Francesca Marcato

A JOURNEY TO IMPROVE ROBUSTNESS OF VEAL CALVES

Propositions

1. Feeding milk prior to transport and a short transport duration make the journey of calves to the veal farm less challenging.
   (this thesis)

2. High colostrum IgG levels have long-term effects on the robustness of veal calves.
   (this thesis)

3. Large datasets are useful to identify biomarkers of diseases only when correct statistical approaches are used.

4. The "five freedoms" (Brambell Report, 1965) should be modified and customized to the welfare needs of each individual animal species.
   Brambell Report, 1965. Report of the Technical Committee to enquire into the welfare of animals kept under intensive livestock husbandry systems. Chairman: Professor F. W. Rogers Brambell. Cmnd. 2836, December 3 1965. Her Majesty's Stationery Office, London.

5. Art paves the way to positivity by opening the mind, cherishing creativity and enriching with colours.

6. Challenging training sessions and too high expectations are the Achilles heel of elite athletes.

Propositions belonging to the thesis entitled:
"A journey to improve robustness of veal calves"

Francesca Marcato

Wageningen, The Netherlands, 18 June 2021

A journey to improve robustness of veal calves

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A journey to improve robustness of veal calves

Francesca Marcato

Thesis

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CHAPTER 1

General Introduction

The veal sector and the use of antimicrobials

The Netherlands is the largest producer of veal in the European Union, accounting for a production volume of approximately 230,000 metric tons. In the last years, around 1.5 million calves have been slaughtered in The Netherlands on an annual basis and ≈ 64 % of calves for veal production are white veal and another 36 % are rosé calves (CBS statline, 2021). In 2020, approximately 764,700 calves were imported in the Netherlands and the majority (77%) were imported from Germany (RVO, 2021). An overview of total number of veal farms and veal calves in The Netherlands is shown in Figure 1.

The veal industry is an important market for male calves born on dairy farms (Bokma et al., 2020). These male calves together with some surplus female calves might be considered by-products for the dairy sector because they have a low financial value and they may not represent a priority (Devant and Marti, 2020). As a consequence, these animals might receive poor post-natal care, including feeding low amounts of colostrum of moderate quality and poor navel disinfection after birth (Mee et al., 2008; Renaud et al., 2017). Providing sufficient amounts of good quality colostrum is essential

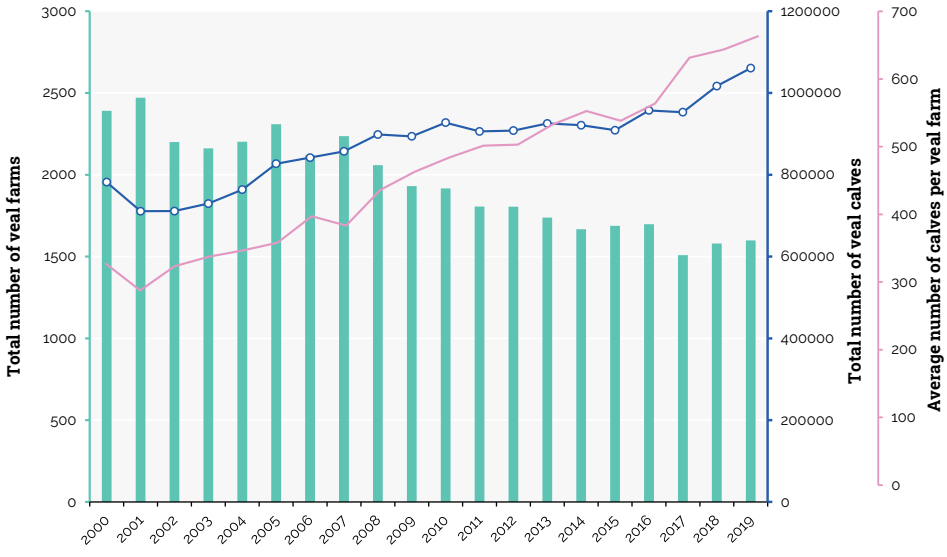

Figure 1. **Overview of total number of veal farms, total number of veal calves and average number of calves per veal farm in the Netherlands (CBS statline, 2021).**

to calves, because it confers the maternal immunity via passive transfer of immunoglobulins (Godden et al., 2009; Dunn et al., 2018). However, failure of passive transfer is a common problem among male calves that enter the veal industry (Wilson et al., 2000; Pardon et al., 2015; Renaud et al., 2017). Other challenges that future veal calves endure in their early life (14-20 days) include commingling with calves from many different dairy farms at a collection center, and (sometimes long term) transportation – from the dairy farm to a collection center, and from a collection center to a veal farm (von Konigslow et al., 2020). These challenges are an integral part of the current veal chain, and may result in the exposure of calves to a multitude of pathogens and stressors (Pardon et al., 2011; Hay et al., 2014; Pardon et al., 2014). Young calves are still developing their gastrointestinal tract, they have a low ability to regulate their body temperature and still have immature physiological and immunological systems (Tao and Dahl, 2005; Marcato et al., 2018). All these challenges between birth and arrival of calves at the veal farm may contribute to a high incidence of diseases at the veal farm. Bovine respiratory disease (BRD) and enteric diseases are the most prevalent (Pardon et al., 2012a, 2013). Among the strategies employed by the veal sector to prevent the spreading of these diseases antimicrobial use (AMU) plays an important role. However, in 2008, the Dutch government requested the livestock industry to reduce AMU by 70% in 2015, using 2009 as the reference year. In the Netherlands,

AMU is monitored by the Dutch Veterinary Medicine Authority that makes annual reports on the national trends on AMU (Holstege et al., 2018). The veal sector in the Netherlands has been able to reduce AMU by 44% in a short period of time, but reductions seemed to have slowed down since 2013 and 2014 (Bokma et al., 2020; MARAN, 2020). Moreover, as stated by Pardon et al. (2012a) and von Konigslow et al. (2020), herd treatments with antimicrobials are still commonly administered in the first weeks upon arrival of calves at the veal farm. In a recent Dutch study, for example, with 32 batches of calves, Heeres et al. (2017) reported the application of an average of 4.5 herd treatments per batch during the rearing period at the veal farm. Additionally, an average of 31% of calves per batch were also individually treated with antibiotics. Bosman et al. (2014) showed that especially the antibiotic class of tetracyclines in combination with Colistin are largely used as oral treatments delivered in the first week upon arrival at the veal farm, and their use subsequently decreases over time until the end of the rearing period (Figure 2).

Additionally, antimicrobial resistance has been reported to increase over the years, and the prevalence of antimicrobial resistance, particularly for (ESC-R) indicative of Extended Spectrum β-Lactamase (ESBLs) and Plasmid-mediated AmpC (pAmpC), in white veal calves is still 40% (MARAN 2020). The increase in antimicrobial resistance in

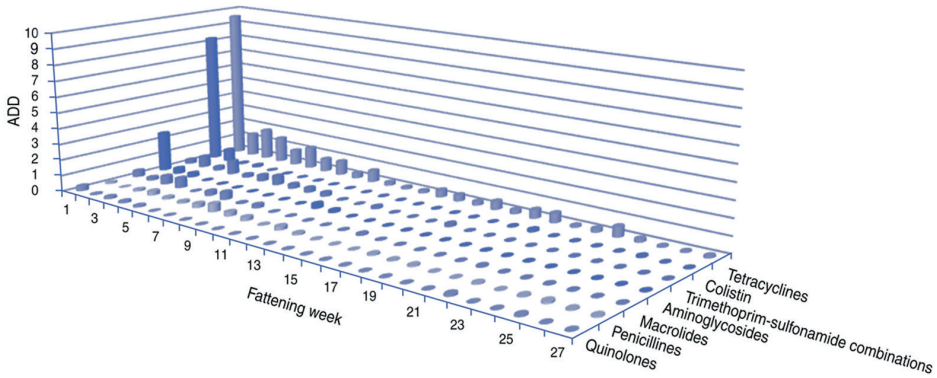

Figure 2. **The average animal daily dosage (ADD) of oral antimicrobial drugs delivered during the rearing period in 48 veal calf herds** *(© Cambridge University Press 2013. Originally published in "Antimicrobial resistance in commensal Escherichia Coli in veal calves is associated with antimicrobial drug use". Available from Bosman et al. (2014), 142(9), 1893-1904).*

commensal and zoonotic bacteria represents a threat for both animal and human health (Wernli et al., 2011). To prevent these problems related to AMU, the veal sector could focus on alternative strategies to reduce the use of AMU in the first weeks upon arrival of calves at the veal farm. One of these alternative strategies is to improve robustness of calves before their arrival at the veal farm.

Robustness

Robustness can be defined as the capacity of an animal to cope with environmental challenges and to bounce back rapidly after challenges occur (Colditz and Hine, 2016). Robust animals are better equipped to cope with endemic infections and to fight diseases and thus probably have a lower need for antimicrobials. Robustness can be measured in terms of physiological indicators (such as fluctuations in glucose levels and body temperature), which reflect the capacity of an animal to regulate the functions of the body in relation to external stimuli (Scheffer et al., 2018). This in turn might have an effect on morbidity, mortality and antimicrobial use, which represent additional, and more ultimate, measures of robustness. As shown in Figure 3, robustness can be shaped by several environmental factors and animal-related characteristics. These factors can affect the functional reserves of an animal and cause changes in the homeostasis (Scheffer et al., 2018).

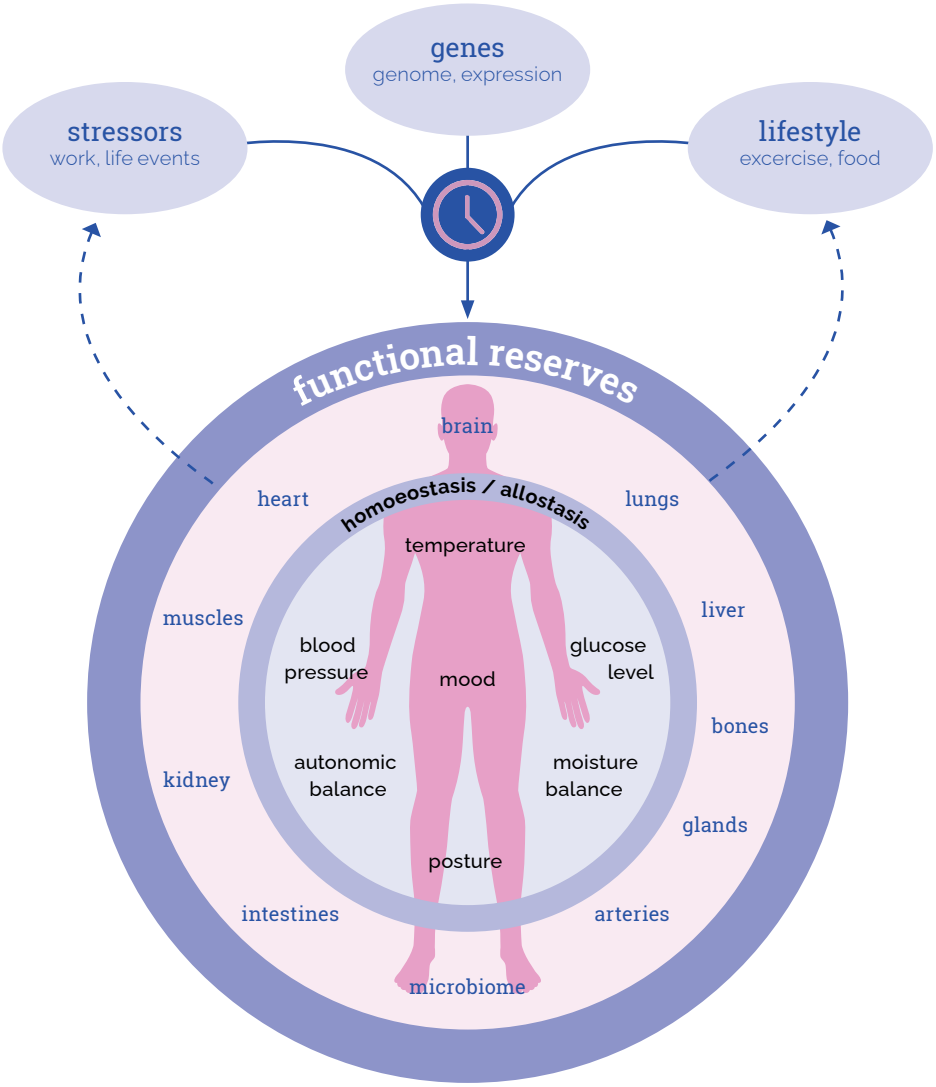

Figure 3. **Mechanisms related to systemic resilience in humans and animals** *The resilience of humans and animals depends on the resilience of subsystems that regulate vital parameters, such as temperature, glucose level, and mood. These parameters in turn depend, among other things, on the functional reserves (overcapacity) of organs that inevitably wear down with aging, depending on stressors, lifestyle, and genetic make-up (© 2018. Originally published in "Quantifying resilience of humans and other animals" under the license 4.0 (CC BY-NC-ND). Available from Scheffer et al. (2018), 115(47), 11883-11890).*

With regard to veal calves, environmental factors that may have an influence on robustness include dairy farm management on the dairy farm of origin, and transport-related factors, whereas factors inherent to the animal include calf and cow characteristics. Since there are not many studies conducted on ways to improve robustness of young veal calves (Pardon et al., 2015; Renaud et al., 2018b; Goetz et al., 2021), this thesis will investigate some factors which are hypothesized to affect robustness of veal calves. In this thesis, robustness was assumed to be reflected in short-term effects, i.e. how calves immediately coped with the challenges they were subjected to, as well as in long term effects, i.e. what were the carry-over effects of early life challenges or predisposing biological factors in the longer run. Thus, robustness was measured in terms of potential biomarkers of (later) health and performance (such as, for example, blood IgG, glucose or body weight) of calves, and in terms of prevalences of health problems, the level of use of herd and individual antibiotic and medical treatments during the rearing phase of calves.

Aim and outline of the thesis

The overall aim of the thesis is to investigate effects of different environmental and animal-related factors on robustness of veal calves. To reach this aim, the thesis will start with a review on biomarkers of health and performance in veal calves (**Chapter 2**). Then, the subsequent 3 chapters of the thesis describe effects of transport-related factors (pre-transport diet, transport duration and type of vehicle) on physiology and metabolism (**Chapter 3**), immunology (**Chapter 4**) and health and performance data of calves (**Chapter 5**). **Chapter 6** and **7** describe effects of calf characteristics (sex and breed) and cow characteristics (parity, ease of birth) and transport age of calves (14 d vs. 28 d of age) on immunity, health and performance of calves at the veal farm. ■

CHAPTER 2

Evaluating potential biomarkers

of health and performance in veal calves

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Abstract

Veal calves undergo many challenges in the early stages of their life. Such challenges, including mixing procedures and transportation of calves to the veal farm, may have a negative influence on growth rate, feed intake, metabolism, immunity and disease susceptibility of calves. As a consequence, many hematological, physiological, metabolic and immunological parameters of stressed calves might be altered on arrival at the veal farm. Some of these response variables might be useful as biomarkers of performance of calves at the veal farm as they might provide information about an ongoing disease process, or may predict future diseases. Biomarkers might be helpful to group and manage calves in different risk categories after arrival. By adopting treatment decisions and protocols on a risk-group or individual basis, it would be possible to improve animal health and reduce both disease incidence and antibiotic use. Moreover, the use of biomarkers might be an economically feasible approach as some of them do not need invasive techniques and others can be measured in blood already taken during routine checks. Previous literature mainly assessed the physiological responses of calves to transportation. However, information on the link between on-farm arrival data and future health and performance of veal calves is limited. This review, therefore, examined a wide range of papers and aimed to identify potential biomarkers of future health and performance.

Keywords: veal calves, challenges, health, diseases, biomarkers, stress

Introduction

The veal industry plays an important role worldwide as side market of the dairy industry (Pardon et al., 2014). Europe is the main veal producer, accounting for 82% of the global production in 2010. Within the European context France, the Netherlands and Italy are the leading veal producing countries with a global market share of 27, 25, and 16%, respectively (Brown and Claxton, 2011). Belgium and Germany represent 6 and 5% of global veal production, whereas other European countries either have a small veal sector such as Switzerland, or no veal production due to animal welfare restrictions such as the Scandinavian countries. Outside Europe, veal production is relatively limited; main veal producing countries outside Europe include the United States, with Canada, Australia and New Zealand each accounting for 3 to 6% of global production (Pardon et al., 2014). The current review will focus on the European scenario because Europe is the leader in veal production.

White veal calves face many challenges in the pre-weaning period (Hulbert and Moisa, 2016). These challenges include birth, transportation, mixing procedures, inappropriate management conditions and new housing environments (Brscic et al., 2012). At the dairy farm, separation of calves from their dams usually occurs immediately after birth. Subsequently, when calves are 14–20 days of age, they are gathered from different dairy farms and transported to a collection center, followed by another transport to the veal farm (Brscic et al., 2012). During these phases, calves from different farms are mixed and are exposed to new environmental conditions and management practices. All these challenges occur at an age at which the calf is immature and several physiological systems are still developing. For example, young calves are still developing their gastrointestinal tract (GIT), and their thermoregulatory (Tao and Dahl, 2013) and acquired immune systems (Warriss, 2004) are not completely functional yet. During the first week of life calves may be exposed to pathogens against which they may not have (maternal) antibodies (Autio et al., 2007; Adenkola and Ayo, 2010). The combination of the indicated challenges and the immature physiological systems of the calves may explain the high susceptibility of calves to infections. As a result, calf health and performance at the veal farm are affected (Van de Water et al., 2003; Hulbert and Moisa, 2016). Calves may develop diseases, among which respiratory diseases (e.g., bovine respiratory diseases, BRD) and enteric diseases are most frequently observed (Nonnecke et al., 2009; Pardon et al., 2012a, 2013).

Respiratory diseases are common health disorders in veal calves, which have a severe impact on both animal welfare and the income of producers, because they are the most important causes of morbidity and mortality (Woolums et al., 2005; Nikunen et al., 2007).

According to Pardon et al. (2013), BRD incidence in veal calves during the rearing period ranges between 4.6 and 43.8%, with an average of 17%. The same authors reported that of the 5.7% of veal calves which died before the end of the production cycle, 27.1% had suffered of pneumonia. Approximately two thirds of calves diagnosed with pneumonia were individually treated for BRD. Post-mortem analysis of lungs at slaughter (Brscic et al., 2012) revealed that 21.4% of veal calves showed signs of pleuritis and 13.9 and 7.7% signs of pneumonia, respectively. Bovine respiratory disease is characterized by many clinical signs, including nasal discharge, coughing, fever, inappetence, apathy and hampered respiration (Brscic et al., 2012). Both subclinical and clinical signs hamper the growth and welfare of infected calves compared with healthy animals (Snowder et al., 2006). Bovine respiratory disease is a complex disease that depends on different interacting factors. The etiology of this respiratory disorder involves several infectious agents, such as bacteria, mycoplasma and viruses, that act in synergy with stressors, like weaning, transportation, nutrition and rearing environment (Arcangioli et al., 2008; Radaelli et al., 2008). Viruses which contribute to the outspread of BRD are mainly bovine respiratory syncytial virus (BRSV), para-influenza-3 virus (PI3V) and bovine viral diarrhea virus (BVDV) (Arcangioli et al., 2008).

Enteritis is another disease that is frequently diagnosed during early stages of life and it is particularly seen in the first 3 weeks after arrival of calves at the veal farm (Pardon et al., 2013). Pardon et al. (2013) showed that, of 5.7% of calves which died, 7.5% had suffered from enteritis. Different microorganisms, including bacteria, viruses, protozoa and yeasts are responsible for enteric diseases (Stoltenow and Vincent, 2003). *Escherichia coli, Salmonella* sp., and rotavirus are the most common microorganisms, causing enteric diseases and diarrhea in young calves (Liang, 2015).

In an attempt to counteract the negative effects of diseases, the use of therapeutic treatments has become widespread (Verstegen and Williams, 2002). The use of antimicrobial growth promoters has been banned in Europe since 2006 (European Commission, 2003), but since then the use of therapeutic antimicrobials increased (Mevius et al., 2009a). A recent study demonstrated that antimicrobial use in veal calves is the highest of all food producing animals (Pardon et al., 2012a). Pardon et al. (2012b) reported that in Belgium the antimicrobial consumption in white veal calves is approximately 25.2 tons per year. In the Netherlands, one of the main veal producing countries in Europe, that has similar veal production systems as Belgium, a reduction in antimicrobial use in veal production has already been achieved during recent years. However, the usage of antimicrobials is considered still high (Mevius et al., 2009b; Pardon et al., 2012a). There is growing public concern about the consequences of feeding antibiotics (especially oral treatments) to farm animals, including veal calves, for both human and animal

health (e.g., a massive use of antibiotics may cause antibiotic resistance) (Verstegen and Williams, 2002; Di Labio et al., 2007). Therefore, there is a strong need for management strategies in the veal sector that may help to reduce the incidence of diseases and, consequently, antibiotic use.

Future implications of clinical utility of potential biomarkers

This review builds on the idea that response variables obtained in calves on arrival at the veal farm may be used as predictors or biomarkers of later health and performance. A biomarker, per definition, is a marker of a biological process or state and it can provide information on a current status or future risk of disease of an individual (Pletcher and Pignone, 2011). The availability of such biomarkers would be helpful, for example, to identify individual calves at an early stage with an enhanced probability to develop disease, and to take preventive measures before clinical problems occur. At herd level, biomarkers might be used for profiling calves according to the magnitude of stress they have experienced and their predisposition to develop future diseases (Wilson et al., 2017). Grouping of calves in different risk categories should help the farmer in managing calves at arrival. By adopting handling procedures, treatment decisions and protocols on a risk-group or individual basis, farmers might be able to better meet individual animal needs and improve the health and welfare of calves throughout the veal production chain (see also Renaud et al., 2018a). Collectively, this may reduce the incidence of disease as well as the use of antibiotics.

Previous studies (Knowles et al., 1997, 1999a; Grigor et al., 2001; Jongman and Butler, 2014) examined effects of different transport conditions and duration on calf blood constituents and performance of calves at their arrival at the veal farm. Only a limited number of studies (Mormede et al., 1982; Knowles et al., 1997, 1999b) assessed relationships between on-arrival blood constituents and future performance of calves at the veal farm. By examining a much wider range of papers, this review aimed to identify potential on-farm biomarkers of health and performance of calves at the veal farm.

Environmental challenges

Effects on physiological pathways and on biomarkers

Environmental challenges, including road transportation, are known to affect metabolic (Fazio and Ferlazzo, 2003), physiological (Minka and Ayo, 2010), immunological (Sporer et al., 2007; Hulbert et al., 2011a, b) and behavioral responses (Broom, 2003; Minka and Ayo, 2010) of calves. As illustrated in Figure 1, exposure of the animal to environmental challenges can be short-term or prolonged.

In both cases, an increase in plasma concentrations of glucocorticoids and cortisol is observed. In case of short exposure, a peak production in glucocorticoids determines an acute stress response. As a result, a calf might experience changes in its biological functions, with shifts in energy sources that allow the animal to better cope with the stressor. Moreover, an activation of the immune system, including enhanced cell function, cell-mediated, humoral and innate immunity, might protect calf health (Dhabhar and Mcewen, 1997). All these changes might restore homeostasis in the short-term and not affect animal health and welfare on the long run. In case of prolonged exposure, persistent higher concentrations of glucocorticoids may lead to prolonged/chronic stress response (Figure 1). Under these circumstances, the activation of the hypothalamic-pituitary-adrenal (HPA) axis is responsible for long-lasting effects on the animal body. Effects include changes in catecholamine release, growth hormone (GH) secretion and modulation of thyroid-stimulating hormones. Additionally, calves might experience BW losses due to increased dehydration and nutrient mobilization accompanied by changes in rectal temperature, enzymes concentrations in the blood and a suppression of the immune function. As a consequence of prolonged stress exposure, calves may experience changes of biological functions to an extent that the risk of developing diseases is increased (Moberg, 2000). This review will first focus on the main effects of HPA axis activation as one of the main pathways between exposure to environmental challenges and susceptibility to disease will be discussed. Therefore, a description in changes in cortisol and BW will be reported. The current review will first discuss dehydration-related variables and then variables associated with nutrient metabolism. Then changes in rectal temperature, immune cells and enzyme concentrations will be discussed. Interactions between different physiological variables and details about the corresponding biological mechanisms involved will be also explained. All these effects will be discussed in association with disease development in young veal calves in the first 3 weeks at the veal farm. Moreover, it will be discussed which parameters might be the most important biomarkers that could be used at on-farm arrival to predict health and welfare of calves at the veal farm. For achieving these goals, each paragraph of this review will contain a description of the physiological role of the variable of interest, how it is affected by environmental challenges, and the possible association of the variable with an ongoing disease process or later disease susceptibility. Finally, some conclusive remarks on the potential use of the variables as biomarker will be made and some advices for future studies will be given.

Cortisol

Cortisol is a glucocorticoid hormone derived from cholesterol. Cortisol is the primary hormone involved in the stress response and is regulated by the HPA axis. The main action of cortisol consists of activating biological functions to respond to stress and

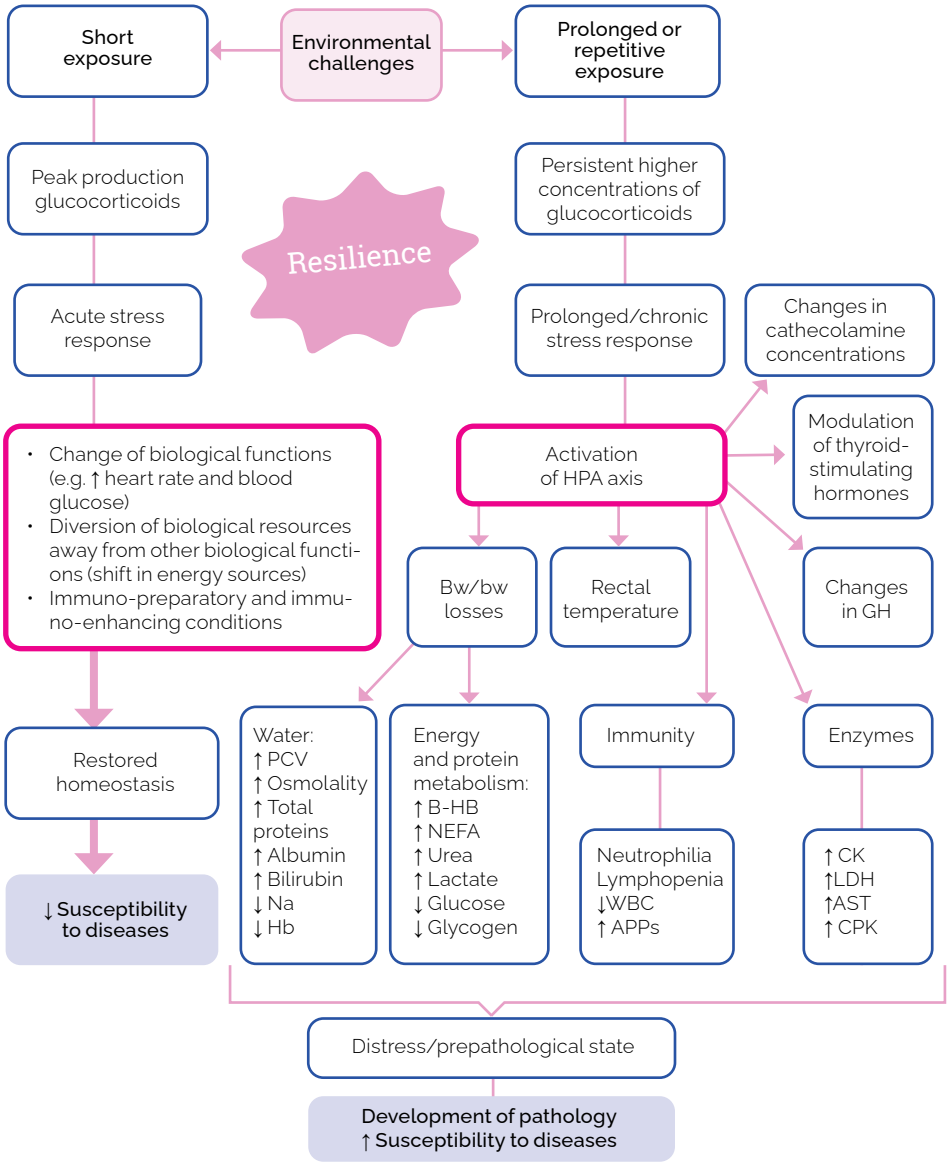


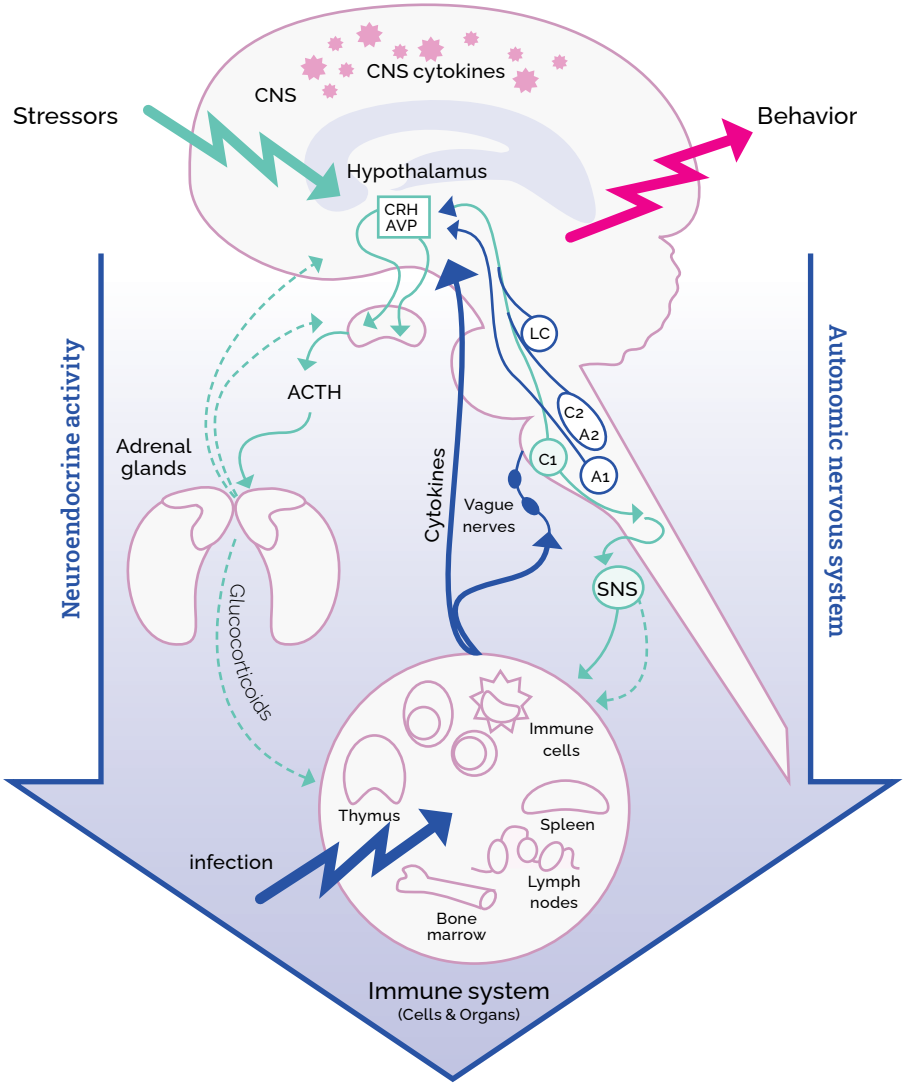
Figure 1. **Effects of short and long term exposure to environmental challenges on disease susceptibility.** *HPA axis = hypothalamic-pituitary-adrenal axis; GH = growth hormone; BW = body weight; PCV = packed cell volume; Hb = hemoglobin; β-HB = β- hydroxybutyrate; NEFA = non esterified fatty acids; WBC = white blood cells; APPs = acute phase proteins; CK = creatine kinase; LDH = lactate dehydrogenase; AST = aspartate aminotransferase; CPK = creatine phosphokinase.*

restoring homeostasis after exposure to stress (Mormede et al., 2007). Transportation of calves to the veal farm, as well as management of calves at the dairy farm of origin and at the collection center, are challenges that increase the activity of the HPA axis (Minka and Ayo, 2010). A rise in plasma cortisol concentrations was often observed in transported calves and it is the main indicator of psychological/physiological stress (Figure 1) (Alberghina et al., 2001; Odore et al., 2004). The increase in plasma cortisol concentrations can be transient when normal levels are restored in 1–2 days or chronic when hyper-cortisolemia continues for at least 4–5 days (Fujiwara, 1996). Grigor et al. (2001) found significantly higher concentrations of cortisol in transported 10-days-old calves (up to 25.2 nmol/l) compared with non-transported calves (up to 16.3 nmol/l). Bernardini et al. (2012) also observed an increase in cortisol (up to 23.7 nM) in young calves (37 ± 6 days of age) following transportation for 19 h. However, cortisol concentrations were not extremely high compared to normal levels (18.4 nM) and calves restored their basal cortisol levels within 2 days.

Aich et al. (2009) found considerably higher cortisol levels the day prior to BRD infection in animals that died compared to those surviving a synergic viral-bacterial infection. However, these concentrations (150 mmol/l cortisol in serum) are indicative of the current health status of the animal and not maintained beyond that day. In all the previously mentioned studies the rise in cortisol concentrations was observed on a short-term and there was no information on chronic elevations of cortisol.

Different studies (Fujiwara, 1996; Rosmond, 2005) reported that prolonged high cortisol concentrations can result in increased glucose metabolism, insulin resistance, inhibition of glycogen synthase in the skeletal muscle and visceral obesity. As a consequence of these adverse health effects, the animal might be less resilient to diseases. These metabolic changes might lead to problems, including hyperglycemia, insulin resistance, glucosuria and reduced energy utilization at the end of the producing cycle at the veal farm. Moreover, changes in circulating glucocorticoids concentrations, and thus cortisol, are responsible for changes in cytokine levels and the production by leukocytes (DeRijk et al., 1996; DeRijk and Sternberg, 1997; Deuster et al., 1999). Therefore, when calves are stressed and have high cortisol levels for a prolonged period, calves are at risk for an altered immune function (Vegas et al., 2011). Figure 2 shows the pathways through which glucocorticoids affect the immune system and, thus susceptibility to diseases (Vegas et al., 2011).

Cortisol, by impairing the immune functionality, might be used as biomarker for predicting future diseases. Calves with chronic hyper-cortisolemia and changes in their immune cell parameters (e.g., neutrophils, lymphocytes and acute phase proteins, APPs) might



Adapted from
Webster et al, 2002

Figure 2. **Diagram of routes of communication between the brain and the immune system, including HPA axis, sympathetic nervous system, and cytokine feedback to the brain**
(© 2011. Oscar Vegas, Larraitz Garmendia, Amaia Arregi and Arantza Azpiroz. Originally published in "Effects of social stress on immunomodulation and tumor development" under CC BY 3.0 license. Available from Vegas et al. (2011)).

be profiled as high-risk calves. Further research is needed to establish a relationship between chronic high cortisol concentrations upon arrival at the veal farm and incidences of health and metabolic disorders during the subsequent fattening period (4–5 months). Chronic hyper-cortisolemia might be measured by taking repetitive blood samples, for example, in the first 2 weeks after arrival at the veal farm. Then, it should be checked whether the overall cortisol levels in serum are consistently increased during the period of blood collection. Moreover, future studies should address the relationship between chronic hyper-cortisolemia and functionality of the immune system. There is a need to clarify what type of immune cells have the greatest effects on the health of veal calves, and especially on the probability of developing respiratory or enteric diseases. All this information will provide useful data for clarifying the role of cortisol as biomarker of future diseases. However, chronic hyper-cortisolemia can only be assessed by repeated measurements. With regards to feasibility, it would be preferable to use non-invasive techniques such as collecting saliva (Negrao et al., 2004) or hair (Cook, 2012) samples instead of blood to prevent induction of acute stress response during sampling.

Body weight (BW) and BW losses

Measurement of BW losses, defined as the differences in BW before and after transport, is an indicator of the hydration status and/or body nutrient mobilization (e.g., fat or proteins). Environmental challenges, and especially long transport durations, are important causes of dehydration, fat mobilization, muscle protein degradation and thus loss in BW (Figure 1) (Minka and Ayo, 2010). Therefore, BW before transport and upon arrival at the veal farm may reflect the dehydration and metabolic state of the animal. Body weight per se, is related to the condition and birth weight of the animal. Calf BW on arrival at the farm might also influence the performance of the animal in the first weeks of the producing cycle.

It has been reported that calves may lose between 3 and 11% of their BW during transport to the farm (Warriss, 1990). Bernardini et al. (2012) found that calves unloaded at the veal farm after 19 h transport had 6.4% BW losses compared with non-transported calves. Calves may lose BW after long transport durations because of stress coupled with fasting and mild dehydration (Tarrant et al., 1992; Von Borell, 2001). Calves that lose more than 8% of BW in 1 day are depressed (clinical symptoms: skin tenting >10 s, eyes very sunken, dry gums and the calf lays down) and require intravenous treatment; BW losses >14% might even lead to calf death (Naylor, 1989). There is evidence that BW loss is highly influenced by feeding and water provision prior to transport of calves to the veal farm (Knowles et al., 1997; Bernardini et al., 2012). Moreover, an increase in plasma protein, albumin, osmolality and packed cell volume (PCV) in calves with high BW loss, suggests that BW losses are particularly due to dehydration of calves.

Body weight losses, resulting from dehydration might reduce the adaptive capacity of the animal. According to Renaud et al. (2018a) the risk of mortality increases when calves are >10% dehydrated. However, mechanisms underlying the loss of BW as a reflection of dehydration have to be further explored.

Table 1. **Some associations between body weight (BW) and future risk of respiratory diseases**

BW & future risks of respiratory deseases				
Reference	**Mean arrival BW (kg)**	**Prevalence (predicted means) of respiratory diseases**		***P*-value**
Brscic et al. (2012)	≤ 43 43-47 48-51 > 51	7.6 % 6.1 % 6.6 % 2.7 %		0.004
Reference	**BW at arrival**	**Hazard ratio for early mortality (< 21 days after arrival at the veal farm)**		***P*-value**
Renaud et al. (2018b)	Per 1 kg increase	0.93		< 0.01
Reference	**BW at arrival**	**Hazard ratio for early mortality (< 21 days after arrival at the veal farm)**		***P*-value**
Winder et al. (2016)	Per pound	0.99		0.03
Reference	**Mean arrival BW (kg)**	**Incidence Rate ratio (IRR) for BRD[1] morbidity**	**IRR for Mortality**	***P*-value**
Cernicchiaro et al. (2012)	272 - 317 318 – 362 > 362	1.08 0.69 0.55	0.99 0.71 0.52	< 0.05
Reference	**Mean arrival BW (kg)**	**Cumulative mortality risk for BRD**		**P > χ2**
Babcock et al. (2010)	363 – 408 318 – 362 272 – 317 227- 271 182 – 226 < 182	0.02 0.12 - 0.01 0.04 0.40 0.44		0.0012

[1]*Bovine respiratory disease.*

It was reported that BW at arrival is associated with the prevalence of respiratory diseases at 3 weeks upon arrival at the farm (Brscic et al., 2012). As shown in Table 1, veal calves with BW > 51 kg had the lowest probability to develop respiratory diseases at the veal farm (adjusted R^2 = 25%) (Brscic et al., 2012). In recent Canadian studies (Winder et al., 2016; Renaud et al., 2018a, b) body weight of calves at arrival at the veal farm was also inversely associated with early mortality (Table 1). Other studies on feedlot cattle used mean cohort arrival body weight as a useful predictor of future diseases and health problems (Lechtenberg et al., 1998; Babcock et al., 2010; Cernicchiaro et al., 2012). Lighter-weight cattle presented a higher incidence of BRD morbidity and overall mortality compared to heavier cattle (Cernicchiaro et al., 2012) (Table 1).

From these findings, it can be concluded that BW might be a useful parameter to monitor and to predict the health status of calves at the veal farm. At this stage, BW upon arrival appears to be the most reliable predictor for future diseases at the veal farm. However, it is still unclear whether lower BW values at arrival are due to lower birth weights of calves or due to substantial BW losses during transportation, or a combination of both. This should be elucidated in future research. As shown in Table 1, it seems that differences in BW in the study of Brscic et al. (2012) were likely related to different factors (e.g. BW losses, age, colostrum management), whereas in the other studies on feedlot cattle the difference in BW is mainly caused by a different developmental stage of calves. Breed is another important factor that might be related to differences in BW. For example, there is an increasing demand in the veal industry for Holstein Friesian-beef breed cross breeds. Moreover, future studies should investigate whether BW losses are also predictive of the future health state of veal calves. With regard to feasibility, in case of an equal predictive value of both BW losses and BW, BW values are simpler to obtain than those for BW losses. In fact, for measuring BW, the animal is just weighed one time upon arrival at the veal farm, whereas in case of BW losses it is necessary to record BW before and after transportation of calves to the veal farm. Therefore, BW might be a more practical and feasible biomarker compared with BW losses. On the basis of BW values, a farmer might profile veal calves by separating putatively high-risk calves with a lower BW from the rest of animals. Then, lighter calves might receive a special daily care and management in order to avoid the spread of diseases and a lower production performance compared with healthy calves.

Dehydration-related variables

PCV

Part of BW losses or low BW of calves may be due to dehydration. Some specific variables may be related to dehydration, among which packed cell volume (PCV). PCV is a variable related to the number of red blood cells (RBC) in an animal. By definition, PCV is

the ratio of the red blood cells to the volume of whole blood (which contains also white blood cells and plasma). PCV can be influenced by environmental challenges, such as transportation of calves to the veal farm. PCV values recorded by Knowles et al. (1997) range from 39.8% in control non-transported calves to 40.8% in 24-h transported calves. This finding was confirmed in other studies (Tadich et al., 2005; Minka and Ayo, 2010) on long-term transport of young cattle. The increase in PCV can also be found in transported calves in combination with fasting of 48–72 h. This is associated with a greater water loss during transportation as well as the stress of adapting to a new environment (Odore et al., 2004; Minka and Ayo, 2010). By contrast, other studies demonstrated a decrease in PCV in animals transported by road (Broom et al., 1996; Knowles et al., 2003). After 8 h transport, calves showed a significantly lower PCV value (33.7%) compared with the control non-transported calves (38.2%) (Knowles et al., 1997). These results might be due to restraint and handling procedures before transport or stressors during transport (Parrot et al., 1988). In fact, an increased cortisol concentration seems to move water from the rumen into the plasma resulting in a decrease in PCV values (Broom et al., 1996).

Both higher and lower PCV values might be good indicators of an ongoing disease process. Calves with diarrhea might experience excessive fluid losses that lead to higher PCV values (Pare et al., 1993). By contrast, lower PCV can be used for the diagnosis of anemia or other health problems (Turkson and Ganyo, 2015).

As explained previously, PCV values can indicate the extent of dehydration of a calf on arrival at the veal farm. When a calf is dehydrated, it may experience a weight loss. Moreover, if not treated immediately, consequences of dehydration might be still visible in the first weeks at the veal farm. Therefore, the ADG and gain:feed ratio may be negatively affected in the first weeks at the veal farm (Cole et al., 1988). Seifi et al. (2006) demonstrated that calves up to 14 days of age with PCV values at arrival above 44.17% were 4 times more likely to die. This was in agreement with Klee et al. (1979) who observed a reduction in treatment efficacy in calves with diarrhea when PCV was above 50%. An accurate analyses of PCV values may, therefore, provide information on calves that need to receive extra care in order to minimize body weight losses and reduction in performance due to water losses in the first weeks at the veal farm. Therefore, by identifying potentially high-risk calves with significantly higher (above 43%) PCV values, PCV might be a reliable biomarker of diseases at the veal farm.

Total protein

Alongside with PCV, an increase in total protein (TP) and albumin concentrations in the plasma are also measurements reflecting dehydration of the animal (Swanson and Morrow-Tesch, 2001). TP is also an important indicator of the amount of colostral

proteins in young calves, that is reflecting the immune state of these animals (Wilson et al., 1994). This parameter can be influenced by environmental challenges, including transport duration. Bernardini et al. (2012) found significantly higher plasma total protein concentrations (63.9 g/l) in young calves (37 ± 6 d of age) subjected to 19 h transport at their arrival at the veal farm compared to non-transported calves.

Values of TP are important, especially for predicting mortality in the first weeks at the veal farm (Wilson et al., 2000). Naylor et al. (1977) observed a significantly lower mortality in the first 5 weeks of age in calves with TP > 6.1 g/dl. Moreover, Rea et al. (1996) reported that calves with TP < 4.5 g/dl had a higher risk of dying in the first weeks at the farm. By contrast, other studies suggested that TP cannot be considered a reliable indicator of diseases and mortality in calves (Wilson et al., 1994; Pardon et al., 2015). Hence, TP is difficult to interpret in terms of risks for calf health and performance. Higher levels of TP may indicate higher levels of colostral proteins, which is a positive sign. However, high levels of TP may also be indicative of dehydration, which is a negative sign.

Albumin

Albumin is the major negative acute phase protein (APP). During the acute phase response, albumin concentrations decrease for the synthesis of positive APP. Hence, albumin is a main source of amino acids that animals can use when necessary and it plays an important role in plasma osmotic pressure (Tothova et al., 2014). As shown by the study of Knowles et al. (1999a), albumin concentrations increased from 39.8 to 43.1 g/l in calves subjected to transport as a result of dehydration.
Albumin might be used not only as a measure of dehydration, but also as a prognostic marker or to assess the severity of diseases (Horadagoda et al., 1999; Humblet et al., 2004; Schneider et al., 2013). For example, low albumin concentrations in dairy cattle were associated with uterine infections (Schneider et al., 2013) and inflammation (Jacobsen et al., 2004; Petersen et al., 2004).

Limited research (Pardon et al., 2015) has been done in young veal calves, especially on the predictive value of albumin. The available evidence suggests both high and low albumin values may indicate a risk. Thus, it is necessary to understand to what extent an increase or a decrease in albumin values is associated with diseases or future health problems in veal calves.

Bilirubin

Bilirubin is a product of heme degradation and it functions as antioxidant (Stocker et al., 1987). This variable is influenced by environmental challenges. Mormede et al. (1982) found an increase in plasma bilirubin (5–10 µmol/l) in young calves after being

transported to the veal farm; this increase was more pronounced in calves subjected to long-transport duration.

The higher concentrations of this variable might be determined by an increased dehydration of the animals subjected to transport. However, their increase could also be related to a compromised health status of calves. Higher bilirubin concentrations were indicators of impaired hepatic function in dairy cows in a negative energy balance situation and with inflammation (Bertoni et al., 2008). Extrapolated to calves, this would imply that calves might experience metabolic changes in the liver as a consequence of stress during transport. However, until now no studies on veal calves examined bilirubin as a possible biomarker of ongoing health problems or predictor of future diseases.

Electrolytes and minerals

Electrolytes and minerals are responsible for maintaining a good water balance and for normal functioning of essential biochemical processes in the animal body (Spears, 1999). Calves may experience changes in their electrolyte and mineral balance when they are transported under stressful conditions (Minka and Ayo, 2010). Cattle have a substantial blood buffering ability but during transport they show plasma electrolyte and mineral changes, including sodium, potassium, chloride, calcium and magnesium (Schaefer et al., 1990, 1997; Parker et al., 2003). In a state of stress, there are higher concentrations of calcium in the extracellular fluids that lead to a greater contractility of skeletal and heart muscles (Davidson et al., 2004). Moreover, Grigor et al. (2004) found significantly higher plasma sodium concentrations after 5.25 h of transport in fed calves (136 mmol/l) compared with the unfed controls (133 mmol/l), but it is unclear what this relatively small difference in plasma sodium means.

Changes in sodium values might be useful as indicators of calf diarrhea. Calves with diarrhea generally have significantly lower concentrations of serum sodium than healthy controls (Klinkon and Ježek, 2012). This is in accordance with Maach et al. (1992), who found that calves with acute diarrhea had lower plasma concentrations of sodium and chlorine (131.2 ± 6.8 mmol/l, 95.6 ± 6.9 mmol/l, respectively) in their serum compared with healthy calves (140.0 ± 9.9 mmol/l, 103.3 ± 6.9 mmol/l, respectively). Calves with diarrhea can also have hyperkalemia as a consequence of the dysfunction of the Na+/K+ ATPase (Constable, 2002).

Seifi et al. (2006) found that serum potassium concentrations >5.63 mEq/l in calves with diarrhea were associated with 4-fold increase risk of mortality. Moreover, hyper--kalemia (K > 5.8 mmol/l) can cause severe dehydration and can be associated with

changes in body temperature homeostasis (Trefz et al., 2013). As a consequence of the alteration of physiological mechanisms, calves might be more predisposed to develop severe diseases.

Osmolality

Transported calves may also experience changes in their plasma osmolality, which is an indicator of the osmotic pressure of the plasma (Brownlow and Hutchins, 1982; Knowles et al., 1997, 1999b; Grigor et al., 2001). Knowles et al. (1997) observed the highest values of plasma osmolality in young calves transported for 24 h (278 mOsm/kg) compared with control calves (275.4 mOsm/kg). These results indicated that calves become more dehydrated when they are subjected to a longer transport duration. The effect is likely to be more pronounced when calves are deprived of food and water prior to transport (Knowles et al., 1997). Osmolality is, therefore, an important indicator of the hydration status of the animal (Grigor et al., 2001; Kaneko et al., 2008).

The dehydration status of a calf might be related with an increased likelihood to develop diseases at the veal farm (Wilson et al., 2017). Griffin et al. (2010) assigned calves suffering from dehydration, combined with malnourishment and exhaustion to a high-risk class, with a greater probability of becoming sick. Moreover, Renaud et al. (2018a) reported that the degree of dehydration at arrival is an important predictor of mortality in the first 21 days after arrival at the veal farm.

Hemoglobin

The white veal calf industry has always raised calves with a low hemoglobin (Hb) status. Hb acts as a transporter of oxygen from the lungs to the tissues and as a transporter of carbon dioxide from the tissues back to the lungs. This function depends on the molecular structure of hemoglobin, which contains four heme groups, each with a central iron molecule (Rifkind et al., 1988). Due to the specific diet based on milk replacers and solid feeds with low iron content for several weeks, calves might develop anemia, which has a negative impact on growth and feed conversion ratio (Reece and Hotchkiss, 1987; Gygax et al., 1993). However, in the interim period or at the end of the fattening cycle, lower Hb concentrations are desirable because they are associated with low myoglobin and pale meat (Miltenburg et al., 1991).

Nowadays, the industry pays more attention in maintaining Hb values a certain range. In the Netherlands, all calves are monitored within the first 2 weeks upon arrival at the veal farm and calves with Hb levels below a certain threshold are treated with supplemental iron. Another systematic monitoring is done between 12 and 14 weeks of fattening (EFSA, 2012). However, outside these moments blood Hb (or iron) values of veal

calves are usually not systematically monitored, and it is likely that some animals may develop subclinical anemia. Therefore, measurement of Hb can be used to assess on-farm anemic state of calves.

Hb might also be used as predictor of diseases in calves. As a consequence of their anemic status, both health and robustness of calves are affected and calves are more vulnerable to diseases (Bami et al., 2008). In animals iron deficiency (with blood Hb lower than 6.0 g/dl) is known to affect both humoral and cell-mediated immunity (Oppenheimer, 2001). As a consequence of the impaired immune functionality, calves might be more susceptible to infectious diseases. Steinhardt and Thielscher (2005) observed that the growth of calves with low Hb values at arrival at the veal farm was lower compared with calves with normal range of Hb. Gygax et al. (1993) reported also that iron deficiency can lead to higher infection rates, especially in the respiratory and the gastrointestinal tract. Therefore, these findings suggested that Hb values upon arrival at the veal farm may be good biomarkers for predicting calf health and welfare status after transportation to the veal farm; lower Hb levels are indeed more likely to be associated with a major risk of poor welfare and a higher incidence of diseases (Blum and Hammon, 1999; Enjalbert, 2009). Hence, the deliberate attempt by the veal industry to avoid anemia in young calves is through the administration of iron.

Conclusions on dehydration-related variables

All variables discussed in this paragraph are correlated with dehydration and PCV seems to be the most suitable and practical on farm biomarker. However, their association with disease status may not only be based on dehydration status, but also on factors like stress, colostrum intake, acute phase proteins and antioxidative status. Therefore, more research on correlations among these variables and the occurrence of respiratory or enteric diseases would be necessary to perform. Based on research currently reviewed, it can be disputed which dehydration related variable is most reliable as on-farm biomarker.

Energy and protein metabolism

Lactate

Besides dehydration, losses of glycogen, protein and fat may affect metabolism and future diseases in calves. In order to understand the consequences of body glycogen, protein and fat mobilization on future health problems, different variables can be measured. Calves may experience a rise in lactate levels, especially after a long-distance transport (Todd et al., 2000). This might be the result of the degradation of muscle glycogen due to stress or exhaustion, that causes the liberation of catecholamines and a rapid glycogenolysis and gluconeogenesis (Chacon et al., 2005).

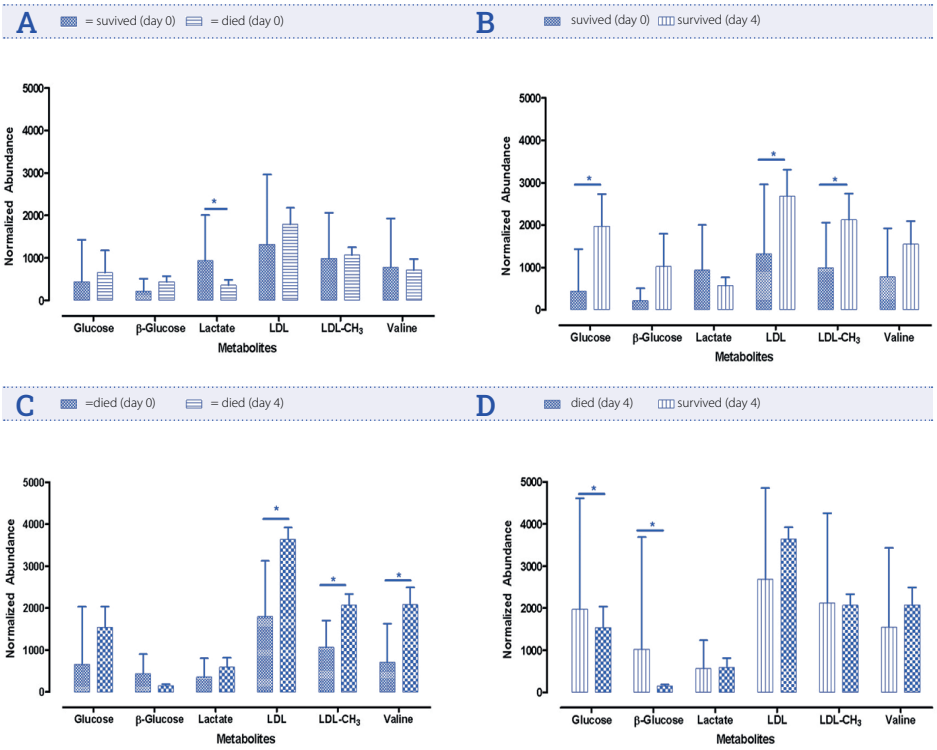


Figure 3. A-D: **Metabolite profiles for animals that survived or died.** *Bar charts for distribution profile of identified metabolites from 1H-NMR studies for animals that died or survived following synergic viral–bacterial infection are shown in bar chart form. Error bars shown indicate 1-standard deviation. Metabolite IDs are shown on the x-axis (The publisher for this copyrighted material is Mary Ann Liebert, Inc. publishers (Aich et al., 2009)).*

In a study in cattle (Aich et al., 2009), L-lactate levels were higher prior to viral infection. Moreover, higher L-lactate values are related with higher risk of disease occurrence, like displaced abomasum and volvulus, in different animal species (Johnston et al., 2007; Zacher et al., 2010; Boulay et al., 2014), and especially in calves with diarrhea (Lorenz 2004, 2009; Lorenz and Vogt, 2006). A positive correlation (r = 0.55) between acidosis (determined by L-lactate) and dehydration (determined by PCV) was found in calves with diarrhea (Naylor, 1987).

Associations between lactate concentrations and measures of later calf health were also reported by different studies, with contradictory results. Aich et al. (2009) found that calves which survived had significantly higher lactate concentrations prior to a viral infection compared to calves which died (Figure 3A). In contrast, in a study by Coghe et

al. (2000), relatively high lactate levels (>4 mmol/l) in BRD affected calves were associated with an increased likelihood of mortality in the following 24 h.

Buczinski et al. (2015) reported that for each 1-unit increase of the log-lactemia there was an increase of 36.5 in hazard of dying of BRD. In general, enhanced L-lactate levels are associated with hypoxemia (Tyler and Ramsey, 1991) and /or endotoxemia (Morris et al., 1986) that characterizes ongoing BRD episodes. Lower oxygen levels in the lungs are known to reduce macrophage activity, so pathogens can multiply at higher rates. As a result, the animal might be more exposed to the action of pathogens and it might develop respiratory diseases (Guterbock, 2014).

Thus, L-lactate might be considered a biomarker for assessing an ongoing disease process, such as pneumonia (based on clinical signs and hypoxemia) and for predicting death of clinically ill calves within 24 h. However, contradictory results merit further research into the use of L-lactate and lactate as biomarkers for respiratory diseases in young veal calves. At the same time, practical application of this biomarker might be highly feasible, because it can be measured by a portable analyser at a relatively low cost and results are available in 60 s (Buczinski et al., 2015).

β-Hydroxybutyrate (β-HB) and Non-esterified fatty acids (NEFA)

Environmental challenges, as depicted in Figure 1, are the main cause of mobilization of fat resources in young calves (Frohli and Blum, 1988). The mobilization of the adipose tissue is associated with an increase in plasma concentration of free fatty acids and β-hydroxybutyrate (β-HB) (Grigor et al., 2001; Winder et al., 2016). Transportation as well as fasting increase the energy demands of young veal calves, so animals experience hypoglycaemia and a lack of C3 units in mitochondria (Cole et al., 1986; Ishiwata et al., 2008). As a result, C2 units accumulate in the mitochondria and are removed via production of ketone bodies. Bernardini et al. (2012) found an increase in NEFA and β-HB above the normal values (0.13–0.20 mmol) at the end of transportation. These results were in accordance to Radostits et al. (2007) and Knowles et al. (1997), who found concentrations up to 0.46 mmol/l β-HB and 0.55 mmol/l NEFA in young calves (between 1 and 2 weeks of age) transported for 24 h. Moreover, the increase in NEFA and β-HB appears to be greater in unfed calves compared with calves that receive feeding prior to transport (Knowles et al., 1997).

β-hydroxybutyrate and NEFA values represent useful indicators of calf energy balance and of body fat mobilization during and immediately after transportation of calves to the veal farm (Knowles et al., 1999b).

Changes in energy balance of calves might be associated with changes in biochemical, endocrinological and metabolic pathways underpinning production, maintenance of health and ability to cope with disease challenges (Van Eerdenburg and Adewuyi, 2005). According to Wilson et al. (2000) and Renaud et al. (2018a) many male calves entering the veal facility are experiencing suboptimal energy status and low body fat cover. A suboptimal energy status and higher levels of NEFA in the serum facilitate disease development and suppress the immune function (Collard et al., 2000). Future studies should continue to investigate the complex link between the immune system responses associated with increase in blood NEFA levels and β-HB concentrations. So far, associations between negative energy balance, NEFA and β-HB have only been studied in adult dairy cattle (Fenwick et al., 2008), and there is little information available on the effects of these parameters on disease incidence later in life of young veal calves. Renaud et al. (2018a) reported that NEFA and β-HB might be used as markers of the energy status, but in this study they were not associated with morbidity or mortality. However, this study was based on short transport duration, thus more research is needed to explore the effects of long transport duration on energy status.

Urea

Plasma urea concentrations might also be affected by environmental challenges (Figure 1), including transportation (Minka and Ayo, 2010). Higher plasma urea values are indicators of protein and nucleic acids breakdown in the muscles as a result of increasing cortisol concentrations and prolonged fasting (Knowles et al., 1999a). Knowles et al. (1997) observed that young calves (<1 month old) receiving 1 l of glucose/electrolyte solution during transport showed lower urea (3.58 g/l) values after 24 h transport compared with the control group (4.50 g/l). In another experiment, these authors found higher plasma urea values in calves subjected to the same transport duration (5.61 g/l in 24 h) when not being fed during the journey compared with control calves (5.34 g/l) (Knowles et al., 1997). These results suggest that feeding during long transport durations may help calves in reducing muscle protein degradation during their journey to the veal farm.

Urea concentrations might be used to assess the acute disease state of calves. In fact, protein catabolism, growth retardation and excessive nitrogen excretion might be the consequences of an ongoing disease process (Seppa-Lassila et al., 2017). However, additional information is needed to understand the underlying physiological and immune mechanisms involved in disease incidence.

Fayet and Overwater (1978) analyzed several biochemical parameters of newborn calves and associated these variables with future survival of calves. Among these variables, blood urea concentration was a reliable predictor of survival rate of calves with

80% accuracy. The authors found significant differences in average urea concentrations between surviving calves (68.5 ± 35.7 mg/dl) and dead calves (141.4 ± 78.0 mg/dl). Seifi et al. (2006) conducted another study in young calves (up 14 days of age) with diarrhea. The authors aimed to investigate the associations between serum biochemical variables and future survival of calves. The results of this study showed that blood urea nitrogen (BUN) concentrations can be used as valid prognostic indicators. Calves with diarrhea were 5.6 times more likely to die when their BUN concentrations were higher than 13.07 mmol/l. Correspondingly, Klee et al. (1979) also demonstrated that the efficacy of treatment in calves with diarrhea was lower when BUN concentrations were above 28.56 mmol/l. However, given these differences in threshold levels, additional research is necessary before urea could be used in practice as biomarker.

Glucose

As indicated in Figure 1, higher cortisol and glucocorticoids concentrations following transportation or other challenges might cause changes in plasma glucose. Previous studies on changes of plasma glucose concentrations in transported calves revealed different outcomes. At one hand, some authors (Kent and Ewbank, 1983, 1986a, b; Swanson and Morrow-Tesch, 2001) found higher plasma glucose levels which may be a result of elevated stress levels that activate the hypothalamic-pituitary-adrenal axis (HPA). On the other hand, Trunkfield and Broom (1990) showed that calves experienced hypoglycaemia after transportation, which may be a consequence of the activation of HPA axis, higher glucocorticoids and catecholamine concentrations and because of the higher energy requirements during the journey and restriction of food and water intake before, during and after transport (Tadich et al., 2005). Mormede et al. (1982) reported a decrease of 38 and 54%, respectively in plasma glucose level in young calves after short and long transportation. Alteration in plasma glucose concentrations are highly influenced by the time and plane of feeding before and after transportation of calves (Knowles et al., 1999a). Differences in this respect may explain the great variability among studies on glucose in calves after transport. On a short-term and shortly after feeding, higher cortisol concentrations can cause an increase in plasma glucose, whereas on a longer-term, glycogen stores can be depleted and thus blood glucose concentration is lower.

Studies in cattle reported that a disease challenge initially resulted in hyperglycemia, followed by a period of hypoglycemia (Steiger et al., 1999; Kushibiki et al., 2000). Montgomery et al. (2009) found lower plasma glucose levels than normal (5.3 ± 0.07 mmol) in an experiment with heifers treated for BRD. Moreover, hypoglycemia was found to be related to neonatal calf diarrhea and endotoxaemia in calves (Trefz et al., 2016). However, more research should be done to investigate the association between hypoglycemia and acute diarrhea in veal calves.

Cusack et al. (2003) found that low plasma glucose levels in calves on arrival at the feedlot were associated with a greater probability of developing severe BRD at later stages. This is in accordance with another study on steers transported for 12 h, in which low blood glucose levels after transport increased the incidence of morbidity and mortality in these animals in the following 56 days (Cole et al., 1988). Additionally, in the study of Trefz et al. (2016) calves with severe hypoglycemia had a lower survival rate (20.6%) compared with calves with normal plasma glucose concentrations (74.0%). Mormede et al. (1982) also reported that hypoglycaemia after transport in combination with lower growth rate in the first weeks after transport may even affect performance at later stages. Accordingly, Aich et al. (2009) demonstrated with metabolomic analyses that higher glucose concentrations in calves of 6 months of age, following a viral BRD infection predicted survival of the animals. Against this background, several studies showed the potential of glucose as important predictor. Therefore, lower glucose concentrations might be used as on-farm biomarker of future diseases in veal calves.

Body temperature

Temperature homeostasis is important in order to guarantee the functionality of the main physiological mechanisms in the animal body. Young calves have a limited ability to regulate their body temperature, especially during transportation (Hemsworth et al., 1995; Borderas et al., 2009). According to Hemsworth et al. (1995), the thermal comfort zone of young calves is between 13 and 26°C, and they are sensitive to both heat and cold stress (Knowles, 1995). Elmer and Reinhold (2002) observed that young calves up to 6 weeks of age are the least tolerant to high ambient temperature (35°C) compared to older animals, especially during long journeys. Changes in rectal temperature can be caused by acute or chronic secretion of catecholamines and glucocorticoids (Burdick et al., 2010). In case of acute stressors, such as handling and loading procedures prior to transport, rectal temperature can increase as a result of peak production in glucocorticoids and cathecolamines (Burdick et al., 2010), whereas, prolonged transport, can decrease rectal temperature to normal temperature (Behrends et al., 2009). Garcia et al. (2015) classified calves with pneumonia based on rectal temperature ≥39.5°C accompanied by clinical signs of respiratory disease (e.g., mucopurulent nasal discharge, cough, increased respiratory rate). McGuirk and Ruegg (2017) also reported that rectal temperature higher than 39.4°C for two successive days in combination with a slower, lower or lack of milk intake are indicators of diseases. Particularly, rectal temperatures >41°C are associated with pneumonia (McGuirk and Ruegg, 2017). However, as reported by Galyean et al. (1995), rectal temperature might be influenced by other factors as well, including processing order, crowding, ambient temperature and humidity. These factors should be considered when assessing animal performance and, especially BRD incidence, on the basis of rectal temperature.

Grigor et al. (2001) found a positive correlation (r_s = 0.649, $P < 0.01$) between rectal temperature of young calves during the first week after transport and first week post-transport clinical respiratory disease score (points from 0 to 3, and greater scores were associated with a worse health status). Higher rectal temperatures in transported calves seemed to indicate a greater clinical response to either BHV-1 inoculations or to other infections. Calves might also experience hypothermia, which might be considered as an adaptive response related to post-transport severe dehydration and hypoglycemia. Hypothermia might cause impairment of the Na+/K+-ATPase, which is temperature dependent, and as a result animals might be more susceptible to diarrhea or other health problems (Baptiste et al., 2000).

Collectively, these findings show simple associations between rectal temperature and current health status of calves but not a clear predictive value of rectal temperature. However, both high and low rectal temperature might be associated with future health problems; future studies could investigate whether the negative effects are more pronounced in relation to high or low rectal temperature. With regard to feasibility, measurement of rectal temperature is a very easy, quick and non-invasive approach, thus it can be used by farmers on a frequent basis to check the health state of calves.

Immunity

Leukocyte count and other immune responses

As shown in Figure 1, environmental challenges affect circulating glucocorticoids (increase in cortisol levels) in calves. As a consequence of stress-driven higher concentrations of stress hormones, the immune system might be affected. On the one hand, acute stress might result in immuno-preparatory conditions, by helping the animal to reinforce its defense against pathogens; on the other hand, long-term exposure to stress might have immunosuppressive effects by making calves less resilient to diseases (Avitsur et al., 2006). Therefore, in case of chronic stress, the functionality of the immune system might be impaired and calves might have changes in number of leukocytes, neutrophils in the peripheral circulation and other immune cells (Odore et al., 2004; Yun et al., 2014). An increase in neutrophil and mononuclear cell ratio (N:M) was reported (Burton et al., 2005; Gupta et al., 2007). An increase in number of neutrophils was also reported (Avitsur et al., 2006; Yun et al., 2014), but results vary among studies and other authors showed the contrary (Bernardini et al., 2012). Moreover, white blood cells (WBC) decreased after transportation (Chacon et al., 2005).

These changes in different immune parameters in the long-term might indicate an immuno-suppressive effect and they might affect the adaptive capacity of the animal to the environmental circumstances. Due to a low functionality of defense immune

mechanisms, the animal might have a reduction in performance, weight gain and an increase in susceptibility to diseases (especially respiratory diseases, such as BRD) (Villarroel et al., 2003; Odore et al., 2011; Yun et al., 2014). Lower immunoglobulin (Ig) concentrations, especially IgG, of calves upon arrival at the farm was also reported and may contribute to a decreased resilience of calves to diseases (Mormede et al., 1982). Pardon et al. (2015) reported that concentrations of immunoglobulins (Ig) upon arrival at the farm, which are dependent on colostrum intake, may serve to predict BRD hazard in veal calves. Calves with Ig < 7.5 g/l have a greater probability of dying in the first weeks at the farm. However, no relationships were found between Ig concentrations and neonatal calf diarrhea. Interestingly, Renaud et al. (2018a) recently demonstrated that, similar to IgG, also greater concentrations of cholesterol were associated with lower risk for mortality in the first 21 days at the veal farm. Although this association could have multiple explanations, it was argued that cholesterol could be used as a marker of colostrum intake.

Overall, it could be concluded that measures of immunocompetence may be important predictors of later life performance, health and welfare. However, it is unclear which immunological variable or set of variables on-arrival at the veal farm would be the best predictor and thus suitable as biomarker.

Acute phase proteins (APPs)

Acute phase proteins (APPs) are proteins synthesized in the liver. The release of APPs in the bloodstream is induced by cytokines in response to many stressors, including transportation (Tothova et al., 2014). Cytokines are produced by cells of the innate immune system (e.g., macrophages, monocytes) and function as messengers between the local site of injury and the hepatocytes producing APPs (Conner et al., 1988; Petersen et al., 2004). APPs exert defensive roles against pathological damage, they are responsible of restoring the homeostasis and they regulate different stages of inflammation (Tothova et al., 2014). Therefore, APPs can be considered as biomarkers of stress and immunity (Yun et al., 2014).

An increase in APPs is observed in animals with diseases, thus high levels of APPs might be used as quantitative measure for identifying sick calves (Murray et al., 2014). In calves with diseases, the main changes in circulating APPs involve serum amyloid A (SAA), haptoglobin (Hp), lipopolysaccharide binding protein (LPB), fibrinogen (Fb), α-1-acid glycoprotein (AGP) and lactoferrin (Yun et al., 2014). Gånheim et al. (2003) indicated a threshold to distinguish between healthy and calves with diseases (age 9–18 weeks), including 0.13 g/l for Hp, 25.6 mg/l for SAA and 6.45 g/l for fibrinogen. Calves with a clinical or subclinical infection showed prolonged higher APPs concentrations

than healthy calves (Yun et al., 2014). Godson et al. (1996) showed that higher concentrations of Hp were associated with severe bacterial respiratory infections in cattle (r^2 = 0.481). Angen et al. (2009) and Hajimohammadi et al. (2013) used Hp to identify calves with pneumonia and diarrea, respectively. Moreover, Tothova et al. (2010) reported that the increase in some APPs levels (Hp and SAA) was associated not only with acute diseases of the respiratory tract, but also chronic cases ($P < 0.01$). The magnitude and the duration of the acute phase response reflect the severity of the infection (Skinner et al., 1991; Hirvonen et al., 1999; Hulten and Demmers, 2002).

Changes in APPs levels can be used not only as a measurement for early diagnosis and prognosis or for assessing the severity of diseases but also as predictors (Horadagoda et al., 1999; Humblet et al., 2004; Schneider et al., 2013). Different studies suggested that Hp might be the most useful APP for predicting diseases and for discriminating between diseased and healthy calves due to the higher sensitivity in detecting diseases (Carter et al., 2002; Ganheim et al., 2007; Angen et al., 2009). Higher levels of Hp (more than 0.13 g/l) in the first week of life were reported to be related with an increased odds ratio of future treatment for BRD (odds ratio (OR) = 2.66; $P = 0.048$), treatment for other diseases (OR = 12.59; $P < 0.001$) and death (OR = 8.67; $P = 0.001$) (Murray et al., 2014). In contrast, SAA might be a less suitable predictor of health problems due to its higher sensitivity to stimuli other than diseases, such as stress (Alsemgeest et al., 1995; Horadagoda et al., 1999; Heegaard et al., 2000). Therefore, higher values of acute phase proteins, especially Hp, might act either as indicators of an ongoing disease process or as predictors of diseases, thus they might be suitable as biomarkers.

Enzymes

Along with changes in immune functionality, transportation of calves at the veal farm and handling procedures are the main cause of other changes in blood plasma, including higher levels of creatine kinase (CK), creatine phosphate kinase (CPK), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) (Minka and Ayo, 2010). These enzymes play a significant function in energy homeostasis of tissue cells and they ensure a constant ATP levels in the cells (Teixeira and Borges, 2012). Changes in enzyme concentrations may occur due to physical and psychological challenges during transportation, which disrupt the homeostasis and, as a consequence, the metabolism of calves (Montane et al., 2002; Lopez-Olvera et al., 2006; Averos et al., 2008). In particular, higher plasma concentrations of CK, CPK and AST are associated with tissue damage, poor muscular tissue reperfusion, hypoxia and fatigue, and an increased permeability of muscles membrane following handling procedures and transport stress (Warriss et al., 1995; Tadich et al., 2005; Averos et al., 2008; Guardia et al., 2009).

Changes in plasma enzymes concentrations might function as indicators of tissue damage in diseased animals (Boyd, 1983). However, information on the relationships between these enzymes and future diseases is lacking in calves and cattle. In human patients, CK concentrations are useful for the evaluations of disorders involving damage to the myocardium, skeletal muscle and central nervous system (Cuestas, 1980). Moreover, evaluation of blood values of this enzyme might be useful to discriminate between high-risk new-born infants and low-risk new-born (Cuestas, 1980). In other studies in humans, an increase in blood LDH values seemed to be an important indicator of lung damage, pulmonary endothelial cell injury or airways problems (Schultze et al., 1994; Drent et al., 1996). Additionally, AST is strongly related with hepatic function, thus higher AST concentrations may indicate liver problems (Col and Uslu, 2007). Values of AST are also assessed together with CK values for diagnosis of muscle damage (Kaneko et al., 2008). An increase in CK, LDH and AST on arrival of calves at the veal farm was also reported compared with pre-transport values (Mormede et al., 1982; Uetake et al., 2009; Jongman and Butler, 2014). It can be speculated that, as in humans, higher values of these enzymes in veal calves might be associated with health problems, diseases and muscle damage. However, higher levels of these enzymes could also be positive indicators of restored homeostasis after transport or other challenges. In fact, when calves are stressed, they might try to restore their homeostasis by changing several physiological processes, including the enhancement of CK, LDH, and AST.

Conclusions and future perspectives

Among all variables listed in this review, there are some that seem to be good predictors of future diseases because there is information available from published studies. These variables include PCV, BW, lactate, glucose, Ig and Hp. Other variables, including Na, osmolality, neutrophils and enzymes seem to be just indicators of ongoing diseases but they do not show any concrete association with future diseases. Existing literature suggests that variables such as cortisol, albumin, bilirubin, K, Hb, BW loss, β-HB, NEFA, urea and rectal temperature may act as potential biomarkers. However, due to limited data, more studies are needed to confirm their association with future diseases. Furthermore, as already indicated, different parameters may be correlated. In the study of Turkson and Ganyo (2015), Hb was positively correlated with PCV vales (R^2 = 0.5504). Moreover, the authors suggested to use the simplified relationship of Hb (g/dl) = (0.3 PCV) + 3 to estimate Hb concentration from PCV in cattle. PCV measurement is a simple and cheap approach and therefore it can be used by farmers to assess anemic status and dehydration of calves at the veal farm. Blood collection for Hb analyses is performed on routine basis in the veal industry. Different blood samples are collected

at specific time points throughout the rearing period. We suggest that these blood samples may also be used to obtain potential biomarkers, including PCV.

With regards to Hp, concentrations of this APP > 0.13 g/l in combination with higher rectal temperature, increased nasal score and calf depression are indicators of respiratory diseases such as pneumonia (Murray et al., 2014). Overall, generation of big data sets including all the afore mentioned variables would allow to establish correlations between all different parameters. With these datasets, it would be possible in future studies not only to describe associations between variables, but also to find their predictive value for diseases in later life in veal calves. Eventually, the performance of promising biomarkers of health and performance of veal calves should also be investigated in terms of quantitative test characteristics such as sensitivity and specificity.

There are other new alternatives that could be considered to obtain more information on these variables and thus improve on farm health problems. A solution might be the use of post-genomic technologies of transcriptomics, proteomics and metabolomics in order to develop new biomarkers for detecting diseases (Ilyin et al., 2004; Seo and Ginsburg, 2005). So far, only a limited number of post-genomic strategies were applied in veterinary research (Witkamp, 2005), but their use is of growing interest in animal studies (Moore et al., 2007). Transcriptomics might be used for detecting and genotyping animal pathogens and for studying gene mutation in case of diseases (Feilotter, 2004; Moore et al., 2007). Within this field, DNA microarrays and analysis of gene expression are interesting tools for the identification of potential biomarkers of future diseases (Ilyin et al., 2004; Moore et al., 2007). Proteomics might be a useful approach in biomarker discovery by studying protein component of a cell, tissue and organism (Wilkins et al., 1996; Moore et al., 2007). Several techniques have been adopted in proteomic studies and quantitative proteomic strategies are developing with the aim to be applied in biomarker research. For instance, Aich et al. (2009) analyzed the associations between apolipoprotein AI and haptoglobin with the risk of developing respiratory diseases in cattle. Metabolomic studies focus on low molecular weight metabolites and might be used to investigate pathophysiological processes of animal diseases (Oresic et al., 2006; Griffin, 2006). Aich et al. (2009) used, for example, lactate and glucose analyses to predict BRD outcome in cattle. Overall, post-genomic technologies are becoming more accepted in veterinary studies, but due to many problems related to their feasibility, cost and practicality the use of these technologies in practice is still limited. Therefore, further research in these fields is needed to identify biomarkers by using more practical and feasible solutions that can be applied in routine clinical practice. ■

CHAPTER 3

Effects of pre-transport diet, transport duration and type of vehicle

on physiological status of young veal calves

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Abstract

This study aimed to investigate effects of pre-transport diet (rearing milk vs. electrolytes), type of vehicle (open vs. conditioned truck), and transport duration (6 vs. 18 h) on physiological status of young calves upon arrival at the veal farm. A total of 368 calves were transported in 2 consecutive batches from a collection center to a veal farm. Blood samples were collected from calves before transport; immediately post-transport (T0); and 4, 24, and 48 h, and 1, 3, and 5 week post-transport. Blood was analyzed for glucose, urea, lactate, non-esterified fatty acids (NEFA), β-hydroxybutyrate (β-HB), creatine kinase, albumin, total protein, osmolality, calcium, sodium, magnesium, and hematological variables. Body weight, rectal temperature, and skin elasticity were determined before and immediately post-transport. Blood glucose, NEFA, and urea concentrations at T0 showed an interaction between pre-transport diet and transport duration. Milk-fed and electrolyte-fed calves transported for 18 h did not significantly differ in plasma glucose concentration or serum NEFA concentrations. However, after 6 h of transport, milk-fed calves had higher plasma glucose and lower serum NEFA concentrations (4.71 mmol/L and 586.5 µmol/L, respectively) than electrolyte-fed calves (3.56 mmol/L and 916 µmol/L, respectively). After 18 h of transport, milk-fed calves had lower urea concentrations (5.40 mmol/L) than electrolyte-fed calves (7.38 mmol/L). In addition, at T0, after 6 h of transport, milk-fed calves gained weight (Δ = 0.41 kg), whereas electrolyte-fed calves lost weight (Δ = −0.16 kg). After 18 h of transport, both milk-fed and electrolyte-fed calves showed body weight losses (Δ = −0.67 and −0.74 kg, respectively). Type of vehicle had a limited influence on blood parameters. Concentrations of NEFA and β-HB reached the maximum values at T0 and then decreased until week 5 post-transport. The increase in NEFA and β-HB concentrations between prior to and just post-transport (T0) was less pronounced in calves transported for 6 h (746.1 µmol/L and 0.38 mmol/L, respectively) than in calves transported for 18 h (850.6 µmol/L and 0.50 mmol/L). Overall, the recovery rate of calves at the veal farm seemed rapid; all blood parameters returned to (below) pre-transport values within 48 h post-transport. We concluded that feeding milk before short-term transport helps young veal calves cope with transport, whereas this is not the case during long-term transport.

Keywords: transport, diet, transport duration, physiology, veal calf

Introduction

Veal calves have to deal with several challenges, such as mixing procedures and changes in environment, in their early stages of their life (Hulbert and Moisa, 2016). One of the largest challenges is transport of calves from the dairy farm to a collection center (CC) or auction center, followed by a second transport to the veal farm (Renaud et al., 2018a). At the CC, during transport, calves from different farms are mixed together and exposed to new environmental conditions, including new microorganisms, against which they may not have obtained colostral antibodies (Autio et al., 2007). As a result, diseases can spread among veal calves quite easily. Respiratory diseases, and especially bovine respiratory disease, have been shown to have a high (up to 17%) incidence at the veal farms during the entire fattening period (Pardon et al., 2013; Buczinski et al., 2018).

This high disease prevalence might be related to adverse effects of transport on physiology (Grigor et al., 2001; Minka and Ayo, 2010), immunity (Buckham Sporer et al., 2008; Hulbert and Moisa, 2016), and health (Schwartzkopf-Genswein et al., 2007; Earley et al., 2017) of calves. Among cattle, newborn calves are more vulnerable to transport stress than older calves because they have a lower ability to maintain body temperature and osmolality during transport (Schrama et al., 1993; Gebresenbet et al., 2012). Knowles et al. (1997) found relatively greater BW losses and more dehydration in young calves (less than 1 month old) post-transport compared with older calves (6 months old). Regarding metabolism, BW losses were more pronounced in calves transported for 24 h than in calves transported for 8 h (Knowles et al., 1997). Changes in hematological variables post-transport were noticeable in young calves (Mormede et al., 1982; Knowles et al., 1999b). Higher levels of plasma cortisol, non-esterified fatty acids (NEFA), urea, β-HB, packed cell volume, neutrophils, basophils, and total white blood cell numbers are commonly found in calves upon arrival at the veal farm (Knowles et al., 1997; Bernardini et al., 2012) compared with pre-transport values. These responses are often greater in calves transported for more than 8 h (Knowles et al., 1997; Knowles et al., 1999b) compared with calves subjected to short-term transport.

Differences in the hematological profile of calves after transport might be related to different factors, such as age of calves at first transport, handling, pre-transport nutrition, transport conditions, type of vehicle, transport duration (Schaefer et al., 1997), and recovery time upon arrival at the veal farm (Pardon et al., 2015; Renaud et al., 2018a). Besides transport duration, to our knowledge no studies have been done on the effects of a pre-transport diet and type of vehicle on metabolic status of young calves. Knowles et al. (1999b) investigated effects of feeding during long-distance transport, but the authors did not include a contrast in diet before transport and they

also did not investigate effects on calf metabolic status. Moreover, Bernardini et al. (2012) investigated effects of environmental conditions on health of calves, but not the conditions inside the truck.

The aim of the current study was to investigate effects of a pre-transport diet (rearing milk vs. electrolytes), transport duration (6 vs. 18 h), and transport condition (open truck vs. conditioned truck) on metabolic and physiological variables of young calves upon arrival at the veal farm. Blood parameters were analyzed until week 5 post-transport to study the recovery time of calves after arrival. We hypothesized that a shorter transport duration has less of an effect on metabolic and physiological parameters of young calves than a longer transport duration; moreover, feeding milk before transport combined with transport in a conditioned truck was assumed to help calves recover faster upon arrival at the veal farm.

Materials and methods

The current experiment was done under commercial conditions and followed common procedures (calves were transported from different German dairy farms to a German CC and additionally to a Dutch veal farm). All calves were complying with minimal weight and health status requirements (BW >36 kg; age: minimum 14 d; no signs of disease and injury; SBK, 2018). The experimental design was approved by the Central Committee on Animal Experiments (The Hague, the Netherlands; approval number 2017.D-0029). The study was conducted in January 2018 in 2 consecutive batches with a 1-week interval in between. Calves were transported from a CC, located in Bocholt-Barlo, Germany, to a veal farm in Veghel, the Netherlands.

Experimental design and animals

The experiment was set up as a complete 2 × 2 × 2 factorial arrangement with 3 factors: (1) provision of rearing milk or electrolytes before transport, (2) transport duration (6 or 18 h), and (3) transport condition (open truck or conditioned truck). This resulted in 8 treatments: (1) milk, 6 h of transport, conditioned truck; (2) milk, 18 h of transport, conditioned truck; (3) milk, 6 h of transport, open truck; (4) milk, 18 h of transport, open truck; (5) electrolytes, 6 h of transport, conditioned truck; (6) electrolytes, 18 h of transport, conditioned truck; (7) electrolytes, 6 h of transport, open truck; and (8) electrolytes, 18 h of transport, open truck. The study included 368 male Holstein-Friesian and crossbred calves (18 ± 4 d; 45.3 ± 3.3 kg of BW; ± SD), divided over 2 consecutive batches (n = 184/batch). The sample size was based on the availability of resources and no formal sample size calculation was completed. However, based on previous comparable experiments we expected that the number of replicates was sufficient to obtain reliable results.

Measurements at the CC and transport of calves to the veal farm

At the CC, calves were randomly allocated by the manager of the CC to 1 of the 8 treatment groups. Within each batch, 8 calves per treatment group were randomly selected for blood sampling (completed by personnel that were blinded to the treatment groups). After blood sampling, calves were weighed with a digital weighing scale, and rectal temperature (RT) and skin elasticity were determined. Skin elasticity was performed according to Constable et al. (1998) to measure the degree of clinical dehydration of calves and was scored as 0 or 1 with 0 = normal, skin tent <1 s, and 1 = dehydrated, skin tent >1 s. Thereafter, calves were fed via bucket with nipples, with 1.5 l of rearing milk (125 g of milk powder/L; ME = 4,028 kcal/kg, CP = 190 g/kg, digestible lysine = 18.7 g/kg; Tentofok KO, Tentego, the Netherlands) or a mixture of electrolytes (20 g of electrolytes/L of water; Navobi, Staverden, the Netherlands) dissolved in 1.5 L of water. All calves ingested the respective amount of milk or electrolytes, thus no refusals were observed.

After feeding, calves rested for approximately 2 h and thereafter they were loaded on the vehicle that was equipped with straw bedding. The vehicle consisted of 2 parts: the truck was conditioned, which means it was provided with a side-ventilation system, it was isolated, and the climate was controlled with regard to inlet and outlet of air (KVM Livestock Transport System, Kleventa BV, Lichtenvoorde, the Netherlands). Settings were according to those provided by the manufacturer and applied by the transporter. The trailer was regular, open, and lacking a ventilation system or climate control. In Table 1, the actual temperatures and relative humidities during transport in the conditioned and open trucks are presented.

Table 1. **Mean and range of actual temperature and relative humidity inside the conditioned and open trucks during short[1] or long[2] transportation of young calves to the veal farm.**

	Conditioned truck				Open truck			
	Batch 1		Batch 2		Batch 1		Batch 2	
	T(°C)[3]	RH (%)[4]	T(°C)	RH (%)	T(°C)	RH (%)	T(°C)	RH (%)
6 h	9.2 (8.2 – 10.3)	66.0 (61.0 – 75.1)	13.0 (11.7 – 13.9)	74.1 (65.0 – 81.0)	7.4 (6.2 – 9.1)	74.1 (66.3 – 84.2)	11.5 (10.4 – 12.5)	80.3 (67.6 – 88.8)
18 h	7.8 (4.5 – 11.2)	68.2 (58.5 – 78.9)	13.6 (11.2 – 16.3)	77.9 (65.4 – 83.9)	6.6 (3.9 – 9.6)	75.8 (66.3 – 86.5)	14.0 (10.8 – 16.6)	77.3 (66.2 – 86.2)

[1] *6 h;* [2] *18 h;* [3] *T = temperature;* [4] *RH = relative humidity.*

The settings of the climate control system as applied in practice did not result in notable differences in temperature or relative humidity inside the truck between the conditioned and open vehicles. The actual temperature and relative humidity inside the vehicles were recorded by loggers (n = 8; Escort imini, Cryopak Verification Technologies Inc., Buchanan, VA). Each logger was positioned in the middle of each compartment of truck and trailer, and recorded temperature and humidity every 10 min. Both truck and trailer were divided into 4 compartments with straw bedding: 2 at the lower deck (3.60 m length × 2.45 m width × 1.35 m height) and 2 at the upper deck (3.60 m length × 2.45 m width × 1.45 m height). Each compartment contained 23 calves and had the same stocking density (0.383 m^2 per calf). Each compartment contained calves of 1 of the 8 treatments. Treatments were distributed in the vehicle according to a design that allows for estimation of all main effects and relevant interactions, while avoiding any other confounding (Appendix 1A, B). After loading, transport started with 2 drivers. Drivers switched every 3 h. No food or water were provided to calves during transport. After 6 h of transport, the truck arrived at the veal farm and all calves were unloaded. Calves assigned to 6 h of transport were placed in the veal farm, whereas the calves assigned to 18 h of transport were reloaded on the truck and trailer (in the same compartments as before) and transported for another 12 h. The time invested for the unloading and reloading procedures took approximately 1 h.

Measurements at the veal farm

At the veal farm, a total of 64 pens were available, divided over 8 similar compartments, each containing 8 pens, with 5 or 6 calves per pen. Within each compartment, all treatments were randomly distributed across pens. For the first 3 week after arrival at the veal farm, calves were individually housed within each pen. After 3 week, the temporary partitions were removed and calves were effectively kept in groups thereafter. Upon arrival at the veal farm, calves (n = 368) were weighed and placed in pens. Immediately after placement (T0) and 4 h (T4) later, 2 calves per pen were selected for blood sampling (same calves as at the CC) and the individual calf was considered as the experimental unit. In between these 2 blood sampling moments, RT and skin elasticity were measured for all animals. All calves received electrolytes (20 g of electrolytes/L of water; Navobi, Staverden, the Netherlands) dissolved in 3 L of water after blood sampling. Calves selected for blood collection, transported for either 6 or 18 h, were subjected to blood sampling again 24 h (T24) and 48 h (T48) after arrival at the veal farm. Furthermore, blood samples of these calves were collected after 1, 3, and 5 week after arrival at the veal farm.

In the current experiment, the times of feeding at the collection and of departure of the vehicle from the CC (14.00 h) were the same for all calves. This means that calves

in the 6 h group arrived in the evening (20.00 h) at the veal farm, whereas calves in the 18 h group arrived in the morning (08.00 h of the next day). Despite the difference in the actual clock time, for both groups of calves, provision of electrolytes dissolved in water always took place directly post-transport and the first milk replacer meal was given 9 h post-transport. Therefore, intervals between feeding times and blood sampling times were the same for both transport durations. At the veal farm, calves were also fed milk replacer (125 g of milk powder/L; ME = 4,028 kcal/kg, CP = 210 g/kg, lysine = 26.0 g/kg; Navobi).

Blood sampling and analyses

Blood samples were taken via jugular venipuncture at the CC before transport, T0, T4, T24, T48, and week 1, 3, and 5 after arrival at the veal farm. Blood was collected in different vacutainer tubes (Vacuette, Greiner BioOne, Kremsmunster, Austria), including heparin and EDTA for plasma and serum. All samples were stored at 4˚C before the analyses, with the exception of serum samples that were stored at room temperature. Part of the samples was then analyzed by fluorescence flow cytometry (XT1800VET, Sysmex Europe GmbH, Norderstedt, Germany) for hemoglobin (Hb), hematocrit (Ht), red blood cells (RBC), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW). These analyses were only done on samples collected at the CC and at T0. The other blood samples were centrifuged (3,000 × g for 10 min, 4˚C) and plasma or serum was decanted and stored at −20˚C. Plasma samples were analyzed for glucose, lactate, urea, albumin, total protein (TP), calcium, and magnesium. Serum samples were analyzed for NEFA, β-HB, creatine kinase (CK), and sodium. Glucose was analyzed with a commercially available kit (Roche Diagnostics Nederland B.V., Almere, the Netherlands). Mass spectrophotometry based on commercially available kits was used for analyzing lactate (Diagnostic Systems GmbH, Holzheim, Germany), urea, albumin, TP, calcium, magnesium, sodium (Human, Wiesbaden, Germany), and NEFA (Wako Chemicals GmbH, Neuss, Germany). Osmolality was calculated as osmolality (mosmol/kg) = 2 × sodium (mmol/L) + urea (mmol/L) + glucose (mmol/L).

Statistical analyses

All statistical analyses were performed with SAS 9.4 (SAS Institute Inc., Cary, NC). First, the analyses of data on immediate post-transport (T0), including hematological data (Hb, Ht, RBC, MCH, MCV, and MCHC), physiological data (TP, albumin, osmolality, glucose, urea, lactate, NEFA, and minerals), BW, and RT were analyzed. Continuous data, such as BW, were analyzed with a linear mixed model (analysis with restricted maximum likelihood with SAS procedure PROC MIXED). Residuals were checked for normality and homogeneity of variance and variables were log-transformed when needed. Data

expressed as proportions, such as Ht, were analyzed with a generalized linear mixed model (analysis with penalized quasi-likelihood with SAS procedure GLIMMIX), with a logit link function, specifying the "error" variance as a multiple of the binomial variance. Both the linear mixed model and the generalized linear mixed model comprised the following fixed effects in the systematic part of the model (the linear predictor part):

$$\mu + Batch_i + Uplo_j + Bafr_k + Diet_l + Type_m + Duration_n + (Diet_l \times Duration_n) + (Diet_l \times Type_m) + (Duration_n \times Type_m) + (Diet_l \times Type_m \times Duration_n) \quad [1]$$

where μ is the base level, and Batch (i = 1, 2), $Uplo_j$ = position in the vehicle (j = upper or lower deck), $Bafr_k$ = position in the vehicle (k = front or back), $Diet_l$ = diet before transport (l = rearing milk or electrolytes), $Type_m$ = type of vehicle (m = open or conditioned truck), and $Duration_n$ = transport duration (n = 6 or 18 h). The model also comprised 2- and 3-way interactions between diet, type of vehicle, and transport duration. Interactions were considered not significant when $P > 0.05$. In addition, random effects for pen and compartment at the veal farm were included (in the linear predictor). Here, and in the subsequent analyses, for all fixed effects, approximate *F*-tests were used (Kenward and Roger, 1997). Interactions that were not significant were excluded from the model (when higher order interactions were already excluded, i.e., respecting the hierarchy of interaction terms) and subsequent pairwise comparisons were done with Fisher's least significant difference method.

Second, physiological data (TP, albumin, osmolality, glucose, urea, lactate, NEFA, CK, β-HB, and minerals) and hematological data (Ht and Hb) from T0 until week 3 were analyzed with a linear mixed model for continuous data or with a generalized linear mixed model for proportional data. The systematic part of these models comprised the following fixed effects:

$$\mu + Batch_i + Uplo_j + Bafr_k + Diet_l + Type_m + Duration_n + Time_o + (Diet_l \times Duration_n) + (Diet_l \times Type_m) + (Duration_n \times Type_m) + (Diet_l \times Time_o) + (Duration_n \times Time_o) + (Type_m \times Time_o) + (Diet_l \times Type_m \times Duration_n) \quad [2]$$

where μ is the base level, and Batch (i = 1, 2), $Uplo_j$ = position in the vehicle (j = upper or lower deck), $Bafr_k$ = position in the vehicle (k = front or back), $Diet_l$ = diet before transport (l = rearing milk or electrolytes), $Type_m$ = type of vehicle (m = open or conditioned truck), $Duration_n$ = transport duration (n = 6 or 18 h), and $Time_o$ = sampling moment (o = CC, T0, T4, T24, T48, week 1, week 3). Three-way interactions between diet, type of vehicle, transport duration, and 2-way interactions between diet, type of vehicle, transport duration, and time were also included in the model. Interactions were considered

not significant when $P > 0.05$. The model comprised random pen, compartment, and animal effects. For the animal effects, a first-order autoregressive model (based on the actual distance between time points) was adopted to introduce correlation in the model between repeated measurements on the same animal.

Third, physiological data and hematological data (Ht and Hb) of week 5 were analyzed similar to the analysis of data immediate post-transport with a linear mixed model. Although calves were housed individually in baby boxes until week 3, they were in group pens from week 5 onward, so random pen effects were included in the model.

Finally, differences between pre- and post-transport measurements (deltas, Δ = T0 – CC) were calculated for hematological data (Hb, Ht, RBC, MCH, MCV, and MCHC), physiological data (TP, albumin, osmolality, glucose, urea, lactate, NEFA, CK, β-HB, and minerals), BW, and RT. These differences were also analyzed similar to the data immediately post-transport with a linear mixed model.

A statistical test was performed to determine whether values at the CC were comparable between treatment groups. The statistical model was similar to the one used to describe effects at T0, but without interactions. No statistical differences were found between treatment groups.

Results

The average values of BW, RT, and all blood parameters measured at the CC are shown in Table 2. Moreover, the skin elasticity test results showed that 70% of calves at the CC were scored as dehydrated. The 3-way interactions and 2-way interaction between transport duration and type of vehicle were not significant in this study. Therefore, results will be shown in 4 main parts: 1) 2-way interaction between pre-transport diet and transport duration (Table 3); 2) 2-way interaction between pre-transport diet and type of vehicle (Table 4); 3) main effects of pre-transport diet, type of vehicle, and transport duration, only if interactions are not significant (Table 5); and 4) effects of treatment in time until 5 week post-transport (Figure 1, Figure 2). Within the first 3 parts, results will be presented at 2 levels: 1) effects of treatments immediately post-transport (T0; Table 3, Table 5), and 2) effects of treatments on the difference between pre- and post-transport measurements (deltas, Δ = T0 – CC; Tables 3, 4, and 6).

Table 2. Mean and range of body weight, rectal temperature and blood parameters measured at the collection center and comparison of these values with the reference values of calves of 14 days of age in other studies.

Parameters	Mean and range at the collection center	SEM[1]	Knowles et al. (2000)	Mohri et al. (2007)
Body weight, kg	45.3 (36.0 – 56.4)	0.2		
Rectal temperature, °C	38.6 (36.0 – 40.1)	0.03		
Hemoglobin, mmol/l	6.0 (3.5 – 8.5)	0.1		
Hematocrit, %	28.8 (18.5 – 40.8)	0.5	37.0	
Red blood cells, 10^{12}/l	8.1 (5.3 – 10.7)	0.1	9.5	7.5
MCH[2], amol	735.7 (554.0 – 901.5)	5.4	776.0	
MCV[3], fl	35.5 (29.0 – 44.3)	0.3	40.0	34.0
MCHC[4], mmol/l	20.7 (17.2 – 22.8)	0.1	19.5	
Albumin, g/l	44.0 (32.3 – 61.4)	0.5	29.0	32.0
Lactate, mmol/l	1.3 (0.6 – 4.3)	0.05		
β-HB[5], mmol/l	0.29 (0.06 – 0.73)	0.01	0.12	
Creatine kinase, U/l	87.1 (16.0 – 375.0)	4.0	100.0	
Glucose, mmol/l	3.7 (2.3 – 6.3)	0.1	6.0	4.3
Urea, mmol/l	5.1 (2.1 – 18.4)	0.2	2.7	4.7
NEFA[6], µmol/l	676.0 (162.5 – 1875.0)	21.4	250.0	
Total protein, g/l	64.0 (45.1 – 92.4)	0.6	58.0	60.0
Osmolality, mosmol/kg	306.1 (239.5 – 388.7)	2.2		
Calcium, mmol/l	2.48 (2.01 – 3.33)	0.02		2.2
Sodium, mmol/l	148.6 (115.6 – 191.4)	1.1		139.0
Magnesium, mmol/l	0.86 (0.64 – 1.11)	0.01		0.95

[1]*SEM = pooled standard error;* [2]*MCH = mean corpuscular hemoglobin;* [3]*MCV = mean corpuscular volume;* [4]*MCHC = mean corpuscular hemoglobin concentration;* [5]*β-HB = beta-hydroxybutyrate;* [6]*NEFA = non-esterified fatty acids.*

Table 3. Interaction between pre-transport diet[1] and transport duration[2] detected in blood parameters measured immediately post-transport[3] and on the difference[4] between pre-transport[5] and post-transport measurements in young veal calves (LMS).

Immediate post-transport						
Parameter	Milk		Electrolytes		SEM[6]	*P*-value Interaction
	6 h	18 h	6 h	18 h		
Glucose, mmol/l	4.71^{a}	3.10^{b}	3.56^{b}	3.35^{b}	0.14	< 0.01
Urea, mmol/l	5.51^{b}	5.40^{b}	5.64^{b}	7.38^{a}	0.42	0.04
NEFA[7], µmol/l	586.5^{b}	853.2^{a}	916.0^{a}	837.7^{a}	54.8	< 0.01
Total protein, g/l	66.90^{a}	59.62^{b}	59.81^{b}	64.77^{a}	1.65	< 0.01
Osmolality, mosmol/kg	310.6^{ab}	297.5^{b}	300.6^{ab}	312.0^{a}	4.5	0.02
Ca, mmol/l	2.56^{a}	2.27^{b}	2.45^{a}	2.42^{a}	2.43	0.02
Δ = To – CC						
Parameter	Milk		Electrolytes		SEM	*P*-value Interaction
	6 h	18 h	6 h	18 h		
Glucose, mmol/l	1.19^{a}	-0.60^{b}	-0.21^{b}	-0.62^{b}	0.15	< 0.01
NEFA, µmol/l	-114.6^{b}	199.3^{a}	251.1^{a}	153.7^{a}	63.0	< 0.01
Total protein, g/l	3.60^{b}	-4.34^{a}	-2.92^{a}	-0.65^{a}	1.56	< 0.01
BW[8] losses, kg	0.41^{a}	-0.67^{c}	-0.16^{b}	-0.74^{c}	0.09	0.03

[1]milk vs. electrolytes; [2]6 h vs. 18 h; [3]0 h, To; [4] deltas, Δ = To – CC; [5] collection center, CC ; [6]SEM = pooled standard error; [7]NEFA = non-esterified fatty acids; [8]BW = body weight. [a-d] Least square means within a row lacking a common superscript differ (P ≤ 0.05).

Table 4. Interaction between pre-transport diet[1] and type of vehicle[2] on the difference[3] between pre-transport[4] and post-transport[5] measurements in young veal calves.

pre-transport and post-transport						
Parameter	Milk		Electrolytes		SEM[6]	*P*-value interaction
	Conditioned	Open	Conditioned	Open		
Lactate, mmol/l	0.10^{a}	-0.41^{b}	0.14^{a}	0.19^{a}	0.11	0.01
Albumin, g/l	-2.27^{ab}	-3.43^{a}	-2.50^{a}	-0.40^{b}	0.72	0.03

[1] milk vs. electrolytes; [2] conditioned vs. open truck; [3] deltas, Δ = To – CC; [4] collection center, CC; [5] 0 h, To. [6]SEM = pooled standard error; [a-d] Least square means within a row lacking a common superscript differ (P < 0.05).

Table 5. Effects of diet composition[1] before to transport, type of vehicle[2] and transport duration[3] on blood parameters, body weight and rectal temperature of young calves at the moment of arrival at the veal farm (LSM).

	Pre-transport diet				Type of vehicle				Transport duration			
Parameter	Electrolytes	Milk	SEM[4]	*P*-value	Conditioned	Open	SEM	*P*-value	6 h	18 h	SEM	*P*-value
Hemoglobin, mmol/l	6.07	6.00	0.15	0.74	6.12	5.95	0.15	0.44	6.02	6.04	0.19	0.96
Hematocrit, %	29.34	28.69	0.65	0.95	29.43	28.59	0.65	0.93	28.71	29.32	0.65	0.97
Red blood cells, 10^{12}/l	8.22	8.26	0.18	0.84	8.38	8.10	0.18	0.24	8.33	8.16	0.22	0.61
MCH[5], amol	735.5	722.7	8.9	0.25	727.7	730.5	8.9	0.80	719.8	738.5	10.4	0.23
MCV[6], fl	35.65	34.71	0.45	0.07	35.11	35.26	0.45	0.78	34.49	35.88	0.52	0.06
MCHC[7], mmol/l	20.64	20.84	0.10	0.14	20.74	20.73	0.10	0.95	20.88	20.60	0.12	0.15
Albumin, g/l	41.38	42.29	0.63	0.32	42.52	41.15	0.63	0.13	40.81	42.86	0.78	0.11
Lactate, mmol/l	1.33	1.29	0.05	0.61	1.39	1.22	0.05	0.02	1.33	1.28	0.05	0.19
β-HB[8], mmol/l	0.546	0.333	0.028	< 0.01	0.411	0.468	0.028	0.10	0.403	0.476	0.033	0.13
Creatine kinase, U/l	104.8	93.8	5.4	0.16	89.7	108.9	5.4	0.08	99.3	99.2	5.4	0.15
Magnesium, mmol/l	0.85	0.88	0.01	0.08	0.86	0.87	0.01	0.53	0.87	0.85	0.02	0.32
Sodium, mmol/l	148.1	147.2	1.4	0.60	149.2	146.2	1.4	0.10	147.8	147.5	1.6	0.92
Body weight, kg	45.18	44.83	0.27	0.37	44.90	45.11	0.27	0.60	45.28	44.73	0.33	0.31
RT[9], °C	38.83	38.83	0.09	0.95	38.87	38.78	0.09	0.17	38.88	38.78	0.10	0.26

[1] *milk vs. electrolytes;* [2] *conditioned vs. open truck;* [3] *6 vs. 18 h.;* [4] *SEM = pooled standard error;* [5] *MCH = mean corpuscular hemoglobin;* [6] *MCV = mean corpuscular volume;* [7] *MCHC = mean corpuscular hemoglobin concentration;* [8] *β-HB = beta-hydroxybutyrate;* [9] *RT = rectal temperature.*

Table 6. Effects of diet composition[1] prior to transport, type of vehicle[2] and transport duration[3] on the difference[4] between pre-transport[5] and post-transport[6] measurements of young veal calves (LSM).

	Pre-transport diet				Type of vehicle				Transport duration			
Parameter	Electrolytes	Milk	SEM[7]	*P*-value	Conditioned	Open	SEM	*P*-value	6 h	18 h	SEM	*P*-value
Hemoglobin, mmol/l	0.067	0.041	0.054	0.66	0.064	0.044	0.054	0.74	0.071	0.037	0.062	0.69
Hematocrit, %	0.42	0.04	0.21	0.98	0.39	0.08	0.21	0.98	-0.05	0.51	0.20	0.99
Red blood cells, 10^{12}/l	0.171	0.122	0.073	0.52	0.167	0.125	0.073	0.58	0.148	0.144	0.083	0.97
MCH[8], amol	-7.51	-5.72	1.56	0.42	-6.82	-6.41	1.56	0.85	-4.51	-8.72	1.90	0.18
MCV[9], fl	-0.25	-0.48	0.08	0.04	-0.27	-0.47	0.08	0.07	-0.46	-0.28	0.10	0.25
MCHC[10], mmol/l	-0.07	0.12	0.05	< 0.01	-0.05	0.10	0.05	0.04	0.14	0.10	0.06	0.01
Osmolality, mosmol/kg	2.91	-5.38	4.04	0.15	5.61	-8.09	4.04	0.02	-1.23	-1.25	4.94	0.99
Urea, mmol/l	1.29	0.42	0.28	0.03	1.15	0.56	0.28	0.14	0.56	1.15	0.34	0.30
β-HB[11], mmol/l	0.254	0.041	0.029	< 0.01	0.144	0.151	0.029	0.84	0.091	0.204	0.033	0.02
Creatine kinase, U/l	21.51	3.68	5.07	0.02	4.16	21.02	5.07	0.04	14.38	10.64	5.07	0.52
Calcium, mmol/l	-0.104	0.007	0.047	< 0.01	-0.025	-0.072	0.047	0.25	0.017	-0.113	0.052	0.03
Magnesium, mmol/l	-0.019	0.018	0.016	< 0.01	-0.002	0.001	0.016	0.80	0.024	-0.025	0.017	0.01
Sodium, mmol/l	1.00	-3.05	2.03	0.16	2.23	-4.48	2.03	0.03	-1.15	-0.90	2.47	0.95
Changes in RT[12], °C	0.07	0.30	0.09	< 0.01	0.28	0.10	0.09	0.02	0.28	0.10	0.10	0.10

[1] *milk vs. electrolytes;* [2] *conditioned vs. open truck;* [3] *6 vs. 18 h;* [4] *deltas, Δ = To – CC;* [5] *collection center, CC;* [6] *0 h, To.* [7] *SEM = pooled standard error;* [8] *MCH = mean corpuscular hemoglobin;* [9] *MCV = mean corpuscular volume;* [10] *MCHC = mean corpuscular hemoglobin concentration;* [11] *β-HB = beta-hydroxybutyrate;* [12] *RT = rectal temperature.*

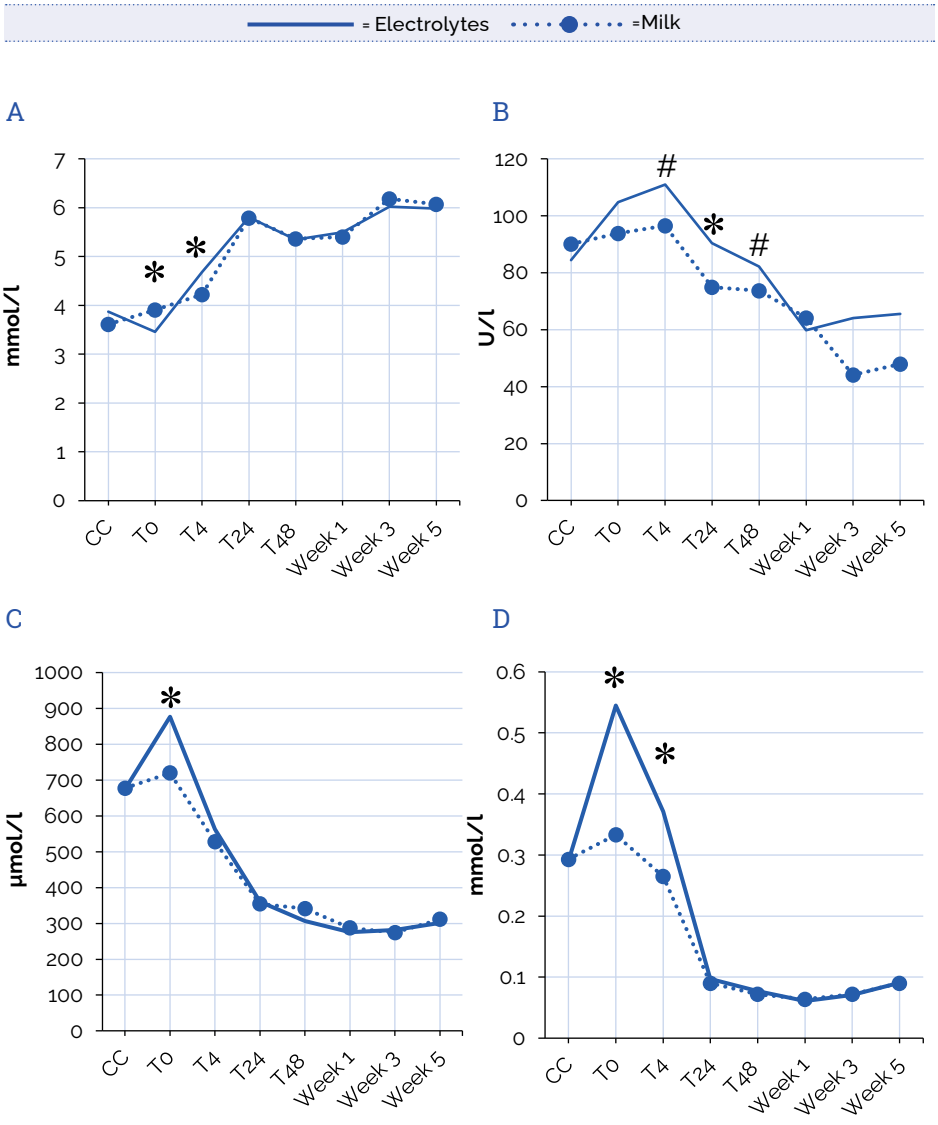

Figure 1. **A-D Interactions between pre-transport diet (milk vs. electrolytes) and time relative to transport for glucose (A), creatine kinase (B), non-esterified fatty acids (NEFA; C), and β-HB (D) in young veal calves.** *Blood samples of these variables were collected before transport at the collection center (CC); at 0 (T0), 4 (T4), 24 (T24), and 48 (T48) h; and 1, 3, and 5 week post-transport. Asterisks indicate significant differences (P ≤ 0.05) between treatments within a sampling moment, whereas hashtags indicate a tendency toward significance (0.05 ≤ P ≤ 0.10).*

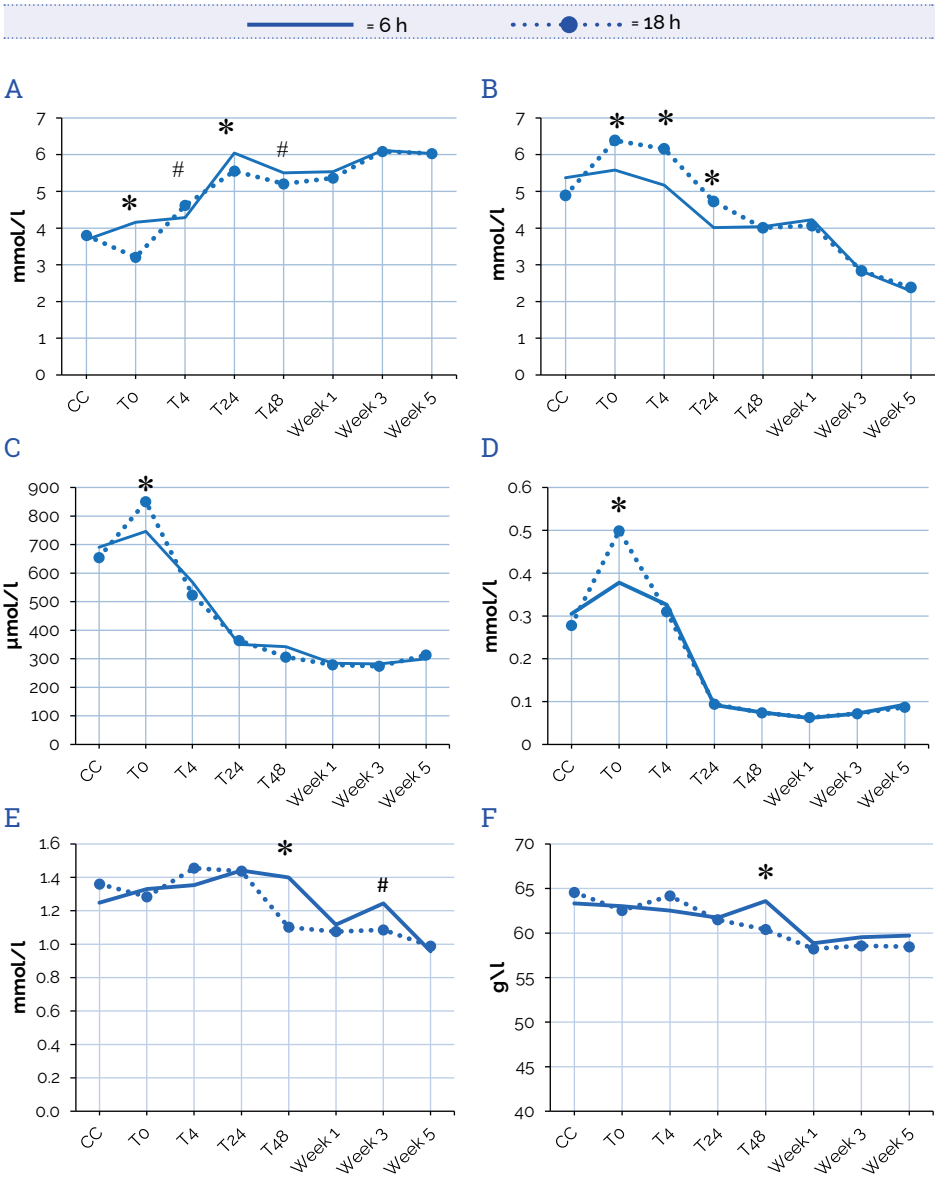

Figure 2. A-F: **Interactions between transport duration (6 vs. 18 h) and time relative to transport for glucose (A), urea (B), non-esterified fatty acids (NEFA; C), β-HB (D), lactate (E), total protein (F) in young veal calves.** *Blood samples of these variables were collected before transport at the collection center (CC); at 0 (T0), 4 (T4), 24 (T24), and 48 (T48) h; and 1, 3, and 5 week post-transport. Asterisks indicate significant differences (P ≤ 0.05) between treatments within a sampling moment, whereas hashtags indicate a tendency toward significance (0.05 ≤ P ≤ 0.10).* *(Continued on next page)*

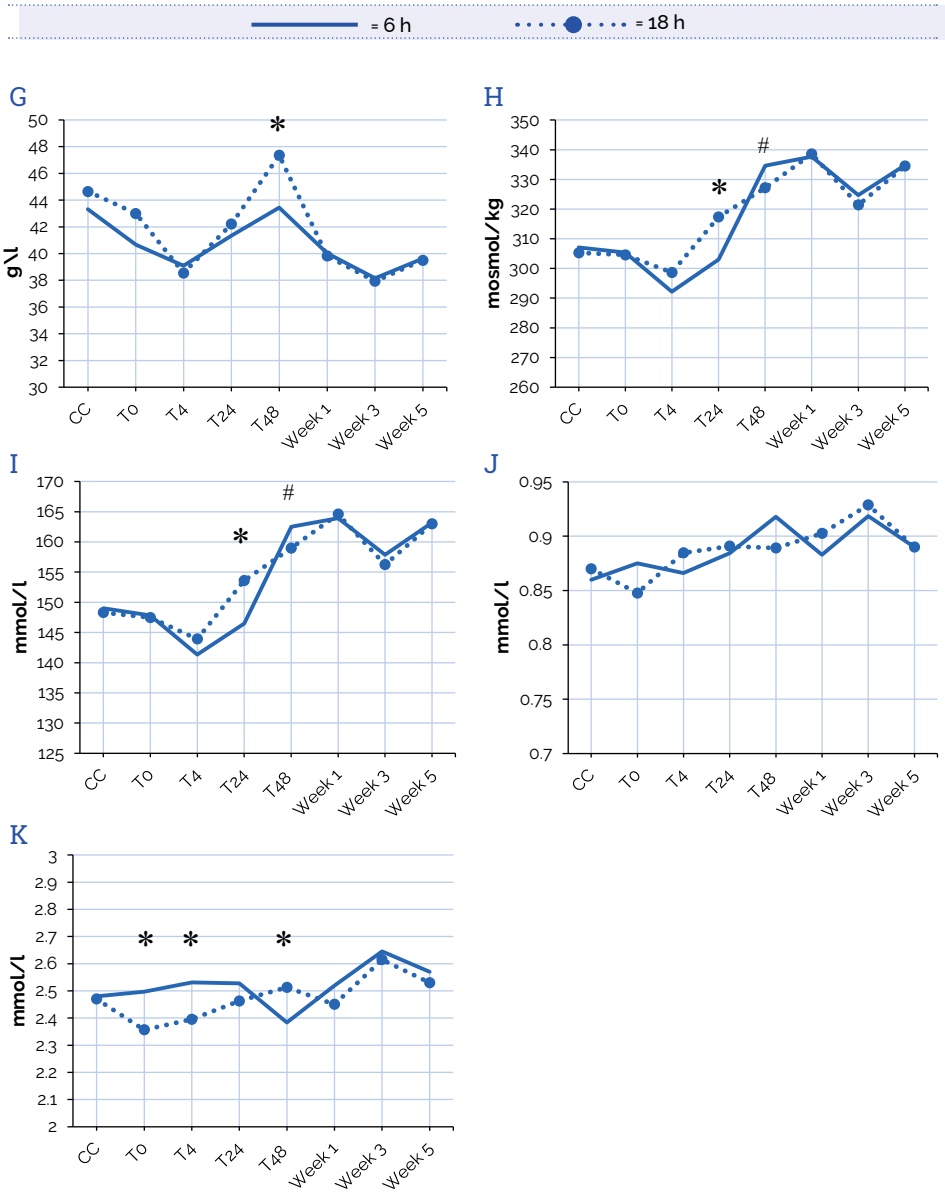

Figure 2. *(Continued)* G-K: **Interactions between transport duration (6 vs. 18 h) and time relative to transport for albumin (G), osmolality (H), sodium (I), magnesium (J), and calcium (K) in young veal calves.** *Blood samples of these variables were collected before transport at the collection center (CC); at 0 (T0), 4 (T4), 24 (T24), and 48 (T48) h; and 1, 3, and 5 week post-transport. Asterisks indicate significant differences ($P \leq 0.05$) between treatments within a sampling moment, whereas hashtags indicate a tendency toward significance ($0.05 \leq P \leq 0.10$).*

Interaction between pre-transport diet and transport duration

Effects on measurements immediately post-transport

An interaction between pre-transport diet and transport duration was detected for glucose, urea, and NEFA (Table 3). Calves fed milk or electrolytes and transported for 18 h did not differ in blood glucose or NEFA concentrations. However, after 6 h of transport, milk-fed calves had higher glucose concentrations than electrolyte-fed calves (Δ = 1.15 mmol/L; $P < 0.0001$) and lower NEFA concentrations (Δ = −329.5 µmol/L; $P = 0.007$). In addition, milk-fed calves transported for 6 h had higher glucose and lower NEFA concentrations than milk-fed calves transported for 18 h (Δ = 1.61 mmo/L; Δ = −266.7 µmol/L, respectively). Blood urea concentration did not differ in milk-fed or electrolyte-fed calves transported for 6 h, but after 18 h of transport, milk-fed calves had lower urea concentrations than electrolyte-fed calves (Δ = −1.98 mmol/L; $P = 0.04$). Moreover, electrolyte-fed calves transported for 6 h had lower urea concentrations than the ones transported for 18 h (Δ = −1.74 mmol/L).

An interaction between pre-transport diet and transport duration was also found for TP, osmolality, and calcium. Higher plasma TP concentrations were found in milk-fed calves than in electrolyte-fed calves after 6 h of transport (Δ = 7.09 g/L), whereas after 18 h of transport, TP concentrations were lower in milk-fed calves than in electrolyte--fed calves (Δ = −5.15 g/L; $P = 0.001$). In addition, milk-fed calves transported for 6 h had higher TP concentrations than the ones transported for 18 h (Δ = 7.28 g/L), whereas electrolyte-fed calves transported for 6 h had lower TP concentrations than the ones transported for 18 h (Δ = −4.96 g/L). Milk-fed and electrolyte-fed calves transported for 6 h did not differ in osmolality and calcium concentrations. However, after 18 h of transport, milk-fed calves had a lower osmolality and calcium concentrations than electrolyte-fed calves (Δ = −14.5 mosmol/kg, $P = 0.02$; Δ = −0.15 mmol/L, $P = 0.02$, respectively). Moreover, milk-fed calves transported for 6 h had higher calcium concentrations than the ones transported for 18 h (Δ = 0.29 mmol/L).

Effects of the difference between pre- and post-transport measurements

An interaction between pre-transport diet and transport duration was identified for glucose, NEFA, and TP (Table 3). Milk-fed and electrolyte-fed calves transported for 18 h decreased to the same degree in glucose concentration during transport. However, calves transported for 6 h differed in their responses; milk-fed calves increased in blood glucose concentration, whereas electrolyte-fed calves decreased in glucose concentration ($P = 0.0001$). Blood NEFA concentrations increased in both milk-fed and electrolyte-fed calves after 18 h of transport. In contrast, after 6 h of transport, milk-fed calves decreased their NEFA concentration, whereas electrolyte-fed calves increased their NEFA concentration ($P = 0.007$). Moreover, milk-fed calves transported for

6 h increased glucose and decreased NEFA concentrations during transport compared with milk-fed calves transported for 18 h. Milk-fed and electrolyte-fed calves did not differ in their change in blood TP concentrations after 18 h of transport, but after 6 h of transport, TP concentrations increased in milk-fed calves and decreased in electrolyte-fed calves ($P = 0.002$). In addition, milk-fed calves transported for 6 h increased TP concentrations during transport, whereas the ones transported for 18 h had decreasing TP concentrations.

An interaction between pre-transport diet and duration was also detected for BW losses (Table 3). Body weight losses were pronounced in both milk-fed or electrolyte-fed calves after 18 h of transport, but calves transported for 6 h and fed with milk increased in BW, whereas electrolyte-fed calves showed BW losses ($P = 0.03$).

Interaction between pre-transport diet and type of vehicle

Effects on the difference between pre- and post-transport measurements

No interactions between pre-transport diet (milk or electrolytes) and type of vehicle (open or conditioned truck) were found for any of the variables determined immediately post-transport (T0).

An interaction between pre-transport diet and type of vehicle was detected for deltas of lactate and albumin (Table 4). Milk-fed and electrolyte-fed calves transported in the conditioned truck increased to the same degree in lactate concentration during transport. However, in the open truck, milk-fed calves decreased lactate concentrations, whereas electrolyte-fed calves increased their lactate concentration ($P = 0.01$).

Albumin concentrations decreased in both milk-fed and electrolyte-fed calves transported in both type of vehicles. The decrease in albumin concentration did not differ between milk-fed or electrolyte-fed calves transported in the conditioned truck. However, milk-fed calves had a greater decline in albumin than electrolyte-fed calves in the open truck ($P = 0.03$).

Main effects

Effects on measurements immediately post-transport

Immediately post-transport, milk-fed calves had lower β-HB levels than electrolyte-fed calves ($\Delta = -0.213$ mmol/L; $P < 0.001$; Table 5). Pre-transport diet, type of vehicle, and transport duration had no significant effects on BW and RT immediately post-transport.

Effects on the difference between pre- and post-transport measurements

Pre-transport diet influenced deltas of blood urea, β-HB, and CK between pre- and post-transport (Table 6). Both milk-fed and electrolyte-fed calves increased urea (P = 0.03), β-HB (P < 0.01), and CK (P < 0.01) during transport. However, the increase was greater in electrolyte-fed calves than in milk-fed calves.

Pre-transport diet had also an effect on MCV, MCHC, calcium, and magnesium values. The MCV decreased in both milk-fed and electrolyte-fed calves during transport, but the decrease was larger in the milk-fed calves (P = 0.04) than in the electrolyte-fed calves. The MCHC, calcium, and magnesium values increased in milk-fed calves during transport, whereas the values decreased in electrolyte-fed calves. In addition, milk-fed calves had a greater increase in RT than electrolyte-fed calves during transport (P < 0.01).

Type of vehicle influenced MCHC values, osmolality, CK, and sodium concentration and change in RT. The MCHC values increased in calves transported in the open truck and decreased in calves in the conditioned truck (P = 0.04). Calves in the open truck showed a decrease in osmolality and sodium values during transport, whereas calves in the conditioned truck increased these values (P < 0.05). Values of CK and RT increased during transport in calves in both the open and the conditioned truck, but the increase in CK was higher in the open truck, whereas the increase in RT was higher in the conditioned truck (both P < 0.05).

Transport duration had an effect on MHCH, β-HB, calcium, and magnesium. After 6 h of transport, MCHC increased to a greater extent than after 18 h (P = 0.01). In case of β-HB, higher concentrations were found in calves transported for 18 h than for 6 h (P = 0.02). Both calcium and magnesium concentrations decreased in calves transported for 18 h, whereas concentrations increased in calves transported for 6 h (P < 0.05).

Effects of treatments in time until week 5 post-transport

Interaction between pre-transport diet and time

Glucose, CK, NEFA, and β-HB showed a significant interaction between pre-transport diet and time (Figure 1A-D). Directly post-transport, milk-fed calves had higher glucose concentrations than electrolyte-fed calves (Figure 1A; P = 0.004). However, 4 h post--transport, milk-fed calves had a lower concentration of glucose than the electrolyte--fed calves (P = 0.003). The amount of CK differed at T24 (P = 0.01), with milk-fed calves showing lower CK values than electrolyte-fed calves (Figure 1B). The increase in NEFA and β-HB directly post-transport was greater in electrolyte-fed calves than in milk-fed calves (Figure 1C, D; P < 0.0001). At T4, concentrations of β-HB were still higher in electrolyte-fed calves than milk-fed calves (P = 0.0002).

Interaction between transport duration and time

An interaction between transport duration and time was found for most of blood metabolites (Figure 2A-H). As depicted in Figure 2A, at T0, calves transported for 18 h had lower glucose concentration than calves transported for 6 h ($P < 0.0001$). At T24, glucose was also higher in calves transported for 6 h than in calves transported for 18 h ($P = 0.007$). Blood urea, β-HB, and NEFA increased post-transport and then they decreased until week 5 (Figure 2B-D). However, the response of calves was different between 6 and 18 h of transport. Calves transported for 18 h presented slightly higher urea concentrations from T0 until T24 than calves transported for 6 h ($P < 0.05$). At T0 only, β-HB and NEFA concentrations were also higher for calves transported for 18 h than the ones transported for 6 h ($P < 0.01$). Lactate, TP, and albumin concentrations differed at T48 (Figure 2E-G) between transport durations. At this sampling moment, calves transported for 6 h had higher lactate ($P < 0.0001$) and TP ($P = 0.03$) than calves transported for 18 h, whereas the opposite was found for albumin concentration ($P = 0.001$). Osmolality decreased until T4 for calves transported either 6 or 18 h and thereafter started to rise; at T24, calves assigned to 18 h of transport showed a higher osmolality than calves assigned to 6 h of transport (Figure 2H; $P = 0.0008$).

A significant interaction between transport duration and time was found for sodium, magnesium, and calcium (Figure 2J-L). Sodium showed the same trend in time as osmolality. Magnesium concentrations fluctuated within a small range. Compared with pre-transport magnesium values, calves transported for 6 h had a small decrease in magnesium post-transport. After T0, magnesium concentrations fluctuated, with peak concentrations at T48 and week 3 post-transport. Calves transported for 18 h had increasing concentrations of magnesium from T0 until week 3. Immediately post-transport and at T4, calves with 6 h of transport had higher calcium values than calves with 18 h of transport ($P = 0.002$). In contrast, at T48, calves with 18 h of transport showed greater calcium concentrations than calves with 6 h of transport ($P = 0.003$).

Discussion

In the current study, transport was a challenge for young veal calves. This is in line with previous studies that showed effects of transport on calf metabolic and physiological status (Knowles et al., 1999b; Minka and Ayo, 2010). This study aimed to investigate effects of transport on young calves whose blood values were already challenged before transport due to previous mixing, handling procedures, and transport to the CC, moving away from baseline values. Values of blood parameters are shown in Table 2 and were compared with reference values for calves of 14 d of age found in other studies. Values recorded at the CC were comparable among treatment groups. Additionally,

for many parameters recorded in the current experiment, especially those related to minerals, fat mobilization, such as NEFA, and energy, such as glucose, the values obtained at the CC were already markedly different from reference values. This strongly suggests that calves used in the current experiment were already challenged when the first blood sample was taken. Indeed, we deliberately used calves in our study as they commonly exist in practice (i.e., animals collected from different dairy farms in Germany, received and mixed at a CC, and then transported toward a veal farm in the Netherlands). Therefore, before arrival at the CC they most likely already had experienced different sorts of stressors, such as handling procedures and transport, as well as different durations of feed and water withdrawal.

Interaction between pre-transport diet and transport duration

Milk-fed calves had higher TP and osmolality after 6 h of transport compared with electrolyte-fed calves. However, after 18 h of transport the effect was reversed, and milk-fed calves had lower TP and osmolality. The differences between the short and the long-term transport might be explained by different effects of dietary energy content on plasma volume (Bachmann et al., 2012). Electrolytes might have provided an immediate rate of hydration due to their quicker absorption rate in the gut of calves compared with milk (Nouri and Constable, 2006; Constable et al., 2009). We hypothesized that the initial rate of hydration might not have been maintained until the end of 18 h of transport. In contrast, the higher energy content of milk might have contributed to a slower rate of plasma expansion (Bachmann et al., 2012), which might explain the lower dehydration level of the milk-fed calves after 18 h of transport compared with the electrolyte-fed calves.

After 18 h of transport, electrolyte-fed calves had higher calcium concentrations than milk-fed calves. The combination of lower energy content and long transport duration might explain the rise in calcium concentrations in electrolyte-fed calves in the present study. Parker et al. (2003) showed higher calcium concentrations in steers after 48 h of transport duration; however, the authors used older animals instead of young calves. Other studies did not show any significant results on calcium, thus more research is needed to investigate how calcium is regulated during long-term transport in young calves.

An interaction between pre-transport diet and transport duration was also identified for BW losses and deltas between pre-transport and immediate post-transport glucose and NEFA concentrations. After 18 h of transport, milk-fed and electrolyte-fed calves experienced BW losses. In accordance to previous studies on long transport duration, BW losses can be highly influenced by feeding and water provision before transport (Tarrant et al., 1992; Knowles et al., 1999b). Bernardini et al. (2012) showed that after 19 h

of transport calves had 6.4% higher BW losses compared with nontransported calves. Moreover, unfed calves or calves provided with only water and no food during transport are more prone to BW losses. In the study of Knowles et al. (1997), unfed calves that were transported for 24 h lost up to 3.2% of their BW. Although diet seemed to have no effects after 18 h of transport, the feeding strategy at the CC played an important role on BW losses after short transport duration. In the current practice, feeding electrolyte solutions before transport is a common approach to counteract expected negative effects of transport on electrolyte metabolism, metabolic acidosis, and viral diarrhea in cattle (Booth and Naylor, 1987; Schaefer et al., 1997). Knowles et al. (1997) used a glucose/electrolyte solution to avoid gastrointestinal problems on arrival at the farm. Despite the important role of electrolytes on calf health, the results of the present study showed that, after 6 h of transport, milk-fed calves gained weight compared with electrolyte-fed calves that lost weight, although they had the same amount of fluid intake. This result suggests that the higher nutrient and energy content of milk seemed to protect calves against the effects of transport on nutrient mobilization and thus on BW losses.

The effect of feeding milk before 6 h of transport was also evident on blood biochemical profile. The higher glucose and lower NEFA concentrations in milk-fed calves post-transport are indicative of a less negative energy balance and might indicate that the nutrient reserve depletion is less serious than in electrolyte-fed calves (Tadich et al., 2005; Marcato et al., 2018). Moreover, the consequences of feeding electrolytes on calf energy and protein metabolism were more pronounced after long distance transport. This is in line with the study of Mormede et al. (1982) who found slightly lower post-transport glucose (–38% for short transport and –54% for long transport), and higher NEFA and urea levels. Mormede et al. (1982) suggested that the results might be due to the combination of food deprivation and the higher energy demands of transport and manipulations. On long journeys, food reserves are used by animals, thus a lower plasma glucose might be an indicator that the animal runs out of fast available energy (Grandin et al., 2014).

Main effects

Pre-transport diet

Feeding electrolytes as pre-transport diet contributed to a significant increase in CK during transport. Higher CK and lactate values might be indicative of calf tissue damage, hypoxia, fatigue, and exhaustion during transport (Chacon et al., 2005; Averos et al., 2008). In our study, the changes observed after short-term transport in milk-fed calves might be associated with the greater energy content of this diet that protects calves from glycogenolysis and gluconeogenesis induced by fear responses during transport.

Diet also influenced RBC parameters. The MCV and MCHC levels depend on Hb concentration. Changes in these blood parameters have been previously described in veal calves (Wilson et al., 2000) in relationship to a low iron dietary regimen at the veal farm, but whether other (transport-related) factors also might play a role is currently not clear.

The higher RT in milk-fed calves might be explained by the higher energy content of their diet compared with electrolytes, and consequently a higher metabolic rate (Scibilia et al., 1987).

In our study, milk-fed calves had an increase in their calcium and magnesium concentration post-transport, whereas electrolyte-fed calves experienced a decrease. Calcium and magnesium are important macrominerals that have key functions in body signaling and enzymes. Magnesium is not stored in the body and needs to be ingested daily. Calcium is well regulated and can derive from bone via the parathyroid hormone. Their main role together with other ions, such as chloride and sodium, is the maintenance of acid-base balance, osmotic pressure, membrane electric potential, and nervous transmission (Mohri et al., 2008). Transportation is known to affect mineral balance in calves (Schaefer et al., 1997; Steinhardt and Hans-Herma, 1998). In the literature, an increase in calcium and magnesium values is generally associated with the activation of the hypothalamic-pituitary-adrenal axis during transport. As a result of high calcium in the extracellular fluids, challenged animals have greater muscle contractility (Minka and Ayo, 2010). In this experiment, the difference in concentrations of these minerals between milk-fed and electrolyte-fed calves might be a consequence of diet instead of the activation of the hypothalamic-pituitary-adrenal axis during transport. Milk contained 9.5 g/kg of calcium and 1.67 g/kg of magnesium, whereas these minerals were not included in the nutrient composition of electrolytes. The greater nutrient content in milk might, therefore, have contributed to the increase in calcium and magnesium.

Transport duration

Long transport duration affected differences in mineral concentrations between pre- and post-transport. Steinhardt and Hans-Herma (1998) found a decrease in calcium and magnesium concentrations after transport of calves. The decrease in these minerals was probably due to fear responses experienced by calves during transport and handling procedures. Lower calcium and magnesium, as well as fear responses, increase catecholamine production, and result in higher cell permeability (Davidson et al., 2004). Moreover, a reduction in magnesium concentrations leads to an increase in glycogen breakdown (Steinhardt and Hans-Herma, 1998). Thus, it is important to maintain mineral

homeostasis to prevent any consequences on calf metabolism and physiology. In our study, a decrease in calcium and magnesium values was observed only after 18 h of transport and not after 6 h of transport. Therefore, long transport duration seemed to exert more negative effects on these minerals compared with short transport duration.

Type of vehicle

Apparently, and contrary to our expectations, in our study the settings of the climate control system as applied in practice did not result in notable differences in temperature or relative humidity between the conditioned and open truck. This might explain the fact that, in the current experiment, the type of vehicle had a small influence on post--transport metabolic parameters of calves. Nevertheless, the type of vehicle influenced deltas of osmolality, RT, and sodium between pre- and post-transport. Perhaps climatic parameters other than those recorded in the present experiment differed between the conditioned and open truck, such as draft, which may have affected the physiological state of our calves. Moreover, a different season (e.g., summer) might have caused more notable differences in physiological measurements; however, to our knowledge, no studies are available on the effects of season in association with different type of vehicle on physiology of young calves. From higher osmolality and sodium values, it can be speculated that calves in the conditioned truck experienced more dehydration, which might have compromised their thermoregulatory abilities, resulting in increased RT (Minka and Ayo, 2010). Follow-up studies are needed to much better and more comprehensively characterize climatic conditions in open and in conditioned vehicles during transport, and to examine which environmental factors inside the truck are (most) relevant for calf physiology and health. In addition, our study indicates that there is a need for the definition of scientifically based reference values of settings for climate control systems for livestock transport trucks, especially those for the transport of young calves.

Interaction between pre-transport diet and type of vehicle

An interaction between pre-transport diet and type of vehicle was detected for albumin. Albumin is a protein synthesized in the liver (Klinton and Ježek, 2012), is responsible for 75% of the osmotic pressure of plasma, and is considered a major negative acute phase protein (Tothova et al., 2014). Therefore, a decrease in albumin could be associated with an ongoing inflammatory process (Petersen et al., 2004). In our study, electrolyte-fed calves exhibited a smaller decrease in albumin during transport than milk-fed ones, but only in the open truck (Table 4). However, we have no indication that these specific treatment groups also demonstrated signs of inflammation. Hence, the reasons behind this particular finding remain to be determined.

Experimental treatments on variables until week 5 post-transport

In our experiment, an interaction between pre-transport diet and time was present for both glucose and NEFA. Around T0, milk-fed calves had higher glucose and slightly lower NEFA in the blood. The feed-related differences in glucose and NEFA remained evident until 4 h post-transport. This is in line with the study of Knowles et al. (1999b). The authors reported that glucose and NEFA concentrations of calves returned to pre-transport values within 4 h of the end of transport. Therefore, the effect of pre-transport diet on these blood measurements was visible only for a short-term post-transport.

An interaction between transport duration and time was detected for energy and protein indicators, dehydration indicators, and minerals. Effects of transport duration on glucose, β-HB, and NEFA concentrations were detected in the first 4 h post-transport. Around T0, calves transported for 6 h had higher glucose and lower β-HB and NEFA in their blood. After T4, the concentrations of these variables were similar for calves transported for 6 or 18 h. The fast recovery time from energy depletion is also observed in the literature (Knowles et al., 1999b). However, calves assigned to long transport duration maintained higher plasma urea concentrations until 48 h post-transport. These results might be also associated with fasting during a long journey to the veal farm. Immediately post-transport, Knowles et al. (1997) found higher urea concentrations (5.61 mmol/L) in unfed calves compared with calves being fed (5.34 mmol/L) during a 24 h journey. Urea concentrations in unfed calves also increased up to 72 h post-transport (Knowles et al., 1997). Thus, results suggest that providing feed to calves before long transport might prevent muscle protein degradation in the first 2 d at the veal farm.

The significant increase in albumin, TP (only for 6 h of transport), and osmolality found 48 h post-transport might be related to the feeding management of calves at the veal farm. The calves in the current study ingested 1.5 L of milk or electrolytes at the CC and then were transported for up to 18 h. At the veal farm, calves received 3 L of water with electrolytes immediately post-transport and only the next day did calves receive milk. This means that calves received only 4.5 L of fluid within 48 h. Thus, the difference in management between the dairy farm and the veal farm might have resulted in a peak of dehydration 2 d post-transport. The effect was more pronounced in calves transported for 18 h due to their longer fasting period (Knowles et al., 1997). Compared with calves transported for 6 h, calves transported for 18 h had lower TP concentrations 48 h post-transport. The lower TP concentration might be also associated with the decrease in other proteins (e.g., globulins). A lower α-globulin accompanied by high albumin concentrations in calves transported for 18 h might be related to water loss (Alam et al., 2012), suggesting that these calves might have experienced dehydration at this time point. A great increase in sodium concentrations was particularly visible from 4 h (141.3 mmol/L) to 48 h (162.5 mmol/L) post-transport. The same trend was also seen in

osmolality and it might suggest that the animals were dehydrated between 4 and 48 h post-transport. Based on glucose and NEFA concentrations, it can be concluded that recovery rate of calves post-transport is quick because concentrations were returning to (below) pre-transport values in a short term post-transport. However, effects of transport duration on urea and dehydration indicators were still evident until 48 h post--transport, and thus for a longer term, which might be related to differences in feeding strategies between dairy farms and veal farms.

Conclusions

In this study, pre-transport diet provided at the collection center and transport duration had a significant effect on the hematological profile and physiological status of young veal calves. Feeding milk before transport and short transport duration reduced utilization of glucose as a primary source of energy and prevented mobilization of fat and protein, and BW losses. However, calves transported for 18 h were more dehydrated, as suggested by increasing concentrations of albumin and osmolality at 48 h post--transport and feeding milk before transport was not able to prevent these increases in blood values. The recovery rate of calves post-transport was quick since calves restored their (below) pre-transport values within 24 h post-transport. Type of vehicle had little influence on calf metabolic parameters. The potential implications of differences in hematological profile and physiological status of calves upon arrival at the veal farm for later clinical health and disease incidence during the rearing period remains to be determined and will be the subject of follow-up research. ■

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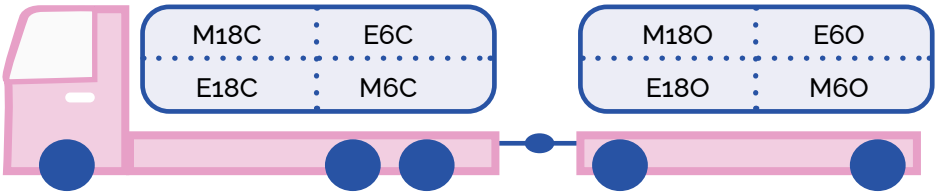
Appendix 1

A



B

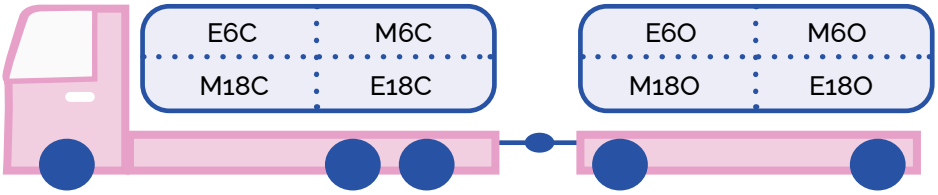


Figure A1 **Design of the truck and the trailer for batch 1 (A) and batch 2 (B) of calves.** *M6C = milk, 6 h transport, conditioned truck; M18C = milk, 18 h transport, conditioned truck; M6O = milk, 6 h transport, open truck; M18O = milk, 18 h transport, open truck; E6C = electrolytes, 6 h transport, conditioned truck; E18C = electrolytes, 18 h transport, conditioned truck; E6O = electrolytes, 6 h transport, open truck; E18O = electrolytes, 18 h transport, open truck.*

CHAPTER 4

Effects of pre-transport diet, transport duration and type of vehicle

on immune cell subsets, haptoglobin, cortisol and bilirubin in young veal calves

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Abstract

The aim of this study was to investigate effects of pre-transport diets, transport durations and transport conditions on immune cell subsets, haptoglobin, cortisol and bilirubin of young calves upon arrival at the veal farm. An experiment was conducted with a 2 × 2 × 2 factorial arrangement with 3 factors: 1) provision of rearing milk or electrolytes at the collection center (CC); 2) transport duration (6 or 18 hours) and 3) transport condition (open truck or conditioned truck). Holstein-Friesian and cross-bred calves were used (N = 368; 18 ± 4 days; 45.3 ± 3.3 kg). Blood samples were collected from calves (N = 128) at the collection center, immediately post-transport (T0) and 4, 24, 48 hours, week 1, 3 and 5 post-transport. Blood was analyzed for cortisol, bilirubin, haptoglobin, IgG and IgM. Moreover, cell counts of neutrophils, lymphocytes, monocytes, basophils and eosinophils were measured in blood samples taken at the collection center and T0. In these same blood samples, different lymphocyte pop ulations were characterized by flow cytometry, including $CD14^+$ cells, NK cells, $\delta\gamma^+$ T cells, $CD8^+$ cells, $CD4^+$ cells and $CD21^+$ cells. Calves transported in the conditioned truck had higher amounts of white blood cell count (WBC) (Δ = 1.39 × 10^9/l; P = 0.01), monocytes (Δ = 0.21 × 10^9/l; P = 0.04), neutrophils (Δ = 0.93 × 10^9/l; P = 0.003), than calves transported in the open truck regardless of pre-transport diet or transport duration. The study showed that transport condition and duration influenced parts of the innate immune system of young veal calves. Cortisol, bilirubin and WBC seemed to be connected by similar underlying mechanisms in relation to transport conditions. However, it is unclear which specific pathways in the immune system of young calves are affected by different transport conditions (e.g. temperature, humidity, draught).

Keywords: transport, diet, transport duration, immunity, veal calf

Introduction

Transport represents a challenge for calves (Brscic et al. 2012). Veal calves are transported when they are 14 to 20 days old, and particularly at this young age, they are vulnerable to transport stress (Swanson and Morrow-Tesch, 2001; Renaud et al., 2018c). In fact, the immune system of young calves is not fully developed and calves lack the time necessary for building immunocompetence compared to older cattle (Swanson and Morrow-Tesch, 2001; Earley et al., 2017). During handling, loading, and commingling around transport, calves may be exposed to microorganisms against which they have no colostral antibodies (Autio et al., 2007). Because transport represents a severe stressor for calves, it may impede immunocompetence and enhance the susceptibility of calves to diseases (Hulbert and Moisa, 2016). Additionally, stress may result in a disruption of the balance between humoral and cellular components of the immune system (Salak-Johnson and McGlone, 2007). To our knowledge, there are limited and contrasting results on which part of the immune system of young calves is affected by transport. Following transport, for example, percentages of γδ T cells, that represent up to 60% of circulating T-cells in young calves, have been reported to increase (Anane et al., 2010; Kanematsu et al., 2017). However, Riondato et al. (2008) showed a decrease in percentages of these cell populations, whereas Baldwin et al. (2000) reported a significant fluctuation in their proportion. Transport related stress may also induce changes in antibody responses. Mackenzie et al. (1997) showed an increase in total IgG_1 and IgG_2 levels following transport and weaning of calves. In contrast, Mormede et al. (1982) found no acute effects of transport on immunoglobulins levels. It is also unknown how specific immune cell subsets of young calves respond in terms of functionality to transport. Moreover, information on the influence of several transport-related factors, namely pre-transport diet, transport duration, and transport conditions on immune cells of young calves is limited. The most common changes observed after transport of all age groups of cattle include an increase in total white blood cell numbers (WBC), basophils, and neutrophils and a decrease in lymphocytes, eosinophils and monocytes (Earley et al., 2017). These changes were mainly investigated in relationship to transport duration, whereas other factors associated with transport, such as pre-transport diet or transport conditions were never studied. Other literature studies showed that also acute phase proteins (APPs) seemed to be influenced by transport duration. Serum concentrations of haptoglobin and serum amyloid-A (SAA) and fibrinogen of cattle were enhanced following transport (Tarrant et al., 1992; Lomborg et al., 2008). After long transport duration (> 24 hours), Murata and Miyamoto (1993) and Arthington et al. (2008) showed increased serum haptoglobin concentrations in calves. In contrast, Buckham Sporer et al. (2008) observed decreased concentrations of haptoglobin and fibrinogen after 9 hours transport of bulls (282 ± 4 days of age). The majority of this type of work

is done on adult animals or at least in older animals. It can, however, be expected that younger calves are more vulnerable to transport-related challenges. This might be expressed in changes in immune related variables, such as immune cell subsets and APPs. The aims of the current study were to investigate immune responses (expressed by changes in immune cell subsets, haptoglobin) and changes in bilirubin and cortisol concentrations in blood of young calves in relation to three transport-related factors (pre-transport diet, transport duration and transport condition).

Materials and methods

Animals and experimental design

The experiment was approved by the Central Committee on Animal Experiments (the Hague, the Netherlands; approval number 2017.D-0029). In total, 368 male Holstein-Friesian and crossbred calves (18 ± 4 days; 45.3 ± 3.3 kg body weight (BW)), divided over two consecutive batches (N = 184/batch) were used. The experiment had a 2 × 2 × 2 factorial arrangement with 3 factors: 1) provision of rearing milk or electrolytes at the collection center (CC); 2) transport duration (6 or 18 hours), and 3) transport condition (open truck or conditioned truck).

In this study we used calves as they normally exist in practice, thus they were collected from different dairy farms in Germany, transported first to a collection center located in Bocholt-Barlo, Germany, and then towards a veal farm in Veghel, The Netherlands. All calves were complying with the minimal weight and health status requirements (BW > 36 kg; age: minimum 14 days; no signs of disease and injury) (SBK, 2018). Since we used animals which followed common procedures of collection, mixing and transport, calves most likely were already challenged prior to their arrival at the collection center (e.g. they had been subjected to feed and water withdrawal, and various handling and transport procedures). Therefore, pre-transport blood values shown in this paper most likely are not representative of baseline values of calves.

Procedures

At the collection center, transport and at the veal farm

At the collection center, calves were randomly allocated by the manager of the collection center to one of eight treatment groups (N = 23 calves per treatment group per batch). Within each batch, 8 calves per treatment group were randomly selected for blood sampling (performed by personnel that were blinded to the treatment groups). After blood sampling, calves were fed via a bucket with nipples with either 1.5 l of rearing milk (125 g of milk powder/l; ME = 4028 kcal/kg of milk powder, CP = 190 g/kg, digestible lysine = 18.7 g/kg; Tentofok KO, Tentego, the Netherlands) or a mixture of electrolytes

(20 g of electrolytes/l of water; Navobi, Staverden, the Netherlands) dissolved in 1.5 l water. No refusals were recorded, thus all calves ingested the expected amount of milk or electrolytes.

After feeding and before transport, calves had the opportunity to rest for 2 hours and after that they were loaded on the vehicle. The vehicle consisted of two parts, a truck and a trailer. The truck was conditioned, which means it was provided with a side-ventilation system, it was insulated and the climate was controlled with regard to in and outlet of air (KVM Livestock Transport System™, Kleventa BV, Lichtenvoorde, the Netherlands). Settings were according to those provided by the manufacturer and applied by the transporter. The trailer was regular, open, and lacked a ventilation system or climate control. Both the truck and trailer were divided into 4 compartments with straw bedding, two at the lower deck (3.60 m length × 2.45 m width × 1.35 m height) and two at the upper deck (3.60 m length × 2.45 m width × 1.45 m height). Additionally, compartments were arranged in both front and back part of the truck and trailer. Each compartment contained 23 calves and had the same stocking density (0.383 m^2 per calf). Each compartment contained calves of one of the eight treatments. Treatments were divided over the truck and the trailer in a way that all main treatments were positioned at the different parts of the truck and trailer (see Figure 1A, B, Appendix 1). The actual temperature and relative humidity inside the truck and trailer were recorded by loggers (N = 8; Escort imini, Cryopak Verification Technologies, Inc.). Each logger was positioned in the middle of each compartment of the truck and trailer and recorded temperature and humidity every 10 minutes. The average (and range in) temperature and relative humidity during transport in the conditioned and open truck are shown in Table 1

Table 1. **Mean and range (between brackets) of actual temperature and relative humidity inside the conditioned and open trucks during short[1] or long[2] transport of young calves to the veal farm.**

	Conditioned truck				Open truck			
	Batch 1		Batch 2		Batch 1		Batch 2	
	T(°C)[3]	RH (%)[4]	T(°C)	RH (%)	T(°C)	RH (%)	T(°C)	RH (%)
6 h	9.2 (8.2 – 10.3)	66.0 (61.0 – 75.1)	13.0 (11.7 – 13.9)	74.1 (65.0 – 81.0)	7.4 (6.2 – 9.1)	74.1 (66.3 – 84.2)	11.5 (10.4 – 12.5)	80.3 (67.6 – 88.8)
18 h	7.8 (4.5 – 11.2)	68.2 (58.5 – 78.9)	13.6 (11.2 – 16.3)	77.9 (65.4 – 83.9)	6.6 (3.9 – 9.6)	75.8 (66.3 – 86.5)	14.0 (10.8 – 16.6)	77.3 (66.2 – 86.2)

[1] *6 h;* [2] *18 h;* [3] *T = temperature;* [4] *RH = relative humidity.*

After loading of calves in the vehicle, transport started with two drivers. Drivers switched every 3 hours. No food or water were provided to calves during transport. After 6 hours transport, the truck arrived at the veal farm and all calves were unloaded. Calves assigned to 6 hours transport were placed in the veal farm, whereas the calves assigned to 18 hours transport were reloaded on the truck and trailer (in the same compartments as before) and transported for another 12 hours. At the veal farm, a total of 64 pens were available, divided over 8 similar compartments, each containing 8 pens, with 5 or 6 calves per pen. Within each compartment, treatments were randomly distributed across pens. Each pen contained 2 calves that were already used for blood sampling at the collection center, and these calves were sampled again immediately after placement at the veal farm (T0). After blood collection at T0, all calves received electrolytes (20 g of electrolytes/l of water; Navobi, Staverden, The Netherlands) dissolved in 3 l of water. For the first 3 weeks, calves were housed individually in temporary partitions positioned within each pen, but these partitions were open, thus calves were able to interact to certain extents with each other. After 3 weeks, the partitions were removed and calves were kept in groups. All calves included in the current research followed the normal production cycle of the veal industry, at the end of which calves were slaughtered and their meat was used for human consumption.

More details on the time and nutrient composition of first feeding at the veal farm are provided by Marcato et al. (2020a).

Blood collection and analysis

To determine effects of transport on subsets of blood immune cells, samples were collected at different sampling moments: at the collection center, prior to feeding (CC), upon arrival at the veal farm prior to provision of electrolytes (T0), after 4, 24, 48 hours (T4, T24, T48) and at week 1, 3 and 5 post-transport. Blood samples (10 ml) were collected from the jugular vein into different vacutainer tubes (Vacuette, Greiner BioOne, Kremsmunster, Austria) and kept at room temperature before centrifugation (3,000 × g for 15 min at 4°C). Plasma and serum were then decanted and stored at – 20°C until analysis. Fluorescence flow cytometry (XT1800VET, IDEXX Bioresearch) was used to determine absolute numbers of different cell types in full blood, including WBC, neutrophils, lymphocytes, monocytes, eosinophils and basophils. Plasma samples were analyzed for levels of immunoglobulins, bilirubin, and haptoglobin, and all measurements were done in duplicate. Immunoglobulin IgG and IgM were measured by an indirect enzyme-linked immunosorbent assay (ELISA) against keyhole limpet hemocyanin (KLH). Plates were coated with 4 µg/ml of KLH (100 µl/well). Natural antibodies of the IgG isotype were detected in plasma, using 1: 20,000 diluted sheep polyclonal anti-bovine IgG-heavy chain conjugated to horseradish PO (Cat. No. E10-118P, Bethyl

Laboratories). Natural antibodies of the IgM isotype were detected in diluted plasma (1:40 as starting dilution) using 1: 20,000 diluted rabbit polyclonal anti-bovine IgM conjugated to horseradish PO (Cat. No. A10-100P, Bethyl Laboratories). Titers were calculated as log2 values of the dilutions, in accordance to Mayasari et al. (2015). Plasma bilirubin concentrations were measured at the mass spectrophotometer, using kit n. 10012, Bilirubin liquicolor, from Human (Wiesbaden, Germany). Plasma haptoglobin concentration was determined with kit n. TP801 from Tridelta Development Ltd. (Maynooth, Ireland). Radioimmunoassay (RIA) (kit n. IM1841, Beckman Coulter, Czech Republic) was used to detect cortisol in plasma samples.

A heparin tube (5 ml) was used for isolation of mononuclear white blood cells. Blood (2 ml) was diluted in phosphate buffered saline (PBS) (2 ml). Diluted blood (2 ml) was then gently added on the top of 2 ml Histopaque (1.083) and samples were centrifuged (1,000 × g, 45 min). White blood cells were then collected from the interphase, resuspended in 2 ml PBS and centrifuged for 20 s at maximum speed (10,000 × g) in the Eppendorf centrifuge. The washing step was repeated twice and the pellet was then suspended in 1 ml fetal calf serum (FCS). An equal volume of freezing medium (1 ml, 10% FCS-RPMI 1640 + 20% DMSO) was added drop by drop to cells. Samples were then stored immediately in a freezing container (Mr. Frosty, Nalgene®) at -80°C.

Flow cytometry

Different combinations of monoclonal antibodies (mAbs) were used to characterize lymphocyte subsets, using flow cytometry (Table 2 on next page).

Mix 1 included mouse anti-bovine CD335-AF488 (AKS1, Bio-Rad, diluted 1:10) and mouse anti-bovine CD8-PE (CC63, Bio-Rad, diluted 1:10). Mix 2 included mouse anti-bovine WC1-FITC (CC15, Bio-Rad, diluted 1:50) and mouse anti-bovine CD4-PE (CC8, Bio-Rad, diluted 1:10). Mix 3 included mouse anti-bovine CD14-FITC (CC-G33, Bio-Rad, diluted 1:50), mouse anti-bovine CD21-PE (CC21, Bio-Rad, diluted 1:10), mouse anti-bovine MHC class II (CC302, Bio-Rad, diluted 1:50) and goat-anti-mouse-IgG2a-APC (diluted 1:2,000).

For the staining of cell surface markers, white blood cells were thawed, resuspended in 15 ml medium (10% FCS-RPMI 1640) and centrifuged (1,300 × rpm, 5 min, 4˚C). Supernatant was discarded and the washing step was repeated. Cells were suspended in 1 ml medium and counted. A total of approximately 500,000 cells/well was transferred to 96 well round bottom plate and PBA (PBS supplemented with 0.5% BSA and 0.005% NaAz) was added. The plate was centrifuged (1,300 × rpm, 2 min, 4˚C), and supernatant was discarded. Each mix of antibodies (50 µl/well) was added to the plate and cells were incubated in the dark for 20 min, on ice.

Table 2. **Composition of the different mixes of monoclonal antibodies used for the flow cytometry analysis.**

	Antibody name	Target
Mix 1	Mouse anti bovine CD8: RPE	CD8+ T cells
	Mouse anti bovine CD335: AF488	NK cells
	Mouse anti human perforin AF647[1]	Perforin[1]
Mix 2	Mouse anti bovine WC1: FITC	gamma delta T cells
	Mouse anti bovine CD4:PE	CD4+ T cells
	Mouse anti human perforin AF647	Perforin[1]
Mix 3	Mouse anti bovine CD14: FITC	monocytes
	Mouse anti bovine CD21:PE	B cells
	Mouse anti bovine MHC Class II UNL	-
	Goat-anti-mouse-IgG2a-APC	IgG2a

[1]*Perforin = it indicates the degranulation of NK cells or T cells, thus is a marker for activation of these cells.*

The plate was then washed with PBA and centrifuged (1,300 × rpm, 2 min, 4˚C). At this stage, cells that were stained with mix 1 and 2 were permeabilized with a permeabilization mix (1 volume FACS permeabilizing solution (BD Pharmingen) + 1 volume FACS lysing solution (BD) + 8 volumes milliQ water). Cells stained with mix 3 remained on ice. To investigate whether the function of CD8+, CD4+ T cells, NK cells and γδ T cells was affected by transport-related stress, intracellular expression of perforin was determined. Therefore, after permeabilization, 50 µl prediluted (1:10) mouse anti-human perforin AF647 (δG9, BD Pharmingen) was added to cells stained with mix 1 and 2. Cells, that had previously received mix 3, received 50 µl prediluted (1:2,000) goat-anti-mouse-IgG2a-APC. Cells were incubated in the dark for 20 min, on ice. Cells were washed with PBA and centrifuged (1,300 × rpm, 2 min, 4˚C) and then resuspended in 200 µl PBA/well.

A minimum of 200,000 cells from a gated lymphocyte population based on FSC and SSC scatter was acquired and measured on a FACS flow cytometer (BD FACSCanto II). Data were then analyzed on FlowJo v10 to obtain percentages of different lymphocyte subsets.

Statistical analyses

All statistical analyses were performed with SAS 9.4 (SAS Inst. Inc., Cary, NC). First, the analyses of data on immediate post-transport (T0), including WBC, neutrophils, lymphocytes, monocytes, eosinophils, basophils, bilirubin, haptoglobin, cortisol, and FACS data (mix 1, 2, 3) are explained. Continuous data, such as bilirubin, were analyzed with a linear mixed model (analysis with restricted maximum likelihood with SAS procedure PROC MIXED). Residuals were checked for normality and homogeneity of variance and variables were log-transformed when needed. Data expressed as proportions, such as FACS data, were analyzed with a generalized linear mixed model (analysis with penalized quasi likelihood with SAS procedure GLIMMIX), with a logit link function, specifying the "error" variance as a multiple of the binomial variance. Both the linear mixed model and the generalized linear mixed model comprised the following fixed effects in the systematic part of the model (the linear predictor part):

$$Y = \mu + Batch_i + Uplo_j + Bafr_k + Diet_l + Type_m + Duration_n + (Diet_l \times Duration_n) + (Diet_l \times Type_m) + (Duration_n \times Type_m) + (Diet_l \times Type_m \times Duration_n) \quad [1]$$

Where: Y = dependent variable, μ is the overall mean, and $Batch_i$ = batch (i = 1, 2), $Uplo_j$ = position in the vehicle (j = upper or lower deck), $Bafr_k$ = position in the vehicle (k = front or back), $Diet_l$ = diet at the collection center (l = rearing milk or electrolytes), $Type_m$ = Transport condition (m = open or conditioned truck), and $Duration_n$ = transport duration (n = 6 or 18 hours). The model also comprised two- and three-way interactions between diet, transport condition and transport duration. Interactions were considered not significant when $P > 0.05$. In addition, random effects for pen and compartment at the veal farm were included (in the linear predictor). Here and in the subsequent analyses, for all fixed effects, approximate F-tests were used (Kenward and Roger, 1997). Interactions that were not significant were excluded from the model (when higher order interactions were already excluded, i.e. respecting the hierarchy of interaction terms) and subsequent pairwise comparisons were done with Fisher's LSD method.

Second, data on bilirubin, haptoglobin and cortisol from T0 until week 3 were analyzed with a linear mixed model for continuous data. The systematic part of this model comprised the following fixed effects:

$$Y = \mu + Batch_i + Uplo_j + Bafr_k + Diet_l + Type_m + Duration_n + Time_o + (Diet_l \times Duration_n) + (Diet_l \times Type_m) + (Duration_n \times Type_m) + (Diet_l \times Time_o) + (Duration_n \times Time_o) + (Type_m \times Time_o) + (Diet_l \times Type_m \times Duration_n) \quad [2]$$

Where: Y = dependent variable, μ is the overall mean, and $Batch_i$ = batch (i = 1, 2), $Uplo_j$ = position in the vehicle (j = upper or lower deck), $Bafr_k$ = position in the vehicle (k = front or back), $Diet_l$ = diet at the collection center (l = rearing milk or electrolytes), $Type_m$ = transport condition (m = open or conditioned truck), $Duration_n$ = transport duration (n = 6 or 18 hours), and $Time_o$ = sampling moment (o = CC, T0, T4, T24, T48, week 1 and 3). Three-way interactions between diet, transport condition, transport duration, and two-way interactions between diet, transport condition, transport duration and time were also included in the model. Interactions were considered not significant when $P > 0.05$. The model comprised random pen, compartment, and animal effects. For the animal effects a first order auto regressive model (based on the actual distance between time points) was adopted to introduce correlation in the model between repeated measurements on the same animal.

Third, data on bilirubin, haptoglobin and cortisol of week 5 were analyzed, using model 2. Although calves were housed individually in baby boxes until week 3, they were in group pens from week 5 onward, so random pen effects were included in the model.

Finally, differences between pre- and post-transport measurements (deltas, Δ = T0 – CC) were calculated for WBC, neutrophils, lymphocytes, monocytes, eosinophils, basophils, bilirubin, haptoglobin, cortisol, and FACS variables. These differences were also analyzed, using model 2.

Although no treatments were applied yet at the collection center, a preliminary statistical test, using model 1 but without interactions, was performed to investigate whether groups of calves already differed before treatments started. No statistical differences were found between treatment groups.

Results

Three-way interactions and two-way interactions between pre-transport diet and transport condition and between transport duration and transport condition were never significant. Consequently, results will be shown as follows: 1) Two-way interaction between pre-transport diet and transport duration (Table 3); 2) Main effects of pre-transport diet, transport condition and transport duration (Table 4-6); 3) Effects of treatments in time until 5 week post-transport (Figure 1, 2, 3A, 3B). Within the first two parts, results will be presented at 2 levels: 1) Effects of treatments immediately post-transport (T0) (Table 3-5); 2) Effects of treatments on differences between pre and post-transport measurements (deltas, Δ = T0 – CC; Table 3 and 6).

Table 3. Interaction between pre-transport diet[1] and transport duration[2] detected in blood parameters measured immediately post-transport (T0) and on the difference[3] between pre-and post-transport measurements in young veal calves (LS means).

A.	Immediate post-transport (T0)					
Parameter	Milk		Electrolytes		SEM[4]	*P*-value Interaction
	6 h	18 h	6 h	18 h		
Bilirubin, µmol/l	8.39^{b}	12.35^{a}	13.24^{a}	12.30^{a}	1.21	0.03
B.	Δ = To – CC					
Parameter	Milk		Electrolytes		SEM	*P*-value Interaction
	6 h	18 h	6 h	18 h		
Basophils, 10^9/l	0.22^{a}	0.15^{ab}	0.19^{b}	0.16^{ab}	0.02	0.03
Bilirubin, µmol/l	-3.75^{b}	1.83^{a}	3.64^{a}	1.50^{a}	1.35	0.02
Haptoglobin, mg/ml	-0.04^{ab}	0.11^{a}	0.03^{ab}	-0.05^{b}	0.05	0.05

[1] *milk vs. electrolytes;* [2] *6 vs. 18 h;* [3] *deltas, Δ = To – CC;* [4]*SEM = pooled standard error.* [a-b] *Least square means within a row lacking a common superscript differ (P ≤ 0.05)*

Interaction between pre-transport diet and transport duration

Effects on variables immediately post-transport (T0)

An interaction between pre-transport diet and transport duration was detected for bilirubin (*P* = 0.03; Table 3). Both milk-fed and electrolyte-fed calves transported for 18 hours and electrolyte-fed calves transported for 6 hours had similar higher bilirubin concentrations than milk-fed calves transported for 6 hours (Δ = 4.24 µmol/l on average) directly post-transport.

Effects on the deltas (Δ = T0 – CC)

An interaction between pre-transport diet and transport duration was found for deltas of basophils, bilirubin and haptoglobin (Table 3). Milk and electrolyte-fed calves transported for 18 hours increased to the same degree their number of basophils and bilirubin concentration. However, after 6 hours transport, milk-fed calves had a stronger increase in the number of basophils than electrolyte-fed calves (*P* = 0.03). In addition, milk-fed calves had a decrease in bilirubin concentration compared with electrolyte--fed calves (*P* = 0.02). Differences in haptoglobin concentrations after 6 hours transport were similar between milk-fed calves and electrolyte-fed calves. However, milk-fed calves and transported for 18 hours increased their haptoglobin values, whereas electrolyte-fed calves and transported for 18 hours had a decrease (*P* = 0.05).

Table 4. Effects of diet composition[1] at the collection center, transport conditions[2] and transport duration[3] on immune parameters of young calves measured directly post-transport (LS means).

	Pre-transport diet				Transport condition				Transport duration			
Parameter	Electrolytes	Milk	SEM[4]	*P*-value	Conditioned	Open	SEM	*P*-value	6 h	18 h	SEM	*P*-value
WBC[5], 10^9/l	8.19	9.44	0.39	0.11	9.51	8.12	0.40	0.01	8.54	9.10	0.41	0.79
Monocytes, 10^9/l	1.18	1.39	0.07	0.03	1.39	1.18	0.07	0.04	1.27	1.30	0.09	0.87
Monocytes, %[6]	14.16	15.13	0.51	0.16	14.60	14.69	0.51	0.89	14.72	14.56	0.63	0.86
Lymphocytes, 10^9/l	3.03	3.27	0.16	0.18	3.15	3.15	0.16	0.99	3.22	3.08	0.18	0.59
Lymphocytes, %	38.22	37.40	1.36	0.77	35.44	40.17	1.36	0.03	38.45	37.15	1.68	0.57
Neutrophils, 10^9/l	3.34	3.94	0.25	0.25	4.10	3.17	0.25	0.003	3.53	3.75	0.31	0.79
Neutrophils, %	39.60	39.55	1.29	0.88	41.48	37.64	1.26	0.02	37.92	41.37	1.27	0.40
Eosinophils, 10^9/l	0.49	0.65	0.09	0.50	0.69	0.45	0.09	0.13	0.55	0.60	0.09	0.54
Eosinophils, %	5.68	5.83	0.59	0.86	6.23	5.27	0.59	0.46	5.49	6.01	0.73	0.80
Basophils, 10^9/l	0.18	0.19	0.02	0.64	0.19	0.17	0.02	0.05	0.21	0.14	0.02	< 0.01
Basophils, %	2.24	2.12	0.15	0.73	2.19	2.18	0.15	0.99	2.64	1.73	0.18	0.26
IgG[7], titer	4.54	4.39	0.18	0.56	4.36	4.58	0.18	0.38	4.60	4.34	0.22	0.47
IgM[8], titer	5.31	5.18	0.21	0.37	5.16	5.33	0.21	0.62	5.12	5.38	0.21	0.59
Haptoglobin, mg/ml	0.34	0.44	0.04	0.20	0.44	0.34	0.04	0.07	0.34	0.44	0.04	0.26
Bilirubin, µmol/l	12.77	10.37	0.62	0.01	12.71	10.43	0.87	0.16	10.82	12.32	0.62	0.05
Cortisol, ng/ml	10.79	8.79	0.99	0.03	10.35	9.23	0.99	0.34	7.95	11.63	0.96	< 0.01

[1] *milk vs. electrolytes;* [2] *conditioned vs. open truck;* [3] *6 vs. 18 h;* [4] *SEM = standard error of the mean;* [5] *WBC = white blood cell count;* [6] *% = all proportions are relative to WBC;* [7] *IgG = immunoglobulin G;* [8] *IgM = immunoglobulin M.*

Table 5. Effects of diet composition[1] at the collection center, transport conditions[2] and transport duration[3] on different cell subsets of young calves analyzed with flow cytometry measured directly post-transport (T0) (LS means).

	Pre-transport diet				Transport condition				Transport duration			
Parameter (%[4])	Electrolytes	Milk	SEM[5]	*P*-value	Conditioned	Open	SEM	*P*-value	6 h	18 h	SEM	*P*-value
CD8+ T cells	8.45	7.26	0.41	0.05	7.63	8.10	0.41	0.35	6.90	8.85	0.50	0.01
CD8+ perf+[6]	1.10	1.24	0.11	0.66	1.17	1.17	0.11	0.99	0.96	1.38	0.14	0.36
NK cells	7.99	7.39	0.54	0.33	7.75	7.63	0.54	0.84	8.72	6.65	0.65	0.07
NK perf+	1.66	1.65	0.07	0.98	1.70	1.61	0.07	0.76	1.65	1.66	0.09	0.95
CD4+ T cells	19.24	18.59	0.93	0.58	18.55	19.28	0.93	0.56	17.55	20.28	1.14	0.13
CD4+ perf+	0.17	0.18	0.02	0.95	0.20	0.15	0.02	0.61	0.17	0.18	0.03	0.86
δγ+ T cells	39.89	42.24	1.69	0.31	41.30	40.83	1.69	0.77	42.93	39.20	2.07	0.25
δγ+ perf+	0.17	0.21	0.02	0.70	0.21	0.17	0.02	0.69	0.18	0.20	0.02	0.87
Monocytes	40.99	45.09	1.80	0.11	44.86	41.22	1.80	0.16	41.62	44.47	2.20	0.44
B cells	2.28	2.69	0.21	0.16	2.50	2.47	0.21	0.90	2.84	2.13	0.25	0.08

[1] *milk vs. electrolytes;* [2] *conditioned vs. open truck;* [3] *6 vs. 18 h.;* [4] *% = proportion relative to lymphocytes;* [5] *SEM = standard error of the means;* [6] *Perf+ = CD8+ T cells, NK cells, CD4+ and δγ+ T cells were stimulated also with perforin to examine the functionality of these cells and how their functionality was affected by the different treatments.*

Table 6. Effects of diet composition[1] at the collection center[2], transport condition[3] and transport duration[4] on the difference between CC and post-transport (T0) immunological measurements[5] of young veal calves (LS means).

	Pre-transport diet				Transport condition				Transport duration			
Parameter	Electrolytes	Milk	SEM[6]	*P*-value	Conditioned	Open	SEM	*P*-value	6 h	18 h	SEM	*P*-value
WBC[7], 10^9/l	-1.90	-1.35	0.51	0.39	-0.98	-2.27	0.51	0.04	-1.91	-1.34	0.60	0.53
Monocytes, 10^9/l	-0.26	-0.19	0.09	0.42	-0.11	-0.34	0.09	< 0.01	-0.26	-0.19	0.10	0.55
Monocytes, %[8]	0.04	-0.11	0.42	0.95	-0.05	-0.03	0.42	0.98	-0.27	0.20	0.42	0.99
Lymphocytes, 10^9/l	-0.40	-0.31	0.11	0.51	-0.29	-0.43	0.11	0.32	-0.40	-0.31	0.13	0.65
Lymphocytes, %	2.72	1.49	1.14	0.92	-0.22	4.45	1.11	0.81	1.81	2.39	1.08	0.99
Neutrophils, 10^9/l	-1.39	-1.01	0.37	0.44	-0.72	-1.68	0.37	0.05	-1.46	-0.93	0.44	0.45
Neutrophils, %	-6.07	-3.46	1.28	0.90	-2.63	-6.91	1.27	0.76	-4.25	-5.26	1.27	0.95
Eosinophils, 10^9/l	0.16	0.21	0.09	0.69	0.25	0.12	0.09	0.34	0.19	0.17	0.11	0.92
Eosinophils, %	2.33	2.14	0.54	0.87	2.21	2.25	0.54	0.97	2.77	1.68	0.54	0.71
Basophils, 10^9/l	-0.06	-0.05	0.02	0.62	-0.07	-0.03	0.02	0.55	-0.05	-0.06	0.02	0.70
Basophils, %	-0.34	-0.05	0.22	0.89	-0.61	0.23	0.22	0.57	-0.06	-0.32	0.22	0.90
IgG[9], titer	-0.15	-0.30	0.05	0.05	-0.25	-0.20	0.05	0.56	-0.21	-0.24	0.06	0.76
IgM[10], titer	0.10	0.18	0.31	0.86	0.09	0.20	0.31	0.81	0.48	-0.19	0.39	0.29
Haptoglobin, mg/ml	-0.01	0.03	0.03	0.26	0.05	-0.03	0.03	0.06	-0.01	0.03	0.04	0.50
Bilirubin, µmol/l	2.57	-0.96	0.78	< 0.01	1.19	0.42	0.78	0.49	-0.06	1.66	0.96	0.28
Cortisol, ng/ml	0.48	-2.01	1.13	0.07	-1.29	-0.23	1.13	0.43	-3.62	2.09	1.32	< 0.01

[1] *milk vs. electrolytes;* [2] *CC;* [3] *conditioned vs. open truck;* [4] *6 vs. 18 h;* [5] *deltas, Δ = T0 – CC.*
[6] *SEM = standard error of the means;* [7] *WBC = white blood cell count;* [8] *% = all proportions are relative to WBC;* [9] *IgG = immunoglobulin G;* [10] *IgM = immunoglobulin M.*

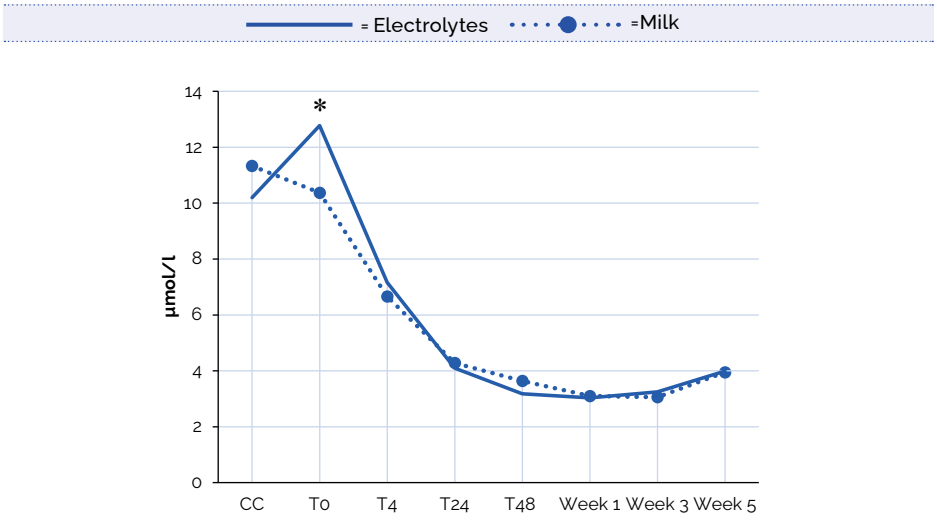

Figure 1. **Interaction between pre-transport diet (milk vs electrolytes) and time relative to transport for bilirubin in young veal calves.** *Blood samples were collected at the collection center (CC), immediately post-transport (To) and 4 (T4), 24 (T24), 48 (T48) hours, and week 1, 3, and 5 post-transport. Asterisks indicate significant differences (P ≤ 0.05) between treatments within a sampling moment.*

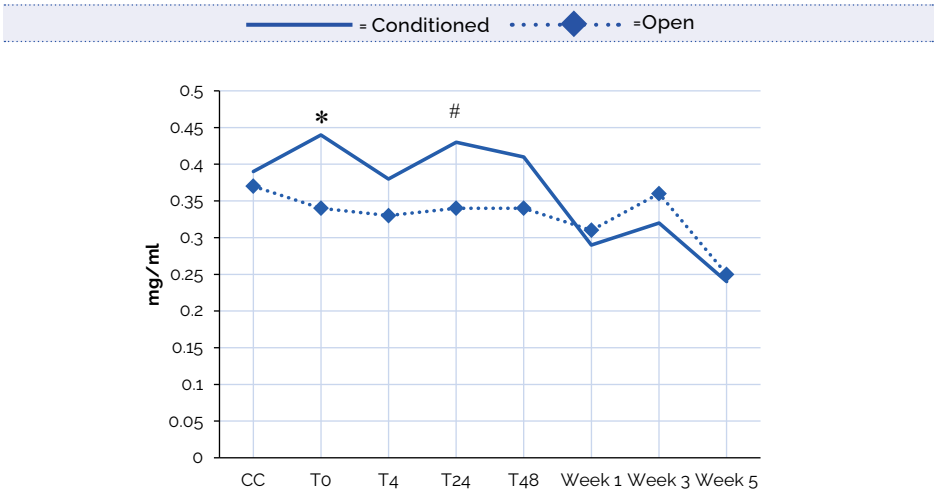

Figure 2. **Interaction between transport condition (conditioned truck vs open truck) and time relative to transport for haptoglobin in young veal calves.** *Blood samples were collected at the collection center (CC), immediately post-transport (To) and 4 (T4), 24 (T24), 48 (T48) hours, and week 1, 3, and 5 post-transport. Asterisks indicate significant differences (P ≤ 0.05) between treatments within a sampling moment, whereas hashtags indicate a tendency towards significance (0.05 ≤ P ≤ 0.10).*

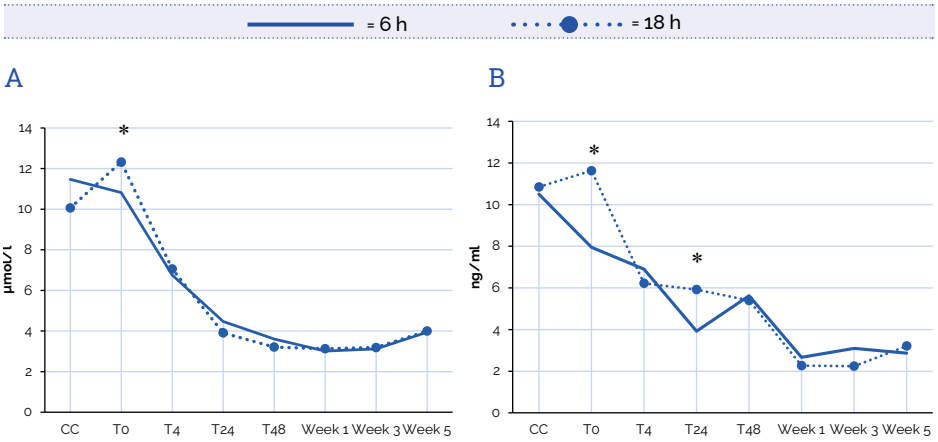

Figure 3 **A, B. Interactions between transport duration (6 hours vs 18 hours) and time relative to transport in young veal calves. (A) Bilirubin and (B) Cortisol.** *Blood samples were collected at the collection center (CC), immediately post-transport (To) and 4 (T4), 24 (T24), 48 (T48) hours, and week 1, 3, and 5 post-transport. Asterisks indicate significant differences (P ≤ 0.05) between treatments within a sampling moment.*

Main effects

Effects on variables immediately post-transport (T0)

As shown in Table 4, diet had an effect on concentrations of monocytes and cortisol at To. Milk-fed calves had higher monocytes concentration (Δ = 0.21 × 10^9/l) and lower cortisol than electrolyte-fed calves (Δ = -2 µmol/l). Moreover, milk-fed calves had a lower proportion of CD8+ lymphocytes than electrolyte-fed calves (Δ = -1.19%, Table 5).

Transport condition influenced absolute numbers of WBC, monocytes, neutrophils, eosinophils and basophils at To, and also proportion of neutrophils and lymphocytes relative to WBC (Table 4). With regards to absolute levels, calves transported in the conditioned truck had higher amount of WBC (Δ = 1.39 × 10^9/l), and higher neutrophils (Δ = 0.93 × 10^9/l), monocytes (Δ = 0.21 × 10^9/l), eosinophils (Δ = 0.24 × 10^9/l) and basophils (Δ = 0.02 × 10^9/l) than calves in the open truck. Despite the higher WBC, the effects of transport conditions on proportions of cells were different compared to those on absolute numbers. In calves transported in the conditioned truck, the higher WBC contributed to an increased proportion of neutrophils (Δ = 3.84%) but to a lower proportion of lymphocytes (Δ = -4.73%) in the blood compared to calves transported in the open truck. Transport condition had no significant effects on proportions of NK cells, B cells, and different T cells subsets upon arrival at To.

Transport duration significantly affected basophils and cortisol concentrations (Table 4). Long-term transport (18 hours) led to lower basophils and higher cortisol values compared to 6 hours transport (Δ = -0.07 × 10^9/l and Δ = 3.68 ng/ml, respectively). In addition, at T0, calves transported for 18 hours had a greater proportion of CD8+ lymphocytes in the blood than calves with 6 hours transport (Δ = 1.95%) (Table 5). No differences were observed in perforin expression in NK cells, γδ T cells, CD8+, CD4+ T cells, indicating that the function of those cell subsets in the blood was not influenced by the different treatments (Table 5).

Effects on the deltas (Δ = T0 – CC)

Pre-transport diet had an effect on differences in IgG titer and bilirubin concentrations between pre and post-transport (Table 6). IgG titer decreased during transport to a greater extent in milk-fed calves than in electrolyte-fed calves. Transport condition influenced the absolute amount of WBC, neutrophils and monocytes. Calves in the conditioned truck had a smaller decrease in WBC, neutrophils and monocytes than calves in the open truck (Table 6). Transport duration had no effects on deltas of differential white blood cell count. Significant effects of pre-transport diet, transport duration or transport condition on deltas of NK cells, B cells, different T cells subsets were not present, nor on perforin expression (Table 1, Appendix 2).

Effects of treatments in time until week 5 post-transport

Interaction between pre-transport diet and time

An interaction between pre-transport diet and time was found for bilirubin (Figure 1). Electrolyte-fed calves showed higher bilirubin concentrations at T0 than milk-fed calves, whereas at the other moments no differences were found.

Interaction between transport condition and time

Haptoglobin showed an interaction between transport condition and time (Figure 2). At T0 ($P < 0.05$) and at T24 ($P < 0.10$) calves transported in the conditioned truck showed higher haptoglobin levels than calves transported in the open truck, whereas at the other moments no significant differences were found between transport conditions.

Interaction between transport duration and time

An interaction between transport duration and time was detected for bilirubin and cortisol (Figure 3A, B). Calves transported for 18 hours had higher bilirubin at T0 and higher cortisol at T0 and T24 than calves transported for 6 hours, whereas at the other moments no differences between transport durations were found.

Discussion

Effects of pre-transport diet

In our experiment, electrolyte-fed calves had a higher cortisol concentration at arrival at the veal farm than milk-fed calves, probably due to the different composition of the pre-transport diet. It has been shown that cortisol can be influenced by the digestive functions that occur after feeding and by intestinal motility (Gardy-Godillot et al., 1989). Plasma glucose and insulin are negatively correlated to plasma ACTH and cortisol (Richet et al., 1985), and, hence, it is expected that glucose and insulin levels are higher and cortisol levels are lower in milk-fed animals in the current study. Moreover, feeding electrolytes might have contributed to a more negative energy balance (NEB) and an enhanced feeling of hunger compared to feeding milk. Hunger increases corticotropin-releasing factor secretion and consequently cortisol secretion in calves (Gardy-Godillot et al., 1989).

In the current experiment, pre-transport diet also had an effect on absolute levels of monocytes and CD8+ lymphocytes at arrival at the veal farm. Nutrition has been shown to play a key role in immune responses of young calves (Nonnecke et al., 2003; Ballou et al., 2015). Especially the energy and protein intake in calves during the first weeks after birth, can influence cell-mediated immunity, cytokine production, phagocytic function and secretory IgA antibody concentrations, but also specific absolute numbers of monocytes (Woodward, 1998). However, these studies were conducted in young calves at the dairy farms, without any challenge or transport, so probably their values were closer to baseline values than in the current study. In addition, Murata et al. (1987) and Earley et al. (2017) reported increased counts of monocytes and differences in lymphocyte subsets post-transport. This effect was attributed to transport duration rather than to pre-transport diet. To our knowledge, there are no other studies conducted on effects of pre-transport diet on subsets of these immune cells of young calves on arrival at the veal farm. It can be hypothesized that the higher counts of monocytes of milk-fed calves may have a protective function against environmental microorganisms, thus reducing the risk of diseases in calves.

Interaction between pre-transport diet and transport duration

Effects on measurements immediately post-transport (T0)

Bilirubin is commonly used as a biomarker of liver status in cattle, especially in dairy cows (Bionaz et al., 2007; Bertoni et al., 2008). In cows, higher bilirubin concentrations are associated with a lower clearance of secretory enzymes in the liver as a response to liver cell damage (Assenat et al., 2004; Bertoni et al., 2008). In addition, bilirubin is positively correlated with the degree of fat infiltration in the liver and high bilirubin levels are

related with a more negative energy balance (NEB) (West, 1990). In our study, the high bilirubin values after prolonged transport in both milk-fed and electrolyte-fed calves might be mainly caused by a prolonged fasting period. Both feeding strategies at the collection center might not have provided calves enough energy to cover the entire period of travel. Therefore, calves were likely in a NEB at T0. This is supported by the electrolyte-fed calves after 6 hours transport, which also demonstrated high bilirubin concentrations, probably because of the low energy intake, whereas milk-fed calves showed lower bilirubin concentrations probably because they had more energy intake, covering the energy needs during a 6 hours transport duration.

Effects on the deltas (Δ = T0 – CC)

Basophils have numerous functions, including a beneficial role in protective immunity against parasitic infections and a role in autoimmune and inflammatory diseases (Karasuyama et al., 2018). Calves have generally a low basophil count in their blood (Brun-Hansen et al., 2006). An increase in basophils can be indicative of a stress response, following transport in adult cattle (Murata et al., 1987; Earley et al., 2017). Transport related-stress appears to be associated with cell trafficking and redistribution of peripheral lymphocytes between different immune compartments (Bauer et al., 2001). Thus, it can be hypothesized that the increase in basophils was triggered by redistribution of lymphocytes in the peripheral blood. However, to our knowledge, there are no studies that investigated effects of transport on these cells in young calves. Additionally, there is no clear evidence that diet could be a factor that contributes to an increase in number of basophils. It could be hypothesized that the higher protein and energy content of milk compared to electrolytes may have modified the adhesion marker expression on T lymphocytes and may have resulted in an increase in basophil counts.

Bovine haptoglobin is an APP that exerts numerous biological functions. In cattle, the primary function of haptoglobin is to form complexes with free hemoglobin in the blood in order to prevent oxidative damages (Ceciliani et al., 2012; Tothova et al., 2014). An increase in haptoglobin has been reported in calves with a higher disease incidence (Ganheim et al., 2007). Moreover, a rise in haptoglobin concentrations was also found in calves after long distance transport (Arthington et al., 2008; Ceciliani et al., 2012). Therefore, these studies suggested that haptoglobin can be used also as a marker of health status or stress in calves. In our study, calves transported for 18 hours and fed with milk or electrolytes showed a different response in terms of haptoglobin concentrations. The increase in haptoglobin after 18 hours transport in milk-fed calves might suggest that these animals were probably experiencing higher post-transport stress than the electrolyte-fed calves. Additionally, in our study, the increase in haptoglobin was relatively small compared to acute values reported in the literature (1.62 ± 0.47 g/l).

Effects of transport duration

In our study, effects of transport duration were also present on absolute values and on deltas of cortisol between T0 and CC. The higher cortisol concentrations after 18 hours transport were in line with previous studies. Knowles et al. (1999a) found increased cortisol concentrations post-transport up to 2.3 µg/100 ml in cattle compared to pre-transport concentrations. Averos et al. (2008) showed that cortisol increased when young bulls were transported for 13 hours from a collection center (6.5 ng/ml) to a growing-finishing farm (12.0 ng/ml). However, other studies on transport related effects on cortisol showed ambiguous results. Odore et al. (2011) found already a significant increase in cortisol concentrations after short-term transport duration. Honkavaara et al. (2003) also observed that animals transported for short journeys had a higher cortisol than animals transported for long journeys (up to 14 hours). Short transport duration can cause acute psychological stress due to the novelty of the transport process, whereas during long transport duration, the exhaustion of the adrenal gland may occur (Odore et al., 2011). Kent and Ewbank (1983), and Warriss et al. (1995) suggested that the decrease in cortisol concentrations during long transport duration might also be due to the adaptation of cattle to the transport.

All these studies used older animals than those included in the current study, which makes a comparison between previous results and the present findings in young calves difficult. Young animals still have to complete their HPA axis development (see Marcato et al. (2018) for overview), and consequently, the responses to stress in young animals might be different and not really consistent as the ones of mature cattle (Mormede et al., 1982; Swanson and Morrow-Tesch, 2001). Overall, in our study, it seems that calves had elevated levels of cortisol directly and 24 hours post-transport.

Following transport, an increase in the adrenal cortex activity might affect the immune system (Odore et al., 2011; Grandin, 2014). Some studies reported that transport can lead to lower number of immune cells in blood, whereas others suggested that transport can have stimulating effects on the immune system, especially after acute stress (Riondato et al., 2008; Grandin, 2014). Lymphocytes express measurable concentrations of glucocorticoid and adrenergic receptors, which can be down-regulated or altered during a stress response (Preisler et al., 2000; Odore et al., 2004; Abraham et al., 2004). Riondato et al. (2008) showed a decrease in proportions of calves' $CD4^+$ and $CD8^+$ T cells in blood after 14 hours transport duration, whereas the decrease in $CD21^+$ B cells occurred just a day later. These data are different from the results of the present study, which demonstrated no effects on $CD4^+$ T cells, but higher proportions of $CD8^+$ T cells after 18 hours transport duration compared to 6 hours transport duration. This difference might reflect the activation of the immune system to redistribute more T cells from the secondary

lymphoid organ into the peripheral blood. Masmeijer et al. (2019) indicated that, in young calves between 14 and 28 days of age, transport-related higher glucocorticoids may cause a robust leukocyte redistribution by inducing increased expression of surface receptors (e.g. CD172a, CD11a) on stress-activated monocytes. A higher humoral and cellular response might be related to a decrease in susceptibility of calves to infections (Murata and Hirose, 1991). In the current experiment, a tendency towards lower amount of NK cells and B cells after 18 h transport was also found compared to 6 hours transport. These data might be explained by higher concentrations of cortisol after long transport duration. However, Ishizaki and Kariya (2010), found a positive correlation (r = 0.704; P < 0.01) between cortisol concentrations and NK cell counts in the blood. The study was conducted on short-term transport and acute stress effects, thus the effects might be different for long-term transport, which might have an inhibitory effect on these cells.

Effects of transport conditions

In the current study, immediately post-transport, a higher number of WBC, a higher proportion of neutrophils, and a lower proportion of lymphocytes were found after transport in the conditioned truck compared to the open truck. Earley et al. (2017) indicated that neutrophilia in conjunction with lymphopenia are common responses observed following transport in all age groups of cattle. Ishizaki and Kariya (2010) reported that higher glucocorticoids levels result in lymphocyte destruction in the thymus cortex and in the extension of the neutrophil half-life. These changes might be indicators of transport stress, but due to a lack of literature on transport conditions, potential reasons for the differences between the open and the conditioned truck remain unknown. In the presence of a pathogen, a higher number of neutrophils might also be the cause of the excessive inflammation and tissues damage found in cattle with bovine respiratory disease following transport (Lekeux, 1995; Wessely-Szponder et al., 2004). Transport stress might also lead to other shifts in immune cell subsets, including an increase in monocytes, eosinophils and basophils (Kent and Ewbank, 1986a; Murata et al., 1987). In fact, transport of young calves (14 until 28 days of age) is associated with an increased production in glucocorticoids, which can cause a robust leukocyte redistribution (Masmeijer et al., 2019). This general mechanism of redistribution may also explain the current effects of the conditioned truck on total WBC and some of the related cells. Perhaps, a range of environmental factors may be involved, such as draught, temperature, relative humidity, or vibrations inside the vehicle. However, this needs to be investigated further.

Effects of factors on variables until week 5 post-transport

In our study, the recovery rate of calves after 6 hours transport was similar to the one after 18 hours transport, since calves restored their pre-transport values within 4 hours post-transport. However, calves transported for 6 hours had a decrease in cortisol concentrations immediately post-transport, suggesting that short transport duration has no effect on the HPA axis response. This result is in agreement with the studies of Cole et al. (1988) and Mitchell et al. (1988) who found lower cortisol concentration post-transport than pre-transport. Moreover, at T24, concentrations of cortisol in calves transported for 6 hours were slightly lower than those of calves transported for 18 hours, suggesting that the recovery rate of calves with long transport duration took a longer time period.

Bilirubin concentrations after 6 and 18 hours transport followed the same trend as cortisol, thus the recovery rate of calves, based on this parameter, seemed also fast. It seems that at 48 hours post-transport all values were similar regardless of treatment, thus these values might represent baseline values. However, since calves were already challenged before transport, it remains unknown whether these values are representative of reference values for homeostasis.

Immediately post-transport, haptoglobin increased in calves transported in the conditioned truck, whereas it decreased in calves transported in the open truck. The same concentrations were also visible 24 hours post-transport, where haptoglobin concentrations were gradually lower until week 5 post-transport. Higher haptoglobin concentrations are associated with tissue injury, inflammation or infection (Murata and Miyamoto, 1993; Joshi et al., 2018). Other studies showed an increase in haptoglobin in response to stress of transport (Murata and Miyamoto, 1993; Lomborg et al., 2008). Thus, the increase in haptoglobin found in calves transported in the conditioned truck might be related with a greater transport stress experienced by calves. The recovery rate of calves was slower compared to the one for cortisol and bilirubin, because haptoglobin tended to be high 24 hours post-transport. However, it is difficult to make a comparison with the recovery rate of calves used in other studies due to a lack of reference values for different time points after transport. Overall, it can be concluded that there are no long-term (> 48 hours post-transport) effects of treatments on bilirubin, cortisol and haptoglobin concentrations.

Conclusions

The current study showed that all treatments (pre-transport diet, transport duration and transport condition) affected different parts of the immune system of young veal calves. The higher energy content of milk relative to electrolytes likely prevented the rise in bilirubin concentrations in calves transported for 6 hours. Long transport duration compared to short transport duration contributed to higher blood cortisol concentrations in calves post-transport. We hypothesized that the higher stress and cortisol concentrations in calves transported for 18 hours may have resulted in a redistribution of leukocytes in their circulation, leading to higher CD8+ T cells than in calves transported for 6 hours. At present it remains unclear which transport factors (e.g. temperature, humidity, draught) are most important in relation to potential effects on the immune system of young veal calves. The potential implications of differences in immune cells upon arrival at the veal farm for later clinical health and disease incidence during the rearing period remains to be determined, and will be the subject of follow-up research ■

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Appendix 1

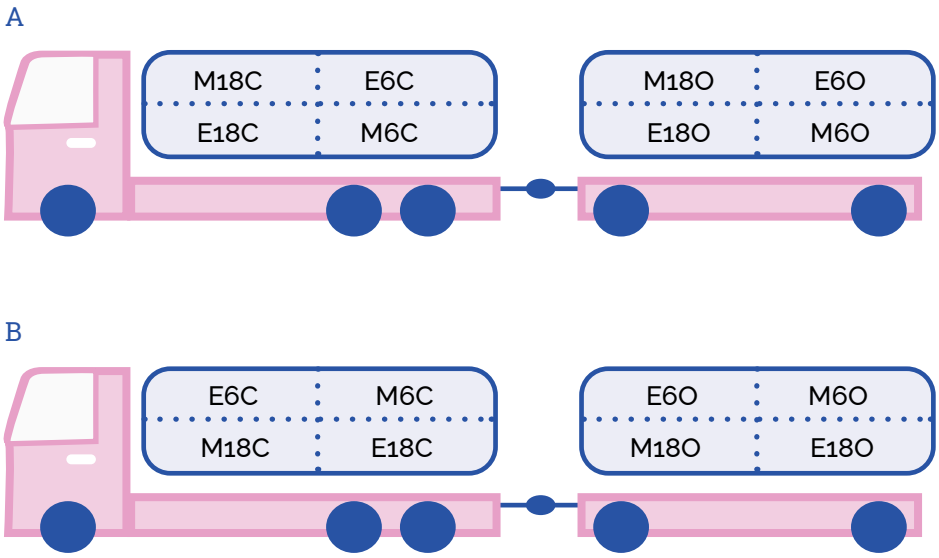

Figure 1 A-B. **Design of the truck and the trailer for batch 1 (A) and batch 2 (B) of calves.** *M6C = milk, 6 h transport, conditioned truck; M18C = milk, 18 h transport, conditioned truck; M6O = milk, 6 h transport, open truck; M18O = milk, 18 h transport, open truck; E6C = electrolytes, 6 h transport, conditioned truck; E18C = electrolytes, 18 h transport, conditioned truck; E6O = electrolytes, 6 h transport, open truck; E18O = electrolytes, 18 h transport, open truck.*

Appendix 2

Table 1. Effects of diet composition[1] at the collection center[2], transport condition[3] and transport duration[4] on the difference[5] between CC and post-transport (T0) measurements of different cell subsets in blood of young veal calves (LS means).

	Pre-transport diet				Transport condition				Transport duration			
Parameter (%)[6]	Electrolytes	Milk	SEM[7]	*P*-value	Conditioned	Open	SEM	*P*-value	6 h	18 h	SEM	*P*-value
CD8+ T cells	-0.67	-1.26	0.25	0.83	-0.76	-1.18	0.25	0.94	-1.56	-0.37	0.25	0.83
CD8+ perf+[8]	-0.12	-0.03	0.13	0.74	0.06	-0.22	0.13	0.99	-0.07	-0.08	0.13	0.91
NK cells	-0.44	-0.46	0.40	0.87	0.00	-0.91	0.40	0.83	-0.77	-0.12	0.40	0.95
NK perf+	0.00	-0.01	0.06	0.94	0.01	-0.02	0.06	0.88	0.01	-0.01	0.06	0.95
CD4+ T cells	0.80	0.95	0.54	0.84	1.61	0.13	0.54	0.68	-0.54	2.31	0.54	0.87
CD4+ perf+	-0.01	-0.36	0.12	0.99	-0.34	-0.04	0.12	0.89	-0.41	0.04	0.12	0.68
δγ+ T cells	6.38	7.53	1.00	0.98	7.04	6.88	1.00	0.99	9.67	4.21	1.00	0.78
δγ+ perf+	0.00	0.05	0.02	0.83	0.04	0.01	0.02	0.97	0.00	0.05	0.02	0.85
Monocytes	1.16	3.02	0.92	0.93	3.42	0.75	0.92	0.87	2.13	2.08	0.92	0.92
B cells	-0.27	0.01	0.20	0.89	-0.19	-0.05	0.20	0.92	-0.01	-0.23	0.20	0.83

[1] milk vs. electrolytes; [2] CC; [3] conditioned vs. open truck; [4] 6 vs. 18 h; [5] deltas, Δ = T0 – CC; [6] % = proportion relative to lymphocytes; [7] SEM = standard error of the means; [8] Perf+ = CD8+ T cells, NK cells, CD4+ and δγ+ T cells were stimulated with perforin to examine the functionality of these cells and how their functionality was affected by the different treatments.

CHAPTER 5

Transport of young veal calves

effects of pre-transport diet, transport duration and type of vehicle on health, behavior, use of medicines and slaughter characteristics

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Abstract

The aim of this study was to investigate effects of different early life transport-related factors on health, behavior, use of medicines and slaughter characteristics of veal calves. An experiment was conducted with a 2 × 2 × 2 factorial arrangement with 3 factors: (1) provision of rearing milk or electrolytes before transport, (2) transport duration (6 or 18 h), and (3) type of vehicle (open truck or conditioned truck). The study included male Holstein-Friesian and cross-bred calves (N = 368; 18 ± 4 days; 45.3 ± 3.3 kg). Data on health status of calves were collected at the collection center and at the veal farm until week 27 post-transport. Behavior of calves was recorded during transport and at the veal farm until week 13 post-transport. Use of herd and individual medical treatments was recorded at the veal farm. The prevalence of loose or liquid manure at the veal farm from day 1 until week 3 post-transport was lower in electrolyte-fed calves transported in the conditioned truck compared to electrolytes-fed calves transported in the open truck or milk-fed calves transported in both the conditioned and open truck (Δ = 11% on average; P = 0.02). In comparison with the open truck, calves transported in the conditioned truck had lower prevalence of navel inflammation in the first 3 weeks post-transport (Δ = 3 %; P = 0.05). More milk-fed calves received individual antibiotic treatments compared to electrolyte-fed calves at the veal farm (P = 0.05). In conclusion, the transport-related factors examined in the present study affected health and behavior of calves in the short-term, but there was no evidence for long-term effects. It remains unknown why no long-term effects were found in this study. Perhaps this absence of transport-related effects was due to multiple use of medical treatments in the first weeks at the veal farm. Alternatively, it might be that the collective effects of the transition from the dairy farm to the veal farm, and of the husbandry conditions during the subsequent rearing period, on the adaptive capacity of calves were so large that effects of individual transport-related factors were overruled.

Keywords: transport, health, behavior, slaughter characteristics, medicine use, veal calves

Introduction

Calves at Dutch veal farms are usually collected from different dairy farms, including dairy farms from other EU-countries (especially Germany) (Hordijk et al., 2012). Collection procedures, which involve mixing of calves from multiple sources, transport to a collection center and subsequent transport to the veal farm result in stress and disease challenges (Mormede et al., 1982; Renaud et al., 2018c). Additionally, placement of calves into a new housing facility and their adaptation to a new feeding regime might also contribute to health problems (Renaud et al., 2018c). Transport normally occurs in the first weeks of the life of calves (14–20 days of age) when they are highly susceptible to microorganisms against which they have no colostral antibodies (Autio et al., 2007; Brscic et al., 2012). Poor condition of calves directly post-transport (calves with failure of passive transfer of immunity, dehydration and navel inflammation) is negatively linked to long-term performance of calves (Pempek et al., 2017; Marcato et al., 2018). A high dehydration score, sunken flanks, diarrhea and navel infection upon arrival at the veal farm are related to mortality in the first 21 days post-transport (Renaud et al., 2018b). Dehydration may result from feed and water withdrawal around transport and is associated with body weight losses. Severe body weight loss during transport (over 10%) increases the risk of lameness and mortality in calves (Gonzalez et al., 2012; Pempek et al., 2017). Transport is also related to incidence of respiratory diseases in calves after arrival at a feedlot (Sanderson et al., 2008). Overall, transport is a challenge for young veal calves, but it remains unknown which specific transport-related factors play a dominant role. Several transport-related factors have an effect on health (Renaud et al., 2018b) and behavior (e.g., standing vs. lying), thus influencing the recovery time of young calves during and in the immediate post-transport period (Jongman and Butler, 2014). In a previous study (Marcato et al., 2020a), we examined the effects of pre-transport diet (milk vs. electrolytes), transport duration (6 vs. 18 h), and type of vehicle (open truck vs. conditioned truck) on the physiological status of young veal calves at the beginning of the rearing period. The aim of the current study was to investigate the effects of these transport-related factors on health (including the use of medicines) during the entire rearing period, behavior and slaughter characteristics of calves at the veal farm. We hypothesized that feeding milk, transportation of calves for 6 h in a conditioned truck, and likely the interaction between these factors, might contribute to less health problems and behavioral signs of discomfort compared to the other treatments.

Materials and methods

Experimental overview

The experiment had a 2 × 2 × 2 factorial arrangement, including the following factors: (1) provision of rearing milk or electrolytes prior to transport; (2) transport duration (6 or 18 h); (3) type of vehicle (open truck or conditioned truck). The experiment included 368 bull Holstein Friesian and crossbred calves [18 ± 4 days; 45.3 ± 3.3 kg body weight (BW)], transported over two consecutive weeks (N = 184 calves/week). Calves were transported from a collection center in Bocholt-Barlo, Germany, where they stayed from 4.00 a.m. until 14.00 p.m., to a Dutch veal farm in Veghel. All animals followed current practices, including handling and mixing procedures at the collection center and transportation, and all calves were in compliance with the minimal weight and health requirements [BW > 36 kg; age: minimum 14 days; no signs of disease and injury (SBK, 2018)]. The experiment was approved by the Central Committee on Animal Experiments (the Hague, the Netherlands; Approval Number 2017.D-0029).

Handling of calves

At the collection center, calves were randomly allocated to one of the eight treatment groups by the manager. Calves were fed *via* a bucket with nipples, with 1.5 l of rearing milk (125 g of milk powder/l; per kg of milk powder: ME = 4028 kcal, CP = 190 g, crude fat = 157 g, digestible lysine = 18.7 g; made with plant-based ingredients; Tentofok KO, Tentego, The Netherlands) or a mixture of electrolytes (20 g of electrolytes/l of water; per 100 g of powder: Na = 7.3 g and moisture = 3.8 g; Navobi, Staverden, The Netherlands) dissolved in 1.5 l water.

After feeding, calves rested for ~2 h and thereafter they were loaded on the vehicle. The vehicle consisted of two parts: the truck was conditioned, which means it was provided with a side-ventilation system, it was isolated, and the climate was controlled regarding in and outlet of air (KVM Livestock Transport System™, Kleventa BV, Lichtenvoorde, The Netherlands). Settings were according to those provided by the manufacturer and applied by the transporter. The trailer was regular, open and lacked a ventilation system or climate control. Temperature and relative humidity in both vehicles are shown in Appendix 1. Both truck and trailer were divided into four compartments with straw bedding, two at the lower deck (3.60 m length × 2.45 m width × 1.35 m height) and two at the upper deck (3.60 m length × 2.45 m width × 1.45 m height). Each compartment contained 23 calves of one treatment group at the same stocking density (0.383 m^2 per calf). Treatments were distributed in the vehicle according to a design that allows for estimation of all main effects and relevant interactions [for details see Marcato et al.

(2020a)]. After loading, transport was conducted by two drivers, switching every 3 h. Neither food nor water was provided to calves during transport. After 6 h transport, the truck arrived at the veal farm and all calves were unloaded. Calves assigned to 6 h transport were placed in the veal farm, whereas the calves assigned to 18 h transport were reloaded on the truck and trailer (in the same compartments as before) and transported for another 12 h. Calves in the 6 and 18 h transport treatment groups were appropriately distributed across the truck and trailer and, therefore, located in both upper and lower decks [see Marcato et al. (2020a)]. Unloading calves located in an upper deck after 6 h of transport required that calves located in the lower deck had to be unloaded first; in some instances these latter animals belonged to the 18 h transport treatment group. In order to avoid unwanted confounding between transport duration and unloading and reloading of calves in part of the calves subjected to 18 h of transport, we decided to unload all calves after 6 h of transport and subsequently reload the animals in the 18 h transport treatment group, placing them in the appropriate compartment. At the veal farm, calves were distributed across 64 pens that were divided over 8 similar compartments. Each compartment included 8 pens, with 5 or 6 calves per pen. Treatments were randomly distributed across pens in every compartment. Calves were housed individually within each pen for the first 3 weeks post-transport. Subsequently, calves were kept in groups.

Calculation of the sample size

The number of experimental units required in the present study was based on a power analysis. Our experimental design was based on the principle that pen (or group) was the basic, independent experimental unit. We have extensive experience with multifactorial experiments with veal calves, and in one of the previous studies we used 16 pens per level of main effects, and 4 pens per treatment combination (Webb et al., 2013). Using this setup, we were able to detect differences between two treatment levels of about one unit standard deviation (SD) with a power of 0.80. However this latter experiment was performed on an experimental farm, under relatively standardized conditions, and using a specific and relatively standardized subset of calves. We anticipated that both the variation in conditions and between calves would be higher during the current experiment which took place under commercial conditions. A recent power analysis that we performed using a very large data set with carcass weights recorded both under experimental and commercial conditions (Engel et al., 2016)) supported this latter assumption, and suggested that under commercial conditions the SD could be 1.5 times higher than under more controlled experimental conditions. Power analysis showed that in order to maintain the same statistical power, the number of experimental units should be approximately doubled. Therefore, in the present experiment, we used 32 pens per level of main effects, and 8 pens per treatment combination.

Table 1. Effects of pre-transport diet, type of vehicle and transport duration on health variables of young veal calves assessed at day 1 after arrival at the veal farm.

	Pre-transport diet				Type of vehicle				Transport duration			
Parameter	Electrolytes	Milk	SE	*P*-value	Conditioned truck	Open truck	SE	*P*-value	6 h	18 h	SE	*P*-value
Navel inflammation	8.9	9.7	2.2	0.21	6.7	11.9	2.1	0.16	9.9	8.6	2.1	0.05
Eye discharge	7.1	6.3	2.1	0.39	5.0	8.4	2.0	0.49	6.1	7.3	2.1	0.76
Sunken eyes	38.3	40.9	3.2	0.63	35.7	43.5	3.2	0.08	36.5	42.8	3.2	0.31
Drooped ears	9.6	12.9	2.0	0.08	11.1	11.3	2.0	0.83	9.7	12.8	2.0	0.69

All parameters are expressed as an average proportion at pen level (N = 5 or 6 calves/pen).
Data are shown as raw means ± SE (Standard Error).

Table 2. Effects of pre-transport diet, type of vehicle and transport duration on health variables of young veal calves assessed from day 1 until week 3 after arrival at the veal farm.

	Pre-transport diet				Type of vehicle				Transport duration			
Parameter	Electrolytes	Milk	SE	*P*-value	Conditioned truck	Open truck	SE	*P*-value	6 h	18 h	SE	*P*-value
Navel inflammation	8.0	7.2	1.2	0.93	5.9	9.2	1.2	0.05	8.3	6.8	1.2	0.04
Joint problems	2.3	1.3	0.8	0.57	1.5	2.1	0.8	0.37	1.9	1.8	0.8	0.17
Loose or liquid manure	22.8	30.7	2.6	< 0.01	24.8	28.7	2.6	0.30	26.1	27.4	2.6	0.15
Eye discharge	7.1	6.2	1.1	0.63	6.5	6.9	1.1	0.75	6.7	6.6	1.1	0.80
Sunken eyes	39.0	40.3	2.5	0.52	38.6	40.7	2.5	0.70	39.8	39.5	2.5	0.71
Drooped ears	13.9	13.3	1.6	0.80	13.4	13.8	1.6	0.92	13.7	13.5	1.6	0.39

All parameters are expressed as an average proportion at pen level (N = 5 or 6 calves/pen).
Data are shown as raw means ± SE (Standard Error).

Health assessment

Health assessment of calves was performed at the collection center (during the resting period), and at the veal farm, on day 1, and in weeks 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, and 27 post-transport by two observers. Inter-observer reliability was tested before the experiment for both health and behavioral observations (k-coefficient = 98%). Two protocols were used for health assessment. The first one, shown in Appendix 2, was used at the collection center and at the veal farm from day 1 until week 3, when the calves were housed individually. The second protocol was according to the one used by Brscic et al. (2012) based on the Welfare Quality® Protocol on veal calves, and appropriate for use at pen level. This latter protocol was used to clinically score calves from week 5 until 27 post-transport (Appendix 3).

Processing of health data

Appendices 2, 3 show a complete list of health variables assessed at the collection center and at the veal farm, throughout the entire rearing period. Each health variable was first expressed at pen level as a percentage reflecting the number of calves displaying a health problem divided by the number of calves in the pen (Table 1, 2).

Table 3. **Severity of health problems at the collection center, on day 1 post-transport and in the first three weeks at the veal farm.**

	Place and time		
Health variables	Collection center: before transport	Veal farm: day 1	Veal farm: day 1 until week 3
Signs of pneumonia	2.7	0.3	2.3
Eye discharge	3.8	6.5	6.6
Nasal discharge	1.9	0.5	3.9
Loose or liquid manure	5.4	5.2	26.7
Navel inflammation	6.5	9.2	7.4
Sunken eyes	18.7	39.7	39.4
Joint problems	2.1	0.5	1.7
Drooped ears	5.4	11.1	13.5

Values represent proportions at batch level calculated as following:
(the number of calves displaying a health problem/ the total number of calves) × 100.

These percentages were averaged per treatment. Prior to statistical analyses, some health variables collected until week 3 post-transport were grouped as follows: (1) navel inflammation = navel with score 1 and 2; (2) loose or liquid manure = loose manure (with score 1) and liquid manure (with score 2); this category includes either infectious diarrhea or feeding-related loose or liquid manure, but it was not possible to make this distinction based on the visual clinical assessment.

To qualitatively compare health data recorded at the collection center with those recorded at the veal farm, a proportion was calculated as follows: (sum of calves displaying a health problem/total number of calves) × 100 (see Table 3 on previous page).

Behavioral observations

The first behavioral observations were conducted at the collection center during the resting hours after the application of the feeding treatment. Two observers conducted behavioral observations, using the scan sampling technique according to Martin et al. (1993). Behavior of calves was assessed every 5 min for 1 h according to an adapted version of the ethogram used by Webb et al. (2012) (Appendix 4). After the rest period, calves were loaded in the truck and trailer according to their respective treatments. Every compartment of both truck and trailer contained a camera that recorded standing and lying behavior throughout the 6 and 18 h of transport. Behavior was also assessed at the veal farm where cameras (N = 8, each positioned in every compartment of the stable) recorded standing vs. lying behavior during the first 24 h after arrival. In addition, two observers assessed behavior of calves by direct observations and using an instantaneous scan sampling technique at 5 min intervals for 1 h. These direct observations were done after arrival of calves, and in weeks 1, 3, 5, 9, and 13 post-transport (always after feeding). Behavioral variables shown in Appendix 4 were grouped into 3 main categories prior to statistical analyses: (1) comfort behavior = licking another calf, self-grooming, rubbing, chewing, eating, and drinking; (2) discomfort behavior = tongue playing, manipulating objects, manipulating another calf, urine drinking, and repetitive calling; (3) playing behavior = mount/leap/jump/back/turn, head-butt, running.

Use of medicines

Use of antibiotics and other medicines during the entire rearing period was recorded at the level of both herd and individual calf. Information on individual treatments included the following data: (1) whether the calf was treated or not with antibiotics or other medicines (this category included products such as anti-inflammatories, multivitamins, anti-coccidiosis, with the exclusion of antibiotics) during the rearing period; (2) single or repeated antibiotic/medical treatments during the rearing period; (3) age at which treatments were applied; (4) type of antibiotic or medication used. Herd treatments

(applied on all calves, *via* the milk) were also recorded, including the age at which they were applied and the type of medication used.

Slaughter characteristics

Slaughter characteristics were assessed per calf and included carcass weight (kg), color of the meat (scale 1–10 points, from pale to dark red color), fat coverage (scale 1–5 points, from low to very high fat coverage) and conformation class (scale 1–15 points, from excellent to poor carcass quality) (European Community, 2003).

Statistical analyses

All data were analyzed in SAS 9.4 (SAS Inst. Inc., Cary, NC). Health and behavioral data (expressed as a proportion of health problems or behaviors per pen) determined on day 1 and directly post-transport, respectively, were analyzed with a generalized linear mixed model with Pseudo Likelihood or equivalently Penalized Quasi Likelihood (PQL) (Breslow and Clayton, 1993), employing SAS procedure GLIMMIX. At this stage, calves were individually housed inside pens. The systematic part of the model comprised the following fixed effects:

$$\mu + Batch_i + Uplo_j + Bafr_k + Diet_l + Type_m + Duration_n + (Diet_l \times Duration_n) + (Diet_l \times Type_m) + (Duration_n \times Type_m) + (Diet_l \times Type_m \times Duration_n) \quad [1]$$

Here, μ is a base level and $Batch_i$ = batch (i = 1, 2), $Uplo_j$ = position in the vehicle (j = upper or lower deck), $Bafr_k$ = position in the vehicle (k = front or back), $Diet_l$ = pre-transport diet (l = rearing milk or electrolytes), $Type_m$ = type of vehicle (m = open or conditioned truck), and $Duration_n$ = transport duration (n = 6 or 18 h) are main effects. The model also comprised two- and three-way interactions between diet, type of vehicle and transport duration. Interactions were considered not significant when $P > 0.05$. In addition, random effects for pen and compartment at the veal farm were included in the linear predictor. The logit link function was used in concert with the variance function of the binomial distribution, which included a multiplicative dispersion factor that was estimated from the data. Here and in subsequent analyses, for all fixed effects, approximate F-tests were used (Kenward and Roger, 1997). Interactions that were not significant were excluded from the model (when higher order interactions were already excluded, i.e., respecting the hierarchy of interaction terms). Subsequent pairwise comparisons were done with Fisher's LSD method.

Health data and direct behavioral observations (expressed as proportion of health problems or behaviors per pen) assessed from the arrival of calves at the veal farm until week 3 post-transport were analyzed with a generalized linear mixed model (again

PQL and GLIMMIX). Until week 3 post-transport, calves were still individually housed inside pens. The systematic part of the model comprised the following fixed effects:

$$\mu + Batch_i + Uplo_j + Bafr_k + Diet_l + Type_m + Duration_n + Time_o + (Diet_l \times Duration_n) + (Diet_l \times Type_m) + (Duration_n \times Type_m) + (Diet_l \times Time_o) + (Duration_n \times Time_o) + (Type_m \times Time_o) + (Diet_l \times Type_m \times Duration_n) \quad [2]$$

in the same notation as before and additionally with $Time_o$ = sampling moment (o = T0 for behavior or day 1 for health, week 1 and 3) as main effect. Three-way interactions between diet, type of vehicle and transport duration, and two-way interactions between pre-transport diet, type of vehicle transport duration and time were also included in the model. Interactions were considered not significant when $P > 0.05$. The model comprised random compartment effects. For the repeated measurements on the same pen a first order auto regressive model (based on the actual distance between time points) was adopted.

Health data assessed from week 5 until 27 and direct behavioral observations assessed from week 5 until 13 were also analyzed with the generalized linear mixed model (Equation 2). During this period, calves were housed in groups instead of individually. Between week 5 and 27 post-transport, the presence of loose or liquid manure, as well as thick and white manure, were recorded as a binary response at pen level (i.e., present or not present). These variables were also analyzed with the generalized linear mixed model (Equation 2).

Data on individual treatments with antibiotics and other medicines during the entire rearing period were expressed as binary data (0 = calf not treated at individual level with antibiotics or medicines; 1 = calf treated at least once at individual level with antibiotics or medicines during the rearing period). These data were analyzed with a generalized linear mixed model (analysis with PQL and GLIMMIX) similar to model 1, but for binary data.

Continuous data on carcass weight at slaughter were analyzed with a linear mixed model (analysis with restricted maximum likelihood with SAS procedure PROC MIXED) with fixed and random effects as in Equation 1 and additional normally distributed error (or residual) terms. Residuals were checked for normality and homogeneity of variance and data were log transformed when deemed necessary.

Carcass weight was also analyzed in relation to the number of individual medical treatments. The number of individual medical treatments was introduced as a qualitative

factor in the model 1, comprising three main levels: 0 = calf not treated; 1 = calf treated once or twice; 2 = calf treated > 2 times.

In all analyses, effects with $P \leq 0.05$ were considered significant, whereas those with $0.05 < P < 0.10$ were considered as a tendency toward significance.

Results

The results of the present study will be shown in four main domains: health, behavior, use of medicines and slaughter characteristics. In each of these domains, effects of main factors, which included pre-transport diet, transport duration and type of vehicle, will be reported. Three-way and two-way interactions were never significant, with the exception of the interaction between pre-transport diet and type of vehicle on loose and liquid manure which is described in the first paragraph.

Health

The day post-transport, there were no significant effects of treatments on individual health parameters (Table 1). Drooped ears tended to be higher in milk-fed calves than in electrolytes-fed calves (Δ = 3.3%; P = 0.08) and sunken eyes tended to be higher in calves transported in the conditioned truck than in calves transported in the open truck (Δ = 7.8%; P = 0.08).

For the average prevalence of loose or liquid manure from day 1 until week 3 post--transport there was an interaction between pre-transport diet and type of vehicle. The percentage of calves with loose or liquid manure was lower (18%) in electrolytes-fed calves transported in the conditioned truck compared to electrolytes-fed calves transported in the open truck (28%) and milk-fed calves transported in both the conditioned and open truck (31% on average; P = 0.02). The percentage of calves with navel inflammation was higher in calves transported in the open truck and calves transported for 6 h compared to calves transported in the conditioned truck and calves transported for 18 h (Δ = 3.3% and Δ = 1.5%, respectively; $P \leq 0.05$; Table 2).

Prevalences of navel inflammation and loose or liquid manure changed significantly in the first 3 weeks post-transport ($P < 0.01$). Navel inflammation decreased from day 1 (9%) until week 3 (4%), whereas loose or liquid manure gradually increased in this period (from 5% on day 1 to 39% in week 3). In addition to the effects of time on health problems in the first 3 weeks post-transport, Table 3 shows the trend of health problems from the collection center until week 3 post-transport. Overall, the prevalence of the majority of health problems gradually increased in the period between the collection center and

week 3 post-transport. Prevalences of loose or liquid manure and sunken eyes in the first 3 weeks post-transport more than doubled compared to the same prevalences at the collection center (Δ = 22% and Δ = 20%, respectively).

Overall, prevalences of health problems from week 5 until 27 were relatively low (<10%), with the exception of coughing (12%), and there were no significant differences due to transport factors. As shown in Figure 1A, the prevalence of coughing changed significantly in this period ($P < 0.01$) and was highest between week 15 and 21 post-transport (15%). Besides coughing, abnormal breathing and nasal discharge, the other two clinical signs of respiratory disease, were below 5%. Raw means for all three signs of respiratory disease beyond week 5 at the veal farm are also shown in Table 4. Figure 1B shows the prevalence of gastrointestinal problems at the veal farm. Thick manure was present only from week 5 until 13 (average prevalence 11%), whereas it disappeared in the remaining part of the fattening period. The average prevalence of loose or liquid manure from week 5 until 13 was 10%, decreased slightly (Δ = −3%) from week 15 until 21, and increased again (Δ = 5%) from week 23 until 27. White manure substantially increased during the entire rearing period (from 5 to 21%)

Behavior

During transport (61 vs. 39%) and directly post-transport (77 vs. 23%), calves spent most of the time lying compared to standing, but no significant differences were found between treatment groups. On the day post-transport, calves transported for 18 h showed more signs of discomfort compared to calves transported for 6 h (9 vs. 6%; $P < 0.01$). Additionally, calves transported in the conditioned truck showed more signs of discomfort behavior compared to calves transported in the open truck (9 vs. 5%; $P = 0.01$).

Table 4. **Raw means recorded for coughing, abnormal breathing and nasal discharge in veal calves between week 5 and 27 post-transport.**

	Weeks post-transport				
Health variable	Week 5	Week 7	Week 9	Week 11	Week 13
Coughing	6.1 ± 1.2	5.3 ± 1.2	9.8 ± 1.5	7.7 ± 1.3	13.7 ± 1.5
Abnormal breathing	2.6 ± 0.8	2.7 ± 1.0	5.4 ± 1.1	0.3 ± 0.3	1.1 ± 0.6
Nasal discharge	2.6 ± 1.0	0.8 ± 0.6	1.3 ± 0.6	0.5 ± 0.4	2.7 ± 0.8

All health variables are expressed as an average proportion at pen level raw means ± SE (Standard Error).

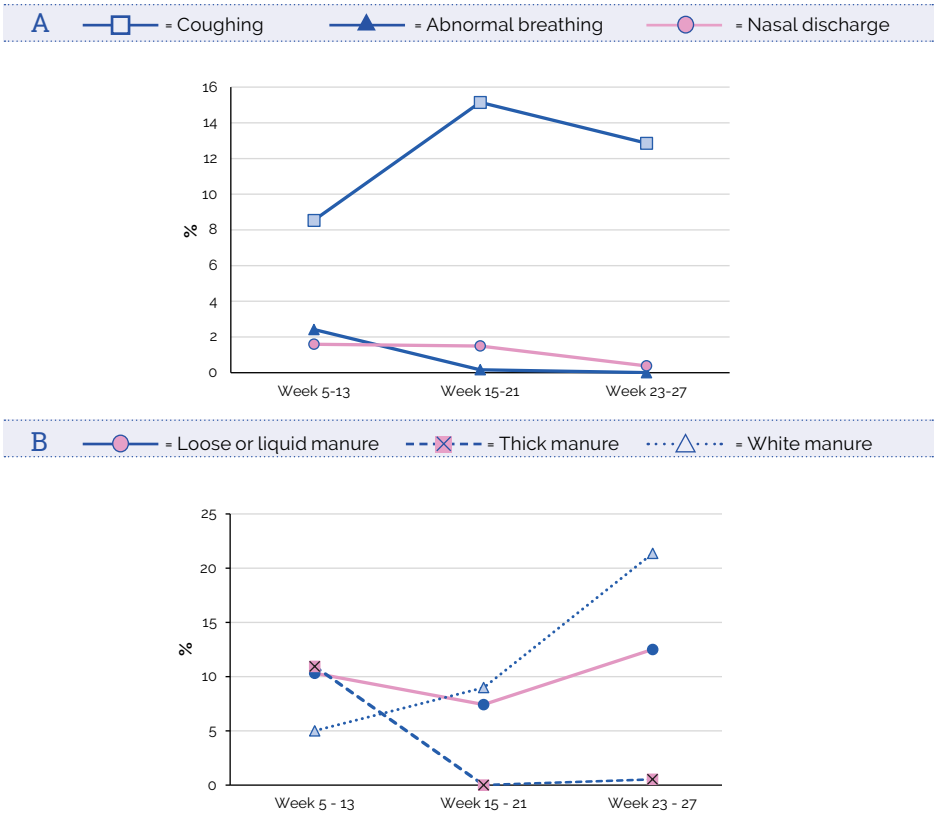

Figure 1 A,B: Prevalence of coughing, abnormal breathing and nasal discharge in veal calves between week 5 and 27 post-transport (expressed at average proportions at pen level) (A) and prevalence of loose or liquid manure, thick manure and white manure in veal calves between week 5 and 27 post-transport (expressed as average proportions of pens) (B)

	Weeks post-transport						
	Week 15	Week 17	Week 19	Week 21	Week 23	Week 25	Week 27
	16.3 ± 1.6	15.1 ± 2.1	15.8 ± 1.8	13.4 ± 1.6	19.1 ± 1.9	10.1 ± 1.7	9.3 ± 1.5
	0.4 ± 0.4	0.3 ± 0.0	0	0	0	0	0
	4.6 ± 1.3	1.1 ± 0.7	0	0.3 ± 0.3	1.1 ± 0.6	0	0

During the first 3 weeks post-transport, calves increased their time in a standing position (from 23% on day 1 to 51% in week 3 post-transport), and calves showed a gradual increase in comfort behavior (from 5% on day 1 to 15% in week 3 post-transport) and a decrease in discomfort behavior within this time frame (from 7% on day 1 to 4% in week 3 post-transport) ($P < 0.01$). In the period between week 5 and 13 post-transport, comfort behavior gradually increased (from 30 to 53%) ($P < 0.01$). Play behavior increased up to a 4% in week 9 and subsequently, it decreased to 1% in week 13 post-transport ($P < 0.01$).

Use of medicines

The percentage of calves individually treated with antibiotics at least once during the rearing period at the veal farm was 33%. Among this fraction of calves, 70% of animals were treated once, 21% were treated twice and 9% were treated more than twice during the rearing period. More milk-fed calves received individual antibiotic treatments compared to electrolyte-fed calves throughout the rearing period (38 vs. 28%, respectively; $P = 0.05$). The percentage of calves that received at least one other medical treatment during the rearing period was 18%. Among this fraction of calves, 69% of animals were treated once, 23% were treated twice and 8% were treated more than twice. No significant differences were found between treatment groups on the use of other medical treatments. In the first 6 weeks at the veal farm, 25% of calves were individually treated with antibiotics and 22% of calves were treated with other medicines. In the following 6 weeks, calves were still individually treated for antibiotics (23%) and for other medicines (4%), but from week 13 until 27 calves were not treated at all, neither individually nor batch-wise. Besides individual treatments, calves were subjected to 5 herd treatments (on day 3, 13, 22, 37, and 47) with oxytetracycline HCl (1.43 g/100 kg/twice a day), doxycycline (1 g/100 kg/day), Tilmovet 250 mg/ml (5.45 ml/100 kg/twice a day), Ampisol 100% (2.26 g/100 kg/day), and doxycycline (0.58 g/100 kg), respectively. These herd treatments were provided *via* the milk for an average of 11 feedings per herd treatment.

Slaughter characteristics

No significant differences were found between treatment groups in relation to carcass weight (164.7 kg ± 18.4; range: 96–215 kg), conformation class (11.9 points ± 1.0; range: 8–15) and color of the meat (5.9 points ± 1.3; range: 2–10) at slaughter. Figure 2 shows a significantly lower carcass weight of calves receiving > 2 individual medical treatments compared to carcass weight of calves not treated or treated once or twice ($P < 0.01$). Figure 3 shows that the color of the meat of calves receiving > 2 individual medical treatments tended to be darker than the meat of calves not treated or treated once or twice ($P = 0.06$).

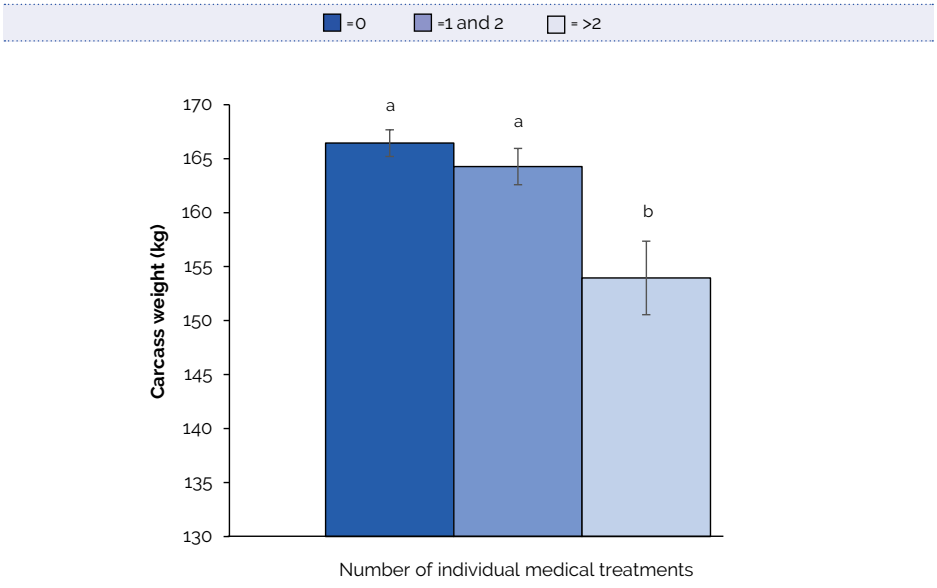

Figure 2. Effects of number of individual medical treatments of veal calves throughout the rearing period on carcass weight at slaughter.

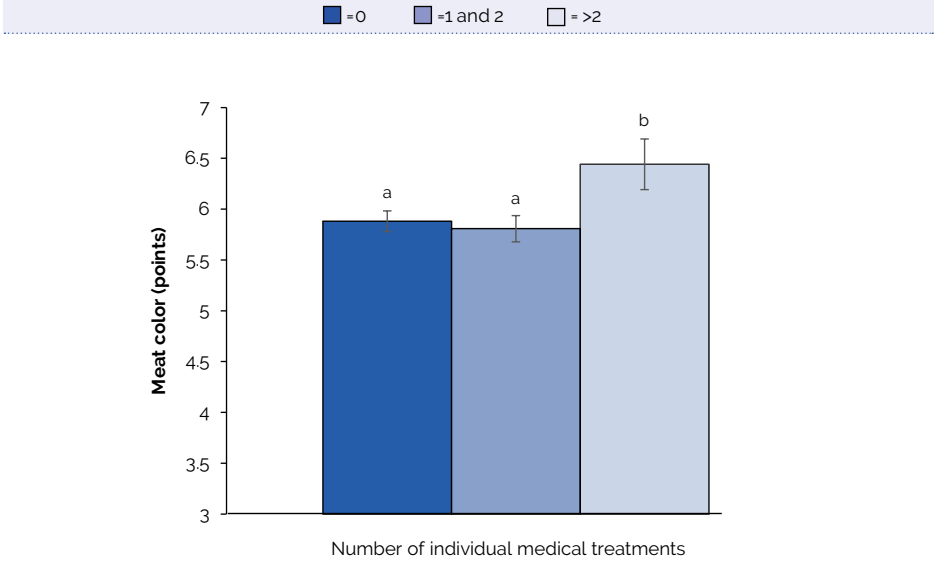

Figure 3. Effects of number of individual medical treatments of veal calves throughout the rearing period on meat color at slaughter.

Discussion

Health and use of medicines

In the current study, health problems in young veal calves increased in the post-transport period compared to pre-transport values at the collection center. Thus, transport (including mixing and handling procedures and feed withdrawal) and the adaptation of calves to the new housing and feeding system at the veal farm were challenges for calves. Health outcomes measured at the veal farm were expressed at pen level, although calves were individually housed in the first 3 weeks post-transport. We used this approach because individually housed calves were not randomly distributed across the barn, but housed pen-wise, thus each pen contained 5 or 6 calves in adjacent baby boxes. Most of the effects of transport-related factors were evident in the first 3 weeks after arrival at the veal farm; this is also the period in which most of the medical treatments were applied. The day post-transport, the prevalence of sunken eyes, which is a clinical characteristic related to dehydration, was lower than the prevalence rate shown by Wilson et al. (2000) (40 vs. 61%, respectively). Dehydration is associated with different factors, including transport and diarrhea (Knowles et al., 1997; Renaud et al., 2018a). In a previous study (Marcato et al., 2020a) we reported that up to 70% of the calves used in the current experiment were dehydrated (based on skin elasticity) already before transport. This explains why application of the treatments resulted in a large number of calves with sunken eyes upon arrival.

Pre-transport diet fed at the collection center had an impact on loose or liquid manure in the first 3 weeks post-transport, where milk-fed calves showed more loose or liquid manure than electrolyte-fed calves. Feeding milk prior to transport is a good remedy against energy depletion or hypoglycemia (Schaefer et al., 1997), which was also visible on most of energy related parameters measured in blood of calves in this experiment (Marcato et al., 2020a). However, time feeding milk may also contribute to alterations in fecal consistency related to transport stress and consequently intestinal atrophy, whereas feeding electrolytes is a good approach to treat calves displaying metabolic acidosis and diarrhea (Booth and Naylor, 1987; Schaefer et al., 1997). The different composition of the pre-transport diet might also explain the higher use of antibiotics in milk-fed calves compared to electrolyte-fed calves in the present experiment. Feeding more nutrients, especially before a challenge, such as transport, may increase fecal abnormalities in pre-weaned calves in the first weeks of life (Brown et al., 2005) and it may result in higher antibiotic use (Quigley et al., 2006). However, since we were not able to make the distinction between infectious or feed-related diarrhea during our clinical assessment, we cannot rule out the possibility that some of the antibiotic treatments in the current study were applied without a proper clinical justification. This may have

affected the difference in antibiotic treatments between milk-fed and electrolyte-fed calves. The significant interaction between pre-transport diet and type of vehicle means that the prevalence of loose or liquid manure was lowest in electrolyte-fed calves transported in the conditioned truck in comparison with electrolyte-fed calves transported in the open truck, and milk-fed calves transported in both the conditioned and open truck. Apparently, in combination with the pre-transport diet, the environment in the conditioned truck exerted some kind of protective effects on the likelihood of calves exhibiting loose or liquid manure during the first 3 weeks of the rearing period. As indicated above, however, we do not know whether this decrease concerns loose or liquid manure with an infectious or non-infectious origin.

In comparison with the open truck, transporting calves in the conditioned truck also reduced the prevalence of navel inflammation in the first weeks post-transport. At present, it remains unknown which environmental factors comprising the conditioned transport in our experiment (such as draft or differences between in and outlet airflow) contributed to these effects on calf health at the beginning of the rearing period; these environmental and climatic factors need to be defined and recorded in more detail in future research. In a recent study by Renaud et al. (2018b), involving close to 5,000 calves, navel inflammation at arrival was associated with early mortality at the veal farm (≤ 21 days post-transport). Therefore, the significant lower navel inflammation in calves transported in the conditioned truck found in the current experiment might be relevant at population level for reducing mortality at the veal farm, provided that conditioned transport would be used at a large scale.

In the first 3 weeks post-transport, navel inflammation decreased significantly and this is in line with Wilson et al. (2000), although the starting prevalence rate in our study was lower (9 vs. 32%, respectively at the first sampling moment). Navel inflammation can be caused by environmental conditions during transport (e.g., lack of bedding on the truck, overcrowding) or by farm management practices before transport to the collection center (e.g., poor hygiene, lack of navel antisepsis) (Mee, 2008; Pempek et al., 2017). In the current experiment, it is likely that navel inflammation was already present at the dairy farm, because all calves were transported on a straw bedding and transport density was not high. Therefore, preventive measures at the dairy farms (high hygiene status, early intake of high-quality colostrum, navel dipping) are necessary to avoid this condition in the veal farm (Pempek et al., 2017). Loose or liquid manure increased over time in the first 3 weeks at the veal farm. The prevalence of loose or liquid manure at day 1 post-transport was lower than reported by Wilson et al. (2000) (5 vs. 16%), but higher in week 3 post-transport (39 vs. 17%). It appeared that, besides transport, calves struggled to adapt to a new feeding regime (based on milk replacer diets)

at the veal farm. In addition to gastrointestinal problems, clinical signs of respiratory disease, gradually increased at the veal farm. Respiratory disease in white veal calves is often of a slow progressive nature, and likely due to presence of maternal immunity and frequently applied metaphylactic antimicrobial therapy (Pardon et al., 2011). The starting prevalence of respiratory disease indicators was in line with the prevalence of bovine respiratory disease (BRD) shown by Pardon et al. (Pardon et al., 2015) in the first 17 days post-transport at the veal farm (<5%). However, in Pardon et al. (2015), 40% of calves showed signs of BRD at day 18 post-transport, a prevalence much higher compared to the 4% in the current experiment. However, prevalences obtained in different studies may be difficult to compare, because of differences in health protocols: the current experiment separately considered nasal discharge, coughing or abnormal breathing as clinical signs of respiratory disease, whereas Pardon et al. (2015) defined BRD cases based on the simultaneous presence of depression, cough, higher rectal temperature and nasal discharge. Pardon et al. (2012b) reported peak prevalences of respiratory disease in veal calves between 2 and 6 weeks post-transport. In the current study, the highest prevalence of coughing occurred at a later stage (between week 15 and 21 post--transport) and this might be due to a reinfection of calves with respiratory pathogens after the first weeks post-transport (Pardon et al., 2011). Next to respiratory disease, the prevalence of white manure and loose or liquid manure in the last weeks of the rearing period indicates that calves might struggle to adapt to the feeding scheme at the veal farm. Besides these health problems, there were no significant differences between the experimental treatments in prevalence of other health problems from week 5 until 27.

The current findings showed that health of calves destined to veal production can be already compromised at the collection center (as indicated by high prevalence of dehydration, sunken eyes and navel inflammation). Thus, in addition to transport-related factors such as examined in the present experiment, further attention on factors and (e.g., early rearing) conditions experienced by veal calves prior to arrival at a collection center is merited. The existence of relatively mild effects of transport factors on health problems in the immediate post-transport period, and the absence of significant effects in the longer term might be due to several reasons. First, it could be suggested that the transport and arrival at the veal farm as applied in the current experiment did not represent a severe enough challenge to significantly disturb the homeostasis of calves. However, this is highly unlikely given the profound overall effects of the experimental treatments on, for example, the physiological status (Marcato et al., 2020a) and a number of aspects of the clinical health of our calves (Table 3). Secondly, the collective effects of the transition of calves from the dairy to the veal farm (including transport and mixing with other calves at the collection center), and of the husbandry conditions during the subsequent rearing period (including dietary changes, and, again,

mixing with other calves) might be so large that they overrule potential effects of individual transport factors as examined in the present experiment on health and adaptive capacity of calves. Thirdly, the high use of antimicrobials and medical treatments both at herd and individual calf level in the first 6 weeks of the rearing period may have masked potential effects of the transport-related factors on the health status of calves in the current experiment.

Behavior

Behavior of calves is influenced by transport (Grigor et al., 2001; Jongman and Butler, 2014). In the current study, calves spent more time lying than standing during transport, which was similar to other studies. Eicher and Morrow (2000) showed that calves had a preference for lying (70% of the trip duration). Knowles et al. (1997) reported that young calves (<1 month old) spent ~80% of their time lying down during 24 h transport duration. Overall, young calves prefer to lie more during transport compared to adult cattle (Eicher, 2001), thus space requirements should account for these preferences. Calves not only showed more lying behavior during transport, but also directly post-transport and up to 24 h post-transport calves spent most of their time lying than standing. This suggests that transported calves might have experienced stress coupled with fatigue after the journey (Grigor et al., 2001). Standing behavior almost doubled a week post--transport, suggesting that calves were beginning to recover from the journey. Calves mainly showed signs of discomfort the day and the week after transport, suggesting that transport caused a disturbance in their homeostasis and calves were able to cope with this challenge toward the end of this period. On the day post-transport, the highest prevalences of discomfort behavior were shown by calves transported in the conditioned truck and by calves transported for 18 h. Apparently, and intuitively logically, long-term transport (18 h) was more challenging to calves than short-term transport (6 h). The fact that calves transported in the conditioned truck exhibited more discomfort behavior in comparison with animals transported in the open truck warrants specific attention. This finding would suggest that transporting calves in a conditioned truck may be favorable for some health characteristics (such as naval inflammation, see above), but unfavorable in terms of behavioral signs of discomfort on the day post-transport. Again, this underlines the need for further research on conditioned transport, and its effect on calf health and behavior. Beyond the first week post-transport, discomfort behavior declined and the gradual increase in comfort behavior might be an indication that calves were adapting to the new environment. Playing behavior significantly increased until week 9; beyond this age the prevalence of this behavior remained relatively low. These changes might be age-related, but may also have been affected by the reduction in space availability in the pen (Ruschen and Passillé, 2014). Transport-related factors did not significantly affect veal calf behavior from week 5 until 13; thus,

similar to health, the various transport-related factors examined in the present study seemed to exert significant effects on behavior in the short-term only.

Slaughter characteristics

In the current study, transport-related factors had no significant effect on either carcass weight, meat color, or conformation class. Notably, carcass weight was negatively related to the number of individual medical treatments. These results are in line with Pardon et al. (2013) who demonstrated that antimicrobial drug use (ADU) was negatively associated with hot carcass weight of veal calves. Every increase in ADU by 1% was associated with 1.5 kg loss in hot carcass weight. Pardon et al. (2013) also showed that carcass weight decreased severely with an increasing number of episodes of bovine respiratory disease and diarrhea. Moreover, Pardon et al. (2013) showed that the odds for undesirable red meat color were lower with an increase in ADU (OR = 0.86 per percentage increase in ADU; 0.95-CI: 0.76–0.98; $P < 0.05$). This was in contrast with the results of the current experiment that revealed a tendency to darker meat color in calves treated >2 times with medicines. It can be hypothesized that calves which received more than two medical treatments were the ones that were more sick and lagging in condition.

Conclusive remarks

The current study shows that pre-transport diet and type of vehicle affected health and behavior of veal calves in the short-term, but had no effects in the long run, including on slaughter characteristics. Perhaps transport-related effects were masked due to multiple use of medical treatments in the first weeks after arrival at the veal farm. Additionally, it might be assumed that the collective effects of the transition from the dairy farm to the veal farm, and of the husbandry conditions during the subsequent rearing period, on the adaptive capacity of calves were so large that the effects of individual transport-related factors were overruled. Despite the lack of treatment effects, the high prevalence of health problems merits more research on strategies to improve health of calves at the veal farm. Further studies are needed on ways to increase the resilience of veal calves during the transition from the dairy farm to the veal farm. These studies should also address transport-related factors in combination with (innovative) husbandry strategies both at the dairy farm and at the veal farm. Correspondingly, there is a need to define and record the (required and appropriate) environmental and climatic conditions and factors during conditioned transport of young calves, and to further study their relationship with calf health and welfare. ■

Acknowledgments

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Appendix

Appendix 1

Table A1 Mean and range (between brackets) of actual temperature and relative humidity inside the conditioned and open trucks during short[1] or long[2] transport of young calves to the veal farm.

	Conditioned truck				Open truck			
	Batch 1		Batch 2		Batch 1		Batch 2	
	T (°C)[3]	RH (%)[4]	T (°C)	RH (%)	T (°C)	RH (%)	T (°C)	RH (%)
6 h	9.2 (8.2 – 10.3)	66.0 (61.0 – 75.1)	13.0 (11.7 – 13.9)	74.1 (65.0 – 81.0)	7.4 (6.2 – 9.1)	74.1 (66.3 – 84.2)	11.5 (10.4 – 12.5)	80.3 (67.6 – 88.8)
18 h	7.8 (4.5 – 11.2)	68.2 (58.5 – 78.9)	13.6 (11.2 – 16.3)	77.9 (65.4 – 83.9)	6.6 (3.9 – 9.6)	75.8 (66.3 – 86.5)	14.0 (10.8 – 16.6)	77.3 (66.2 – 86.2)

[1] *6 h;* [2] *18 h;* [3] *T = temperature;* [4] *RH = relative humidity.*

Appendix 2

Table A2 Health parameters of veal calves at the collection center and at the veal farm (from day 1 until week 3 post-transport). Health parameters were assessed at individual level.

Health parameter	Score	Explanation
Navel inflammation	0	No signs of inflammation
	1	Swollen, without discharge
	2	Swollen, with discharge
Joint inflammation	0	No evidence of joint problems
	1	Evidence of joint inflammation
Loose or liquid manure	0	No loose or liquid manure
	1	Pasty manure
	2	Watery manure
Pneumonia	0	No abnormal breathing, no cough
	1	Abnormal breathing and cough
Eye discharge	0	No eye discharge
	1	Slight watery discharge
Sunken eyes	0	Normal, bright eyes
	1	Eyes markedly recessed into the orbits
Ears	0	Normal
	1	Loop-eared
Nasal discharge	0	No discharge
	1	Watery discharge
	2	Purulent discharge

Appendix 3

Table A3 Health parameters of veal calves at pen level from week 5 until week 27 post-transport.

Health parameters	Explanation	Method of assessment
Milk leftovers	Untouched rests of milk in feeding trough	Yes / No
Roughage leftovers	Untouched rests of roughage in feeding trough	Yes / No
Abnormal breathing	Fast breathing (> 40 breaths/min), excessive abdominal breathing	number of calves
Nose discharge	Presence of discharge from one or both nostrils	number of calves
Coughing	Audible expulsion of air through mouth of calves	number of calves
Loose or liquid manure	Presence of loose or liquid manure in the pen	Yes / No
Thick manure	Thicker and higher consistency manure, often combined with undigested food	Yes / No
White manure	Sticky and higher consistency manure. The colour is white or grey	Yes / No
Bloated calves	Calves with overfilled/bloated belly (upper, lower, right, left and all around)	number of calves
Lame calves	Calves with a different load/ or do not stand on one or more legs	number of calves
Claw problems	Red and swollen skin around the claw, often combined with lameness	number of calves
Joint problems	Clear thickening of one or more joints caused by accumulation of fluids/ synovia. Often painful and combined with lameness	number of calves
Bursa problems	Clear thickening (disc or round shaped) of the joint. Usually not painful and calves are not lame	number of calves
Chewing wounds	Wounds (damaged tail/ ear or skin on the body) caused by other calves in the pen.	number of calves
Skin infection	Skin damage due to infection: presence of round, hairless spots and wrinkled skin	number of calves
Hard skin	Thickened skin (often wrinkled and hairless), especially on the withers	number of calves
Urine suckling	Calves that suckle urines from other calves as well as calves being suckled	number of calves
Condition 15-30	Calves that are 15-30% behind condition (based on weight and size of calves) compared to the other calves in the herd	number of calves
Condition >30	Calves that are >30% behind condition (based on weight and size of calves) compared to the other calves in the herd	number of calves
Wet fur	Calves with a wet fur all along the back line	number of calves
Dull fur	Dull fur with abnormal structure, gloss & length	number of calves
Sick calves	Calves (not scored earlier) that give a general sick impression, depressed calves/ not attentive	number of calves

Appendix 4

Table A4 **Ethogram used to assess behavior of veal calves during transport and at the veal farm.**

Behavior		Description
Posture	Stand	Body elevated from floor and weight supports by legs.
	Lie	Brisket in contact with the floor.
Activity	Tongue play	Tongue playing/rolling. Turning, rolling and unrolling of tongue extended outside or inside mouth.
	Urine drink	Preputial sucking of pen mates ore drinking of urine flow
	Chew	(sham) Chewing/ruminating. Any repetitive movements of lower jaw in lateral plane.
	Manipulate objects	Oral manipulation of the pen structure and bucket/trough.
	Lick another calf	Tongue extending and shifted across any body part of pen mate or nibbling of pen mate.
	Manipulate another calf	Sucking any body parts (ears, tail, face, body) of pen mate excluding prepuce)
	Graze	Grazing pen mate hair situated on back. Mouth nibbles and pulls away from back, sometimes coming away with hair.
	Self-groom	Tongue extending and shifted across any body part of self or nibbling of self.
	Rub	Rub any part of body against substrate.
	Leap/Jump/ Buck/Turn	Forelegs lifted from ground, forepart of body elevated, with or without forward movement, kicking and turning (frolic behavior)
	Mount	Mount other calf from any side.
	Head-butt	Butting of substrate or another calf.
	Head-shake	Head shaken or rotated
	Run	Rapid movement forward with all four legs leaving the floor at one point in time.
	Sniff	Sniff at surroundings including calves.
	Repetitive calling	Repetitive calling with apparent no reason
	Walking	Walk around the pen
	Eat straw	Eating straw in the pen
	Drink water	Drink water from the bucket (from week 3 at the veal farm)
	Other	Any other activity not mentioned above.

CHAPTER 6

Transport age, calf and cow characteristics

affect immunoglobulin titers and hematological parameters of veal calves

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Abstract

The aim of this study was to investigate longitudinal effects of the age of calves when transported from the dairy farm of origin (14 vs 28 d), calf characteristics (sex and breed), and dam characteristics (parity and ease of birth) on immunoglobulin titers and hematological variables of veal calves. Calves (N = 684) that were born over a 9-month period on 13 dairy farms in the Netherlands were transported to 8 veal farms at 14 or 28 d of age. Natural antibodies N-IgG, N-IgM and N-IgA (collectively indicated as N-Ig's) specific for phosphorylcholine conjugated to bovine serum albumin (PC-BSA) were measured in serum of calves in the first week after birth, one day prior to transport and in weeks 2 and 10 post-transport at the veal farm. Maternal N-Ig's were measured in serum of the dams one week prior to calving and in first colostrum just after birth. Hematological variables were assessed in plasma of calves one day prior to transport and in week 2 post-transport. In addition to main effects, relationships between, on the one hand, N-Ig's and hematological variables, and on the other hand, individual treatments with antibiotics and other medicines at the veal farm and carcass weights were examined. One day prior to transport, titers of N-IgG, N-IgM and N-IgA were higher (Δ = 0.75, Δ = 0.71, Δ = 0.62, respectively) in 14 d old calves compared to 28 d old calves. In week 2 at the veal farm, calves transported at 14 d had higher N-IgG titers (Δ = 0.27), but lower N-IgM (Δ = - 0.65) and N-IgA (Δ = - 0.35) titers compared to calves transported at 28 d. In week 1 and one day prior to transport, all three N-Ig isotypes of calves were positively related to N-Ig's in colostrum. At the veal farm, N-IgG of calves was positively related to N-IgG in colostrum. High N-IgG titers in serum of calves were associated with less individual treatments with antibiotics and other medicines at the veal farm. Colostrum obtained from first parity cows had lower N-Ig's titers compared to colostrum of higher parity cows. It can be concluded that transportation of calves at both 14 and 28 d of age still occurred in the immune gap period, but that calves transported at a later age showed a more advanced development of their adaptive immunity than calves transported at 14 d of age. The present findings suggest that quality of colostrum might have long-term consequences for the immunity of veal calves and that N-IgG titers in calf serum might be used as potential biomarker of future performance of veal calves.

Keywords: veal calf; transport age; immunoglobulin; hematology

Introduction

In the Netherlands, male (and surplus female) calves born on dairy farms are typically transported at the legally required minimum age of 14 days, first to a collection center and then to a veal farm, to be raised for meat production (Marcato et al., 2018). Since transport represents a critical event, many studies focused on the effects of transport on health and recovery rate of calves upon arrival (Knowles et al., 1999b; Masmeijer et al., 2019; Marcato et al., 2020a, b) and during the rearing period at the veal farm (Marcato et al., 2020c). Recent observational studies have found associations between the background and early rearing practices on the dairy farm of origin and mortality rate of male calves at the veal farm (Winder et al., 2016; Renaud et al., 2018b). Correspondingly, it has been demonstrated that body weight (Brscic et al., 2012; Scott et al., 2019) or the clinical health condition of calves upon arrival at the veal farm (Renaud et al., 2018a; Scott et al., 2019), as well as certain biomarkers analyzed in a blood sample taken at arrival, including, for example, immunoglobulins (Pardon et al., 2015; Goetz et al., 2021), total protein (Scott et al, 2019; von Konigslow et al., 2020), cholesterol (Renaud et al., 2018a), or specific immune cell counts (von Konigslow et al., 2020), were all significantly correlated with later risks of disease and mortality. Collectively, these studies suggest that both transport and husbandry characteristics of the dairy farm of origin may be important determinants of the biological state of a calf when it arrives at the veal farm. This biological state, in turn, may predispose the animal to disease, poor performance or premature death during the subsequent fattening period. This process could also be described in terms of robustness of calves. Robustness is defined as the ability of an animal to cope with environmental challenges and to bounce back rapidly after challenges occur (Colditz and Hine, 2016). Robust animals are well equipped to cope with endemic infections and to fight diseases and, hence, may have a lower need for antimicrobials. Robustness can be measured in different ways, for example through physiological indicators in the blood (similar to the biomarkers recorded in the observational studies mentioned above) or by the assessment of the health status or mortality rate, which could be considered the ultimate indicators of animal robustness (Marcato et al., 2018; de Almeida et al., 2019). So far, identifying which specific environmental or animal-based factors may play a causal role in influencing robustness of veal calves has hardly been studied.

Transporting a calf from the dairy farm to the veal farm at 14 days of age might be convenient for dairy farmers, because it minimizes the rearing costs of calves on dairy farms. However, at this age, immune components of calves are not completely functional because calves are in the so- called "immune gap period" due to the combination of a decreased passive immunity and the absence of a mature adaptive immune system

(Chase et al., 2008). Transporting calves two weeks later than current practices may allow the adaptive immunity of calves to further develop (Chase et al., 2008), and, thus, calves might be more robust upon arrival at the veal farm. Therefore, the first objective of the current study was to systematically investigate, in a longitudinal fashion, the impact of two different transport ages (14 days vs. 28 days) on potential biomarkers of calf robustness, including natural antibody titers specific for phosphorylcholine conjugated to bovine serum albumin (PC-BSA), and the hematological profile of calves with various cell counts. Phosphorylcholine is an abundant environmental antigen, present on bacterial membranes, fungi and parasites (Pinkert et al., 1989). Pinkert et al. (1989) reported that immune responsiveness to PC is a useful model for pathogen recognition. In the current study, the combination of PC with BSA allowed also to detect natural autoantibodies, because BSA can be considered as a self-antigen for both cows and calves. Since both natural antibodies and natural autoantibodies will be described in the current paper, we will use the term N-Ig's (N-IgG, N-IgM and N-IgA) to indicate both types of antibodies detected against PC-BSA. The second objective of the current research was to investigate the effects of calf characteristics (such as birth weight, sex, breed) and cow characteristics (parity, ease of birth, dry period length (DPL), colostrum quality, milk yield) on these same biomarkers. These calf and cow characteristics are thought to be critical for the development and future performance of calves (Godden et al., 2005; Roland et al., 2016). Finally, we aimed to examine some relationships between our putative early biomarkers of calf robustness and individual treatments with antibiotics and other medicines at the veal farm as well as carcass weight. The latter are assumed to reflect the capacity of the calves to successfully adapt to the conditions at the veal farm. Therefore, all calves were individually followed prospectively from birth at the dairy farm until the end of the fattening period at the veal farm. Findings from this experiment on clinical health and growth performance of calves are reported in a companion paper (Marcato et al., 2021b).

Materials and methods

Experimental design

The experiment was executed between March 2019 and May 2020 and was approved by the Central Committee on Animal Experiments (the Hague, the Netherlands; approval number 2017.D-0029). The experimental design was a matrix, consisting of 13 dairy farms and 8 veal farms. Calves (N= 684) originated from 13 Dutch dairy farms. Within each farm, calves were assigned to one of two treatment groups, meaning that they were transported to a veal farm at either 14 or 28 days of age.

Calves born in the first two weeks from the start of the experiment left the dairy farm at 28 days of age, and calves born in the subsequent two weeks left the dairy farm at 14 days of age. Subsequently, all calves born at all farms within this 4-week timeframe were transported to the same veal farm (see Figure 1). At each transport day, two transporters collected calves from the dairy farms (6 and 7 dairy farms, respectively) and brought these directly to the veal farm, meaning that for each veal farm in total 4 transports were performed. The timeframe shown in Figure 1 was repeated 8 times, meaning that calves born in each timeframe were transported to a different veal farm. The recruitment of calves into the experiment stopped when the last veal farm was filled with calves. At the veal farms (herd size = 1,065 calves on average), calves were individually housed in so-called "baby boxes" for the first three weeks post-transport, after which they were housed in groups (5 or 6 calves per pen). The feeding scheme and the management of calves were similar in all veal farms, and our experimental calves were fully blended in and treated in accordance with the rest of calves present at the veal farm. Additionally, veal farmers were unaware of the background and age of calves.

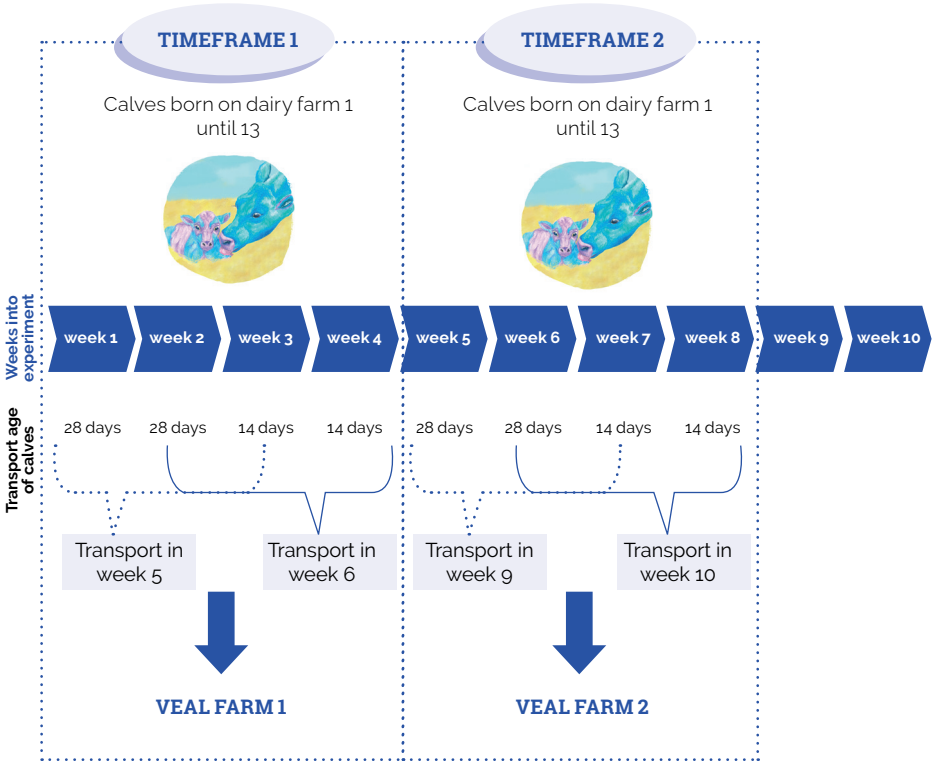

Figure 1. **Representation of the experimental design.**

Measurements

Immunoglobulin titers in serum samples of cows

All pregnant dams at the dairy farms were blood sampled approximately one week prior to the expected day of calving. A sample (10 ml, Vacuette, Greiner BioOne, Kremsmunster, Austria) was obtained from the tail vein of cows. Samples were kept at room temperature until centrifugation (3000 × g for 15 min at 4°C), then serum was decanted and stored at – 20°C until analysis. The titers of N-IgG, N-IgM and N-IgA were measured in serum samples (N = 813) with indirect enzyme-linked immunosorbent assay (ELISA) specific for PC-BSA. Pre-diluted samples (1:10) in PBS mix (PBS + 1% horse serum + 0.05% tween) were coated with different amounts of PC-BSA (PC-1011-10, Bioresearch Technologies, Petaluma, CA): 1 µg/ml for N-IgG and 0.25 µg/ml for N-IgM and N-IgA. Natural antibodies N-IgG and N-IgM were detected using 1: 20000 diluted sheep polyclonal anti-bovine IgG-heavy chain conjugated to horseradish PO (Cat. No. A10-100P, Bethyl Laboratories, Montgomery, US), and 1: 20000 diluted rabbit polyclonal anti-bovine IgM conjugated to horseradish PO (Cat. No. A10-100P, Bethyl Laboratories, Montgomery, US). Natural antibody N-IgA was detected using 1: 10000 diluted sheep polyclonal anti-bovine IgA conjugated to horseradish PO (Cat No. A10-131P, Bethyl Laboratories, Montgomery, US). Starting dilution of standards was 1: 160 for N-IgG, 1:80 for N-IgM, and 1: 20 for N-IgA. Serial dilutions for N-IgG, N-IgM and N-IgA in serum samples started at 1: 40 (4 steps). After the last 1.5 hour incubation at room temperature with the conjugates, plates were washed with demi-water. Each well of the plate was filled with 100 µL of substrate tetra methyl benzine (TMB) (Sigma Aldrich Chemie, Steinheim, Germany), which contained milliQ water, 1% TMB, and 10% TMB buffer. Plates were then incubated for 30 min at room temperature. After the incubation, the reaction was stopped by adding 50 µL of H_2SO_4 solution in each well. Extinctions were measured with a Multiskan reader (Lab Systems, Helsinki, Finland) with a wavelength of 450 nm. Titers were calculated based on log2 values of the dilution that gave extinction closest to 50% of Emax, where Emax represents the highest mean extinction of standard positive serum present on each plate (Ploegaert et al., 2007).

Immunoglobulin titers in colostrum samples

Colostrum samples (15 ml) were collected by the dairy farmer as soon as possible after birth of each calf. Samples were temporarily stored in a freezer (-20°C) on each dairy farm until they were processed in the laboratory and then they were stored at -80°C until analysis. The titers of N-IgG, N-IgM and N-IgA were measured in colostrum samples (N = 490) using the indirect enzyme-linked immunosorbent assay (ELISA) specific for PC-BSA described before. Pre-diluted samples (1:10) in PBS mix (PBS + 1% horse serum + 0.05% tween) were coated with 0.25 µg/ml PC-BSA (PC-1011-10, Bioresearch Technologies, Petaluma, CA) (100 µl/well). Natural antibodies N-IgG and N-IgM were detected, using

1: 20000 diluted sheep polyclonal anti-bovine IgG-heavy chain conjugated to horseradish PO (Cat. No. A10-100P, Bethyl Laboratories), and 1: 20000 diluted rabbit polyclonal anti-bovine IgM conjugated to horseradish PO (Cat. No. A10-100P, Bethyl Laboratories). Natural antibody N-IgA was detected using 1: 10000 diluted sheep polyclonal anti-bovine IgA conjugated to horseradish PO (Cat No. A10-131P, Bethyl Laboratories, Montgomery, US). Starting dilution of standards was 1: 10, and serial dilutions for N-IgG, N-IgM and N-IgA in colostrum samples started at 1: 40 (4 steps). Titers were calculated according to the previous procedure described for cow samples.

Immunoglobulin titers in serum samples of calves

Calves were blood sampled one week after birth, one day before transport, and 2 and 10 weeks post-transport (at the veal farm). Samples (10 ml) were collected from the jugular vein of calves into serum vacutainer tubes (Vacuette, Greiner BioOne, Kremsmunster, Austria). Samples were kept at room temperature until centrifugation (3000 × g for 15 min at 4°C), then serum was decanted and stored at – 20°C until analysis. The titers of N-IgG, N-IgM and N-IgA in serum samples (N = 684) were measured using indirect enzyme-linked immunosorbent assay (ELISA) specific against PC-BSA described before. Pre-diluted samples (1:10) in PBS mix (PBS + 1% horse serum + 0.05% tween) were coated with different amounts of PC-BSA (PC-1011-10, Bioresearch Technologies, Petaluma, CA): 1 µg/ml for N-IgG and 0.25 µg/ml for N-IgM and N-IgA. N-IgG and N-IgM were detected using 1: 20000 diluted sheep polyclonal anti-bovine IgG-heavy chain conjugated to horseradish PO (Cat. No. A10-100P, Bethyl Laboratories), and 1: 20000 diluted rabbit polyclonal anti-bovine IgM conjugated to horseradish PO (Cat. No. A10-100P, Bethyl Laboratories). N-IgA was detected using 1: 10000 diluted sheep polyclonal anti-bovine IgA conjugated to horseradish PO (Cat No. A10-131P, Bethyl Laboratories, Montgomery, US). Starting dilution of standards was 1: 80 for N-IgG and N-IgM, whereas 1: 20 for N-IgA, and serial dilutions for N-IgG, N-IgM and N-IgA in serum samples started at 1: 40 (4 steps). Titers were calculated according to the procedure described for serum samples of cows.

The hematological profile of calves

Blood samples (5 ml) were collected from the jugular vein of calves into EDTA vacutainer tubes (Vacuette, Greiner BioOne, Kremsmunster, Austria) one day prior to transport and in week 2 post-transport. Samples were stored at 4°C and then analysed by fluorescence flow cytometry (XT1800VET, Sysmex Europe GmbH, Germany) for a complete hematological profile, including: hemoglobin (Hb), hematocrit (Ht), red blood cells (RBC), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), red blood cells (RBC), white blood cells (WBC), lymphocytes, neutrophils, monocytes, basophils and eosinophils.

Data on calf and cow characteristics

Characteristics of both calves and cows were obtained by the dairy farmers. Calf characteristics included body weight at birth, breed and sex; cow characteristics included parity, DPL, total milk yield during the previous lactation were, and information on the ease of birth of the calf. Ease of birth was recorded as a binary response: score = 0 referred to a calving process without the assistance of the farmer, and a score = 1 indicated assistance of the farmer during the calving process. Additionally, each dairy farmer assessed the quality of the first colostrum of each cow, using a refractometer (model 101 ATC, MS Schippers, Bladel, The Netherlands). Values obtained from the refractometer (Brix values) are indicative of the amount of N-IgG (Quigley et al., 2013; Bartier et al., 2015).

Performance data

At both dairy and veal farms, individual treatments with antibiotics and other medicines were recorded. Information on individual treatments included the following data: 1) calf treated or not with antibiotics or other medicines (e.g. this latter category referred to products other than antibiotics, such as anti-inflammatories, multivitamins, and anti-coccidiosis); 2) whether single or repeated antibiotic/medical treatments were applied; 3) age at which treatments were applied. Finally, carcass weights were measured at slaughter.

Statistical analyses

All statistical analyses were performed with SAS 9.4 (SAS Inst. Inc., Cary, NC). Continuous data on N-IgG, N-IgM and N-IgA in serum samples of cows were analyzed with a linear mixed model (analysis with restricted maximum likelihood with SAS procedure MIXED). Residuals were checked for normality and homogeneity of variance and variables were log-transformed when needed. The linear mixed model (referred to as model 1) comprised fixed main effects for factors Sex (two levels: bull or heifer) and Breed (three levels: Holstein Friesian, Holstein-Friesian × Belgian Blue, or other crossbreds) of calves, for Parity (four levels: 1, 2, 3, 4-10) of dams, and random effects for dairy farms in addition to random error (residual) terms. Random farm effects account for dependence between observations from calves from the same farm and represent between farm variation and random error terms represent (remaining) variation between calves within the same farm.

Data on N-IgG, N-IgM and N-IgA in colostrum samples were similarly analyzed with a linear mixed model for continuous data. In addition to the fixed and random effects of the aforementioned linear mixed model, this model (referred to as model 2) also included a factor at two levels for ease of birth (0 = unassisted birth; 1 = assistance during birth) and a covariate for birthweight among the fixed effects.

N-IgG, N-IgM and N-IgA measured in serum samples of calves in week 1 were analyzed with a linear mixed model (further referred to as model 3) similar to model 2, but with the days between date of birth and the actual sampling moment in week 1 as an additional covariate. N-Ig's and hematological profile of calves measured one day prior to transport were analyzed with a linear mixed model (further referred to as model 4), which was similar to model 3, but with the inclusion of a factor at two levels for transport age (14 d of age vs. 28 d of age). Data expressed as proportions, such as hematocrit, were analyzed with a generalized linear mixed model (analysis with Penalized Quasi Likelihood with SAS procedure GLIMMIX), with a logit link function, specifying the "error" variance as a multiple of the binomial variance.

Data on N-IgG, N-IgM and N-IgA in serum samples collected at the veal farm were also analyzed with a linear mixed model for continuous data. The linear mixed model used for analysis (referred to as model 5) was more elaborate. The model comprised random effects for dairy farms and for veal farms, and their interaction, random effects for transports, and random effects for animals, in addition to the usual random error (residual) terms. The model comprised the same fixed effects as models 4, but also included fixed main effects for the sampling moment (at 2 or 10 weeks post-transport).

Hematological data collected in week 2 post-transport were analyzed in a similar way to data collected in week 1, but with the inclusion of random veal farm effects, the interaction between dairy and veal farm effects, and random transport effects in the model (model 6). Variables that were expressed as proportions, were analyzed with a generalized linear mixed model as discussed before.

In all previous analyses, approximate F-tests (Kenward and Roger, 1997) were used for fixed effects. Subsequent pairwise comparisons were done with Fisher's LSD method.

All the previously mentioned analyses were also conducted for a subset of samples, excluding first parity cows. These models included DPL (days), total milk yield (kg) and number of days open as additional covariates in order to obtain regression coefficients to test for significant associations between these factors and the respective response variable.

In order to test relationships between N-Ig's in serum of calves with the ones in serum of the dams and in colostrum, N-Ig's in colostrum and cow serum samples were added in the model for data analyzed with models 3, 4 and 5 again to test for significant associations between these covariates and the respective response variables.

To test whether or not Brix values obtained from refractometers were reflecting the actual amount of N-IgG in colostrum, the relationship between these two parameters was analyzed with regression analysis. Brix value was introduced as a covariate in model 2.

Birth weight of calves was also analyzed in relation to sex, breed of calves, parity of the dam, ease of birth and DPL. DPL (expressed in days) was introduced as a qualitative factor in the model comprising three levels: 1 = 0-30 days; 2 = 30 to 60 days; 3 = > 60 days). These levels were chosen in accordance with the classification proposed in a previous literature study (Mayasari et al., 2017).

Relationships between N-Ig's in serum of calves and individual treatments with antibiotics or other medicines, and carcass weights were analyzed with regression analysis. Individual treatments were analyzed as binary data (0 = calf not treated with any antibiotics or other medicines; 1 = calf treated at least once with antibiotics or other medicines during their rearing period). Individual treatments and carcass weight were used as response variables, and N-Ig's or neutrophils and lymphocytes measured at the dairy farm and in week 2 post-transport were introduced as covariates in the model (model 6). In all analyses, interactions between the covariates and the fixed factors in the model were tested to check whether or not slopes were equal for fixed effect levels. In all analyses, effects with $P \leq 0.05$ were considered significant, whereas those with $0.05 < P < 0.10$ were considered as tendencies toward significance.

Results

Effects

Transport age

Transport age significantly influenced the hematological variables of calves measured one day prior to transport and in week 2 post-transport. One day prior to transport, calves that were transported at 28 d had a significantly lower MCV (Δ = -1.67 fl), MCH (Δ = -35.6 amol), RDW (Δ = -1.12%), WBC (Δ = -0.7×10^9/l), neutrophils (cell count (Δ = -1.1×10^9/l) and proportion (Δ = -7.97%)), and monocytes (cell count (Δ = -2.12×10^9/l) and proportion (Δ = -2.14%)) compared to calves transported at 14 d (all $P < 0.05$; Table 1). In addition, calves transported at 28 d had higher RBC (Δ = 0.3×10^{12}/l), lymphocytes (cell count (Δ = 0.63×10^9/l) and proportion (Δ = 8.14%)), basophil count (Δ = 0.02×10^9/l), and eosinophils (cell count (Δ = 0.15×10^9/l) and proportion (Δ =1.19%) compared to calves transported at 14 d (all $P < 0.05$; Table 1). In week 2 post-transport, calves transported at 28 d had a lower MCV (Δ = -1.81 fl), MCH (Δ = -24 amol), WBC (Δ = -1.21×10^9/l), neutrophils (cell count (Δ = -1.61×10^9/l) and proportion (Δ = -12.05%)) and basophil count (Δ = -0.02×10^9/l) compared

to calves transported at 14 d (all $P < 0.05$; Table 2). Additionally, calves transported at 28 d had higher MCHC (Δ = 0.39 mmol/l), lymphocytes (both cell count (Δ = 0.50×10^9/l) and proportion (Δ = 11.9%)) and proportion of monocytes (Δ =0.40×10^9/l) compared to calves transported at 14 d ($P < 0.05$; Table 2).

Table 1. **Effects of transport age, sex and breed of calves on hematological profile measured in plasma of calves one day prior to transport (LS means).**

Parameter	Transport age		SEM[1]	*P*-value	Sex		SEM	*P*-value
	14 d	28 d			Bull	Heifer		
N. of calves	339	316			490	165		
Hemoglobin, mmol/l	6.58	6.47	0.18	0.56	6.37^a	6.68^b	0.16	< 0.01
Hematocrit,%	30.94	30.51	0.78	0.55	30.10^a	31.34^b	0.72	< 0.01
MCV[2], fl	34.76^a	33.09^b	0.42	< 0.01	33.84	34.01	0.40	0.56
MCH[3], amol	734.1^a	698.5^b	9.6	< 0.01	711.4	721.1	8.7	0.08
MCHC[4], mmol/l	21.20	21.14	0.11	0.68	21.09	21.25	0.10	0.09
RDW, SD[5], %	37.85^a	36.73^b	0.20	< 0.01	37.30	37.37	0.23	0.98
RDW, CV[6], %	31.27	32.18	0.16	0.06	31.73	31.64	0.18	0.93
RBC[7], 10^{12}/l	9.0^a	9.3^b	0.15	< 0.01	8.9^a	9.3^b	0.15	0.01
WBC[8], 10^9/l	11.4^a	10.7^b	0.37	0.01	10.9	11.1	0.38	0.59
Neutrophils, 10^9/l	4.68^a	3.58^b	0.13	< 0.01	4.13	4.21	0.14	0.88
Neutrophils, %	40.32^a	32.35^b	0.61	< 0.01	36.50	36.42	0.73	0.39
Lymphocytes*, 10^9/l	4.79^a	5.42^b	0.17	< 0.01	5.09	5.13	0.17	0.79
Lymphocytes, %	43.28	51.42	0.60		47.36	46.76	0.72	
Monocytes, 10^9/l	14.94^a	12.82^b	0.91	< 0.01	13.39	14.37	0.92	0.12
Monocytes, %	15.47^a	13.33^b	0.34	< 0.01	13.28	14.18	0.21	0.14
Basophils, 10^9/l	0.10^a	0.12^b	0.002	< 0.01	0.11	0.12	0.002	0.08
Basophils, %	0.95	1.11	0.02	0.32	1.02	1.06	0.01	0.72
Eosinophils, 10^9/l	0.13^a	0.28^b	0.02	< 0.01	0.21	0.18	0.02	0.57
Eosinophils, %	1.20^a	2.39^b	0.14	< 0.01	1.84^a	1.59^b	0.14	< 0.01

*[1]SEM = pooled standard error; [2]MCV = mean corpuscular volume; [3]MCH = mean corpuscular hemoglobin; [4]MCHC = mean corpuscular hemoglobin concentration; [5]RDW, SD = red blood cell width, standard deviation; [6]RDW, CV = red blood cell width, coefficient of variation; [7]RBC = red blood cells; [8]WBC = white blood cells. *The statistical model for this variable did not converge. [a, b] LSmeans within a factor and line lacking a common superscript differ (P ≤ 0.05).*

Table 2. Effects of transport age, sex and breed of calves on hematological profile measured in plasma of calves in week 2 post-transport (LS means).

Parameter	Transport age		SEM[1]	*P*-value	Sex	
	14 d	28 d			Bull	Heifer
N. of calves	365	320			512	174
Hemoglobin, mmol/l	6.51	6.39	0.23	0.39	6.37	6.54
Hematocrit,%	30.23	29.21	0.93	0.11	29.41	30.04
MCV[3], fl	32.64[a]	30.83[b]	0.43	< 0.01	31.71	31.77
MCH[4], amol	695.6[a]	671.6[b]	10.5	< 0.01	683.4	683.8
MCHC[5], mmol/l	21.24[a]	21.63[b]	0.06	< 0.01	21.44	21.35
RDW, SD[6]*, %	37.39	34.79	0.20		36.14	36.25
RDW, CV[7], %	32.52	32.64	0.16	0.99	32.61	32.46
RBC[8], 10^{12}/l	9.35	9.45	0.24	0.38	9.29	9.51
WBC[9], 10^9/l	10.92[a]	9.71[b]	0.38	< 0.01	10.22	10.42
Neutrophils, 10^9/l	3.23[a]	1.62[b]	0.09	< 0.01	2.50	2.41
Neutrophils*, %	28.20	16.15	0.58		22.69	22.19
Lymphocytes, 10^9/l	5.92[a]	6.42[b]	0.27	0.02	6.13	6.21
Lymphocytes*, %	55.23	67.13	0.57		61.04	60.08
Monocytes, 10^9/l	14.77	14.20	0.75	0.22	13.80[a]	15.16[b]
Monocytes, %	14.10[a]	14.50[b]	0.20	0.02	13.99[a]	15.17[b]
Basophils, 10^9/l	0.15[a]	0.13[b]	0.006	0.02	0.14	0.15
Basophils, %	1.43	1.35	0.05	0.35	1.37	1.46
Eosinophils, 10^9/l	0.11	0.08	0.008	0.12	0.10	0.11
Eosinophils*, %	1.04	0.87	0.08		0.91	1.11

*[1]SEM = pooled standard error; [2]Breed: HF = Holstein Friesian, HF× BB = Holstein Friesian × Belgian Blue crossbreds, O = other crossbreds; [3]MCV = mean corpuscular volume; [4]MCH = mean corpuscular hemoglobin; [5]MCHC = mean corpuscular hemoglobin concentration; [6]RDW, SD = red blood cell width, standard deviation; [7]RDW, CV = red blood cell width, coefficient of variation; [8]RBC = red blood cells; [9]WBC = white blood cells. *The statistical model for these variable did not converge; [a, b] LS means within a factor and line lacking a common superscript differ (P ≤ 0.05).*

SEM	*P*-value	Breed[2]			SEM	*P*-value
		HF	HF×BB	O		
		236	250	200		
0.23	0.12	6.45	6.64	6.28	0.24	0.15
0.90	0.17	29.61	30.38	29.18	0.99	0.22
0.43	0.84	31.42	32.20	31.58	0.54	0.26
10.7	0.95	675.7	694.0	681.2	11.3	0.08
0.09	0.19	21.51	21.30	21.47	0.18	0.42
0.23		35.66	36.25	36.68	0.40	
0.18	0.75	32.41	32.11	33.35	0.34	0.23
0.24	0.11	9.52	9.47	9.21	0.26	0.54
0.39	0.82	10.54	10.49	9.82	0.43	0.32
0.11	0.86	2.26	2.61	2.58	0.36	0.87
0.74		20.08	23.48	24.32	1.80	
0.26	0.69	6.50	6.15	5.86	0.29	0.07
0.73		63.24	59.20	59.94	1.69	
0.75	0.01	15.45[b]	14.9[b]	13.11[a]	0.83	0.02
0.22	0.05	14.49	14.67	13.57	0.43	0.73
0.007	0.20	0.14	0.16	0.13	0.009	0.46
0.05	0.72	1.34	1.50	1.32	0.08	0.51
0.01	0.99	0.09	0.13	0.08	0.02	0.29
0.10		0.85	1.16	0.85	0.22	

Calves' sex

One day prior to transport, bull calves had significantly lower values of hemoglobin (Δ = -0.31 mmol/l), hematocrit (Δ = -1.24%), and RBC (Δ = -0.4×10^{12}/l) and more eosinophils (Δ = 0.25%) compared to female calves (all $P < 0.05$; Table 1). In week 2 post-transport, bull calves had a lower monocyte count (Δ = -1.36×10^{9}/l) and proportion (Δ = -1.18%)($P < 0.05$; Table 2) compared to female calves. Bull calves received colostrum with a lower amount of N-IgG titer compared to female calves (Δ = -0.35; $P = 0.04$), whereas sex did not affect the other titers measured in colostrum, or N-IgG, N-IgM, and N-IgA titers measured in serum samples of cows and calves (Table 3, 4, 5, 6, 9).

Calves' breed

Breed did not significantly affect hematological variables measured one day prior to transport. In week 2 post-transport, Holstein Friesian and Holstein Friesian × Belgian Blue crossbred calves had a higher monocyte count compared to other crossbred calves (Δ = 2.06×10^{9}/l; $P = 0.02$; Table 2). One day prior to transport, N-IgM titers were higher in serum of Holstein Friesian × Belgian Blue calves compared to Holstein Friesian and other crossbred calves (Δ = 0.43 titer on average; $P = 0.02$; Table 6). At the veal farm, N-IgA titers were lower in serum of Holstein Friesian × Belgian Blue calves compared to Holstein Friesian and other crossbred calves (Δ = -0.23 titer on average; $P = 0.02$; Table 9). Breed did not have a significant influence on immunoglobulin titers in colostrum and in serum samples of cows.

Table 3. **Effects of calf sex, calf breed and cow parity on immunoglobulin titers specific for PC-BSA[1] measured in serum of cows one week before calving (LS means).**

Parameter	Sex		SEM[2]	*P*-value	Breed[3]		
	Bull	Heifer			HF	HF×BB	O
N. of calves	374	115			170	177	142
N-IgG	7.31	7.45	0.15	0.37	7.50	7.24	7.39
N-IgM	8.36	8.30	0.11	0.56	8.34	8.27	8.38
N-IgA	6.24	6.20	0.11	0.66	6.32	6.16	6.18

[1]phosphorylcholine conjugated to bovine serum albumin; [2]SEM = pooled standard error; [3]Breed: HF = Holstein Friesian, HF× BB = Holstein Friesian × Belgian Blue crossbreds, O = other crossbreds; [a, b, c] LSmeans within a factor and line lacking a common superscript differ ($P \leq 0.05$).

Parity

First parity cows showed lower amounts of N-IgG, N-IgM and N-IgA in their serum one week prior to calving compared to higher parity cows ($P < 0.01$; Table 3). Colostrum obtained from first parity cows had lower N-IgG, N-IgM and N-IgA titers (all $P < 0.05$; Table 4) compared to colostrum of older parity cows. Calves born from first-parity cows showed lower N-IgG ($P < 0.01$) and N-IgA titers ($P = 0.02$) in week 1 after birth, and lower N-IgG titers one day prior to transport and at the veal farm compared to calves born from cows of higher parities (Table 5, 7 and 8). One day prior to transport, calves born from first parity cows had lower eosinophil proportions compared to calves born from higher parity cows ($P < 0.01$; Table 1, Appendix 1), but no significant effects of parity on hematological profile were present in week 2 post-transport.

Ease of birth

Calves that were born with the assistance of the farmers had more eosinophils ($\Delta = 0.10\%$) in their serum one day prior to transport ($P < 0.01$; Table 1, Appendix 1) compared to calves that were born without assistance. The birth process did not have a significant effect on immunoglobulin titers in colostrum and serum.

Interaction between time and transport age at the veal farm

Transport age significantly influenced the levels of immunoglobulins measured in serum of calves one day prior to transport and in week 2 and 10 at the veal farm (Figure 2A-B). One day prior to transport, calves transported at 14 d had higher N-IgG, N-IgM and N-IgA titers ($\Delta = 0.75$, $\Delta = 0.71$ and $\Delta = 0.62$, respectively) compared to calves transported at 28 d (all $P < 0.01$; Table 6).

SEM	*P*-value	Parity				SEM	*P*-value
		1	2	3	4-10		
		60	119	101	212		
0.18	0.30	6.60^{b}	7.62^{a}	7.60^{a}	7.70^{a}	0.17	< 0.01
0.12	0.60	7.68^{c}	8.44^{a}	8.51^{ab}	8.67^{b}	0.12	< 0.01
0.12	0.23	5.67^{b}	6.41^{a}	6.32^{a}	6.48^{a}	0.12	< 0.01

Table 4. Effects of calf sex, calf breed, cow parity and the ease of birth on immunoglobulin titers specific for PC-BSA[1] measured in colostrum (LS means).

Parameter	Sex		SEM[2]	*P*-value	Breed[3]			SEM	*P*-value
	Bull	Heifer			HF	HF×BB	O		
N. of calves	516	177			240	251	203		
N-IgG	10.42[a]	10.77[b]	0.22	0.04	10.61	10.51	10.66	0.25	0.81
N-IgM	7.11	7.01	0.18	0.49	6.99	7.20	6.98	0.21	0.41
N-IgA	6.41	6.43	0.19	0.91	6.44	6.55	6.26	0.23	0.57

[1] *phosphorylcholine conjugated to bovine serum albumin.*[2]*SEM = pooled standard error;* [3]*Breed: HF = Holstein Friesian, HF× BB = Holstein Friesian × Belgian Blue crossbreds, O = other crossbreds,*[4]*Ease of birth: 0 = unassisted; 1= assisted.* [a, b, c] *LSmeans within a factor and line lacking a common superscript differ (P ≤0.05).*

Table 5. Effects of calf sex, calf breed, cow parity and the ease of birth on immunoglobulin titers specific for PC-BSA[1] in serum of calves one week after birth (LS means).

Parameter	Sex		SEM[2]	*P*-value	Breed[3]			SEM	*P*-value
	Bull	Heifer			HF	HF×BB	O		
N. of calves	508	175			235	246	202		
N-IgG	7.92	7.97	0.24	0.75	8.04	7.98	7.82	0.27	0.82
N-IgM	6.69	6.80	0.24	0.54	6.89	6.99	6.36	0.28	0.20
N-IgA	4.82	4.93	0.21	0.54	5.04	5.06	4.51	0.25	0.24

[1] *phosphorylcholine conjugated to bovine serum albumin.* [2]*SEM = pooled standard error;* [3]*Breed: HF = Holstein Friesian, HF× BB = Holstein Friesian × Belgian Blue crossbreds, O = other crossbreds;* [4]*Ease of birth: 0 = unassisted; 1= assisted.* [a, b, c] *LSmeans within a factor and line lacking a common superscript differ (P ≤ 0.05)*

	Parity				SEM	*P*-value	Ease of birth[4]		SEM	*P*-value
	1	2	3	4-10			0	1		
	89	168	152	257			537	154		
	9.59^{c}	10.69^{a}	11.12^{b}	10.97^{ab}	0.23	< 0.01	10.43	10.76	0.23	0.20
	6.68^{b}	7.04^{ab}	7.36^{a}	7.14^{a}	0.20	0.02	7.12	6.99	0.19	0.53
	5.75^{b}	6.31^{c}	6.78^{a}	6.83^{a}	0.21	< 0.01	6.50	6.33	0.22	0.54

	Parity				SEM	*P*-value	Ease of birth[4]		SEM	*P*-value
	1	2	3	4-10			0	1		
	90	165	150	252			527	153		
	7.24^{c}	7.91^{a}	8.44^{b}	8.19^{ab}	0.26	< 0.01	7.94	7.95	0.25	0.99
	6.62	6.74	6.97	6.66	0.26	0.37	6.92	6.58	0.25	0.16
	4.40^{ac}	4.83^{bc}	5.13^{b}	5.12^{b}	0.24	0.02	4.98	4.77	0.22	0.31

Table 6. Effects of transport age, sex and breed of calves on immunoglobulin titers specific for PC-BSA[1] measured in serum of calves one day prior to transport (LS means).

Parameter	Transport age		SEM[2]	*P*-value	Sex	
	14 d	28 d			Bull	Heifer
N. of calves	363	320			508	175
N-IgG	7.40[a]	6.65[b]	0.17	< 0.01	6.99	7.05
N-IgM	3.29[a]	2.58[b]	0.14	< 0.01	2.94	2.92
N-IgA	1.35[a]	0.73[b]	0.12	< 0.01	1.06	1.02

[1] *phosphorylcholine conjugated to bovine serum albumin.* [2]*SEM = pooled standard error;* [3]*Breed: HF = Holstein Friesian, HF× BB = Holstein Friesian × Belgian Blue crossbreds, O = other crossbreds;*
[a, b] *LSmeans within a factor and line lacking a common superscript differ (P ≤ 0.05).*

Table 7. Effects of parity of cows and ease of birth on immunoglobulin titers specific for PC-BSA[1] measured in serum of calves one day prior to transport (LS means).

Parameter	Parity				SEM[2]	*P*-value
	1	2	3	4-10		
N. of calves	90	165	151	252		
N-IgG	6.26[c]	7.05[a]	7.46[b]	7.32[ab]	0.19	< 0.01
N-IgM	2.81	2.85	3.01	3.06	0.16	0.36
N-IgA	0.84	0.97	1.13	1.22	0.14	0.09

[1] *phosphorylcholine conjugated to bovine serum albumin;* [2]*SEM = pooled standard error.*
[a, b, c] *LSmeans within a factor and line lacking a common superscript differ (P ≤0.05).*

	SEM	*P*-value	Breed[3]			SEM	*P*-value
			HF	HF×BB	O		
			235	246	202		
	0.17	0.72	7.19	7.12	6.76	0.20	0.32
	0.14	0.89	2.95[ab]	3.22[b]	2.62[a]	0.17	0.02
	0.13	0.26	1.26	1.34	0.99	0.13	0.46

	Ease of birth		SEM	*P*-value
	Unassisted	Assisted		
	527	153		
	6.98	7.07	0.17	0.56
	3.04	2.83	0.14	0.12
	1.14	0.95	0.12	0.12

Table 8. Effects of parity of cows and ease of birth on immunoglobulin titers specific for PC-BSA[1] measured in serum of calves at the veal farm [2] (LS means).

Parameter	Parity				SEM[3]	*P*-value
	1	2	3	4-10		
N. of calves	89	166	151	252		
N-IgG	5.51[a]	5.75[ab]	5.94[b]	5.89[b]	0.15	0.02
N-IgM	5.23	5.32	5.22	5.37	0.13	0.53
N-IgA	2.95	3.01	3.03	3.16	0.12	0.18

[1] *phosphorylcholine conjugated to bovine serum albumin;* [2] *week 2 and 10 post-transport;* [3]*SEM = pooled standard error.* [a, b] *LSmeans within a factor and line lacking a common superscript differ (P ≤0.05).*

Table 9. Effects of transport age, sex and breed of calves on immunoglobulins titers specific for PC-BSA[1] measured in serum of calves at the veal farm[2] (LS means).

Parameter	Transport age		SEM[3]	*P*-value	Sex	
	14 d	28 d			Bull	Heifer
N. of calves	363	320			508	175
N-IgG	5.91[a]	5.64[b]	0.14	< 0.01	5.85	5.69
N-IgM	4.96[a]	5.61[b]	0.12	< 0.01	5.28	5.30
N-IgA	2.86[a]	3.21[b]	0.11	< 0.01	3.07	3.00

[1] *phosphorylcholine conjugated to bovine serum albumin;* [2] *week 2 and 10 post-transport;* [3]*SEM = pooled standard error;* [4]*Breed: HF = Holstein Friesian, HF× BB = Holstein Friesian × Belgian Blue crossbreds, O = other crossbreds.* [a, b] *LSmeans within a factor and line lacking a common superscript differ (P ≤ 0.05).*

	Ease of birth		SEM	*P*-value
	Unassisted	Assisted		
	527	153		
	5.76	5.78	0.14	0.81
	5.29	5.29	0.13	0.99
	3.02	3.05	0.11	0.73

SEM	*P*-value	Breed[4]			SEM	*P*-value
		HF	HF×BB	O		
		235	246	202		
0.14	0.11	5.82	5.67	5.82	0.16	0.41
0.12	0.83	5.34	5.15	5.37	0.14	0.19
0.11	0.36	3.11	2.88	3.12	0.12	0.02

Table 10. **Effects of sampling moment and the interaction between sampling moment and transport age on immunoglobulin titers specific for PC-BSA[1] measured in serum of calves at the veal farm (LS means).**

Parameter	Sampling moment		SEM[2]	*P*-value	Sampling moment × Transport age				SEM	*P*-value
	Week 2	Week 10			Week 2		Week 10			
					14 d	28 d	14 d	28 d		
N-IgG	6.23^{a}	5.32^{b}	0.14	< 0.01	6.55^{c}	5.90^{b}	5.26^{a}	5.37^{a}	0.15	< 0.01
N-IgM	2.99^{a}	7.58^{b}	0.12	< 0.01	2.40^{c}	3.59^{b}	7.52^{a}	7.64^{a}	0.14	< 0.01
N-IgA	1.43^{a}	4.64^{b}	0.11	< 0.01	1.15^{c}	1.71^{b}	4.57^{a}	4.72^{a}	0.12	< 0.01

[1] *phosphorylcholine conjugated to bovine serum albumin;* [2]*SEM = pooled standard error.* [a, b, c] *LSmeans within a factor and line lacking a common superscript differ (P ≤ 0.05).*

Calves that were transported at 14 d and sampled in week 2 post-transport had higher N-IgG titers (Δ = 0.65), but lower N-IgM (Δ = -1.19) and N-IgA (Δ = -0.56) compared to calves transported at 28 d and sampled in week 2, whereas N-IgG, N-IgM and N-IgA titers did not differ between calves of both transport ages when sampled in week 10 post-transport.

Relationships between measures

Table 11 shows the regression coefficients (ß) obtained from regression analysis of relations between N-Ig's in serum samples of calves (as response variables) and N-Ig's in colostrum or in serum samples of cows (as the explanatory variable). In week 1 and one day prior to transport, all three N-Ig's isotypes of calves were positively related to N-Ig's in colostrum ($P < 0.05$), and only N-IgG of calves was positively related to N-IgG in cow serum samples ($P < 0.01$). At the veal farm, only N-IgG of calves was positively related to N-IgG in colostrum and N-IgG in serum of cows ($P < 0.01$).

Brix values (as explanatory variable) were positively related with the amount of N-IgG in colostrum (as the response variable) (ß = 0.11, SE = 0.01; $P < 0.01$).

Table 12 shows the regression coefficients (ß) obtained from regression analysis of relations between N-Ig's in calf serum in week 1 after birth, one day prior to transport or in week 2 at the veal farm and individual treatments with antibiotics and other medical treatments at the veal farm. Relationships between N-Ig's in calf serum and carcass weight of calves were never significant.

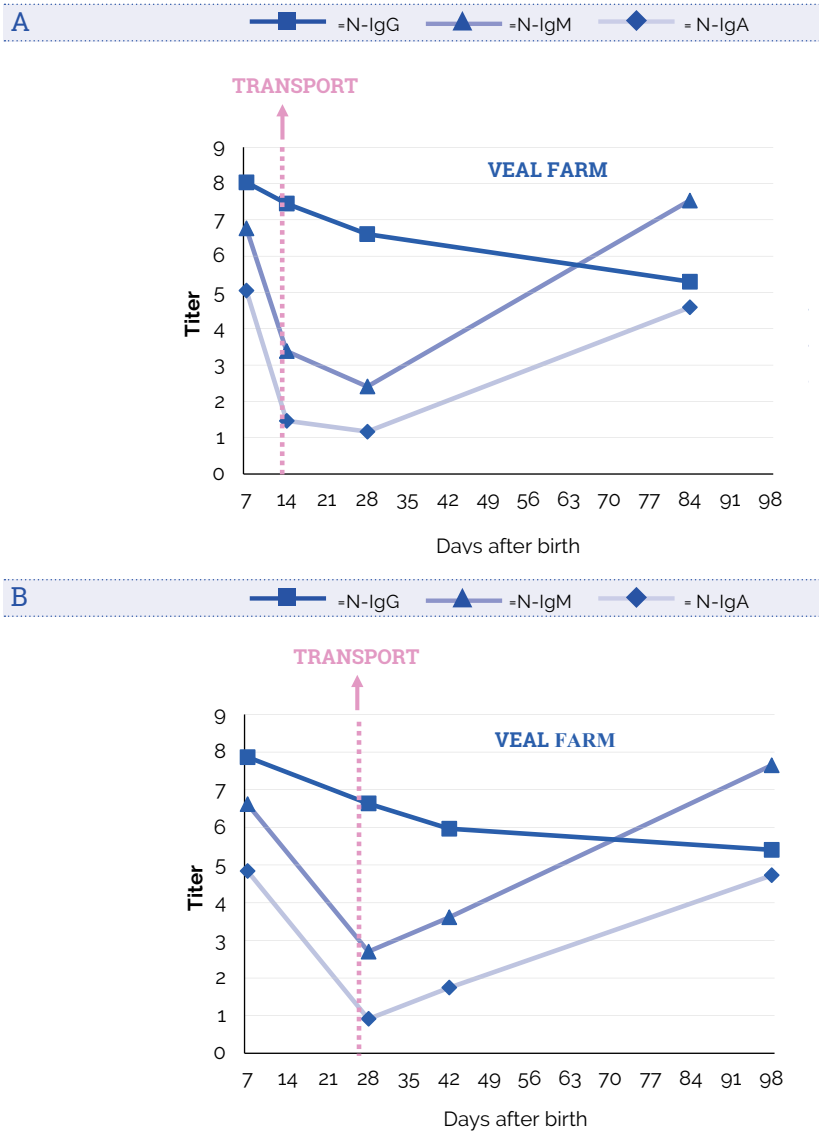

Figure 2 A-B. **Immunoglobulin titers specific for phosphorylcholine conjugated to bovine serum albumin measured in serum of calves one week after birth on dairy farm, one day prior to transport to the veal farm and in week 2 and 10 post-transport**. *Figure 2A illustrates the comparison between the three isotypes in calves transported at 14 d from dairy to veal farms, whereas Figure 2B showed the comparison in calves transported at 28 d of age. In both scenario A and B, calves were transported on the same day to the veal farm, thus calves belonging to the 28 d group (B) were simply two weeks older than the other age group (A).*

Regression coefficients shown in Tables 11 and 12 represent the coefficients obtained from regression models without the interaction term, i.e. on the assumption of homogeneity of regression slopes (slopes are parallel for different ages of transport, parity groups, sexes and breeds). Some of the interactions were statistically significant, but they always reflected proportional differences between levels of a fixed effect in the strength of the relationship between the response variable and the covariable.

Table 11. **Regression coefficients (ß), standard error (in brackets) and significance values of immunoglobulins measured in serum of calves in week 1, one day prior to transport and at the veal farm in relation to those measured in colostrum and cow serum samples.**

Response variables	Explanatory variables	
	Colostrum	Cow serum
Immunoglobulins measured in week 1		
N-IgG	ß = 0.614 (0.06), $P < 0.001$	ß = 0.364 (0.06), $P < 0.001$
N-IgM	ß = 0.576 (0.07), $P < 0.001$	ß = 0.542 (0.09), $P < 0.001$
N-IgA	ß = 0.571 (0.06), $P < 0.001$	ß = 0.639 (0.10), $P < 0.001$
Immunoglobulins measured one day prior to transport		
N-IgG	ß = 0.620 (0.06), $P < 0.001$	ß = 0.385 (0.05), $P < 0.001$
N-IgM	ß = 0.171 (0.06), $P = 0.002$	ß = 0.199 (0.08), $P = 0.01$
N-IgA	ß = 0.170 (0.05), $P < 0.001$	ß = 0.154 (0.08), $P = 0.05$
Immunoglobulins measured at the veal farm (week 2 post-transport)		
N-IgG	ß = 0.676 (0.05), $P < 0.001$	ß = 0.370 (0.05), $P < 0.001$
N-IgM	ß = 0.066 (0.07), $P = 0.33$	ß = 0.086 (0.09), $P = 0.34$
N-IgA	ß = 0.090 (0.04), $P = 0.03$	ß = 0.086 (0.06), $P = 0.17$
Immunoglobulins measured at the veal farm (week 10 post-transport)		
N-IgG	ß = 0.189 (0.05), $P < 0.001$	ß = 0.092 (0.05), $P = 0.05$
N-IgM	ß = -0.030 (0.05), $P = 0.59$	ß = -0.050 (0.06), $P = 0.41$
N-IgA	ß = -0.002 (0.04), $P = 0.96$	ß = 0.018 (0.06), $P = 0.77$

Table 12. Regression coefficients (ß), standard error and significance values of individual treatments with antibiotics and other medicines during the whole rearing period at the veal farm in relation to calf serum immunoglobulin titers measured at different time points.

Explanatory variables	Response variables	
	Antibiotics	Other medicines
N-IgG		
N-IgG_week 1 after birth	ß = - 0.196 (0.06), $P < 0.001$	ß = - 0.192 (0.06), $P < 0.001$
N-IgG_one day prior to transport	ß = - 0.235 (0.07), $P < 0.001$	ß = - 0.212 (0.06), $P < 0.001$
N-IgG_week 2 at the veal farm	ß = - 0.253 (0.06), $P < 0.001$	ß = - 0.196 (0.06), $P = 0.001$
N-IgM		
N-IgM_week 1 after birth	ß = - 0.165 (0.06); $P = 0.03$	ß = - 0.136 (0.06), $P = 0.01$
N-IgM_one day prior to transport	ß = - 0.168 (0.09), $P = 0.05$	ß = - 0.114 (0.08), $P = 0.17$
N-IgM_week 2 at the veal farm	ß = - 0.04 (0.06), $P = 0.57$	ß = - 0.03 (0.07), $P = 0.66$
N-IgA		
N-IgA_week 1 after birth	ß = - 0.148 (0.06), $P < 0.01$	ß = - 0.132 (0.06), $P = 0.02$
N-IgA_one day prior to transport	ß = - 0.160 (0.10), $P = 0.14$	ß = - 0.08 (0.10), $P = 0.43$
N-IgA_week 2 at the veal farm	ß = 0.04 (0.09), $P = 0.68$	ß = 0.04 (0.09), $P = 0.68$

Effects of calf and cow characteristics on birth weight of calves

Calves delivered by first parity cows had a significantly lower birth weight (41.4 kg) compared to calves delivered by multiparous cows (43.6 kg for second parity cows, 45.3 kg for third parity cows, and 46.1 kg for cows with parity 4-10; $P < 0.01$). Birth weight was significantly higher for calves born with assistance during birth compared to calves born with a normal delivery (Δ = 2 kg, $P < 0.01$). Additionally, crossbred calves (Holstein Friesian × Belgian Blue and other crossbreds) were heavier at birth compared to Holstein Friesian calves (Δ = 3.5 kg on average, $P < 0.01$). Birth weight of bull calves was also higher than that of female calves (Δ = 4.3 kg, $P < 0.01$).

Discussion

Effects of transport age

In the current study the hematological profile of calves was significantly affected by transport age. This is in line with previous studies, which indicated that age is a key factor for changes in hematological values, especially in the first weeks after the birth of calves (Brun-Hansen et al., 2006; Panousis et al., 2018). At two weeks of age, which coincided with the transport of the 14 d treatment calves, values of MCV, MCH, WBC, neutrophils and monocytes were higher compared to values at four weeks of age, which coincided with transport of the 28 d treatment. These results are in accordance to previous studies, which indicated that these changes might reflect the replacement of RBC containing fetal hemoglobin (HgbF) with smaller RBC containing the adult type (HgbA) (Egli and Blum, 198; Brun-Hansen, 2006) and the progressively decreasing cortisol concentrations after birth (Knowles et al., 2000; Mohri et al., 2007). The lymphocytes followed an opposite trend compared to neutrophils. This is also in line with previous studies that reported a gradual increase in lymphocytes until 10-12 weeks of age in calves (Brun-Hansen et al., 2006). All these changes, and in particular the higher lymphocyte and lower neutrophil counts might contribute to improved immune responses in calves transported at 28 d compared to calves transported at 14 d. Von Konigslow et al. (2020) showed that lymphocyte counts between 4.6 and 5.8 × 10^9/L were associated with a decreased hazard of mortality compared to lymphocyte counts < 4.6 or > 5.8 × 10^9/L. Additionally, lymphocyte counts > 5.8 × 10^9/L reduced the hazard of morbidity of calves upon arrival at the veal farm compared to lymphocyte counts < 5.8 × 10^9/L, and elevated neutrophil counts (> 6.0 × 10^9/L) increased the hazard of mortality by more than 5 times. These authors proposed that especially an elevated lymphocyte count (> 7 × 10^9/L) might be used as an indicator of resilience to stress, in particular related to transport. These findings may, therefore, suggest that calves transported at 28 d of age might be more robust than calves transported at 14 d of age. This idea will be further substantiated in our companion paper (Marcato et al., 2021b) looking at differences between the two age groups in putative measures of robustness recorded at the veal farm, including treatments with medicines, carcass weight and mortality. The results of the regression analyses reported in the current paper showed that, at least within age groups, differences between calves in cell counts were not associated with differences in individual antibiotic or other medical treatments or carcass weight, which seems to be in contrast with the results of von Konigslow et al. (2020). However, in their study, lymphocyte and neutrophil counts were significantly correlated with morbidity and mortality rates of calves at the veal farm. As will be addressed in our companion paper (Marcato et al., 2021b), these rates were relatively low in the present experiment.

Transport age also had an effect on immunoglobulin titers in calves' serum one day prior to transport. One day before transport, calves transported at 14 d of age had higher serum titers of all three N-Ig isotypes compared to calves transported at 28 d of age. Since calves transported at 14 d were two weeks younger than the other transport group, their serum immunoglobulins likely reflected maternal immunoglobulins obtained from colostrum to a greater extent than the serum of calves transported at 28 d. It is indeed well known that the immune response of calves in the first weeks after birth relies largely on passive immunity transferred from their mothers via colostrum (Barrington and Parish, 2001; Stilwell and Carvalho, 2011). The current study showed a positive relationship between the amount of N-Ig's in colostrum and the N-Ig's in the serum of calves measured one week after birth. Mayasari et al. (2016) also found a positive relationship between the level of natural autoantibodies in plasma of calves in their first weeks of age and natural autoantibodies in colostrum, although this relationship was not maintained after 2 weeks of age. In contrast, the current study showed a positive relationship between N-IgG in serum of calves and N-IgG in colostrum, and this relationship was maintained until week 2 at the veal farm, regardless of age. These results suggest that immune protection via maternal colostrum may last for a longer period. These long-term maternal effects might have a positive effect on robustness of calves at the veal farm. This assumption was supported by the negative relationship between N-IgG in serum of calves and the use of individual antibiotic and other medical treatments at the veal farm. Two weeks after arrival at the veal farm, calves transported at 14 d of age showed lower serum N-IgM and N-IgA levels compared to calves transported at 28 d. This difference between the two transport age groups might reflect the decline of the effectiveness of passive immunity derived from colostrum in 14 d old calves and the activation of endogenous Ig synthesis in 28 d old calves (Burton et al., 1989; Chase et al., 2008). The immune system of calves in the first weeks after birth is still immature and requires time to complete development (Gomes et al., 2014). According to Chase et al. (2008), endogenous production of IgM in calves starts reaching functional levels around 8 d of age, whereas levels of endogenous IgG and IgA do not reach functional levels until 16 to 32 d after birth. Since the adaptive immunity of calves is not completely developed yet, maternal antibodies in colostrum provide neonatal calves passive immunity and protection in the first 2 weeks after birth (Haessig et al., 2007; Yang et al., 2015). Burton et al. (1989) showed that peak concentrations of IgG, IgM and IgA in serum of calves occurred at 24 to 36 h after birth (1801 mg/100 ml, 154 mg/100 ml, and 110 mg/100 ml, respectively) and were associated with colostrum intake. The minimum serum concentrations of these immunoglobulins were found at 3 weeks of age for IgM (36 mg/100 ml) and IgA (27 mg/100 ml) and at 4 weeks for IgG (1213 mg/100 mg). After these moments, concentrations of all three isotypes gradually increased as a result of endogenous production (Burton et al., 1989). In the current experiment, the patterns

over time of serum N-IgM and N-IgA titers were also in line with previous research, thus calves transported at 28 d were older at arrival at the veal farm and this explains their higher N-IgM and N-IgA titers compared to calves transported at 14 d of age. When comparing the titers of immunoglobulins in serum of calves measured in week 1 after birth and one day prior to transport with the titers measured in week 2 and week 10 post-transport, it is evident that the lowest N-IgM and N-IgA titers occurred at the age of 4 weeks, i.e. in week 2 post transport in calves transported at 14 days of age, and one day prior to transport in calves transported at 28 days of age (Figure 2). The lowest N-IgG titers were obtained in week 10 post-transport, i.e. when calves in the two transport age groups were 12 and 14 weeks old, respectively, and until that time the level of N-IgG seemed to only moderately decrease (Figure 2). This particular pattern for N-IgG was shown by both transport age groups, and it notably differs from what is described in the literature. Perhaps, endogenous IgG production was more pronounced in our calves compared to other (e.g. replacement heifer) calves, for example because their adaptive immune system was stimulated to a greater extent, in particular after arrival at the veal farm. However, in our experiment we were not able to discriminate between N-IgG's of maternal or endogenous origin. Future research is, therefore, needed to further characterize patterns over time of immunoglobulins in veal calves.

Our results clearly underline the importance of feeding high quality colostrum to calves. In fact, the amount of N-Ig's in colostrum had a long-term influence on the amount of N-Ig's in serum of calves. Refractometers are easy and practical tools to measure N-IgG in colostrum as indicated by the positive relationship between the Brix value and N-IgG in colostrum. Moreover, the N-IgG concentration in calf serum might be used as an indication of robustness, because high N-IgG titers were associated with less individual antibiotic and other medical treatments at the veal farm and vice versa. The current results also demonstrate that transportation of calves at either 14 d and 28 d is still occurring in a vulnerable moment, because Ig titers were lower compared to those measured in week 1 after birth. On the basis of the levels of N-Ig's measured in week 2 at the veal farm, transportation of calves two weeks later than the usual practice might be more appropriate, because the development of their immunity was more advanced. Given the present finding, N-IgG should be monitored also in the period between week 2 and 10 post-transport, and beyond. This would allow to 1) determine the proportions of maternal and endogenous IgG over this time frame, 2) determine when the adaptive immune system of calves becomes functional, and 3) investigate whether or not N-IgG titers continue to decrease or reach a plateau beyond week 10 post-transport.

Effects of calves' sex

Effects of calf sex on hematological profile are not extensively investigated and they are often controversial. Tennant and Kendrick (1974) did not observe any sex-related differences in calves, whereas Raleigh and Wallace (1962) found higher hemoglobin and hematocrit values in female calves from birth to 25 weeks of age than in male calves.

In the current study, female calves had a higher hemoglobin, hematocrit, and RBC compared to bull calves one day prior to transport. These results are in line with the study of Panousis et al. (2018), although they included only Holstein Friesian calves between 1 and 9 days of age. Differences between hematological characteristics shown by female calves and those shown by bull calves might be related to a different hormonal status or to a difference in birth weight between bull and female calves (46.2 vs 41.9 kg, respectively). In our companion paper (Marcato et al., 2021b) effects of sex on health, medicine use at the veal farm and on carcass weight will be investigated to understand whether or not there is a difference in robustness between bull and female calves.

Effects of calves' breed

The hematological profile of calves has been studied in both beef (Adams et al., 1992; Egli and Blum, 1998) and dairy breeds, especially Holstein Friesian calves (Mohri et al., 2007; Panousis et al., 2018). In the current study, hematological variables did not differ among breeds. This was in contrast with previous studies that indicated differences in blood variables (such as hemoglobin and hematocrit) between dairy and beef breeds. With regard to N-Ig's, Belgian Blue × Holstein Friesian calves had the highest N-IgM titers one day prior to transport and the lowest N-IgA titers at the veal farm compared to the other breeds. Although not significant, the other N-Ig's titers followed the same principle as N-IgM and N-IgA at both time points, respectively. These results might be an indication that the immunity gap occurs at a later stage for Belgian Blue × Holstein Friesian calves compared to the other breeds. Higher N-Ig's at the time of transport might be related to an improved robustness of calves upon arrival at the veal farm. This will be investigated in our companion paper (Marcato et al., 2021b), looking at effects of breed on measures of robustness in the long term.

Effects of parity

Parity had the most evident effects on N-Ig's titers measured in cow samples, colostrum and calf samples in week 1 after birth, whereas parity affected only N-IgG titers measured one day prior to transport and at the veal farm. Results indicated significantly higher N-Ig's titers in older parity cows compared to first parity cows, and this is in line with previous studies. Older cows are likely to be exposed to a greater number of pathogenic antigens in their lifetime, which is likely the cause for higher immunoglobulin titers

in their serum and, sequentially, in colostrum (Conneely et al., 2013). Tyler et al. (1999) reported that colostrum produced by cows of parity 3 or higher contained 19.5 g more IgG compared to colostrum produced by primiparous cows. Aydogdu and Guzelbektes (2018) also showed that colostrum of multiparous cows had a higher IgG concentration than colostrum produced by primiparous cows (117.4 vs. 73.8 g/l, respectively). Another reason for the higher immunoglobulin concentration in colostrum of multiparous cows might be related to the development of the mammary gland. As indicated by Dunn et al. (2017), younger cows might be not fully developed and the transport of immunoglobulins from the blood circulation into the mammary gland may be reduced compared to older cows. Effects of parity were still evident on N-IgG titers measured in serum of calves 10 weeks post-transport, which is a long period after colostrum intake. The relationships between N-Ig's titers, especially N-IgG, in serum of calves and the ones in colostrum and serum of cows indicate a clear connection between calves and their mothers. These results are in line with those shown by Mayasari et al. (2016), who suggested that the level of natural autoantibodies in the plasma of calves in the first 2 weeks after birth reflects the levels of natural autoantibodies in colostrum and in plasma of cows. In that study, this relationship was not maintained beyond week 2, whereas in the current study the relationship was maintained until week 10 post-transport. All these findings suggest that the pre and post-natal period play an important role on the immune development of calves and farmers should provide good quality colostrum in order to increase the immunoglobulin content in serum of calves. Additionally, colostrum obtained from older cows might confer more protection to calves via passive transfer.

Effects of calving process

The calving process is known to have profound effects on hematological profile and immune system of new-born calves (Probo et al., 2012). A longer delivery requiring assistance of the farmer represents an even more stressful event for calves compared to a normal birth. As a result of stress, the immune response is enhanced with higher levels of WBC and neutrophils. Additionally, the severity of the birth process can create disturbance of the gas exchange during calving, and thus, it can affect the acid-base status of newborn calves (Szenci et al., 1989). According to Probo et al. (2012), the level of oxygenation is responsible for changes in hematological profile, such as higher Hb, Ht and RBC. All these observations were done on new-born calves and there is scarce information on the association between the ease of birth and hematological profile and immune system in older calves for veal production. In the current study, ease of birth did not influence the hematological profile. Additionally, N-Ig's titers of calves at different time points were not influenced by ease of birth. This result is in contrast with previous studies, which indicated that the calving process can affect the amount of immunoglobulins in dairy calves (Barrier et al., 2012, 2013). Calves with a high birth weight usually

require assistance of the farmer during delivery and the calving process might be prolonged (Eriksson et al., 2004). In the current study, calves born with assistance of the farmer were heavier than calves born without assistance (45.1 vs 43.1 kg, respectively). As a consequence of a prolonged calving process, these calves might be less vigorous and they might be less willing to ingest sufficient amounts of colostrum compared to calves with a normal delivery (Vasseur et al., 2009; Barrier et al., 2012). This may result in inadequate transfer of immunoglobulins and poorer survivability and higher morbidity compared to calves with a normal birth (Gasparelli et al., 2009; Barrier et al., 2013). To assess whether or not the calving process has long term consequences on robustness of calves, our companion paper (Marcato et al., 2021b) will investigate effects of ease of birth on health and performance of calves at the veal farm.

Effects of other cow-related characteristics

Factors as DPL, milk yield and gestation length of dairy cows have been shown to play an important role on the prenatal life of calves (van Eetvelde and Opsomer, 2020). Prenatal conditions can affect the developmental programming of later health and performance of calves (Astiz et al., 2014; Pinedo and De Vries, 2017). For example, high milk yield during pregnancy leads to a significant loss of nutrients (e.g. protein and glucose) for the fetus, because they are diverted to the mammary gland rather than to the uterus (Opsomer et al., 2017). This in turn affects the birth weight of the calf and high-producing cows (with cumulative milk production during gestation between 7,200 and 11,600 kg) have been shown to deliver 1 kg lighter calves than low-producing cows (Kamal et al., 2014). Since milk yield affects protein partitioning to the fetus, the current study investigated whether or not milk yield also affected immunoglobulin production in calves. In the current study immunoglobulins were not affected by DPL or number of days open. At the same time, calves born from cows with a shorter dry period (0-30 days) had a lower birth weight (42.6 kg) compared to calves born from cows with a longer dry period (44.6 kg for cows with 30-60 days, and 45.5 for cows with > 60 days). Additionally, calves delivered by first parity cows had a significantly lower birth weight (41.4 kg) compared to calves delivered by multiparous cows (43.6 kg for second parity cows, 45.3 kg for third parity cows, and 46.1 kg for cows with parity 4-10). These findings are supported by previous studies, where a shorter DPL and primiparity of cows contributed to a lower birth weight of calves (Kamal et al., 2014; van Eetvelde and Opsomer, 2020). A shorter dry period is often used for high yielding cows with high lactation persistency, thus, as explained above, high milk yield might have a negative impact on fetal development (van Eetvelde and Opsomer, 2020). In first parity cows, pregnancy coincides with continued growth of the dam, thus the fetus might face competition with the nutrients the mother needs for her own development (Opsomer et al., 2017). The current study showed that maternal characteristics can affect calf characteristics at birth. Any

long-term consequences of birth weight on measures of robustness recorded at the veal farm, including treatments with medicines, carcass weight will be further investigated in our companion paper (Marcato et al., 2021b).

Conclusions

Transportation of calves at both 14 and 28 d of age still occurs in the immune gap period. However, in week 2 post-transport, calves transported at 28 d of age had higher N-IgM and N-IgA titers, which might be interpreted as a sign of a more advanced development of their adaptive immunity. Feeding high quality colostrum might have important long-term consequences on the immunity of veal calves, because high colostrum N-Ig's titers, in particular of N-IgG, were associated with high N-Ig's titers in serum of calves, even two weeks after arrival at the veal farm. High N-IgG titers in calf serum were associated with less individual antibiotic and other medical treatments at the veal farm, which might be an indication of improved robustness. ■

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Appendix 1

Table A1. Effects of parity of cows and ease of birth on the hematological profile of calves measured one day prior to transport (LS means).

	Parity						Ease of birth			
Parameter	1	2	3	4-10	SEM[1]	*P*-value	Unassisted	Assisted	SEM	*P*-value
N. of calves	80	148	144	245			501	151		
Hemoglobin, mmol/l	6.68	6.46	6.48	6.48	0.17	0.53	6.54	6.51	0.16	0.80
Hematocrit,%	31.28	30.45	30.62	30.54	0.78	0.65	30.79	30.65	0.73	0.76
MCV[2], fl	33.91	33.82	34.11	33.87	0.44	0.83	33.90	33.95	0.40	0.86
MCH[3], amol	720.2	712.2	717.5	715.1	9.3	0.74	715.6	716.9	8.7	0.82
MCHC[4], mmol/l	21.31	21.15	21.08	21.15	0.11	0.49	21.17	21.17	0.10	0.99
RDW, SD[5], %	37.54	37.08	37.19	37.61	0.32	0.72	37.47	36.83	0.24	0.52
RDW, CV[6], %	32.22	31.72	31.44	31.77	0.26	0.88	31.86	31.19	0.18	0.44
RBC[7], 10^{12}/l	9.28	9.08	9.01	9.07	0.17	0.58	9.11	9.10	0.15	0.94
WBC[8], 10^9/l	11.2	11.0	10.7	11.2	0.44	0.40	10.8	11.2	0.38	0.47
Neutrophils, 10^9/l	4.04	3.97	3.95	4.47	0.21	0.60	4.12	4.24	0.16	0.77
Neutrophils, %	35.35	36.01	36.53	37.25	1.03	0.11	36.57	36.08	0.81	0.84
Lymphocytes, 10^9/l	5.34	5.23	4.78	5.08	0.23	0.27	5.05	5.17	0.18	0.47
Lymphocytes*, %	48.97	47.57	47.65	45.96	1.01		47.08	47.69	0.80	
Monocytes, 10^9/l	14.46	13.60	13.13	14.31	1.00	0.26	13.89	13.87	0.92	0.97
Monocytes, %	13.15	13.54	13.15	13.86	0.29	0.79	13.55	13.35	0.22	0.27
Basophils, 10^9/l	0.11	0.11	0.10	0.11	0.003	0.38	0.11	0.11	0.002	0.91
Basophils, %	1.04	1.04	1.03	1.02	0.03	0.99	1.03	1.02	0.01	0.93
Eosinophils, 10^9/l	0.17	0.22	0.17	0.23	0.03	0.30	0.20^a	0.21^b	0.02	0.03
Eosinophils, %	1.49^a	1.84^b	1.63^{ab}	1.91^b	0.20	< 0.01	1.76^a	1.86^b	0.16	< 0.01

*[1]SEM = pooled standard error; [2]MCV = mean corpuscular volume; [3]MCH = mean corpuscular hemoglobin; [4]MCHC = mean corpuscular hemoglobin concentration; [5]RDW, SD = red blood cell width, standard deviation; [6]RDW, CV = red blood cell width, coefficient of variation; [7]RBC = red blood cells; [8]WBC = white blood cells. *The statistical model for this variable did not converge. [a, b]LSmeans within a factor and line lacking a common superscript differ (P ≤ 0.05).*

Appendix 2

Table A2. Effects parity of cows and ease of birth on the hematological profile of calves measured in week 2 post-transport (LS means).

	Parity				SEM[1]	*P*-value
	1	2	3	4-10		
N. of calves	86	167	152	253		
Hemoglobin, mmol/l	6.71	6.31	6.38	6.41	0.23	0.10
Hematocrit,%	30.66	29.13	29.56	29.54	0.95	0.16
MCV[2], fl	31.82	31.72	31.85	31.56	0.46	0.79
MCH[3], amol	692.5	677.7	684.2	680.0	11.4	0.41
MCHC[4], mmol/l	21.65	21.31	21.26	21.49	0.10	0.07
RDW, SD[5]*, %	35.84	36.20	36.36	36.19	0.31	
RDW, CV[6], %	32.86	32.54	32.58	32.56	0.24	0.95
RBC[7], 10^{12}/l	9.66	9.25	9.29	9.40	0.25	0.18
WBC[8], 10^9/l	10.78	10.16	10.18	10.16	0.44	0.61
Neutrophils, 10^9/l	2.23	2.42	2.47	2.61	0.21	0.98
Neutrophils*, %	20.03	22.81	22.49	23.35	1.02	
Lymphocytes, 10^9/l	6.51	6.02	6.02	6.13	0.28	0.26
Lymphocytes*, %	64.55	60.46	60.54	59.96	1.00	
Monocytes, 10^9/l	14.73	14.88	14.09	14.24	0.88	0.75
Monocytes, %	13.69	14.48	14.22	14.29	0.29	0.50
Basophils, 10^9/l	0.12	0.13	0.16	0.14	0.009	0.65
Basophils, %	1.19	1.34	1.55	1.41	0.07	0.16
Eosinophils, 10^9/l	0.05	0.09	0.13	0.10	0.01	0.06
Eosinophils*, %	0.54	0.90	1.19	0.98	0.10	

*[1]SEM = pooled standard error; [2]MCV = mean corpuscular volume; [3]MCH = mean corpuscular hemoglobin; [4]MCHC = mean corpuscular hemoglobin concentration; [5]RDW, SD = red blood cell width, standard deviation; [6]RDW, CV = red blood cell width, coefficient of variation; [7]RBC = red blood cells; [8]WBC = white blood cells. * The statistical model for these variables did not converge.*

	Ease of birth		SEM	*P*-value
	Unassisted	Assisted		
	529	154		
	6.48	6.43	0.22	0.70
	29.92	29.52	0.91	0.39
	31.93	31.55	0.43	0.18
	686.6	680.6	10.7	0.36
	21.38	21.56	0.07	0.42
	36.15	36.24	0.24	
	32.60	32.47	0.17	0.59
	9.36	9.44	0.24	0.61
	10.42	10.22	0.39	0.44
	2.44	2.60	0.14	0.20
	22.45	22.96	0.8	
	6.28	6.06	0.26	0.25
	60.73	60.98	0.79	
	14.52	14.45	0.75	0.91
	14.39	13.92	0.23	0.95
	0.14	0.13	0.006	0.77
	1.41	1.32	0.05	0.84
	0.11	0.08	0.01	0.42
	1.00	0.82	0.08	

CHAPTER 7

Effects of transport age and calf and cow characteristics

on health and performance of veal calves

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Abstract

The objective of the study was to investigate effects of calf transport age (14 d vs. 28 d) and calf (sex and breed) and dam characteristics (parity and ease of birth) on health and performance of veal calves until slaughter age. Calves (N = 684) originated from 13 dairy farms in the Netherlands and were transported at either 14 d or 28 d of age from the dairy farm to 8 Dutch veal farms. A health assessment of calves was performed on a weekly basis at the dairy farm and in weeks 2, 10, 18 and 24 at the veal farm. Body weight of calves was measured one day prior to transport and upon arrival at the veal farm. At the veal farm, use of antibiotics, other medicines during the rearing period (both at herd and individual level) and carcass weights were recorded. Calves transported at 28 d had a higher body weight upon arrival (Δ =11.8 kg), a higher carcass weight at slaughter (Δ = 14.8 kg), received less individual treatments with medicines other than antibiotics (Δ = -5.4%), and had a lower mortality rate (Δ = -3.1%) at the veal farm compared to calves transported at 14 d. Crossbreds other than Belgian Blue × Holstein Friesian received a higher number of individual treatments with antibiotics and other medicines (Δ = 14.8% and Δ = 15.1%, respectively) at the veal farm compared to Belgian Blue × Holstein Friesian calves. One day prior to transport, body weight of calves born from first parity cows was lower (Δ = -4.7 kg on average) compared to calves born from higher parity cows. The results show that both calf and dam characteristics influenced later health and performance of veal calves.

Key words: veal calf, transport age, health, performance

Introduction

In the Netherlands, as well as in other European countries, veal calves are collected from dairy farms and are transported to a collection center before entering the veal sector (Damiaans et al., 2019). In the collection center, calves are sorted by body weight, breed or conformation and thereafter they are transported to a veal farm. The legally required minimum age at which calves are transported and enter the Dutch veal sector is 14 d (SBK, 2018). Upon arrival at the veal farm, calves frequently exhibit health problems (Bähler et al., 2012; Pempek et al., 2017; Renaud et al., 2018b), some of which are associated with a higher risk of mortality (Pempek et al., 2017; Renaud et al., 2018b). The sometimes high disease and mortality rates in veal calves are likely related to the various challenges these animals are exposed to, including transport, irregular meals and mixing with other animals (Pempek et al., 2017). Most importantly, in comparison with older animals, young calves have a higher risk of infection with pathogens because of an immature immune system and an additional risk of failure of passive transfer due to insufficient colostrum intake (Autio et al., 2007; Pardon et al., 2015); they could, therefore, be considered less 'robust' than older animals. Robustness can be defined as the ability of calves to cope with environmental challenges and to bounce back rapidly when challenges occur (Colditz and Hine, 2016). Robust calves are better equipped to cope with endemic infections and fight diseases and thus probably have a lower need for antimicrobials. In a companion paper (Marcato et al., 2021a) we looked at potential early indicators of robustness measured in blood of calves, including immunoglobulins (Pardon et al., 2015; Goetz et al., 2021) and hematological parameters (von Konigslow et al., 2020).

The current study will examine the clinical health status and mortality rate of calves, and will quantify the number of antibiotic and other medical treatments as more ultimate measures of robustness (Marcato et al., 2018; de Almeida et al., 2019). Our central hypothesis was that calves transported at 28 days of age from the dairy farm to the veal farm are more robust, have a greater adaptive capacity and, therefore, show less clinical health problems compared to calves transported at 14 days of age. An improved health status of calves transported at 28 days of age might contribute to a lower number of medical treatments and, possibly, a higher carcass weight compared to calves transported at 14 days of age. Thus, the main aim of the current study was to examine – in a longitudinal fashion – the effects of transport age on measures of health and performance, and the mortality rate of calves at the veal farm. In addition, we aimed to investigate the extent to which characteristics related to the calves (breed and sex) and their dams (parity and ease of birth) might affect the robustness of veal calves. Recent studies provided support for the relevance of these characteristics for

health and performance of beef cattle (Diana et al., 2020) and female offspring of dairy cows (Astiz et al., 2014; Pinedo and De Vries, 2017). In the present experiment, calves were individually followed prospectively from birth at the dairy farm until the end of the fattening period at the veal farm.

Materials and methods

Experimental design

The experiment was conducted between March 2019 and May 2020, and was approved by the Central Committee on Animal Experiments (the Hague, the Netherlands; approval number 2017.D-0029). The experimental design was a matrix, consisting of 13 dairy farms and 8 veal farms (see Marcato et al., 2021a). Calves (N= 684) originated from 13 different Dutch dairy farms. Within each dairy farm, calves were assigned to one or two treatment groups, i.e. transport to the veal farm at either 14 or 28 d of age. Calves of all 13 dairy farms were transported to 8 veal farms. At each transport day, two transporters collected calves from the dairy farms (6 and 7 dairy farms, respectively) and brought these directly to the veal farm, meaning that for each veal farm in total 4 transports were performed. More details on the experimental design are described in our companion paper (Marcato et al., 2021a).

Health assessment

Health assessments of calves were performed at the dairy farms on a weekly basis until one day prior to transport. All calves were examined individually on the basis of a fixed protocol (see protocol 1, Appendix 1). During each clinical assessment, the rectal temperature was also recorded. At the veal farm, health assessments took place in weeks 2, 6, 10, 18 and 24 post-transport. In week 2 post-transport, calves were housed individually and their health was examined using protocol 1; at all other moments, calves were group housed in pens, but calves were still individually examined on the basis of the Welfare Quality® Protocol on veal calves (Appendix 2; Brscic et al. (2012)). Presence of loose or liquid manure, thick and white manure were the only items scored at pen level. Health assessments of calves at the dairy and veal farms were performed by in total 5 different observers. These observers were thoroughly trained, theoretically as well as practically (on-farm), and inter-observer reliability was tested at the end of the training period (k-coefficient = 98%).

Antibiotics and other medical treatments

At both dairy and veal farms, the use of antibiotics and other medical treatments was recorded at individual calf level. Information on individual treatments included the following data: 1) calf treated or not with antibiotics or other medicines (this latter category

referred to products, other than antibiotics such as anti-inflammatories, multivitamins, and anti-coccidiosis; 2) whether single or repeated antibiotic/medical treatments were applied; 3) age at which treatments were applied. Herd treatments (applied on all calves, via the milk) were also recorded during the rearing period at the veal farm, as well as the age at which the herd treatments were applied and the type of medication that was used.

Body weight, average daily gain (ADG) and carcass weight

All calves at the dairy farms were weighed on an portable scale directly after birth and weekly thereafter until one day prior to transport. Subsequently, the body weight of each calf was recorded upon arrival at the veal farm and carcass weight was finally recorded at slaughter. ADG at the dairy farm was calculated on a weekly basis.

Calf and cow characteristics

Characteristics of calves and their dams were recorded at the dairy farm. With regard to calves, body weight at birth, breed and sex were recorded; with regard to cow characteristics, parity of the dam and ease of birth of calves were recorded. Ease of birth was recorded as a binary response, where a score = 0 indicated a calving process without the assistance of the farmer, and a score = 1 indicated that assistance of the farmer was required during the calving process.

Mortality rate at the veal farm

An index of mortality rate at the veal farm was calculated separately for calves transported either at 14 or 28 d of age, as well as for different breeds and sexes of calves. Total mortality was calculated as the sum of the numbers of calves that died, were euthanized by the veterinarian for health reasons, or were prematurely slaughtered because of poor performance (i.e. did not successfully complete the rearing period at the veal farm).

Data processing and statistical analyses

All statistical analyses were performed with SAS 9.4 (SAS Inst. Inc., Cary, NC). In the current study, variables were either analyzed as binary variables or as continuous variables. Binary variables were analyzed with a generalized linear mixed model comprising a logit link function, whereas continuous response variables were analyzed with a linear mixed model. In the latter case, residuals were checked for normality and homogeneity of variance and variables were log-transformed when needed. Binary variables were analyzed with a linear mixed model with Pseudo Likelihood or equivalently Penalized Quasi Likelihood (PQL), employing SAS procedure GLIMMIX, whereas continuous variables were analyzed with a linear mixed model with restricted maximum likelihood, employing SAS procedure MIXED.

Health parameters were analyzed as binary data with score 0 vs score 1 and 2. Individual treatments with antibiotics or medicines were also expressed as binary data (0 = calf not treated at individual level with antibiotics; 1 = calf treated at least once at individual level with antibiotics). Rectal temperature was assessed as a continuous variable, but analyzed as a binary variable (0 = calves with temperature < 39.5° C; 1 = calves with temperature ≥ 39.5° C) to investigate prevalence of fever. Body weight and carcass weights were analyzed as continuous variables

In week 1 after birth of calves at the dairy farm, health parameters and body weight were analyzed with a linear mixed model (referred to as model 1), which comprised fixed main effects for factors Sex (two levels: bull or heifer) and Breed (four levels: Holstein Friesian, Holstein-Friesian × Belgian Blue, or other crossbreds) of calves, for Parity (four levels: 1, 2, 3, 4-10) of dams, for Ease of birth (two levels: without assistance (score 0) or with assistance of the farmer (score 1)), a covariate for calf birthweight among fixed effects, and random effects for dairy farms in addition to random error (residual) terms. Random farm effects account for dependence between observations from calves from the same farm and represent between farm variation and random error terms represent (remaining) variation between calves within the same farm.

One day prior to transport, health parameters and body weight were analyzed with a linear mixed model (further referred to as model 2) similarly to model 1, but with the inclusion of a factor at two levels for transport age (14 d of age vs. 28 d of age).

Health data collected in week 2, 6, 10, 18 and 24 post-transport at the veal farm were analyzed similarly to model 2, but the linear mixed model used for analysis (referred to as model 3) was more elaborate. The model comprised random effects for dairy farms, for veal farms, and their interaction, random effects for transports, and random effects for animals, in addition to the usual random error (residual) terms.

Data on individual treatments with antibiotics during the rearing period at the dairy and the veal farm were analyzed with the same model as was used for health data assessed at the veal farm (model 3). The total usage of antibiotics and other medicines at the dairy and veal farms was analyzed with model 2.

Carcass weights at slaughter were analyzed with a linear mixed model with the same fixed and random effects in the model used for health data assessed at the veal farm (model 3).

Carcass weights were also analyzed in relation to the number of individual treatments with antibiotics or other medicines at both dairy and veal farms, which was introduced as a qualitative factor in model 2, comprising five main levels: 0 = calf not treated; 1 = calf treated once; 2 = calf treated 2 times; 3 = calf treated 3 times; 4 = calf treated ≥ 4 times.

Average daily gain at the dairy farm and body weight at arrival at the veal farm were also analyzed in relation to individual treatments with antibiotics or other medicines, and carcass weights. Individual treatments and carcass weight were used as response variables, and ADG and body weight at arrival at the veal farm were introduced as covariates in the model (same as model 3). Interactions between the covariates and the fixed factors in the model were tested to check whether or not slopes were equal for fixed effect levels.

In all analyses, approximate F-tests (Kenward and Roger, 1997) were used for fixed effects. Subsequent pairwise comparisons were done with Fisher's LSD method. Effects with $P \leq 0.05$ were considered significant.

Mortality rates were too low to allow for a parametric analysis as described above. Therefore, the non-parametric Wilcoxon Signed Rank Test was performed to test for differences in mortality rate at the veal farm between calves transported at either 14 or 28 d of age and for different breeds and sexes of calves. For each veal farm, two matched (dependent) observations were available, i.e. the mortality rates among calves transported at 14 and 28 d, or among calves of Holstein Friesian an a mixed breed, or among male and female calves, respectively. Effects with $P \leq 0.05$ were considered significant.

Results

Effects

Transport age

One day prior to transport, the percentage of calves with loose or liquid manure was significantly higher (Δ = 16.6%) in calves transported at 14 d compared to calves transported at 28 d ($P < 0.01$; Table 1). The percentage of calves with an impaired skin elasticity (score 1 and 2; Δ = 6%) was higher in the 14 d group compared to the 28 d group, whereas body weight at transport was lower (Δ = -11.8 kg) in calves transported at 14 d than calves transported at 28 d ($P < 0.05$; Table 1). Transport age did not have significant effects on health parameters assessed two weeks after arrival at the veal farm (Table 2). Prevalences of health problems beyond week 2 at the veal farm were low (< 10%), thus effects of transport age, as well as for breed, sex, parity and ease of birth could not be statistically tested. The percentage of calves which received at least one individual

medical treatment other than antibiotics at the veal farm was higher (Δ = 5.4%) in 14 d calves compared to 28 d calves (P = 0.02; Table 3). Total amount of antibiotics or other medicines administered individually to calves at both the dairy and the veal farm was not significantly different among the two age groups (Table 3). Calves transported at 14 d had a lower carcass weight at slaughter (Δ = -14.8 kg) compared to calves transported at 28 d (P < 0.01; Table 4).

Calf sex

Body weight measured in week 1 after birth and a day prior to transport was significantly higher (Δ = 1.8 kg and Δ = 5.8 kg, respectively) in bull calves compared to female calves (both P < 0.01; Table 5 and Table 1, respectively). Calf sex did not affect health parameters assessed at the dairy farm (week 1 and one day prior to transport), but bull calves showed more signs of navel inflammation (Δ = 7.3%) compared to female calves in week 2 post-transport (P = 0.02; Table 2). Beyond week 6 post-transport, sex did not affect health. In terms of individual antibiotic and other medical treatments, no significant differences were found between bull and females calves (Table 3). Carcass weights at slaughter were higher (Δ = 16.8 kg, respectively) in bull calves compared to female calves (P < 0.01; Table 4).

Calf breed

Belgian Blue crossbred calves had a higher body weight in week 1 after birth (Δ = 1.5 kg on average) and a day prior to transport (Δ = 4.6 kg on average) compared to Holstein Friesian calves and other crossbreds (both P < 0.01; Table 5 and 1, respectively). Breed did not affect clinical health parameters assessed at the dairy and veal farms. Total use of individual antibiotic and other medicine treatments in calves at the dairy and veal farms was higher in other crossbreds calves (Δ = 17.3% and Δ = 20.2% on average respectively) compared to Holstein Friesian calves and Belgian Blue crossbreds (both P < 0.05; Table 3). The prevalence of calves individually treated with antibiotics and other medicines at the veal farm was also higher in other crossbred calves (Δ = 13.6 % and Δ = 13.4% on average, respectively) compared to Holstein Friesian calves and Belgian Blue crossbreds (both P = 0.04; Table 3). Breed had a significant influence on carcass weight at slaughter (P < 0.05. Table 4). Belgian Blue crossbreds had a higher carcass weight compared to other crossbreds (Δ = 9.7 kg) and Holstein Friesian calves (Δ = 17.9 kg, P < 0.01).

Parity

In week 1 after birth, calves born from second parity cows showed less signs of navel inflammation than calves from third parity cows (Δ = -12.1%, P = 0.02; Table 5) and other parity classes. One day prior to transport, body weight of calves born from first parity cows (Δ = -4.7 kg on average) was significantly lower compared to calves born from

higher parity cows ($P < 0.01$; Table 6). Parity did not significantly affect health parameters assessed at the veal farm, or the individual use of antibiotics and other medical treatments (Table 7). In addition, carcass weights at slaughter were not significantly influenced by parity (Table 8).

Ease of birth

Calves born with assistance of the farmer had a higher body weight a day prior to transport (Δ = 2.5 kg; $P < 0.01$; Table 6). Ease of birth did not affect any health parameters assessed at the dairy and veal farms and did not have an influence on individual use of antibiotics and other medical treatments (Table 5, 7, 9). Carcass weights were also not significantly affected by ease of birth (Table 8).

Herd treatments with antibiotics and medicines

Besides individual treatments, calves were subjected to an average of 4.4 herd treatments with antibiotics and 3.9 herd treatments with other medicines at the veal farm. The antibiotic treatments were provided via the milk for an average of 10 feedings over 5 days per treatment.

Relationships

Between individual use of antibiotics or other medicines and carcass weight

Calves that received three or more individual treatments with antibiotics or other medicines had a significantly lower carcass weight at slaughter (Δ = -13.6 kg on average for antibiotics and Δ = 15.0 kg on average for other medicines, respectively) compared to calves treated once or twice ($P < 0.01$;Table 10). Additionally, calves not treated at all had a significantly higher carcass weight compared to calves individually treated at least once with antibiotics (Δ = 19.6 kg on average) and other medicines (Δ =16.1 kg on average). The results on individual treatments with antibiotics and other medicines at the veal farm were also in line with the results on total use (Table 10).

Between body weight upon arrival or ADG at dairy farm and later performance

The relationship between use of antibiotics or other medicines and body weight upon arrival was not significant. The relationship between use of antibiotics or other medicines and ADG at the dairy farm arrival was also not significant. Body weight upon arrival and ADG at the dairy farm were both positively associated with carcass weight at slaughter (slope ß = 0.228, SE = 0.05, and slope ß = 15.1, SE = 3.12, respectively; $P <$ 0.01). These regression coefficients were obtained from regression models without the interaction term, i.e. on the assumption of homogeneity of regression slopes (slopes are parallel for different ages of transport, parity groups, sexes and breeds). The interaction between body weight upon arrival and the fixed effect of transport age was

significant ($P < 0.01$). This implies that the slopes of the covariable body weight upon arrival were not the same for the two age groups. The effect of body weight upon arrival on carcass weight was much higher for calves transported at 28 d of age (slope ß = 0.85, SE = 0.13) than for calves transported at 14 d of age (slope ß = 0.13, SE = 0.05).

Table 1. **Effects of calf transport age, sex and breed on prevalence of health problems (% of calves) and body weight of calves assessed one day prior to transport from the dairy farm to the veal farm**[1]

Parameter	Transport age		SEM[2]	*P*-value	Sex	
	14 d	28 d			Bull	Heifer
N. of calves	372	326			522	176
Navel inflammation	19.6	12.9	2.0	0.23	17.0	14.8
Loose or liquid manure	23.6	7.0	1.8	< 0.01	15.5	17.0
Joint problems	3.2	5.5	1.3	0.15	5.0	2.3
Coughing	3.8	4.9	1.3	0.47	4.2	4.5
Eye discharge	5.4	3.7	1.3	0.67	4.6	4.5
Sunken eyes	2.7	1.2	0.8	0.32	1.7	2.8
Nose discharge	2.4	4.0	0.9	0.38	3.8	1.1
Skin elasticity	7.2	1.2	1.2	<0.01	4.8	2.8
Rectal temperature*	5.6	3.4	1.1		4.0	6.2
Body weight, kg	56.3	68.1	1.9	<0.01	65.1	59.3

[1] *LSmeans ± SEM;* [2] *SEM = pooled standard error of the mean;* [3] *Breed: HF = Holstein Friesian, HF× BB = Holstein Friesian × Belgian Blue crossbreds, O = other crossbreds;* [a, b, c] *LSmeans within a factor and line* >>

Table 2. **Effects of calf transport age, sex and breed on prevalence of health problems (% of calves) of calves assessed in week 2 post-transport at the veal farm**[1].

Parameter	Transport age		SEM[2]	*P*-value	Sex	
	14 d	28 d			Bull	Heifer
N. of calves	365	320			512	173
Navel inflammation	15.1	10.1	1.8	0.14	14.2	6.9
Loose or liquid manure	34.5	37.2	2.6	0.25	35.7	35.8
Joint problems	4.4	2.2	0.9	0.06	3.9	1.7
Coughing	4.1	6.9	1.2	0.31	4.9	6.9
Sunken eyes	3.6	3.1	1.0	0.60	3.3	3.4
Looped ears	3.8	2.5	0.9	0.15	2.9	4.0

[1] *LSmeans ± SEM.* [2]*SEM = pooled standard error of the mean;* [3]*Breed: HF = Holstein Friesian, HF× BB = Holstein Friesian × Belgian Blue crossbreds, O = other crossbreds.*

At the same time, after introduction of body weight upon arrival as a covariable in the model, the effect of transport age on carcass weight remained statistically significant (*P* < 0.01). Carcass weights corrected for body weight upon arrival of calves transported at either 14 or 28 d of age were 152.9 ± 4.6 and 165.7 ± 4.7 kg, respectively (LSmeans ± SEM).

SEM	*P*-value	Breed[3]			SEM	*P*-value
		HF	HF× BB	O		
		240	252	206		
2.2	0.24	12.1	15.5	22.8	2.4	0.32
2.2	0.71	15.8	17.1	14.5	2.4	0.95
1.3	0.33	2.9	1.6	9.7	1.5	0.50
1.5	0.49	4.6	3.6	4.8	1.6	0.80
1.5	0.86	7.9	2.7	1.4	1.4	0.51
1.0	0.10	3.7	1.2	1.0	0.9	0.14
0.8	0.20	5	2.8	1.4	1.1	0.55
1.3	0.43	2.5	1.6	10.2	1.4	0.29
1.3		4.6	5.5	3.4	1.3	
1.9	<0.01	59.8[a]	65.3[b]	61.5[a]	2.0	<0.01

*<< lacking a common superscript differ (P ≤ 0.05). *The statistical model did not converge and this explains the lack of P-values for rectal temperature.*

SEM	*P*-value	Breed[3]			SEM	*P*-value
		HF	HF× BB	O		
		236	246	203		
1.9	0.02	10.6	14.6	13.8	2.4	0.12
2.9	0.64	12.3	9.3	13.8	3.4	0.93
0.9	0.32	2.9	2.8	4.4	1.4	0.86
1.4	0.27	7.2	4.9	3.9	1.4	0.14
1.1	0.81	4.2	2.8	2.9	1.2	0.84
1.1	0.83	3.3	3.6	2.4	1.1	0.93

Table 3. Effects of calf transport age, sex and breed on prevalence (%) of calves individually treated with antibiotics and other medicines[1].

Parameter	Transport age		SEM[2]	*P*-value	Sex	
	14 d	28 d			Bull	Heifer
N. of calves	362	318			507	173
Total use of antibiotics[4]	30.4	28.9	2.5	0.79	31.1	25.4
Total use of medicines[5]	34.0	32.1	2.6	0.62	34.3	29.5
At the veal farm:						
Use of antibiotics	24.3	21.4	2.3	0.14	23.9	20.2
Use of other medicines	27.1	21.7	2.3	0.02	25.4	22.0

[1] *LSmeans ± SEM.* [2]*SEM = pooled standard error of the mean;* [3]*Breed: HF = Holstein Friesian, HF× BB = Holstein Friesian × Belgian Blue crossbreds, O = other crossbreds;* [4, 5] *Total use of antibiotics or* >>

Table 4. Effects of calf transport age, sex and breed on carcass weight of calves at slaughter[1].

Parameter	Transport age		SEM[2]	*P*-value	Sex	
	14 d	28 d			Bull	Heifer
N. of calves	350	315			469	169
Carcass weight, kg	152.0	166.8	4.5	< 0.01	167.8	151.0

[1] *LSmeans ± SEM.*[2]*SEM = pooled standard error of the mean;* [3]*Breed: HF = Holstein Friesian, HF× BB = Holstein Friesian × Belgian Blue crossbreds, O = other crossbreds;*>>

Table 5. Effects of calf sex and breed, parity of dam and ease of birth on prevalence of health problems (% of calves) and body weight of calves in week 1 after birth at the dairy farm[1].

Parameter	Sex		SEM[2]	*P*-value	Breed[3]		
	Bull	Heifer			HF	HF× BB	O
N. of calves	536	189			259	258	208
Navel inflammation	36.4	29.6	2.7	0.34	30.1	32.1	42.3
Loose or liquid manure	18.6	22.2	2.4	0.13	15.0	20.9	23.5
Joint problems	6.7	3.7	1.3	0.75	6.5	3.5	8.1
Nose discharge	3.9	2.1	1.1	0.43	3.9	5.0	1.0
Skin elasticity	13.2	7.4	1.7	0.15	6.9	8.5	21.6
RT[5]	9.5	11.6	1.7		5.8	12.8	11.9
Body weight, kg	48.7	46.9	0.66	< 0.01	47.3[a]	48.8[b]	47.4[ab]

[1] *LSmeans ± SEM.* [2]*SEM = pooled standard error of the mean;* [3]*Breed: HF = Holstein Friesian, HF× BB = Holstein Friesian × Belgian Blue crossbreds, O = other crossbreds;* [4]*Ease of birth: 0 = unassisted;* >>

SEM	*P*-value	Breed[3]			SEM	*P*-value
		HF	HF× BB	O		
		229	248	203		
2.7	0.42	25.3^{a}	23.8^{a}	41.9^{b}	3.0	0.02
2.8	0.68	28.4^{ab}	25.8^{a}	47.3^{b}	3.1	0.03
2.5	0.85	20.1^{ab}	17.7^{a}	32.5^{b}	2.8	0.04
2.5	0.95	22.2^{ab}	18.9^{a}	34.0^{b}	2.9	0.04

<< medicines refers to the sum of antibiotics or other medical treatments given at the dairy and veal farm. [a, b, c] *LSmeans within a factor and line lacking a common superscript differ (P ≤0.05).*

SEM	*P-value*	Breed[3]			SEM	*P*-value
		HF	HF× BB	O		
		209	207	197		
4.5	< 0.01	150.7^{b}	168.6^{c}	158.9^{a}	4.7	< 0.01

<< [a, b, c] *LSmeans within a factor and line lacking a common superscript differ (P ≤0.05).*

SEM	*P*-value	Parity				SEM	*P*-value	Ease of birth[4]		SEM	*P*-value
		1	2	3	4-10			0	1		
		98	169	153	273			536	155		
3.1	0.34	35.7^{ab}	27.8^{a}	39.9^{b}	36.6^{ab}	3.8	0.02	34.5	34.8	2.9	0.67
2.6	0.89	14.3	16.6	18.3	22.7	3.0	0.25	19.8	19.3	2.5	0.79
1.6	0.44	10.2	7.7	6.5	3.3	2.2	0.47	5.6	5.2	1.4	0.59
1.4	0.50	4.0	3.6	5.2	2.6	1.8	0.86	4.1	1.9	1.1	0.41
2.2	0.77	11.2	18.9	6.5	9.9	2.6	0.11	12.3	10.3	2.0	0.66
1.9		4.0	8.3	7.8	14.6	2.7		10.2	9.0	1.8	
0.76	< 0.01	47.8	47.6	48.3	47.7	0.70	0.52	47.7	47.9	0.65	0.61

<< 1= assisted by the farmer; [5] *RT = rectal temperature.* [a, b, c] *LSmeans within a factor and line lacking a common superscript differ (P ≤ 0.05).*

Table 6. Effects of parity of the dam and ease of birth on prevalence of health problems (% of calves), body weight and rectal temperature of calves assessed a day prior to transport[1].

Parameter	Parity				SEM[2]	*P*-value
	1	2	3	4-10		
N. of calves	91	167	151	259		
Navel inflammation	11.0	16.8	14.6	19.7	2.9	0.66
Loose or liquid manure	16.5	12.6	16.5	17.8	3.0	0.49
Joint problems	3.3	6.6	2.0	4.6	1.8	0.19
Coughing	6.6	1.2	6.0	5.0	2.1	0.20
Eye discharge	4.4	5.4	2.6	4.2	2.1	0.22
Sunken eyes	1.0	4.2	0.7	1.9	1.1	0.34
Nose discharge	2.2	1.8	3.9	4.2	1.4	0.34
Skin elasticity	6.6	4.2	4.0	4.6	2.0	0.72
Body weight, kg	58.7^{c}	62.5^{a}	63.4^{ab}	64.2^{b}	1.9	<0.01
Rectal temperature*	4.4	3.6	4.6	5.4	1.6	

[1] *LSmeans ± SEM;* [2]*SEM = pooled standard error of the mean.* [a, b, c] *LSmeans within a factor and line lacking a common superscript differ (P ≤0.05).* **The statistical model did not converge and this explains the lack of P-values for rectal temperature.*

Table 7. Effects of parity and ease of birth on prevalence (% of calves) of individual treatments with antibiotics and other medicines[1].

Parameter	Parity				SEM[2]	*P*-value
	1	2	3	4-10		
N. of calves	88	166	146	254		
Total use of antibiotics[3]	38.6	32.5	23.3	28.7	3.8	0.20
Total use of medicines[4]	42.0	36.7	28.8	30.7	3.9	0.40
At the veal farm:						
Use of antibiotics	31.8	21.7	18.5	23.6	3.5	0.47
Use of other medicines	32.9	22.9	22.6	24.4	3.6	0.59

[1] *LSmeans ± SEM;* [2]*SEM = pooled standard error of the mean;* [3, 4] *Total use of antibiotics or medicines refers to the sum of antibiotics or other medical treatments given at the dairy and veal farm.*

Table 8. Effects of parity of the dam and ease of birth on carcass weight at slaughter[1].

Parameter	Parity				SEM[2]	*P*-value
	1	2	3	4-10		
N. of calves	87	164	145	244		
Carcass weight, kg	154.8^{a}	160.7^{ab}	159.1^{ab}	163.0^{b}	4.74	0.07

[1] *LSmeans ± SEM;* [2]*SEM = pooled standard error of the mean.* [a, b, c] *LSmeans within a factor and line lacking a common superscript differ (P ≤0.05).*

	Ease of birth		SEM	*P*-value
	Unassisted	Assisted		
	539	156		
	15.6	18.6	2.3	0.23
	14.6	19.9	2.4	0.49
	3.7	6.4	1.5	0.03
	4.1	5.1	1.4	0.43
	4.1	5.8	1.6	0.74
	2.4	0.6	0.7	0.16
	3.0	3.8	1.1	0.48
	4.8	3.2	1.2	0.39
	61.0	63.5	1.9	<0.01
	4.6	3.8	1.2	

	Ease of birth		SEM	*P*-value
	Unassisted	Assisted		
	526	151		
	28.7	33.8	2.9	0.41
	32.1	37.1	3.0	0.55
	21.9	27.1	2.7	0.67
	23.4	29.1	2.8	0.55

	Ease of birth		SEM	*P*-value
	Unassisted	Assisted		
	514	148		
	158.6	160.2	4.5	0.49

Table 9. Effects of parity of the dam and ease of birth on prevalence of health problems (% of calves) observed in week 2 post-transport at the veal farm[1].

Parameter	Parity				SEM[2]	*P*-value
	1	2	3	4-10		
N. of calves	90	166	149	252		
Navel inflammation	13.3	15.1	14.1	11.5	2.8	0.77
Loose or liquid manure	34.4	43.4	31.5	34.5	3.9	0.15
Joint problems	5.5	2.4	3.3	3.2	1.6	0.29
Coughing	4.4	7.8	3.3	5.5	1.8	0.28
Sunken eyes	3.3	4.2	4.0	2.4	1.5	0.69
Looped ears	1.1	2.4	4.0	3.6	1.3	0.60

[1] *LSmeans ± SEM;* [2]*SEM = pooled standard error of the mean.*

Table 10. Association between carcass weight of calves and the use of individual treatments with antibiotics or other medicines.

	Parameter	N. calves	Carcass weight (kg)	SEM	*P*-value
Dairy + veal farm	Total use of antibiotics			4.14	< 0.01
	0	478	163.4^{d}		
	1	118	151.9^{c}		
	2	39	149.2^{bc}		
	3	19	139.2^{ab}		
	≥ 4	26	134.7^{a}		
	Total use of medicines			3.67	< 0.01
	0	455	163.8^{c}		
	1	57	155.8^{b}		
	2	82	154.6^{b}		
	3	19	140.9^{a}		
	≥ 4	67	139.4^{a}		
Veal farm	Use of antibiotics			5.72	< 0.01
	0	524	162.7^{c}		
	1	84	149.9^{b}		
	2	35	144.3^{ab}		
	3	17	144.1^{ab}		
	≥ 4	20	135.2^{a}		

	Ease of birth		SEM	*P*-value
	Unassisted	Assisted		
	529	153		
	13.2	12.4	2.1	0.34
	35.0	37.9	3.0	0.55
	3.4	2.6	1.0	0.16
	5.7	4.6	1.4	0.49
	3.6	2.6	1.1	0.47
	3.2	3.3	1.1	0.88

Table 10. Continued

	Parameter	N. calves	Carcass weight (kg)	SEM	*P*-value
Veal farm	Use of medicines			5.69	< 0.01
	0	513	162.8^{d}		
	1	19	155.7^{cd}		
	2	71	151.4^{bc}		
	3	15	141.5^{ab}		
	≥ 4	62	141.0^{a}		

$^{a, b, c, d}$ *LSmeans within a factor and line lacking a common superscript differ (P ≤0.05).*

Mortality rate at the veal farm

Mortality at the veal farm was higher in calves transported at 14 d of age compared to calves transported at 28 d of age (5.9 % vs. 2.8 %, respectively, $P < 0.05$, Wilcoxon Signed Rank Test). Notably, on 5 out of the total of 8 veal farms involved in the present experiment no mortality at all occurred during the entire rearing period among calves were transported at 28 d of age, whereas among calves transported at 14 d of age this only held for 1 veal farm. No significant differences in mortality rate were found between different breeds or sexes of calves.

Discussion

Effects

Transport age

To the best of our knowledge the current study is the first to systematically examine and demonstrate effects of different ages of calves at transport from the dairy farm to the veal farm on health and performance of veal calves. When calves in the Netherlands are usually transported to the veal farm, i.e. around 14 days of age, the animals are in the so called "immune gap period" (Hulbert and Moisa, 2016), in which maternal antibodies decrease while the calf own immune system is still immature (Hulbert and Moisa, 2016). Results of our companion paper (Marcato et al., 2021a) demonstrated that the "immune gap" exists in both age groups (14 d and 28 d) at the moment of transport, but the developmental stage of the adaptive immune system might be different between age groups. In this paper it is suggested that the adaptive immune system of calves transported at 28 d of age might be more developed compared to that of calves transported at 14 d of age. To try to understand whether or not a possible difference in adaptive immunity between transport age groups is reflected in differences in the health status of calves, the current study investigated the effects of transport age on various measures of robustness of calves at the veal farm. One day prior to transport, calves transported at 14 d of age had a higher prevalence of loose or liquid manure and more signs of dehydration (as indicated by skin elasticity) compared to calves transported at 28 d of age. Calves transported at 14 d also had a lower body weight at transport and upon arrival at the veal farm compared to calves transported at 28 d. These pre-transport signs shown by 14 d calves might be all linked to a lower robustness of calves upon arrival at the veal farm. Smith et al. (2009) indicated that diarrhea might be responsible for creating dehydration, possibly resulting in a higher mortality at the veal farm (Berchtold, 2009; Renaud et al., 2018a). However, this latter study did not find a relationship between fecal score and dehydration, thus they could not conclude that diarrhea is associated with dehydration. Moreover, this experiment was not conducted with calves transported at different ages, so potential relationships between transport age, diarrhea, dehydration and mortality could not be established.

In the present experiment, in spite of a clear health difference between transport age groups one day prior to transport, we did not find any significant effects of transport age on measures of clinical health at the veal farm. In the second week upon arrival prevalences of loose and liquid manure were not only similar between transport age groups, but also much higher (over 35% on average) than at the dairy farm one day prior to transport (15% on average). Apparently, all calves, regardless of age, had difficulty coping with the transition from the dairy farm to the veal farm, in adjusting their digestive

system. Since it was not possible to distinguish between infectious or feed-related loose or liquid manure, it remains unknown the extent to which exposure to pathogens or, for example, a change in dietary conditions was the defining factor here. The prevalences of loose or liquid manure observed at the veal farm are in line with previous studies in veal calves (Wilson et al., 2000; Marcato et al., 2020c). In the study of Marcato et al. (2020c) the prevalence of this condition also sharply increased in the first three weeks post-transport (from 5% upon arrival at the veal farm to 39% in week 3). Beyond week 2, prevalences of clinical signs of respiratory problems were generally quite low (< 5% at the beginning of the rearing period, and always < 10 %, Figure 1), which is in contrast with other studies (e.g. Pardon et al. 2015).

The prevalences of all other clinical parameters were even lower (< 5 %). In view of the high number of herd treatments with antibiotics applied on the veal farms that participated in the current experiment (i.e., on average 4.4 herd treatments per farm), it seems likely that bacterial infections were largely suppressed, which may also have masked differences in (overt) clinical consequences between treatment groups. Interestingly, despite an overall high level of antimicrobial use in this study, in comparison with calves transported to the veal farm at 28 days of age, a significantly higher proportion of calves transported at 14 days of age received individual medical treatments other than antibiotics. This could be interpreted as an indication of reduced vigor in calves transported at 14 days of age. Two additional findings from the current study seem to support this idea. First, even after correcting carcass weight for body weight upon arrival, the effect of age of transport on carcass weight remained statistically significant. This suggests that the ultimate difference in carcass weight between age groups at the end of the rearing period was not merely the consequence of maintaining an age-related body weight difference that existed at the time of arrival at the veal farm. Moreover, the slope of the curve between carcass weight and body weight upon arrival at the veal farm was considerably steeper for calves transported at 28 d of age than for calves transported at 14 d of age, as indicated by different regression coefficients (0.85 vs. 0.13). This would suggest that calves transported at 28 d also exhibited a higher growth rate during the rearing period in comparison with calves transported at 14 d. However, further research would be necessary to characterize growth curves of calves from arrival at the veal farm until slaughter in more detail. Secondly, and most importantly, the mortality rate among calves transported to the veal farm at 14 d of age was higher than the mortality rate among calves transported at 28 d (5.9 vs. 2.8 %). Since mortality rate is generally considered the ultimate measure of robustness (Marcato et al., 2018; de Almeida et al., 2019), we tentatively propose that transportation of calves from the dairy farm to the veal farm at 28 rather than 14 d of age might contribute to a higher robustness of the animals. A more advanced adaptive immune system in calves transported at 28 d relative to

animals transported at 14 d, among other things reflected in differences between age groups in immunoglobulin isotypes and various immune cell counts (see Marcato et al. 2021a) might be one of the influencing factors behind this difference.

Breed

As reported by Diana et al. (2020), breed is an important factor with a strong impact on performance traits, antimicrobial use, and risk of mortality in beef cattle. These authors found that beef breeds with a higher body weight at the beginning of the fattening cycle had a lower likelihood to be treated with antimicrobials. In our study, Belgian Blue × Holstein Friesian calves were heavier upon arrival at the veal farm compared to other crossbreds and Holstein Friesian calves. This can be attributed to the genetics of Belgian Blue, which is a breed with exaggerated muscular growth (Fiems et al., 2013), rather than a characteristic associated with improved robustness. Total use of antibiotics was higher in other crossbreds compared to both Holstein Friesian and Belgian Blue × Holstein Friesian calves. These results are in contrast to the idea that crossbreds are more robust than pure breeds (Clasen et al., 2017, 2019), and they indicate that there might be a difference in robustness among different types of crossbreds. Moreover, previous studies reported that double muscled breeds, such as Belgian Blue, may even have an impaired immunity due to their lower amount of body fat, which may affect their susceptibility to diseases and performance (Fiems, 2012; Pardon et al., 2012b). In the current study, differences in clinical health status among breeds were not present. However, our study showed that the use of antibiotics and other medicines at the veal farm was higher in other crossbreds compared to Belgian Blue × Holstein Friesian calves. These results might be an indication that crossbreds other than Belgian Blue × Holstein Friesian calves are less robust, but this would need confirmation in future research.

Sex

Sex of the calves is another factor that was not extensively investigated in veal calves and might affect their production traits and robustness. Previous studies showed that, already in early life, sex-related differences in the immune system and sex hormone milieu in humans and animals are contributing factors for a higher disease susceptibility and mortality among males compared to females (Baxter et al., 2012; Zarulli et al., 2018; Pradhan and Olsson, 2020). In support of these findings, the prevalence of navel inflammation in week 2 post transport was higher among bull calves compared to heifers calves. However, sex did not significantly affect the mortality rate at the veal farm. The high prevalence of inflamed navels in bull calves upon arrival at the veal farm is a common problem in the veal industry (Wilson et al., 2000; Renaud et al., 2018b). Wilson et al. (2000) showed that the percentage of bull calves with unacceptable navel scores (scores 1 and 2) were 32% and 23.7% upon arrival and 28 d post transport, respectively.

Moreover, Renaud et al. (2018b) showed that bull calves with enlarged and inflamed navels (score 3) upon arrival at the veal farm are at a higher risk for early (< 21 d; OR = 2.4) and late mortality (> 21 d; OR = 1.8) than the reference category with navel score 0 or 1. Eventually this infection can spread to other parts of the body and affect multiple organs. To prevent this condition and the corresponding use of antibiotic treatments, hygiene should be monitored and the navel of all bull and surplus female calves, should be carefully dipped into disinfecting solutions after birth at the dairy farms of origin (Mee, 2008; Renaud et al., 2018b).

Parity

The current study as well as results of our companion paper (Marcato et al., 2021a) showed that parity affected calf performance not only around birth, but also in the longer term. In fact, calves born from first parity cows had a significantly lower body weight in week 1 after birth and one day prior to transport compared to calves born from cows of older parity. These effects might be explained by the difference in nutrient availability in late gestation between primiparous and multiparous cows (Carvalho et al., 2020). Effects of maternal characteristics on future performance of calves are well reported in dairy cattle (Astiz et al., 2014; Pinedo and De Vries, 2017), but there are no studies in veal calves. Besides the weight differences due to parity, there were no additional long--term effects of parity on health status and performance of calves. However, it might be interesting to investigate whether rearing regimes of calves at the dairy and veal farm need to be adjusted, or perhaps could be optimized, based on the parity of the dam.

Calving process

In the current study, ease of birth was only associated with the body weight of calves one day prior to transport, but no significant effects of this factor were found on the clinical health status of calves. Previous studies have reported significant effects of ease of birth on post-natal health of calves (Probo et al., 2012; Barrier et al., 2012), but no studies investigated long-term effects of ease of birth in veal calves. The current experiment provided no support for the idea that ease of birth may have long-term consequences on health and performance of calves.

Use of body weight as an indicator of robustness

Body weight of calves upon arrival at the veal farm is believed to be a reliable indicator of robustness (Marcato et al., 2018), because it has been associated with later risks of disease, in particular respiratory disorders (Brscic et al., 2012), and mortality at the veal farm. Recent observational studies showed that every 1 kg increase in body weight of calves upon arrival is associated with a decreased hazard of mortality in the first 21 d (OR = 0.93; Renaud et al. (2018b)) and 78 d (OR = 0.925; Goetz et al., (2020)) at the veal

farm, respectively. The current study showed that the difference in body weight upon arrival between the two age groups was associated with a difference in several putative measures of robustness, including neutrophil and lymphocyte counts, levels and patterns of N-IgA and N-IgM, the prevalence of individual treatments with medicines other than antibiotics, growth rate, and mortality rate. However, significant weight differences between other treatment groups of calves, e.g. between calves born from dams with different parities, or between calves born with or without assistance during calving, did not necessarily coincide with differences in any other putative measure of robustness. Likewise, a higher average weight upon arrival at the veal farm due to breed such as, for example, was observed in Holstein Friesian × Belgian Blue crossbreds in comparison with Holstein Friesian calves, was not associated with significant differences in individual treatments with antibiotics or mortality rate. Therefore, the current study seems to suggest that weight upon arrival *per se* might not be a reliable indicator or predictor of robustness, but should be considered in combination with other factors such as, for example, age (which may likely be associated with underlying characteristics related to the development of the adaptive immune system) or breed (i.e., genetic background). The results of the current study clearly support previous findings that antimicrobial use is negatively related to carcass weight of calves at slaughter. Calves which received > 4 individual antibiotic treatments had on average a 27.5 kg lower carcass weight compared to calves without antibiotic treatments. In accordance, Pardon et al. (2013) demonstrated that for every 1% increase in antimicrobial drug use the carcass weight decreased by 1.5 kg. In a previous study, we also found that carcass weight was negatively related to the number of individual treatments with antibiotics (Marcato et al., 2020c). In this latter study, the average carcass weight of calves receiving > 2 treatments was 12.5 kg lower compared to that of calves with no treatments. Thus, predicting robustness of calves based on body weight upon arrival might be more complicated. However, retrospectively interpreting robustness of calves in terms of treatments with antibiotics based on differences in carcass weight seems to be feasible across factors such as age and breed.

Table 11. **Prevalence of health problems[1] across weeks.**

	Sampling moments					
	Dairy farm (after birth)					Veal farm
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 2 post-transport
Calves with at least one health problem	53.1% (388/730)	55.1% (398/722)	45.8% (326/712)	33.0% (110/333)	31.6% (104/329)	52.8% (362/685)

[1] *% of calves with at least one health issue over the total amount of calves.*

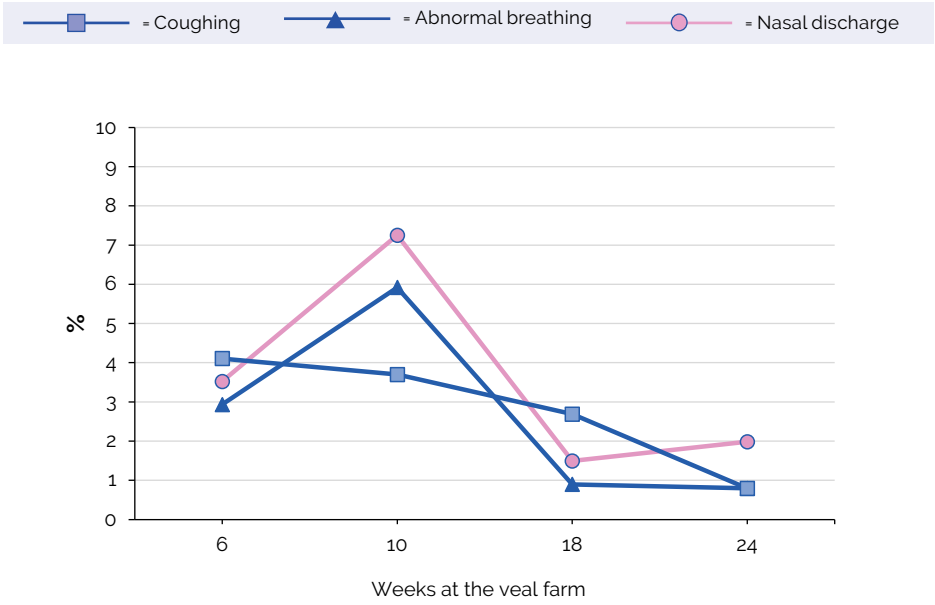

Figure 1. **Prevalence of abnormal breathing, nasal discharge and coughing in veal calves in week 6, 10, 18 and 24 at the veal farm (expressed as proportion of calves).**

Conclusions

This study showed that health and performance of veal calves was significantly affected by transport age and breed. Transportation of calves from the dairy farm to the veal farm at 28 d of age resulted in less individual treatments with medicines other than antibiotics at the veal farm and a higher carcass weight compared to transportation at 14 d of age. Mortality rate at the veal farm was lower in calves transported at 28 d than in calves transported at 14 d. More crossbreds other than Holstein Friesian × Belgian Blue calves received individual treatments with antibiotics and medicines other than antibiotics at the veal farm compared to Holstein Friesian × Belgian Blue calves. Collectively, these findings suggest a higher robustness of calves transported at 28 d of age in comparison with calves transported at 14 d of age. Additionally, Holstein Friesian × Belgian Blue calves seemed more robust in comparison with other crossbreds. The potential readouts of robustness recorded in this study and in the previous companion paper might be helpful for the veal sector in developing strategies to improve calf health and reduce antimicrobial use at the veal farm. ■

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Appendix 1

Table A1 Health parameters of veal calves assessed on a weekly basis at the dairy farm and in week 2 at the veal farm. Health parameters were assessed at individual level.

Health parameter	Score	Explanation
Navel inflammation	0	No signs of inflammation
	1	Swollen, without discharge
	2	Swollen, with discharge
Joint inflammation	0	No evidence of joint problems
	1	Slight swelling, not warm or painful
	2	Swelling with pain, heat, slight lameness
Loose or liquid manure	0	No loose or liquid manure
	1	Pasty manure
	2	Watery manure
Coughing	0	No coughing
	1	Induced single coughing
	2	Repeated coughing
Eye discharge	0	No eye discharge
	1	Slight watery discharge
	2	Moderate amount of bilateral ocular discharge
Sunken eyes	0	Normal, bright eyes
	1	Eyes markedly recessed into the orbits
Ears	0	Normal
	1	Slight unilateral droop
	2	Head tilt or bilateral droop
Nasal discharge	0	No discharge
	1	Watery discharge
	2	Purulent discharge
Skin elasticity	0	Skin tent returns to normal < 2 s
	1	Skin tent returns to normal in 2-4 s
	2	Skin tent returns to normal in > 8-10 s

Appendix 2

Table A2 Health parameters of veal calves assessed at pen level in week 6, 10, 18 and 24 post-transport at the veal farm.

Health parameters	Explanation	Method of assessment
Milk leftovers	Untouched rests of milk in the feeding trough	Yes / No
Roughage leftovers	Untouched rests of roughage in the feeding trough	Yes / No
Abnormal breathing	Fast breathing (> 40 breaths/min), excessive abdominal breathing	number of calves
Nose discharge	Presence of discharge from one or both nostrils	number of calves
Coughing	Audible expulsion of air through the mouth of calves	number of calves
Loose or liquid manure	Presence of loose or liquid manure in the pen	Yes / No
Thick manure	Thicker and higher consistency manure, often combined with undigested food	Yes / No
White manure	Sticky and higher consistency manure. The colour is white or grey	Yes / No
Bloated calves	Calves with overfilled/bloated belly (upper, lower, right, left and all around)	number of calves
Lame calves	Calves with a different load/ or do not stand on one or more legs	number of calves
Claw problems	Red and swollen skin around the claw, often combined with lameness	number of calves
Joint problems	Clear thickening of one or more joints caused by accumulation of fluids/ synovia. Often painful and combined with lameness	number of calves
Bursa problems	Clear thickening (disc or round shaped) of the joint. Usually not painful and calves are not lame	number of calves
Chewing wounds	Wounds (damaged tail/ ear or skin on the body) caused by other calves in the pen.	number of calves
Skin infection	Skin damage due to infection: presence of round, hairless spots and wrinkled skin	number of calves
Hard skin	Thickened skin (often wrinkled and hairless), especially on the withers	number of calves
Urine suckling	Calves that suckle urines from other calves as well as calves being suckled	number of calves
Condition 15-30	Calves that are 15-30% behind condition (based on weight and size of calves) compared to the other calves in the herd	number of calves
Condition >30	Calves that are >30% behind condition (based on weight and size of calves) compared to the other calves in the herd	number of calves
Wet fur	Calves with a wet fur all along the back line	number of calves
Dull fur	Dull fur with abnormal structure, gloss and length	number of calves
Sick calves	Calves (not scored earlier) that give a general sick impression, depressed calves/ not attentive	number of calves

CHAPTER 8

General Discussion

Introduction

Robustness can be defined as the capacity of an animal to cope with environmental challenges and to bounce back rapidly after challenges occur (Colditz and Hine, 2016). Robust animals are well equipped to cope with endemic infections and to fight diseases and, thus, probably have a lower need for antimicrobials. Robustness can be measured in terms of physiological indicators (such as fluctuations in glucose levels and body temperature), which reflect the capacity of an animal to regulate the functions of the body in relation to external stimuli (Scheffer et al., 2018). This in turn might have an effect on morbidity, mortality rate of animals and antimicrobial use, which represent additional, and more ultimate, measures of robustness. Robustness is an animal characteristic that is shaped by early-life environmental factors and by animal characteristics (such as body weight, breed or age) (Scheffer et al., 2018). The aim of this thesis was to investigate the role of different animal and environmental factors in robustness of veal calves. With regard to environmental factors, **Chapter 3**, **4** and **5** described effects of transport-related factors (including pre-transport diet, transport duration and type of vehicle) on readouts of robustness of calves. **Chapter 6** and **7** described effects of early rearing conditions of future veal calves at the dairy farm (such as assistance during calving, colostrum management, dry cow management). In addition, these two chapters discussed effects of calf characteristics (including transport age, sex and breed) and dam characteristics (including parity) on robustness of calves. In this thesis, robustness was assumed to be reflected in short-term effects, i.e. how calves immediately coped with the challenges they were subjected to, as well as in long-term effects, i.e. what were the carry-over effects of early life challenges or predisposing biological factors in the longer run. Robustness was quantified in terms of the prevalence of health problems, the number of herd and individual antibiotic and medical treatments, and the mortality rate during the rearing phase of veal calves. Moreover, this thesis focused on blood variables that might be used as biomarkers (or predictors) of robustness. The first part of the General Discussion will discuss the main findings of **Chapter 3-7** in relation to robustness. **Chapter 2**, which is a review on potential biomarkers of robustness in veal calves, will also be used to support the results found in both experiments. The second part of the General Discussion will provide suggestions and practical implications to the veal sector on ways to improve robustness of veal calves. Finally, some suggestions on future studies will be made.

Effects of transport-related factors on robustness of calves

Transport represents one of the largest challenges for veal calves (Brscic et al., 2012; Renaud et al., 2018b) and thus it probably can affect their robustness (**Chapters 3**, **4** and **5**). Calves are mostly transported between 14 and 20 days of age, commingled and placed in new housing environments (Brscic et al., 2012; Goetz et al., 2021). As indicated in **Chapter 2**, all these factors may have negative consequences on physiology (Grigor et al., 2001; Minka and Ayo, 2010) and health of calves (Earley et al., 2017). As a result, pathogens may easily spread among veal calves which may lead to high levels of morbidity (Pardon et al., 2012b), mortality (Bähler et al., 2012), antimicrobial use (Bos et al., 2013) and, as an undesirable side-effect, antimicrobial resistance (Catry et al., 2016) in the veal industry. The need for practical interventions to alleviate or prevent these negative effects was the motivation behind the investigation into three transport-related factors that were hypothesized to have a large influence on the health of calves: 1) pre-transport diet, 2) transport duration and 3) type of vehicle used.

Pre-transport diet

Feeding practices before transport have not been extensively studied (Devant and Marti, 2020) and there has always been a debate on what to feed calves at the collection center prior to transport. Previous research mainly addressed effects of administration of electrolytes, mineral and glucose solutions (Knowles et al., 1997; Schaefer et al., 1997). However, none of the previous studies investigated the use of milk as a pre-transport meal in young veal calves. **Chapters 3**, **4** and **5** provide the comparison between the use of milk and electrolytes. Moreover, calves in the experiment were exposed to procedures as they exist in common practice: they were collected from different dairy farms in Germany, received and mixed at a collection center and transported to a veal farm in the Netherlands. Since Germany represents a major source of calves for veal production in The Netherlands (≈ 50% of calves) (Bos et al., 2013), this study was expected to provide results that were relevant for the Dutch situation. This approach was in contrast with some previous studies, where calves were not challenged prior to transport to the veal farm (Knowles et al., 1997; Knowles et al., 1999b). In fact, in these latter studies calves were allowed to rest and recover from the first transport to the auction for at least a week prior to the start of the experiment.

The current findings demonstrated that feeding milk prior to transport may have various beneficial effects compared to feeding electrolytes. First, **Chapter 3** showed that milk, having a higher nutrient and energy content compared to electrolytes, seemed to protect calves against nutrient mobilization and, thus, body weight losses. This was

also reflected in the higher glucose (3.9 vs 3.4 mmol/l) and lower NEFA (876.9 vs 719.9 µmol/l), urea (5.4 vs 6.5 mmol/l) and ß-HB (0.3 vs 0.5 mmol/l) concentrations directly post-transport in calves fed with milk (Tadich et al., 2005; **Chapter 2**). Second, **Chapter 4** demonstrated that, upon arrival at the veal farm, feeding milk prior to transport contributed to lower cortisol (8.8 vs 10.8 ng/ml) and bilirubin levels (10.4 vs 12.8 µmol/l), and to higher levels of monocytes (45.1 % vs 41.0 %) compared to feeding electrolytes. These blood characteristics are signs of a lower stress response and better immune function as a result of the higher energy intake of milk compared to electrolytes (Woodward, 1998; Ballou et al., 2015; **Chapter 2**). Despite these short-term beneficial effects, **Chapter 5** showed that feeding milk prior to transport, increased fecal abnormalities in calves at the veal farm (Brown et al., 2005). In the first 3 weeks post-transport, milk-fed calves had a higher prevalence of loose or liquid manure compared to calves electrolyte--fed (30.7% vs 22.8%, respectively). This higher prevalence may have contributed to the higher antibiotic use in milk-fed calves compared to electrolyte-fed calves at the veal farm (milk-fed calves treated = 38% vs electrolyte-fed calves treated = 28%). Overall, the results showed that pre-transport diet influenced a number of blood variables directly post-transport, whereas no significant effects were present in the longer term. Health status of calves beyond week 5 post-transport was also not affected by pre-transport diet, indicating that long-term effects were not present.

Transport duration

Although transport itself is a challenge for young calves, the duration of the transport might make this challenge more or less severe (Eicher, 2001). Long transport durations of young calves (> 8 hour transport) have been associated with dehydration, weight loss and mobilization of body reserves (Kent and Ewbank, 1986b; Knowles et al., 1997). Whether these effects are due to the length of transport or the duration of feed and water withdrawal (Grandin et al., 2014; **Chapter 2**) is unclear. The length of transport (up to 800 km) was found to be positively correlated to mortality of young veal calves upon arrival at the farm (Cave et al., 2005). Despite the potential relevance of this topic, there are very few studies conducted on transport duration in young veal calves in Europe (Knowles et al., 1999b; Bernardini et al., 2012). Moreover, the available studies have mainly focused on physiological indicators, whereas information on the immune system, and more long-term effects on health, behavior and performance of calves was largely lacking.

Chapters 3, **4** and **5** investigated physiological, immunological and health responses of calves in relation to both short and long-term transport under current practices. All findings in these chapters led to the same conclusion: long transport duration (18 h) had more short-term detrimental effects compared to short transport duration (6 h).

This conclusion was based on lower concentrations of glucose (3.2 vs 4.1 mmol/l), Ca (2.3 vs 2.5 mmol/l) and higher concentrations of ß-HB (0.48 vs 0.40 mmol/l) and NEFA (845.5 vs 751.3 µmol/l) post-transport in calves transported for 18 h compared to calves transported for 6 h. High urea concentrations, an indicator of protein utilization, were also maintained until 24 h post-transport in calves with 18 h transport (**Chapter 3**).

Additionally, 18 h transport duration provoked higher bilirubin (12.3 vs 10.8 µmol/l) and cortisol (11.6 vs 7.9 ng/ml) concentrations than 6 h transport duration (**Chapter 4**), which could be related to prolonged fasting (West, 1990; Averos et al., 2008). The higher cortisol values probably determined also a leukocyte redistribution (higher CD8+ cells) into the peripheral blood, in line with previous studies (Masmeijer et al., 2019). In line with **Chapter 2** and previous studies (Knowles et al., 1999b; Wilson et al., 2000; Renaud et al., 2018a), **Chapter 3** and **4** indicated that ß-HB, NEFA, bilirubin, glucose and urea are the parameters most affected in the short-term post-transport. Effects of transport duration also interacted with pre-transport diet. In fact, the combination of feeding electrolytes and long transport duration caused more severe body weight losses (-0.74 kg vs 0.41 kg) and greater short-term changes in blood variables of calves compared to feeding milk and a shorter transport duration. Overall, the findings showed that effects of transport duration on blood variables and body weight were evident immediately post-transport, but they did not affect long-term clinical health or growth performance.

Type of vehicle

Along with pre-transport diet and transport duration, the type of vehicle represents another factor that may affect robustness, and therefore health, of calves upon arrival at the veal farm (**Chapter 3**, **4**, **5**). Effects of transport conditions on health of young veal calves have never been investigated. Previous studies looked at effects of season (Knowles et al., 1999b; Giespert et al., 2000) in which calves were transported and effects of environmental temperature on thermoregulation of these animals (Bernardini et al., 2012), but effects of type of vehicles were never described. Results described in **Chapter 3, 4 and 5** showed inconsistent and contradictory results on the effects of the type of vehicle. In **Chapter 3**, an increase in post-transport osmolality (Δ = 5.6 vs Δ = -8.0 mosmol/kg), Na (Δ = 2.2 vs Δ = -4.5 mmol/l) and rectal temperature (Δ = 0.3 vs Δ = 0.1 °C) compared to their pre-transport values was found in calves transported in the conditioned truck compared to calves transported in the open truck. These changes might be the result of dehydration and compromised thermoregulatory abilities (Minka and Ayo, 2010). **Chapter 4** demonstrated that transportation of calves in the conditioned truck led to a higher post-transport white blood cell count (9.5 vs 8.1 × 10^9/l), a higher proportion of neutrophils (41.5% vs 37.6 %) and a lower proportion of lymphocytes (35.4% vs 40.2 %) compared to transportation of calves in the open truck. These results might be

indicative of a shift in immune cell subsets and a leucocyte redistribution in the peripheral blood as a consequence of higher stress experienced by calves transported in the conditioned truck compared to calves transported in the open truck (Murata et al., 1987; Masmeijer et al., 2019). In line with **Chapter 3** and **4**, **Chapter 5** showed that the day post-transport, calves transported in the conditioned truck showed the highest prevalence of discomfort behavior (9 %). However, in the first 3 weeks post-transport, calves transported in the conditioned truck showed a lower prevalence of navel inflammation compared to calves transported in the open truck (5.9 % vs 9.2%). The lower prevalence of navel inflammation is an important sign of robustness, because in a recent observational study (Renaud et al., 2018b) this variable was associated with early mortality (< 21 d post-transport) at the veal farm. Moreover, transportation of calves in the conditioned truck in combination with feeding electrolytes before transport was the treatment that contributed to the lowest prevalence of loose and liquid manure (18 %) during the first 3 weeks post-transport. These ambivalent results showed that type of vehicle might have effects on calf robustness but exact vehicle settings should be further investigated. In fact, in our study, using the settings of the climate control system according to those provided by the manufacturer and applied by the transporter, there were no significant differences in temperature and relative humidity between the conditioned and open truck. Thus, at present, it remains unknown which environmental factors in each type of vehicle contributed to the effects found in the experiment.

Conclusions on transport-related factors

Chapters 3 and **4** showed that effects of transport-related factors were temporary and affected the physiology and metabolism of calves only in the short-term. Most of the blood parameters returned to their likely baseline values within 24 h post-transport, with the exception of albumin, total protein and osmolality concentrations, which were affected until 48 h post-transport. In addition, **Chapter 5** showed that effects of transport-related treatments on health status of calves were evident within the first 3 weeks post-transport, but not thereafter, except for effects of pre-transport diet on antibiotic use. However, it was not possible to rule out the possibility that some of these antibiotic treatments were applied without a proper clinical justification because it was not possible to distinguish between feeding related and infectious diarrhea. An overview of effects of treatments immediately after arrival, in the short and in the long-term is shown in Table 1.

Table 1. **Measurements in veal calves affected by their experimental treatments[1] and their interactions immediately post-transport, in the short and long-term**

Measurements in calves				
Experimental treatments	Immediate effects (upon arrival until 48 h post-transport at veal farm)		Short-term effects (1-3 weeks post-transport)	Long-term effects (> week 3 post-transport)
Pre-transport diet (Milk vs. Electrolytes)	• glucose • monocytes • NEFA • ß-HB	• urea • cortisol • bilirubin	• Loose or liquid manure	• Individual treatments with antibiotics
Transport duration (6 h vs. 18 h)	• glucose • Ca • NEFA • ß-HB • urea • cortisol	• bilirubin • CD8+ T cells • albumin • total protein • osmolality • Na	-	-
Type of vehicle (Conditioned truck vs. Open truck)	• osmolality • Na • white blood cells • haptoglobin	neutrophils lymphocytes discomfort behaviour	• Navel inflammation	-
Pre-transport diet × transport duration	glucose urea NEFA total protein	Ca osmolality bilirubin	-	-
Pre-transport diet × type of vehicle	-	-	• Loose or liquid manure	-

[1]*pre-transport diet, transport duration and type of vehicle. Table based on Chapter 3, 4 and 5*

The lack of long term effects on health status might be due to the following reasons:

- Calves are robust, thus they have the ability to recover quickly after transport. Despite their relative immature physiological system (Schrama et al., 1993; Hulbert and Moisa, 2016), calves responded to transport in the short term, indicating a quick recovery.

- The collective effects of the transition of calves from the dairy farm to the veal farm (including transport and commingling with other calves at the collection center), and of the husbandry conditions during the subsequent rearing period (including dietary changes, and, again, commingling with other calves) might be so large that they overrule potential effects of individual transport factors on health and adaptive capacity of calves.

- High use of herd and individual treatments with antibiotics within the first 6 weeks post-transport masked potential long-term effects of transport-related factors on health status of calves. In the experiment, calves received 5 herd treatments with antibiotics within the first 2 months after arrival at the veal farm (**Chapter 5**). Moreover 25% calves were individually treated with antibiotics and 22% of calves were individually treated with medicines (**Chapter 5**). This experiment shows that there is a high use of antibiotics. This is supported by observations in the second experiment (**Chapter 6** and **7**). That experiment included 8 different veal farms and the data was also based on a larger sample size (680 calves). Again, calves received on average 4.4 herd treatments with antibiotics and 3.9 herd treatments with other medicines. On the top of that, 23% calves were individually treated with antibiotics and 25% of calves were individually treated with medicines within the first two months post-transport.

  The results of this first part of the discussion showed that transport-related factors affected health of calves in the short-term, especially during the first hours post-transport. The first two options indicated above were likely not the main causes for the lack of treatment effects on the health status of calves in the long-term. In fact, health problems were still present during the rearing period at veal farm, suggesting that robustness of calves was affected and the transition to the veal farm represented a challenge. Therefore, the most logic explanation for the lack of treatment effects would be the high use of herd and individual treatments with antibiotics and medicines.

Effects of management factors on dairy farms on calf robustness

Dairy farm management and, in particular, colostrum management might also affect robustness of calves. Most calves that enter the veal industry are male calves from dairy production and surplus female calves, which have long been considered as by-products (Devant and Marti, 2020). These animals may not represent a priority for the dairy sector. As a consequence, they might receive little attention and run the risk of poor post-natal care, in particular insufficient intake or quality of colostrum (Cave et al., 2005). A second experiment (**Chapter 6 and 7**) confirmed that colostrum is extremely important for passive transfer of Ig's to calves in accordance with previous studies (Godden et al., 2009; Dunn et al., 2018). **Chapter 6** determined natural antibodies specific for phosphorylcholine conjugated to bovine serum albumin (PC-BSA). Phosphorylcholine is an abundant environmental antigen, present on bacterial membranes, fungi and parasites (Pinkert et al., 1989). Pinkert et al. (1989) reported that immune responsiveness to

PC is a useful model for pathogen recognition and, in combination with BSA, allows to detect also natural autoantibodies. Since **Chapter 6** determined both natural antibodies and natural autoantibodies, the term **N-Ig's** (N-IgG, N-IgM and N-IgA) was utilized to indicate natural antibodies detected against PC-BSA. Notably, in **Chapter 6**, a positive relationship was found between the amount of N-Ig's in colostrum and N-Ig's in the serum of calves measured in week 1 after birth (ß = 0.61 (SE = 0.06), ß = 0.58 (SE = 0.07), ß = 0.57 (SE = 0.06) for N-IgG, N-IgM and N-IgA respectively; $P < 0.05$), and one day prior to transport to the veal farm (ß = 0.62 (SE = 0.06), ß = 0.17 (SE = 0.06), ß = 0.17 (SE = 0.05), for N-IgG, N-IgM and N-IgA respectively; $P < 0.05$). Interestingly, N-IgG in colostrum was even significantly associated with IgG in calf serum in week 2 (ß = 0.68 (SE = 0.05); $P < 0.05$) and week 10 (ß = 0.19 (SE = 0.05); $P < 0.05$) after arrival at the veal farm. These long-term relationships were not found beyond week 2 after birth in replacement heifer calves (Mayasari et al., 2016). This suggests that the level of maternal N-Ig's in colostrum seemed to have long-term effects on serum N-Ig's levels in veal calves. **Chapter 6** showed also that N-Ig's concentrations, especially N-IgG, were negatively related to individual use of antibiotics and other medicines at the veal farm (Table 2). These results suggest that maternal N-Ig's, in particular N-IgG, exert long-term effects on health and may be used as a biomarker of robustness of veal calves.

Table 2. **Regression coefficients, standard error, and significance values of individual treatments with antibiotics and other medicines during the whole rearing period at the veal farm in relation to calf serum immunoglobulin titers measured at different time points.**

	Antibiotics	Other medicines
N-IgG		
week 1 after birth	ß = -0.196 (0.06), $P < 0.001$	ß = -0.192 (0.06), $P < 0.001$
one day prior to transport	ß = -0.235 (0.07), $P < 0.001$	ß = -0.212 (0.06), $P < 0.001$
week 2 at the veal farm	ß = -0.253 (0.06), $P < 0.001$	ß = -0.196 (0.06), $P = 0.001$
N-IgM		
week 1 after birth	ß = -0.165 (0.06); $P = 0.03$	ß = -0.136 (0.06), $P = 0.01$
one day prior to transport	ß = -0.168 (0.09), $P = 0.05$	ß = -0.114 (0.08), $P = 0.17$
week 2 at the veal farm	ß = -0.04 (0.06), $P = 0.57$	ß = -0.03 (0.07), $P = 0.66$
N-IgA		
week 1 after birth	ß = -0.148 (0.06), $P < 0.01$	ß = -0.132 (0.06), $P = 0.02$
one day prior to transport	ß = -0.160 (0.10), $P = 0.14$	ß = -0.08 (0.10), $P = 0.43$
week 2 at the veal farm	ß = 0.04 (0.09), $P = 0.68$	ß = 0.04 (0.09), $P = 0.68$

The birth process is another factor known to affect the hematological profile and immune system of new-born calves (Probo et al., 2012), which might affect robustness of calves at a later stage. Calves with a higher birth weight more often require assistance of the farmer during birth and the calving process might be prolonged (Eriksson et a., 2004). Heavy calves might be less vigorous (due to hypoxia) after the calving process requiring assistance of the farmer, and thus they might drink less colostrum compared to calves born without assistance (Vasseur et al., 2009; Barrier et al., 2012). A lower colostrum intake may result in inadequate transfer of immunoglobulins and to a poorer survivability of calves (Lombard et al., 2007; Gasparelli et al., 2009). In line with previous studies, **Chapter 6** showed that calves born with assistance had a higher birth weight compared to calves born without assistance (45.1 vs 43.1 kg, respectively). However, no differences in various N-Ig's were found between calves that received assistance during birth and calves with a normal delivery. **Chapter 7** also showed that ease of birth also had no significant long term effects on antibiotic treatments and health problems at the veal farm.

Chapter 6 showed that calves born from cows with a shorter dry period length (0-30 days) had a lower birth weight (42.6 kg) compared to calves born from cows with a longer dry period length (44.6 kg for cows with 30-60 days, and 45.5 for cows with > 60 days). This result was in line with the study of Kamal et al. (2014) and it suggests that dry period management might be also a factor with a potential influence on future robustness of calves. Therefore, more research is needed to understand the role of dry period length on robustness of veal calves as well as replacement heifer calves.

Conclusions on management factors on dairy farms

Results found in **Chapter 6** and **7** showed that dairy farm management might be a predisposing factor of robustness of calves. In particular, colostrum management appears to be important, because N-Ig's in colostrum protect the calf via passive transfer and they are negatively related to individual treatments with antibiotics and other medicines in the long-term. Consequently, monitoring colostrum quality by dairy farmers before feeding it to calves, e.g. using a refractometer, is strongly recommended. Possible associations between other management factors, including dry period length, and calf robustness need further investigations.

Factors inherent to the animal

The previous sections described the impact of different environmental factors and management factors at dairy farms, on predisposing factors of robustness of veal calves. Another way to improve robustness of calves is by looking at factors inherent to the animals. The following paragraphs discuss the main findings of **Chapter 6** and **7**,

thus how calf characteristics (age, breed and sex), and characteristics of the dam (parity) can impact robustness of calves.

Transport age of calves

Under current practices, calves are transported at an age between 14 and 20 days (Hulbert and Moisa, 2016; Devant and Marti, 2020) and that was also the case in the first experiment (**Chapter 3**, **4** and **5**). At this young age, the immune system of calves is not fully developed because their adaptive immunity becomes functional at 3-6 weeks of age (Heinrichs and Elizondo-Salazar, 2009). The early intake of sufficient amounts of good quality colostrum is crucially important to establish early protection to calves, due to the passive transfer of immunoglobulins, and other components such as leukocytes, hormones and cytokines (Silva et al., 2013; Gomes et al., 2014). However, maternal immune protection gradually declines over time and at two weeks of age calves are known to be in the most critical period, the so-called "immune gap", due to the combination of a decreased passive immunity and the absence of a mature adaptive immune system of calves (Chase et al., 2008; Figure 1 on previous page).

In this window, calves are at the highest risk of enteric and respiratory diseases (Hulbert and Moisa, 2016). It has been suggested that transportation of calves at an older age (> 3 weeks of age) would reduce the incidence of diseases, because of a more developed adaptive immune system of the calf itself (Devant and Marti, 2020). Given this background, an experiment (described in **Chapter 6** and **7**) was conducted to compare effects of two different age groups on robustness of calves that enter the veal industry: 1) 14 d, the conventional transport age, and 2) 28 d, a delayed transport age. Indicators of robustness were measured on the basis of different variables:

- Immunoglobulin titers specific for phosphorylcholine conjugated to bovine serum albumin (PC-BSA) (named as N-IgG, N-IgM and N-IgA, and collectively as N-Ig's). These titers were measured in serum of calves at different time points (week 1 after birth, day prior to transport, week 2 and 10 post-transport). Pardon et al. (2015) indicated that 40% of calves that enter the veal industry suffer of failure of passive transfer (FPT) and their study showed that calves with serum Ig < 7.5 g/L at arrival at the veal farm had a substantial higher risk for bovine respiratory disease (BRD) (see Figure 2).

  Renaud et al. (2018a) compared blood IgG values of calves that survived and those of calves that died during the first 21 d at the veal farm. These authors found that higher IgG concentrations upon arrival were associated with lower odds of mortality (OR = 0.94 every 1 g/L increase in IgG). The advantage of measuring

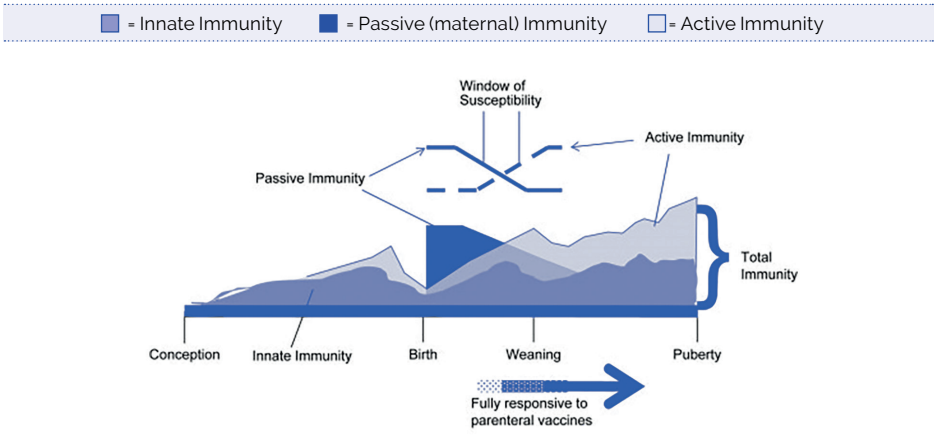


Figure 1. **Development of the immune response in calves: from conception to puberty. The window of susceptibility represents the immune gap period** *(reprinted from Chase et al. (2008) "Neonatal immune development in the calf and its impact on vaccine response", 24, 87-104. © 2008 Elsevier B.V. With permission from Elsevier).*

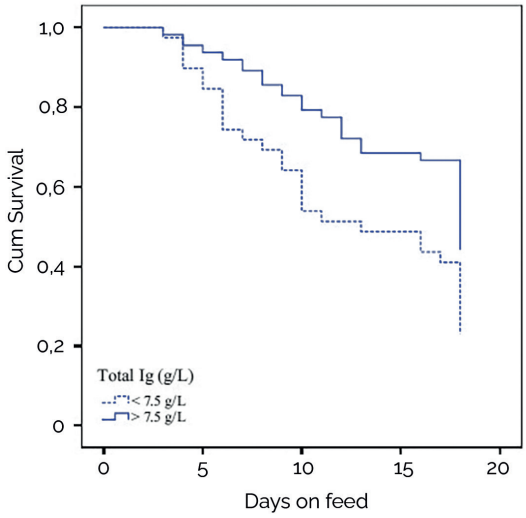


Figure 2. **Survival graph for occurrence of bovine respiratory disease (BRD) in rosé veal calves in the first weeks after arrival, according to the immunoglobulin (Ig(g/L)) concentration measured upon arrival** *(reprinted from Pardon et al. (2015) "Prediction of respiratory disease and diarrhea in veal calves based on immunoglobulin levels and the serostatus for respiratory pathogens measured at arrival", 120, 169-176. © 2015 Elsevier B.V. With permission from Elsevier).*

Ig's only upon arrival might be to create high and low-risk groups of calves at that particular moment. In order to understand when the "immune gap" occurs and whether it is more or less pronounced in calves transported at 28 d compared to 14 d, it is of interest how the pattern of immunoglobulin concentrations develops over time. The results described in **Chapter 6** showed that the lowest N-IgM and N-IgA titers occurred at the age of 4 weeks, i.e. in week 2 post transport in calves transported at 14 d of age, and one day prior to transport in calves transported at 28 d of age. This suggested that the adaptive immune system of calves was not yet functional when calves were transported to the veal farm, thus transport at either 14 d or 28 d still took place during the most susceptible time window. **Chapter 6** showed also that the lowest N-IgG titers were obtained in week 10 post-transport in both transport age groups, and until that time the level of N-IgG seemed to only moderately decrease. These results were notably different from previous studies (Chase et al., 2008; Hulbert and Moisa, 2016). The results might reflect a more pronounced endogenous IgG production in calves in the experiment compared to other calves due to the greater activation of their adaptive immunity following arrival at the veal farm. However, it was not feasible to discriminate between N-IgG's of maternal or endogenous origin. Future research is, therefore, needed to further characterize patterns over time of immunoglobulins in veal calves.

In week 2 post-transport, titers of N-IgA and N-IgM in calves transported at 28 d were higher compared to those of calves transported at 14 d (**Chapter 6**). This difference between the two transport age groups might reflect the decline of the effectiveness of passive immunity derived from colostrum in 14 d-old calves and the activation of endogenous Ig synthesis in 28 day-old calves (Burton et al., 1989; Chase et al., 2008). Although calves still appeared to be in the immune gap, it can be hypothesized that transport of calves at 28 d might be more appropriate than to transport them at 14 d of age, because the development of their adaptive immunity was more advanced, which could result in a higher robustness to diseases.

- Health status. **Chapter 7** showed that, one day prior to transport, calves transported at 14 d of age had a higher prevalence of loose or liquid manure (24 % vs. 7 %) and more signs of dehydration (7 % vs. 1 %) compared to calves transported at 28 d of age. Despite these health differences between age groups one day prior to transport, transport age did not affect health status of calves in the long-term at the veal farm. In week 2 post-transport, prevalences of loose and liquid manure were similar between age groups, but much higher (over 35% on

average) than at the dairy farm one day prior to transport (15% on average). These results were in line with previous studies (Wilson et al., 2000; **Chapter 5**) and they may reflect the difficulty of calves to cope with the transition from the dairy farm to the veal farm. Beyond week 2 post-transport, prevalences of health problems were < 10 %, especially clinical signs related to respiratory problems. This may be the cause of the high number of herd treatments (4.4 on average per veal farm), which likely suppressed bacterial infections and masked differences in clinical problems between treatment groups.

- Hematological profile a day prior to transport and two weeks post-transport. As indicated in **Chapter 2**, recent studies suggested that hematological parameters, such as neutrophils or lymphocytes, can be used as biomarkers of health in veal calves. Von Konigslow et al. (2020) showed that lymphocyte counts between 4.6 and 5.8 × 10^9/L were associated with a decreased hazard of mortality compared to lymphocyte counts < 4.6 or > 5.8 × 10^9/L, whereas lymphocyte counts > 5.8 × 10^9/L reduced the hazard of morbidity of calves upon arrival at the veal farm compared to lymphocyte counts between 4.6 and 5.8 × 10^9/L or < 4.6 × 10^9/L. Elevated neutrophil counts (> 6.0 × 10^9/L) increased the hazard of mortality by more than 5 times. As indicated in **Chapter 6**, calves transported at 14 d and 28 d had lymphocyte counts > 5.8 × 10^9/L in week 2 post-transport, but calves transported at 28 d had significantly higher lymphocyte and lower neutrophil counts (Δ = 0.5 × 10^9/L and Δ = -1.6 × 10^9/L, respectively) compared to calves transported at 14 d. This could mean that calves transported at 28 d are more robust than calves transported at 14 d. However, results of the regression analyses showed that the difference between age groups in these cell counts were not associated with long-term differences in medical treatments or carcass weights (**Chapter 7**). These results were thus in contrast with the previous research (von Konigslow et al., 2020).

- Performance data. **Chapter 7** showed that calves transported at 14 d had a lower carcass weight (Δ = -14.8 kg) and more animals received medical treatments other than antibiotics (Δ = 5.4 %) than calves transported at 28 d, which is an indication of a reduced vigour. Differences in carcass weights between the two age groups were still significant, even after correcting carcass weight for body weight upon arrival. This suggests that the ultimate difference in carcass weight between age groups at the end of the rearing period was not merely the consequence of maintaining an age-related body weight difference that existed at the time of arrival at the veal farm. Moreover, the slope of the curve between carcass weight and body weight upon arrival at the veal farm was considerably steeper

for calves transported at 28 d of age than for calves transported at 14 d of age (ß = 0.85 (SE = 0.13) vs. ß = 0.13 (SE = 0.05), respectively), which suggest that calves transported at 28 d also exhibited a healthier growth during the rearing period in comparison with calves transported at 14 d. However, further research would be necessary to characterize in more detail growth curves of calves arriving at the veal farm at different ages. Mortality rate at the veal farm was also higher in calves transported at 14 d compared to mortality rate of calves transported at 28 d (5.9 % vs. 2.8 %, respectively) (**Chapter 7**). Notably, in this experiment on 5 out of the involved 8 veal farms no mortality at all occurred during the whole rearing period when calves were transported at 28 d of age, whereas in calves transported at 14 d of age this only held for 1 veal farm. Since mortality rate is generally considered the ultimate measure of robustness (Marcato et al., 2018; de Almeida et al., 2019), it was suggested that transportation of calves from the dairy farm to the veal farm at 28 rather than 14 d might contribute to a higher robustness of the animals.

Collectively, the findings shown in **Chapter 6** and **7** suggest that transportation of calves at 28 d of age might contribute to a higher robustness for the following 4 reasons: 1) More developed adaptive immunity in week 2 post-transport; 2) lower use of medicines other than antibiotics at the veal farm; 3) lower mortality at the veal farm; 4) higher carcass weight at slaughter (even after correcting for weight differences at the beginning of the rearing period).

Other characteristics related to calves and their dams

Other calf and dam characteristics might play a role on robustness of calves at the veal farm as well, but there is not a lot of information on this subject. **Chapter 6** and **7** provided an overview on how different factors, such as breed or sex of calves, parity of the dam may affect the robustness of veal calves.

With regard to breed, it seemed that crossbreds other than Belgian Blue × Holstein Friesian calves were less robust, which was based on the following long term indicators:

- Higher total (at both dairy farm and veal farm) use of antibiotics and other medicines (Δ = 17.2 % and Δ = 20.2 % on average, respectively) compared to both Holstein Friesian and Belgian Blue × Holstein Friesian calves.

- Higher use of antibiotics and other medicines at the veal farm (Δ = 14.8 % and Δ = 15.1 %, respectively) in comparison to Belgian Blue × Holstein Friesian calves.

These results are in contrast to the knowledge that crossbreds are more robust than pure breeds (Clasen et al., 2017, 2019) and it indicates that there might be a difference in robustness among crossbreds.

With regards to sex, female calves had higher hemoglobin, hematocrit, and RBC than bull calves one day prior to transport (**Chapter 6**). These traits in combination with sex-related hormones might contribute to a difference in disease susceptibility among the two sexes, with males being more vulnerable than females (Baxter et al., 2012; Pradhan and Olsson, 2020). However, **Chapter 7** showed no long-term effects of sex on mortality rate and on the majority of health problems at the veal farm. The only health problem that was significantly more prevalent in bull calves than in female calves (Δ = 7.3%) was navel inflammation in week 2 post-transport. The higher prevalence of this health condition in bull calves is often reported in literature, but mainly upon arrival at the veal farm (Wilson et al., 2000; Bähler et al., 2012; Renaud et al., 2018b). Renaud et al. (2018b) showed that male calves with enlarged and inflamed navels (score 3) upon arrival at the veal farm are at higher risk for early mortality (< 21 d; OR = 2.4) and late mortality (> 21 d; OR = 1.8) compared to calves with a normal navel (score 0 or 1). In fact, the infection can spread to the other parts of the body and it can affect multiple organs. To prevent this condition and the use of antibiotic treatment, hygiene should be monitored and the navel of bull calves should be carefully dipped into specific solutions after birth at the dairy farms of origin (Mee, 2008; Renaud et al., 2018b).

Another way to look at robustness of calves is to consider effects of maternal characteristics on the offspring. There is a growing evidence that maternal factors (such as parity) alter conceptus development and programming in utero and this could result in short and long-term consequences on post-natal offspring health (Fleming et al., 2015). As indicated by Carvalho et al. (2020), milk production levels and health status during lactation are two major factors responsible for changes in oocyte quality and uterine environment of dairy cows. Additionally, there is a profound difference in metabolism and endocrinology between primiparous and multiparous cows due to effects of higher lactation requirements of multiparous cows, which are visible in the level of glucose, IGF-1, NEFA, and steroid hormones (Cerri et al., 2012; Maillo et al., 2012; Valour et al., 2014). Changes in these metabolites can program the development of the offspring (Opsomer at al., 2017). In **Chapter 6**, calves born from first parity cows had lower N-Ig's titers in their serum from one week after birth until 10 weeks post-transport compared to calves born from higher parity cows. Mayasari et al. (2016) suggested that the level of Ig's in the plasma of calves in the first 2 weeks after birth reflects the Ig's levels in colostrum and in plasma of cows, which in the second experiment were lower in first parity cows compared to higher parity cows (**Chapter 6**). In **Chapter 6**, these relationships were also

maintained beyond week 2 after arrival at the veal farm, which supports the notion that effects of maternal immunity can result in long-term protection to calves. Moreover, it can be hypothesized that maternal immune protection might be higher in calves born from older parity cows than calves born from first parity cows, because of their higher quality of colostrum. This might suggest to provide calves born from primiparous cows with colostrum of older parity cows instead of colostrum from their own mothers (when the measured colostrum IgG content is not sufficient).

Calves born from first parity cows had a lower birth weight and body weight one day prior to transport compared to calves born from higher parity cows (**Chapter 6** and **7**). These results were in line with previous literature studies. Carvalho et al. (2020) found that daughters of primiparous cows had a lower birth weight compared to daughters of multiparous cows (35.6 kg vs. 41.1 kg, respectively). The lower birth weight can be explained by the difference in nutrient availability in late gestation. On the one hand, in late gestation, heifers are still growing and other body tissues compete with the pregnant uterus for nutrients. On the other hand, older parity cows are normally dried off at that stage and there is little competition of nutrients with the mammary gland (Carvalho et al., 2020). Long-term effects of parity of the dam on growth, health and performance has never been investigated before in veal calves. **Chapter 7** showed that parity of the dam affected weight of calves in the longer-term. First parity cows delivered calves with a lower body weight in week 1 after birth and one day prior to transport compared to calves delivered by multiparous cows. There was also a tendency towards lower carcass weight of calves born from first parity cows compared to higher parity cows. Besides these weight differences due to parity, there were no additional long-term effects on calf health status or antibiotic use. Overall, effects of parity are unavoidable, but it appears interesting to investigate whether rearing regimes of calves at the dairy and veal farm need to be adjusted, based on the parity of the dam. Moreover, future studies might investigate effects of dietary feeding interventions during the dry period on colostrum components such as IgG, insulin and fatty acids, which play a role on calf immune system and the maturation of calf gastrointestinal, respectively (Hammon et al., 2013; Mann et al., 2016).

Body weight as an indicator of robustness

Body weight (BW) of calves upon arrival at the veal farm is a known indicator of robustness (**Chapter 2**), because low BW is a risk factor for the onset of diseases, especially respiratory disorders (Brscic et al., 2012) and because it affects mortality at the veal farm (Renaud et al., 2018b) (see Table 3).

Moreover, Pempek et al. (2017) showed that calves with a low BW had a more severe

Table 3. **Associations between body weight upon arrival and odds ratio for respiratory disorders and mortality at the veal farm.**

Author	Body weight	Odds ratio (for respiratory diseases)
Brscic et al. (2012)	< 43 kg	3.13
	44-47 kg	2.43
	48-51 kg	2.68
	> 51 kg	-
Winder et al. (2016)	Per pound	0.97[1]
Renaud et al. (2018b)	Per 1 kg increase	0.93[1]
Goetz et al. (2021)	Per 1 kg increase	0.92[2]

[1]*Odds ratio for early mortality (< 21 d);* [2]*Odds ratio for mortality in the first 78 d post-transport.*

depression score (indicated by weak, lethargic calves, with altered gait) upon arrival at the veal farm (OR = 0.95) than calves without signs of depression. Factors including age, feeding methods at the dairy farm may be the causal underlying factors related to differences in body weight of calves upon arrival at the veal farm. **Chapter 7** showed that calves transported at 28 d entered the veal industry with 10.4 more kg of BW compared to calves transported at 14 d. This difference is likely to be related to age, but **Chapter 7** also showed that a higher body weight upon arrival was positively associated with carcass weight (ß = 0.228 (SE = 0.05), $P < 0.01$). **Chapters 6** and **7** showed also that the difference in body weight upon arrival between the two age groups was associated with a difference in putative measures of robustness (including neutrophil and lymphocytes (von Konigslow et al., 2020), N-IgA and N-IgM, individual treatments with medicines other than antibiotics, carcass weight, and mortality rate). Although there were significant weight differences between other groups of calves, for example between calves born from dams with different parities, these weight differences did not coincide with differences in any other measure of robustness. Likewise, a higher average weight upon arrival at the veal farm due to breed such as, for example, was observed in Holstein Friesian × Belgian Blue crossbreds in comparison with Holstein Friesian calves, was not associated with a significant difference in individual treatments with antibiotics or mortality rate. Collectively, results of **Chapter 6** and **7** suggest that weight *per se* might be not a reliable indicator of robustness, but it might be an indicator of robustness in combination with other factors, such as age or breed.

Results of this thesis supported previous findings that antimicrobial use is negatively correlated to carcass weight of calves at slaughter (**Chapter 5** and **7**). **Chapter 5** showed that calves not treated were on average 13 kg heavier at slaughter compared to calves treated individually > 2 times with antibiotics. These results were in line with the second experiment (**Chapter 7**) that showed that calves not treated had a 23 kg higher carcass weight than the one of calves treated individually > 2 times with antibiotics. These findings suggest that, the relationship between treatments with antibiotics and differences in carcass weight might be used as a feasible measure of robustness of calves.

Conclusions on factors inherent to the animal

The previous section showed that transport age of calves and different calf and dam characteristics may be potential factors affecting robustness of calves. In particular, calves transported at 28 d showed signs of a more developed adaptive immune system, needed less individual medical treatments and had a lower mortality rate at the veal farm compared to calves transported at 14 d, which are indications of long-term effects on robustness. Use of antibiotics and medicines was also higher in crossbreds other than Belgian Blue × Holstein Friesian, suggesting that they might be less resilient compared to Belgian Blue × Holstein Friesian calves. Parity affected birth weight and weight of calves one day prior to transport, but besides the weight differences, parity did not affect health or antibiotic use in the long-term. The veal sector might use these results to investigate whether rearing regimes of calves at the dairy and veal farm need to be adjusted, based on calf and dam characteristics.

On the basis of the results described in this thesis, an overview with the main factors affecting calf robustness in the short and long-term was created (see Figure 3 on page 216-217), which might have practical relevance for the veal sector.

Suggestions for the veal sector

To reduce the incidence of diseases (especially BRD and diarrhea), the veal sector uses considerable amounts of antimicrobials. Herd treatments with antimicrobials are commonly administered in the first weeks at the veal farm (Pardon et al., 2012b; von Konigslow et al., 2020; **Chapter 5** and **7**). However, the high use of antimicrobials is not a long-term sustainable strategy, because they can be associated with antibiotic resistance, which represents a threat for animal and human health (Bos et al., 2013). In the last 6 years, the veal sector was able to decrease antimicrobial use by 22% (Veterinary Medicine Institute, 2019), but prevalence of antimicrobial resistance, particularly for (ESC-R) indicative of Extended Spectrum β-Lactamase (ESBLs) and Plasmid-mediated

AmpC (pAmpC) in veal calves is still 40% (MARAN, 2020). Therefore, the veal sector might consider alternative approaches, such as the following suggestions, to improve calf robustness and reduce antimicrobial use and antimicrobial resistance:

- Role of the collection center. Future research is useful to evaluate the impact of the transition phase at the collection center on robustness of calves. Future research might investigate the comparison between two groups of calves: one directly transported from the dairy to the veal farm, and the other one transported first to the collection center and then to a veal farm. It might be useful to include this comparison in the context of the second experiment described in **Chapter 6** and **7**. Since these chapters showed that transport age can influence the immune system and performance of calves in the long-term, it would be useful to investigate whether or not age differences are still visible with the inclusion of a collection center as an in-between step.

- Development of a "track and trace" system for calves. In **Chapters 3-5,** it was clear that calves were already challenged when they arrive at the collection center. Unfortunately, in this experiment details on the history of these animals were not available, but this knowledge is important, because early life rearing conditions may have long-term effects on calf robustness (as seen in **Chapter 6** and **7**). In the current practices, the veal sector uses a system, called "Kalf Volg Systeem" (SBK, 2018), to gather information on transport age, body weight, health status, breed and sex of calves. In addition to these variables, a track and trace system may include the following information before the arrival of calves at the collection center: 1) last meal prior to the first transport to the collection center (e.g. composition, amount and timing of intake) 2) colostrum intake, 3) number of calves originating from each dairy farm that are commingled during the first transport to the collection center, and 4) duration of the journey from the dairy to the collection center. Dairy farmers may fill in the information of the first two points in the system. Then the transporter may provide information under point 3 and 4. With this approach, the system may provide the personnel of the collection center with an overview of the calves arriving at their facility and, additionally, the veal farmer of the calves that arrives on her/his farm. The "calf passport" might be useful to the veal sector in order to distinguish between high and low-risk profile calves upon arrival at the veal farm (**Chapter 2**). Risk categories may be established already at the collection center on the basis of parameters such as body weight (Renaud et al., 2018b) or serum concentration of IgG (Pardon et al., 2015). In this way, veal farmers may be more prepared when receiving calves of different and known risk profiles, and intervene in the most

useful way. Based on such a classification, calves could, for example, be housed in different compartments and they can be treated in different ways without a high use of herd antibiotic treatments and prevent transmission of diseases within a farm (Damiaans et al., 2019).

- Identification of reliable predictors of health and performance of calves. To classify calves into different risk categories at the collection center, the veal sector can identify biomarkers of future health and performance of calves (**Chapter 2**). Currently, Renaud et al. (2018b), Goetz et al. (2021), and von Konigslow et al. (2020) identified statistical associations between biomarkers measured upon arrival at the veal farm and early or later morbidity and mortality of calves in the production cycle. However, they did not investigate these associations at a collection centre or at dairy farms. Appropriate statistical methods should be used to identify the best predictors of a risk profile of (individual or groups) calves. Principle component analysis (PCA) is an example of a statistical method to identify blood variables that reflect underlying characteristics of calves. With the PCA, it is possible not only to measure intercorrelations between blood variables underlying specific characteristics of calves, but also to look at consistency of the individual differences over time. For example, a PCA was conducted on blood parameters measured at different timepoints in the transport experiment (**Chapters 3, 4** and **5**). The results showed that there was consistency over time of some individual differences, such as for hemoglobin (Hb) and hematocrit (Ht). This suggests that part of the variation in potential biomarkers reflects stable underlying characteristics of the individual animal. In this sense, the biomarkers obtained in the first experiment (**Chapter 3, 4** and **5**) meet an important precondition. In addition to PCA, there are other statistical and quantitative techniques available to obtain useful predictors for establishing risk categories and to estimate the risk profile of individual or groups of calves (for example machine learning). Due to the limited dataset obtained in the first experiment (**Chapter 3 to 5**), accuracy of prediction models was relatively poor and it was not feasible to test whether or not blood parameters could be used as biomarkers. Therefore, these statistical techniques require a much larger sample size than the one used in the first experiment (**Chapter 3** to **5**) and they may find predictors that may be animal-based or environment-based.

  As indicated in **Chapter 2** to **5**, glucose, NEFA, ß-HB and bilirubin seem to be reliable indicators of the energy status of calves in line with previous literature studies (Renaud et al., 2018a). These indicators could also be investigated in addition to the blood parameters measured in the experiment described in

Chapter 6 and **7**. With their inclusion, it might be tested whether or not calves with delayed transport (28 d) have an improved energy status compared to calves with conventional transport (14 d). As discussed in **Chapter 2**, an improved energy status before transport and at the veal farm might contribute to a higher robustness of calves. Other blood parameters that might have been interesting to include in **Chapter 6** and **7** are IGF-1, insulin and cholesterol. IGF-1 and insulin are related to development of the gastrointestinal tract (GIT) of calves and to their growth, which in turn might determine robustness of calves. Both IGF-1 and insulin can markedly differ due to age and, especially, to delay or omission of colostrum intake or different planes of nutrition (e.g. high-intensity vs. low intensity colostrum diet, or milk replacer diet) in the rearing period at the dairy farm (Hammon and Blum, 1997; Hammon et al., 2000; Ontsouka et al., 2016). It might be interesting to investigate whether or not a different transport age can affect the level of these hormones in the blood of calves and to test whether or not both IGF-1 and insulin are associated with disease incidence at the veal farm. It could be speculated that calves with higher IGF-1 and insulin concentrations have a more developed GIT and, thus, cope better with challenges during transport and in the rearing phase at the veal farm. This may also result in a reduction of health problems at the veal farm (less diarrhea and lung infections, the two most common problems in veal calves). Therefore, these hormones might be used as potential predictors of future health in veal calves. Cholesterol concentrations also change in relation to age of calves and their feeding regimen (especially related to colostrum intake) (Kühne et al., 2000; Piccione et al., 2010; Herosimczyk et al., 2013). A higher cholesterol concentration may help calves to fulfill elevated demands, associated with post-natal development and growth (Gofflot et al., 2003; Pfrieger, 2003). In addition, cholesterol is likely a relevant biomarker of health of veal calves and a predictor of early mortality. In fact, calves with low levels of cholesterol upon arrival at the veal farm are at greater risk for early mortality (Renaud et al., 2018a).

- Closer link between the dairy and the veal sector. Since robustness has an economic impact at the veal farm (due to costs for antibiotics and carcass weight losses related to poor growth), it might be good to invest more on robustness of calves at the dairy farm. As indicated before, male calves born on dairy farms have a low financial value for dairy farmers (Devans and Marti, 2020) and as such they may not provide optimal care to the animals. The current Dutch veal sector has already developed some programs to establish collaborations between the dairy and the veal sector. Examples of this collaboration are "Programmakalf" (Denkavit Nederland BV, 2021) and "MKD Driehoek" (MKD

Driehoek, 2021) developed by the companies Denkavit Nederland BV and MKD Driehoek, respectively. The veal sector might stimulate the production of robust calves by raising the financial value of these calves. Both dairy and veal farmer might benefit from this bi-directional collaboration. On the one hand, dairy farmers might be more motivated to deliver a good quality calf, because of the higher contribution that allows them to sustain the feeding and husbandry costs. On the other hand, veal farmers obtain more robust animals, which might allow the veal sector to reduce antimicrobial use. Among management practices at the dairy farm, the veal sector should especially promote and stimulate hygiene (including navel dipping; Mee et al., 2008) after birth and feeding of high quality colostrum (> 40-50 mg/ml of IgG or Brix value between 20 and 30; Quigley et al., 2013). To measure whether or not colostrum provided to calves is of sufficient quality, farmers should always test it. A practical tool that dairy farmers can use is the refractometer, which gives a value (Brix value) that is associated with the amount of IgG in colostrum (Bielmann et al., 2010; Quigley et al., 2013). In line with previous studies (Dunn et al., 2018), **Chapter 6** showed that Brix value was positively related to IgG in colostrum (ß = 0.11 (SE = 0.01); $P < 0.01$).

- Other husbandry strategies upon arrival at the veal farm. To prevent the onset of health problems at the veal farm (**Chapter 5**), veal farmers already use different strategies upon arrival of calves at the veal farm. For example, 1) provision of a warm and high energy meal directly post-transport in combination with warmth (especially in the winter months) to prevent calves from suffering from negative energy balance effects and hypothermia. 2) Provision of extra warmth via the bedding in the baby boxes (e.g. wooden crates with heating system), or via heating lamps installed above the baby-boxes. 3) Implementation of strict biosecurity measures to prevent the spread of pathogens inside the herd and ensure internal biosecurity. In fact, the high degree of commingling of calves during transport can lead to high transmission rates resulting in health issues and economic losses at the veal farm (Damiaans et al., 2019). Biosecurity can be considered a cost-effective method of prevention and can partially prevent these losses (Van Schaik et al., 2001; Roca et al., 2015) through cleaning, disinfection and grouping animals with the same disease status in the same compartment to limit contamination of the other calves and the environment (Bokma et al., 2019; Damiaans et al., 2019). The use of stricter entrance protocols and prevention of rodents to reduce the risk of *methicillin-resistant Staphylococcus aureus* (MRSA) carriage (Van de Giessen et al., 2009; Bos et al., 2012) are other important measures. With high level of biosecurity, antimicrobial use and antimicrobial resistance can be reduced (Postma et al., 2016; Collineau et al., 2017).

- **Research on transport conditions.** Effects of type of vehicle remained unclear, because of the lack of understanding of factors (other than temperature and humidity) that contributed to the effects found in the experiment (**Chapter 3, 4 and 5**). In fact, due to the inconsistency of results found in these chapters, there is a need for follow-up studies to better and comprehensively characterize climatic conditions in open and conditioned vehicles during transport and to examine which environmental factors inside the truck (such as draught, differences between in and outlet airflow or vibrations) are most relevant for calf health and its immunological and physiological system. Additionally, research on transport conditions is also necessary to define scientifically based reference values of settings for climate control systems for transport of young veal calves.

- **Epidemiological studies to assess robustness of calves.** The experiment described in **Chapter 3**, **4** and **5** showed the effects of transport-related factors on robustness of calves. This approach focused mainly on effects of experimental treatments on physiological mechanisms of robustness. Epidemiology might be used as a different angle to assess robustness of calves. Epidemiological studies can be performed to quantify antimicrobial use, morbidity and mortality rates in veal farms at cohort level, with thousands of calves, and with the involvement of different veal farms. For example, Bokma et al. (2019) quantified antimicrobial use over 2014 to 2016 in veal calves in Belgium and generated data from 83 veal farms, 342 production cycles and 167,629 calves belonging to twelve independent veal companies. Another example is shown by Renaud et al. (2018b) who evaluated a total of 4,825 calves between November 2015 and September 2016 and estimated the mortality risks of calves at the veal farm. Moreover, epidemiology can be used as an approach to establish the link between the dairy farms of origin and future health and performance of calves at the veal farm. Epidemiological studies might be useful to identify risk factors of calf health and thus, they may help to define useful interventions and remedial strategies to improve veal calf health.

- **Role of maternal effects and epigenetics on future robustness of calves.** The experiment described in **Chapter 6** and **7** showed various effects of calf and cow characteristics on robustness of veal calves. In addition to these results, future research may focus on effects of feeding interventions in late gestation on neonatal development and long-term health of calves (Abuelo, 2020). This topic might be relevant for both the dairy and veal sector because feeding interventions during the dry period of cows can have long-term consequences on health of veal calves. In fact, maternal nutritional status during pregnancy (especially

in the last months) is a major factor that might program metabolism and growth of the offspring (Reynolds and Caton, 2012; Elolimy et al., 2019). Nutrient availability in late pregnancy can also alter placental function. This, in turn, can result in changes of nutrient availability to the fetus and it can affect fetal growth and long-term health of the offspring (Gaccioli et al., 2013). Colostrogenesis also takes place during the last weeks of pregnancy and thus it is influenced by the nutritional status of the dam. Feeding a high energy diet during the dry period can result, for example, in lower colostral IgG (Mann et al., 2016). A lower amount of Ig's in colostrum may be associated with a higher risk of failure of passive transfer and thus it may affect calf immune development (Abuelo, 2020). Feeding interventions during the dry period can also influence other colostrum components such as insulin and fatty acids, which play a role on the maturation of the gastrointestinal tract of calves (Hammon et al., 2013). All these topics have never been studied in relation to future performance of veal calves. Therefore, more research might be useful to find appropriate dry cow feeding interventions associated with positive long-term effects on gut maturation, metabolism, growth and health of veal calves. The role of epigenetics needs to be also addressed by future research in this field. As indicated by Carvalho et al. (2020), associations between maternal characteristics and performance of calves might be the result of developmental programming, potentially mediated by epigenetic changes. A better understanding of these events and their biological mechanisms would be important to find new management strategies to improve robustness of calves at the veal farm.

- Development of a new protocol to assess loose or liquid manure. The protocol used to assess health status of calves in **Chapter 5** did not include the distinction between feeding related and infectious diarrhea. As a consequence, it could not be firmly concluded whether or not antibiotics were used properly based on diarrhea with infectious origin. Thus, it was not possible to rule out the possibility that some antibiotic treatments were applied without a clinical justification.

  Additional research on IgG levels. The experiment described in **Chapter 6** represented the first study focussing on the "immune gap period" in veal calves. In future studies, immunoglobulin concentrations, in particular IgG, should be monitored on a weekly basis over the entire rearing period at the veal farm. This approach might be useful for the following reasons: 1) determine the proportions

of maternal and endogenous IgG at the veal farm, 2) determine when the adaptive immune system of calves becomes functional, and 3) investigate whether or not IgG titers continue to decrease or reach a plateau beyond week 10 post-transport.

Conclusions

The aim of this thesis was to investigate the role of different environmental and animal-related factors on robustness of veal calves on both the short-term and the long-term. This thesis showed that feeding milk prior to transport and 6 h transport duration made the journey of calves to the veal farm less challenging in the short-term. In the long-term, transport-related factors did not affected robustness of calves mainly due to the high use of antibiotic treatments upon arrival at the veal farm. Transportation of calves at 28 d of age resulted in an improved robustness, as shown by the lower number of medical treatments other than antibiotics, a more advanced adaptive immunity, a higher carcass weight and lower mortality at the veal farm than calves transported at 14 d of age. The readouts of robustness measured in this thesis such as blood IgG titers or body weight of calves, might be useful to the veal sector to identify biomarkers related to future health and performance of calves. The availability of such biomarkers would be helpful, for example, to identify individual calves at an early stage with an enhanced probability to develop disease, and to take preventive measures (such as profiling calves in different risk categories) before clinical problems occur. This strategy would be helpful to the veal sector in order to reduce use of antimicrobials at the veal farm.

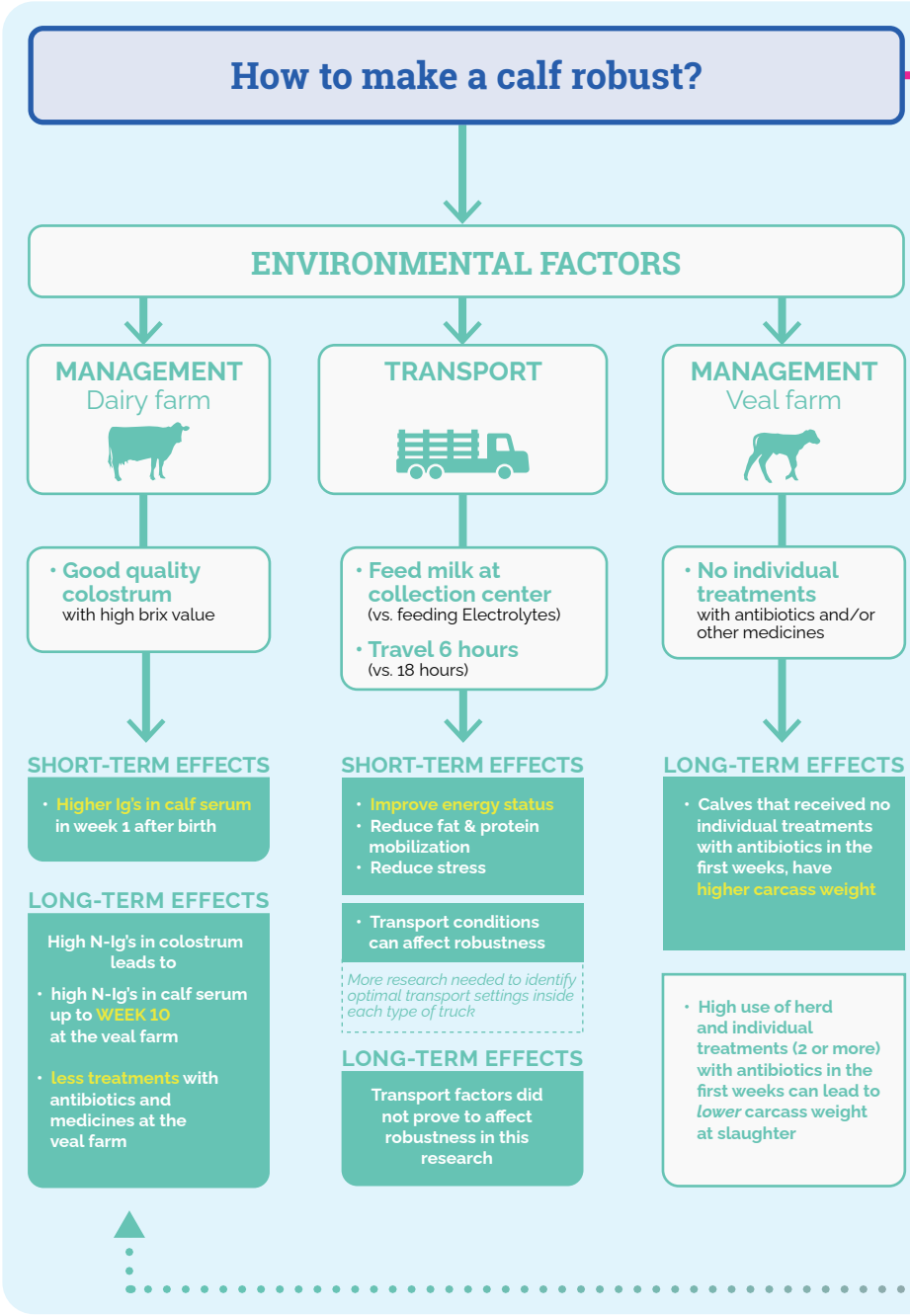


Figure 3. Overview of factors affecting robustness of veal calves based on the results discussed in the thesis. ■

INHERENT CHARACTERISTICS

MATERNAL characteristics

- **Multiparous cows** (vs. first parity cows)

LONG-TERM EFFECTS

The higher colostrum quality (N-Ig's content) in multiparous cows leads to

- higher maternal immune protection in calves

CALF characteristics

- **Transport age 28 days** (vs. 14 days)

LONG-TERM EFFECTS

Calves transported at 28 days of age have

- a more advanced active immunity in week 2 at the veal farm
- received less individual medication treatments other than antibiotics
- a lower mortality rate
- a higher carcass weight

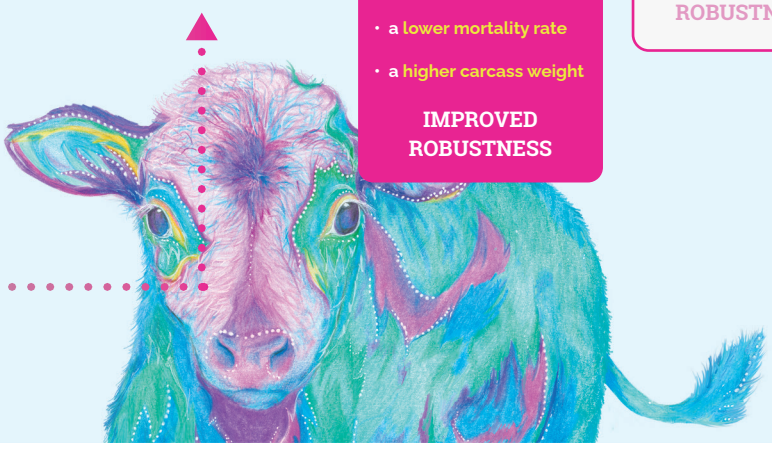
IMPROVED ROBUSTNESS

- **Breed**

LONG-TERM EFFECTS

Use of antibiotics and other medicines is *higher* in crossbreds OTHER THAN Holstein Friesian × Belgian Blue

LOWER ROBUSTNESS



ADDENDUM

Summary

Veal calves are mostly male calves and surplus female calves born on dairy farm. These animals may be considered by-products and they may not represent a priority for the dairy sector. As a consequence, these animals might receive poor post-natal care on dairy farms (including feeding low amounts of colostrum of moderate quality and poor navel disinfection after birth). These calves undergo many challenges before entering the veal industry, such as transportation at 14-20 d of age, commingling at a collection center, exposure to many pathogens, and changes in housing and management conditions upon arrival at the veal farm. All these challenges can result in high morbidity at the veal farm. To reduce morbidity and mortality rate, veal farmers make use of antimicrobial treatments in the first rearing period. However, a high use of antimicrobials is not a long-term sustainable strategy because they can be associated with antibiotic resistance. Therefore, alternative strategies to reduce antimicrobial use and antimicrobial resistance are needed for the veal sector. A strategy to reduce antimicrobial use is to improve the robustness of calves. Robustness can be defined as the capacity of an animal to cope with environmental challenges and to bounce back rapidly after challenges occur. Robust animals are well equipped to cope with endemic infections and to fight diseases and thus have probably a reduced need for antimicrobials. Robustness can be measured in terms of physiological indicators, which reflect the capacity of an animal to regulate the functions of the body in relation to external stimuli. This in turn might have an effect on morbidity, mortality rate of animals and antimicrobial use, which also represent additional measurements of robustness. Robustness can be shaped by early-life environmental factors and by animal functional reserves. The aim of this thesis was to investigate the role of different environmental and animal-related factors on robustness of veal calves. With regard to environmental factors,

Chapter 3, 4 and 5 described effects of transport-related factors (including pre-transport diet, transport duration and type of vehicle) on readouts of robustness of calves. Chapter 6 and 7 described effects of early rearing conditions of future veal calves at the dairy farm (such as assistance during calving, colostrum management, dry cow management) on readouts of robustness. In addition, these two chapters discussed effects of calf characteristics (including transport age, sex and breed) and dam characteristics (including parity) on robustness of calves.

In this thesis, robustness was measured as short-term effects, thus immediate response to the challenge, and long-term effects, thus what were the carry-over effects of early life challenges in the longer run. Long-term effects were measured in terms of prevalence of health problems and quantification of herd and individual antibiotic and medical treatments during the rearing phase of calves. Chapter 2 is a review which provided a detailed overview on potential biomarkers of health and performance of calves that were used in the subsequent chapters. Chapter 2 showed a wide range of biomarkers related, for example, to the energy and protein metabolism and immunity. The availability of such biomarkers would be helpful, for example, to profile calves at the veal farm according to the magnitude of stress they have experienced and their predisposition to develop future diseases. Grouping of calves in different risk categories might help the farmer in managing calves at arrival. By adopting handling procedures, treatment decisions and protocols on a risk-group or individual basis, farmers might be able to better meet individual animal needs and improve the health and welfare of calves. Some blood variables described in Chapter 2 were measured in Chapter 3-5, which described effects of transport related factors on robustness of calves. These chapters showed that effects of pre-transport diet, transport duration and type of vehicle on health of calves were visible in the short-term post-transport.

Compared to feeding electrolytes, feeding milk prior to transport contributed to higher glucose (3.9 vs 3.4 mmol/l) and lower NEFA (876.9 vs 719.9 µmol/l), urea (5.4 vs 6.5 mmol/l) and ß-HB (0.3 vs 0.5 mmol/l) concentrations directly post-transport, which are indicators of energy balance, nutrient reserve depletion and protein utilization (Chapter 3). Feeding milk contributed to lower cortisol (8.8 vs 10.8 ng/ml), bilirubin levels (10.4 vs 12.8 µmol/l) and higher levels of monocytes (45.1 % vs 41.0 %) compared to feeding electrolytes (Chapter 4). In the first 3 weeks post-transport there was a higher prevalence in loose or liquid manure among calves fed with milk compared to calves fed with electrolytes (30.7% vs 22.8%, respectively) (Chapter 5). Long transport duration (18 h) had more pronounced short-term detrimental effects compared to short transport duration (6 h). This conclusion was supported by post-transport lower amount of glucose (3.2 vs 4.1 mmol/l), Ca (2.3 vs 2.5 mmol/l) and higher values of ß-HB (0.48 vs 0.40 mmol/l) and

NEFA (845.5 vs 751.3 µmol/l), cortisol (11.6 vs 7.9 ng/ml) in calves transported for 18 h compared to calves transported for 6 h (**Chapter 3** and **4**). High urea concentrations, an indicator of protein utilization, were also maintained until 24 h post-transport in calves with 18 h transport (**Chapter 3**).

The combination of feeding electrolytes and long transport duration caused more profound body weight losses (-0.74 kg vs 0.41 kg) and greater short-term changes in blood variables of calves compared to feeding milk and a shorter transport duration (**Chapter 3**). Effects of the type of vehicle were inconsistent and contradictory. Calves transported in the conditioned truck had an increase in post-transport osmolality (Δ = 5.6 vs Δ = -8.0 mosmol/kg), Na (Δ = 2.2 vs Δ = -4.5 mmol/l) and higher post-transport white blood cell count (9.5 vs 8.1 × 10^9/l), proportion of neutrophils (41.5% vs 37.6 %) and a lower proportion of lymphocytes (35.4% vs 40.2 %) compared to calves transported in the open truck (**Chapter 3** and **4**). These results might be indicative of dehydration and shift in immune cell subsets after transport. However, in the first 3 weeks post-transport, calves transported in the conditioned truck showed a lower prevalence of navel inflammation compared to calves in the open truck (5.9 % vs 9.2%) (**Chapter 5**). Moreover, transportation of calves in the conditioned truck in combination with feeding electrolytes before transport contributed to the lowest prevalence of loose and liquid manure (18 %) during the first 3 weeks post-transport (**Chapter 5**).

Effects of pre-transport treatments on health status of calves were not present beyond week 5 post-transport. This result might be mainly attributed to the high use of herd and individual treatments with antibiotics within the first period at the veal farm, which masked potential effects of transport-related factors on health status of calves. In fact, calves received 5 herd treatments with antibiotics within the first 2 months after arrival at the veal farm (**Chapter 5**). Moreover 25% calves were individually treated with antibiotics and another 22% of calves were individually treated with medicines (**Chapter 5**). To test whether or not blood parameters assessed in **Chapter 3** to **5** could be used as biomarkers or predictors, they were analyzed in relation to future health and performance of calves. However, the accuracy of prediction was relatively poor due to the limited amount of data, thus future analyses should be performed with a much larger sample size. With regards to other environmental factors,

Chapter 6 and **7** showed that dairy farm management and, in particular, colostrum management may be predisposing factors of calf robustness. **Chapter 6** found a positive relationship between the amount of N-Ig's (N-IgG, N-IgM and N-IgA) in colostrum and the N-Ig's in the serum of calves measured in week 1 after birth, one day prior to transport, and N-IgG in week 2 and 10 at the veal farm. These results showed that immune

protection via maternal colostrum may last for a longer period, thus it might have a positive effect on robustness of calves. Besides environmental factors, calf characteristics (such as age, breed and sex), characteristics of the dam (such as parity) may also be predisposing factors of robustness. In week 2 post-transport, transportation of calves at 28 d of age resulted in higher lymphocyte and lower neutrophil count (Δ = 0.5 × 10^9/L and Δ = -1.6 × 10^9/L, respectively) compared to calves transported at 14 d (**Chapter 6**).

According to the literature, a higher lymphocyte count and lower neutrophil count are linked to lower morbidity and mortality at the veal farm. However, in **Chapter 6**, the differences in cell counts between age groups were not associated with long-term differences in medical treatments or carcass weights. In week 2 post-transport, titers of N-IgA and N-IgM in calves transported at 28 d were higher compared to those of calves transported at 14 d (**Chapter 6**). This might be an indication of the decline of the effectiveness of passive immunity derived from colostrum in 14 d old calves and the activation of endogenous N-Ig synthesis in 28 d old calves. Moreover, **Chapter 6** showed that, N-Ig's and, especially N-IgG, in calf serum might be used as biomarkers of future performance, because they were negatively related to individual use of antibiotics and other medicines at the veal farm. Performance data also showed that calves transported at 28 d had a higher carcass weight (Δ = 14.8 kg) and received less individual medical treatments, other than antibiotics (Δ = -5.4%) compared to calves transported at 14 d (**Chapter 7**). Mortality rate was lower among calves transported at 28 days (2.8%) relative to calves transported at 14 days (5.9 %), which is an indication of improved robustness.

With regards to calf breed, crossbreds other than Belgian Blue × Holstein Friesian received more individual treatments with antibiotics and medicines (Δ = 14.8 % and Δ = 15.1 %, respectively) compared to Belgian Blue × Holstein Friesian calves (**Chapter 7**). This might be interpreted as an indication of lower robustness. Parity of the dams significantly affected N-Ig's (N-IgG, N-IgM and N-IgA) concentration in serum of cows one week prior to calving and N-Ig's concentrations in colostrum and serum of calves (**Chapter 6**). Calves born from first parity cows had lower N-Ig's titers in their serum from one week after birth until 10 weeks post-transport compared to calves born from higher parity cows. These results indicated that effects of maternal immunity may confer long-term protection to calves.

Effects of calf and cow characteristics described in **Chapter 6** and **7** were not present on long-term health problems at the veal farm, but the prevalence of health problems in week 2 post-transport was higher compared to prevalences on the dairy farm. This might reflect the difficulty of calves to cope with the transition from the dairy to the veal farm. Beyond week 2 post-transport, prevalences of health problems were < 10 %, especially

clinical signs related to respiratory problems. This might be due to the high number of herd treatments (4.4 on average per veal farm), which likely suppressed bacterial infections and masked differences in clinical problems between treatment groups

Conclusions

This thesis showed that robustness of calves can be affected by both environmental factors (such as transport-related factors) and animal-related factors (such as breed, age of calves and parity of the dam). With regard to transport-related factors, feeding milk prior to transport and 6 h transport duration made the journey of calves to the veal farm less challenging in the short-term. In the long-term, transport-related factors did not affected robustness of calves mainly due to the high use of antibiotic treatments upon arrival at the veal farm. With regards to animal-related characteristics, transportation of calves at 28 d of age resulted in an improved robustness, as shown by the lower number of medical treatments other than antibiotics, a more advanced adaptive immunity, a higher carcass weight and lower mortality at the veal farm than calves transported at 14 d of age. ■

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About the author

Curriculum Vitae

Francesca Marcato was born on the 12th of August 1992 in Ancona, Italy. She has done her BSc in Animal Sciences and Technologies at the University of Padova. Her thesis focused on effects of mycotoxins on health of dairy cows and she achieved her BSc degree with a final mark of 110/110 cum laude. In 2014, she started the MSc programme at Wageningen University, in the Netherlands. During her MSc, she has done two major thesis. The first one was conducted in collaboration with the Animal Nutrition Group, and she investigated effects of structural features of condensed tannins on rumen biohydrogenation in vitro. The second thesis was conducted in the Adaptation Physiology Group and focused on effects of dry period length on lactation persistency of dairy cows. She graduated in 2016 and afterwards she was employed as a PhD candidate at the Adaptation Physiology Group/Animal Health and Welfare Group. During her PhD she looked at different factors affecting measures of robustness of veal calves. The main factors that were investigated included the following: 1) transport-related factors (pre-transport diet, transport duration and type of vehicle), 2) animal-related characteristics (calf transport age sex and breed, and cow characteristics such as parity, dry period length and ease of birth). The results of her PhD project are presented in this thesis.

List of publications

Peer reviewed scientific papers

Marcato, F., H. van den Brand, B. Kemp, and K. van Reenen. 2018. Evaluating potential biomarkers of health and performance in veal calves. Front. Vet. Sci. 5:133. https://doi.org/ 10.3389/fvets.2018.00133.

Marcato, F., H. van den Brand, B. Kemp, B. Engel, M. Wolthuis-Fillerup, and K. van Reenen. 2020. Effects of pre-transport diet, transport duration, and type of vehicle on physiological status of young veal calves. J. Dairy Sci. 103(4):3505-3520. https://doi.org/10.3168/jds.2019-17445.

Marcato, F., H. van den Brand, C.A. Jansen, V.P.M.G. Rutten, B. Kemp, B. Engel, M. Wolthuis-Fillerup, and K. van Reenen. 2021. Effects of pre-transport diet, transport duration and transport condition on immune cell subsets, haptoglobin, cortisol and bilirubin in young veal calves. Plos One. 16(2), e0246959. https://doi.org/10.1371/journal.pone.0246959.

Marcato, F., H. van den Brand, B. Kemp, B. Engel, M. Wolthuis-Fillerup, and K. van Reenen. 2020. Transport of young veal calves: effects of pre-transport diet, transport duration and type of vehicle on health, behavior, use of medicines, and slaughter characteristics. Front. Vet. Sci. 7, 1076. https://doi.org/10.3389/fvets.2020.576469.

Expected journal publications

Marcato, F., H. van den Brand, B. Kemp, B. Engel, S. Schnabel, C.A. Jansen, V.P.M.G. Rutten, F.A. Hoorweg, A. Wulansari, M. Wolthuis-Fillerup, and K. van Reenen. 2021. Transport age and calf and cow characteristics affect immunoglobulin titers and hematological parameters of veal calves (submitted to Journal of Dairy Science).

Marcato, F., H. van den Brand, B. Kemp, B. Engel, S. Schanbel, F.A. Hoorweg, M. Wolthuis-Fillerup, and K. van Reenen. 2021. Effects of transport age and calf and cow characteristics on health and performance of veal calves (submitted to Journal of Dairy Science).

Conference proceedings and abstracts

Marcato, F., H. van den Brand, C.A. Jansen, V.P.M.G. Rutten, B. Kemp, B. Engel, M. Wolthuis-Fillerup, and K. van Reenen. 2019. Changes in metabolic and immune parameters of young veal calves following different pre-transport diets, transport durations and transport conditions (poster). In Proceedings of International Symposium on Ruminant Physiology (ISRP), 3-6 September, Leipzig, Germany. p.580.

Marcato, F., H. van den Brand, B. Kemp, B. Engel, M. Wolthuis-Fillerup, and K. van Reenen. 2019. Effects of pre-transport diet, transport duration and type of vehicle on metabolism and immunity of young veal calves. In Proceedings of the 17th International Conference on Production Diseases in Farm Animals (ICPD), 27-29 June, Bern, Switzerland. p.97.

Marcato, F., H. van den Brand, B. Kemp, B. Engel, M. Wolthuis-Fillerup, and K. van Reenen. 2019. Effects of pre-transport diet, transport duration and conditions on performance of calves at the veal farm. In Trade-offs in science – Keeping the balance: Abstract of the WIAS Science Day, 18 March, Lunteren, the Netherlands.

Marcato, F., H. van den Brand, B. Kemp, B. Engel, M. Wolthuis-Fillerup, and K. van Reenen. 2020. Transport of young veal calves: effects of pre-transport diet, transport duration and condition on clinical health, behaviour and antibiotic treatments. Frontiers in Animal Science – lessons from the past, challenges of the present, aspirations for the future: Abstract of the WIAS Science Day, 13-14 February, Lunteren, the Netherlands.

WIAS Education & training certificate

Completed in fulfilment of the requirements of the Wageningen Institute of Animal Sciences

Description	Year
The Basic Package (3 ECTS)	
WIAS Introduction Day	2016
WGS course Research Integrity and Ethics in Animal Sciences	2020
WIAS course Essential Skills	2017
Disciplinary competences (15 ECTS)	
Writing research proposal	2016
Animal Health and Immunology Discussion Group	2016-2018
Course Design of Experiments	2016
Laboratory Course on Animal Experiments	2017
Successful Dairy Heifer Rearing: Feeding and Management	2017
The Fundamentals of Animal Emotions	2019
Epigenesis and Epigenetics	2017
Professional Competences (9.3 ECTS)	
Information Literacy Including Endnote Introduction	2016
Supervising BSc and MSc Thesis Students	2019
Shaping Future Animal Systems: Exploring Practices Through Dialogue	2017
The Essential of Scientific Writing and Presenting	2016
WGS PhD Workshop Carousel	2019
Member of the WAPS Council	2016-2019

Description	Year
Professional Competences (9.3 ECTS) *(continued)*	
Member of the advisory committee (BAC)	2020
Review of Proposals (Research Master Cluster)	2020
The Final Touch	2020
Career Perspectives	2020
Presentation Skills (4 ECTS)	
WIAS Science Day, Lunteren, The Netherlands	2019
WIAS Science Day, Lunteren, The Netherlands	2020
17th International Conference on Production Diseases in Farm Animals (ICPD), Bern, Switzerland	2019
International Symposium on Ruminant Physiology (ISRP), Leipzig, Germany	2019
Teaching competences (6 ECTS)	
Supervision of 6 MSc students	2018-2021

Total 37.2 ECTS
(One ECTS credit equals a study load of 28 hours)

Colophon

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