)	
WGS course Writing grant proposals, Wageningen, the Netherlands	2019
WIAS course The final touch: writing the general introduction and discussion, Wageningen, the Netherlands	2019
NWO Pump your career, Utrecht, the Netherlands	2018
WGS course Effective behaviour in your professional surroundings, Wageningen, the Netherlands	2017
WGS course Presenting with impact, Wageningen, the Netherlands	2017
WGS course Project and time management, Wageningen, the Netherlands	2016
ESD course Teaching and supervising thesis students, Wageningen, the Netherlands	2016
WGS course Stress identification management, Wageningen, the Netherlands	2016
Research Skills Training (8.0 ECTS)	
WIAS Ph.D. Research Proposal, Wageningen, the Netherlands	2016
External Training Period, University of Guelph, Canada	2015

Management Skills Training (2.5 ECTS)	
Organizing member of WIAS Science day 2017	2016-2017
Secretary of Wageningen Evolution and Ecology Seminars (WEES)	2015-2016
Didactic Skills Training (6.0 ECTS)	
Supervising 9 MSc students	2015-2019
Assisting practical of BSc course Animal Behaviour	2017-2019
Assisting practical of MSc course Behavioural Ecology	2018
Lecture in MSc course Health, Welfare and Management	2018
Lecture in BSc course Immunology and Thermoregulation	2017
Reviewing research proposals of Research Master Cluster students	2017
Supervising 2 BSc students	2016-2017
Assisting practical of MSc course Adaptation Physiology	2016-2017
Presentations (4.0 ECTS)	
Oral presentations	
70th Annual meeting of the European Federation of Animal Science (EAAP), Ghent, Belgium	2019
53rd International Congress of the International Society for Applied Ethology (ISAE), Bergen, Norway	2019
WIAS Science day, Lunteren, the Netherlands	2019
52nd International ISAE Congress, Charlottetown, Canada	2018
25th Annual Meeting of the Netherlands Society for Behavioural Biology (NVG), Soesterberg, the Netherlands	2017
Benelux ISAE Congress, Hoogeloon, the Netherlands	2017
51st International ISAE Congress, Aarhus, Denmark	2017
50th International ISAE Congress, Edinburgh, Scotland	2016
Benelux ISAE Congress, Berlicum, the Netherlands	2016
16th International Conference on Production Diseases in Farm Animals (ICPD), Wageningen, the Netherlands	2016
Poster presentations	
10th European Symposium on Poultry Welfare, Ploufragan, France	2017
1st International Conference on Microbiota-gut-brain axis, Amsterdam, the Netherlands	2016
WIAS Science day, Wageningen, the Netherlands	2016

[1] one ECTS credit equals a study load of approximately 28 hours

Colophon

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