Disease resilience in farm animals

Ingrid van Dixhoorn

Propositions

1. Disease resilience improves when animals' behavioural needs are met.
   (this thesis)

2. Dynamic indicators of resilience predict disease duration and intensity upon disruption.
   (this thesis)

3. Explainable artificial intelligence (XAI) refutes the proposition of the Danish philosopher Søren Kierkegaard (1813-1855),

   'Life can only be understood backwards; but it must be lived forwards'.

4. A change from 'faculty of medicine' to 'faculty of health' is vital to prioritise prevention over cure.

5. Struggling with procedures and formats is an indicator of a creative mind.

6. In the Netherlands, the wolf takes its ecological role too seriously.

7. The success of a rowing crew is determined outside the boat.

Propositions belonging to the thesis, entitled
'Disease resilience in farm animals'

Ingrid van Dixhoorn
Wageningen, 2 February 2024

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Ingrid Danielle Ellen van Dixhoorn

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Disease resilience in farm animals

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Thesis

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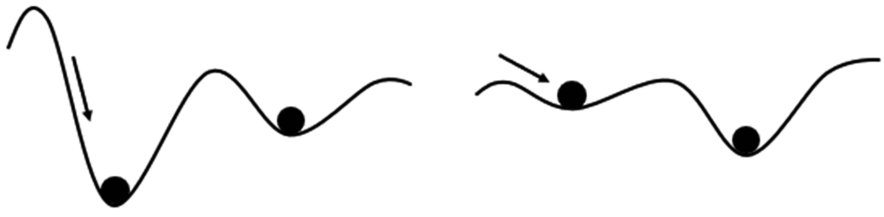
Abstract

To diminish the detrimental impact of diseases and to improve farm animal health, it has been suggested to enhance disease resilience of the animals. Good disease resilience can be described as the ability of animals to be minimally affected by challenges that can cause disease and, if affected, to recover quickly. At this moment, knowledge is limited on how to operationalise disease resilience into farm management, in favour of animal health. Knowledge is required whether disease resilience can be influenced and even predicted, so we know how animals will respond to disease challenges they may encounter. The aim of this thesis was to investigate potential influencing factors and possible predictive indicators of resilience to typical multifactorial production related diseases in livestock animals. Disease resilience was quantified in pigs by measuring the duration, recovery and severity of the symptoms after a challenge with a respiratory co-infection model of Porcine Respiratory Reproductive and Respiratory Syndrome Virus (PRRSV) and *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*). The effect of housing condition on disease resilience was studied by the introduction of social and environmental enrichment to one study group in comparison to the resilience of pigs that were conventionally housed. The application of enrichment benefitted resilience of pigs to the co-infection, by an accelerated viral clearance and a reduction of the risk and severity of lung lesions. In dairy cows the calving was seen as challenge and the period after transition of the dry to the lactation phase was used to monitor various clinical deviations as proxies of resilience, because this is the time when cows become most susceptible to diseases. To test whether (dynamic aspects of) variables could predict disease outcome, a number of physiological, immunological and behavioural variables were measured in pigs and cows prior to the challenges and tested as (dynamic) indicators of resilience ((D)IORs). The indicators of resilience that were found could consist of single measurements, the average values of multiple measurements (IORs), or the dynamic features of continuous measurements (DIORs). In pigs a higher level of lymphocytes, naïve T helper cells, memory T cells and higher relative levels of granulocytes and raised concentrations of natural (auto-) antibodies (N(A)Abs) were found as predicting IORs for the severity of the co-infection. In dairy cows, high average of eating time, high variance in ear temperature and strict regularity in behaviour including rumination and activity, were found as (D)IORs for duration and severity of post-partum diseases, summarised as a Total Deficit Score. This thesis shows that disease resilience of animals improves when they are kept under conditions that better meet their needs. Subsequently, indicators were found that can predict how animals will respond to disease challenges. These (D)IORs provide insight in the disease resilience upon perturbations. As a consequence, the influencing factors and (D)IORs will help to assess and improve animal disease resilience on farms and will form a basis for future development of (D)IORs in farm management application studies.



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CHAPTER 1

General Introduction

1.1 The relevance of animal resilience in relation to diseases

Because increased vulnerability to challenges is observed within a great number of bio-based production systems, a stronger adoption of resilience thinking has been suggested as strategy to address the increased vulnerabilities and to face future challenges (Ge et al., 2016). Over the last five decades, livestock production systems in the western world have been developed on the premise of a need to supply more food with the greatest efficiency and least risks for production losses and compromised animal health. Therefore, these systems have been designed to maximise productivity and efficiency. Due to the standardised conditions, efficient use of living space with high stocking densities, low complexity of the living space and limited use of enrichment materials, highly efficient production levels can be achieved in these systems. Genetic selection and feeding strategies were aligned with these housing conditions and were focused on increasing production efficiency. To control diseases in livestock animals, a biosecurity control strategy has been the focus for many years and has shown to be effective in many cases, although the importance of biosecurity is still not fully recognised (Morgans et al., 2021). Farm biosecurity has been defined as 'the set of practices that stop the spread of disease onto or out of an area where farm animals are present' (Shortall et al., 2018). Biosecurity constitutes a range of practices to avoid introduction in the farm (external biosecurity) and spread around the farm (internal biosecurity) and can consist of restriction of movements between infected and non-infected areas, vaccination and antibiotic treatments, cleansing and disinfecting and whole herd health plans (Morgans et al., 2021; Shortall et al., 2018). However, we also face the side effects and limitations of the primarily focus on maximum production and efficiency. Moreover, biosecurity cannot guarantee full prevention of diseases, although the level of biosecurity can definitely be improved in many cases in the livestock sector (Brennan & Christley, 2013; Gröndal et al., 2023; Maye & Chan, 2021). In addition, vaccination and the use of antibiotics have proven to be effective ways to prevent or cure specific diseases, but not for all diseases that occur in livestock effective vaccines or antibiotics have been developed (Kimman et al., 2009). Often an unknown complex and varying mixture of pathogens is involved in the expression of diseases, which may include novel or mutated pathogens, which additionally hampers effective vaccination or other biosecurity strategies. Added to that, antimicrobial resistance forces us to search for alternative solutions to ensure the health of animals in livestock production systems.

The measures that are introduced from the maximum production and efficiency point of view, such as a stimulus-poor environment with limited enrichment materials and high stocking densities, impose severe constraints on the development and expression of normal species-specific behaviour, often leading to harmful redirected behaviours like navel sucking, feather pecking and tail biting (Haskell et al., 1996; Van De Weerd et al., 2005; Wemelsfelder, 1993). The last decades, awareness is growing on the fact that the current housing and management conditions are demanding for the animals. Our high producing farm animals are permanently challenged by a variety of factors: the housing systems that hamper the expression of species-specific behaviour, chronic stress and high productivity which requires the intake of enough feed of high quality with adequate nutritional components (Sundrum, 2015). These (combinations of) challenging factors make animals vulnerable to diseases, despite the biosecurity measures (Mulligan & Doherty, 2008). Many health issues in farm animals are seen as production diseases, i.e. the outcome of the modern livestock industry that is pushing for maximization of profit (Mulligan & Doherty, 2008; Nir, 2003; Rauw et al., 1998). Many

of the diseases in livestock are caused by a combination of multiple infectious agents and environmental stressors or challenges that are inherent in the husbandry system. In spite of the biosecurity strategies and partly due to current systems themselves, the incidence of multifactorial production diseases is persistently high, and this is considered as a sign that the farm animals' ability to adapt to the demanding circumstances and high production output is overstressed (Opriessnig et al., 2011; Sundrum, 2015; Wisnieski et al., 2019). This strongly suggests that important issues that affect the development of diseases are ignored and that additional strategies are required to cope with livestock diseases (Hassan et al., 2022; Knaus, 2009; Lopez, 2022; Nakov et al., 2019; Proudfoot & Habing, 2015; Sundrum, 2015). As the most important issue, it is suggested that little attention has been paid to the animal's capacity to cope with disturbances and that allow them to bounce back to normal functioning after disturbances (Nakov et al., 2019; Saborido & Moreno, 2015; Scheffer et al., 2018). This capacity to cope with disturbances and quickly resume normal functioning after disturbances, is also known as animal resilience (Colditz & Hine, 2016; Ge et al., 2016). If animal resilience is reduced, the risks of developing diseases are expected to increase and, moreover, the clinical symptoms are expected to be more severe (Scheffer et al., 2018). Therefore, fostering animal disease resilience is suggested as approach to diminish the occurrence and severity of diseases in future livestock production systems while maintaining or even increasing production levels (Ge et al., 2016).

At this moment knowledge is limited on how to quantify the disease resilience in farm animals. In addition, it is not known which factors can influence this resilience in daily management nor if specific indicators can be found that predict this resilience. If we want to increase animal resilience, we need to find **proxies of resilience** that reflect (parts of) the response(s) of animals after challenges. Then we can test if specific (management) **factors** can influence these proxies of resilience and if **indicators** that are measured prior to the challenges can predict the proxies of resilience. The three elements: proxies, influencing factors and indicators of resilience can help to know what the causes are of reduced resilience and how farmers can improve animal resilience within livestock production systems to reduce the incidence and severity of clinical diseases.

1.2 Resilience and related concepts

To describe how animals cope with challenges, including diseases, four resilience-related concepts are commonly used: Robustness, disease resistance, disease tolerance and disease resilience (Nakov et al., 2019). These concepts are characterised by a highly multidimensional nature and have been defined as inherent mechanisms of complex dynamic systems (Urruty et al., 2016). Although the concepts have been developed in the fields of ecology and engineering, they are of universal applicability, but have a context-specific interpretation (Nakov et al., 2019).

When applied to animals, resilience at the level of the individual has been defined as the ability to react, adapt to changing environmental conditions or potentially stressful challenges with a minimum loss of function and adequate and or quick recovery (Colditz & Hine, 2016; Rutter, 2006). Others have defined animal (including human) resilience as the capacity to be minimally affected by and recover swiftly after challenge (Colditz, 2022; Gijzel, 2020; Scheffer et al., 2018; Wu et al., 2013), or as the capacity to bounce back to normal functioning after perturbation (Colditz & Hine, 2016). Friggens et al. (2022) defined resilient animals as animals that respond well to environmental

challenges and have a decreased probability of needing assistance to overcome them. Resilience is often used in combination with adaptive capacity or recovery potential in the context of stressors or adversity, where it resembles the ability to absorb change and to anticipate to future perturbations through adaptive capacity (Gijzel, 2020).

With regards to the variety of definitions for the resilience related concepts, the concept of resilience itself was considered as the broadest concept that includes all aspects that are described by other concepts separately: the magnitude of the impact of the challenge, a timescale approach and the recovery process after perturbation. In this thesis disease resilience in farm animals was defined as the capacity to remain undisturbed or quickly recover from disease related challenges. The response after disease challenges was assessed by measuring the severity of the disease symptoms, the duration of the symptoms and or the degree of recovery.

Robustness has been defined by biologists as the ability of living systems to maintain specific functionalities despite environmental or genetic perturbations (Kitano, 2004). For livestock specifically, robustness has been defined as the ability to carry on doing the various things that the animal needs to do in the face of environmental constraints in favour of its future ability to (re)produce (Friggens et al., 2017). Robustness has also been defined as 'the ability to maintain homeostasis or 'the ability of an animal to function well in the environment it lives in as well as in a wide range of climates and production systems' (Klopčič et al., 2009).

Disease resistance in animals has been defined as the ability to actively diminish the burden of a pathogen or prevalence of clinical disease through inhibition of the infection process or the reduction of pathogen replication rate (Bishop & Woolliams, 2014). Also the level of control that the host can have over the pathogen's lifecycle falls within the disease resistance definition (Bishop & Woolliams, 2014; Nakov et al., 2019).

Disease tolerance has been defined as the adaptive ability in preserving homeostasis and at the same time limiting the detrimental impact that infection can inflict on an animal's health and performance without affecting pathogen burden per se (Nakov et al., 2019). So high disease tolerance might limit the possible clinical symptoms (Guy et al., 2012) or the net impact on performance of a given level of infection (Bishop & Woolliams, 2014).

Previous studies state that resilience differs from robustness in the sense that robustness covers the ability of animals to function and maintain homeostasis at a certain level, whereas resilience is typically associated with the capacity to adapt to or recover (fast) from changes, challenges and perturbations which could contain short term as well as long term responses (Colditz & Hine, 2016; Knap & Doeschl-Wilson, 2020; Napel ten et al., 2010). This means that good resilience also includes an adequate and quick recovery process when homeostasis is shorty deviated in case of diseases. It was therefore previously suggested that a combination of disease resistance, tolerance and robustness together form disease resilience in animals when faced with pathogens (Harlizius et al., 2020; Poppe, 2022).

1.2.1 Tipping points, positive feedback loops, critical slowing down and (dynamic) indicators of resilience

The concept of resilience can be described with the use of stability landscape illustrations and at the same time it can be supported mathematically with the dynamical systems theory and tipping points, which is part of a wider theory called dynamical systems theory (Scheffer, 2009). An example of a stability landscape is given in Figure 1. This (two dimensional) stability landscape is useful for describing and communicating the phenomena of tipping points and relevant aspects of resilience (Beisner et al., 2003; Gunderson, 2000; Holling, 1973; Scheffer et al., 2009). The stability landscape can be described by its landscape configuration (the line) and a ball. When applied to individual animals, the position of the ball in the landscape represents the animal's state at a certain timepoint (1) and can be defined by a variable (e.g., body temperature). The position of the ball can be changed by external forces (perturbations or challenges e.g., an infection, represented by a black arrow (P) in the figure). The size of the arrow indicates the force on the ball and shows the degree of perturbation the animal is facing that can cause a disease. After small perturbation, gravity will pull the ball downwards into a valley, representing stability (example A in panel 1a, also referred to as equilibrium or basins of attraction) and in case of animals representing the normal value (homeostasis) of the variable when the animal is healthy (e.g. a body temperature in cows of 38.5°C). The landscape configuration, which represents resilience, is dynamic and the depth of the valley, the steepness of the slopes and the height of the peaks are determined by external factors (e.g. husbandry conditions) and/or by animal factors (e.g. age of the animal and its genetic background) (van Nes et al., 2016). Consequently, a change in landscape influences the size of the perturbation which is needed to shift to a different state. When the slopes are steep and the valley is deep, representing high resilience, only a very large force (being a disturbance larger than the arrow P e.g., a highly infectious pathogen) will cause the ball to move to the adjacent valley which represents a diseases state (example A in panel 1a). However, if the landscape is different with less steep slopes, representing lower resilience, the perturbation (P) can cause the ball to shift to the adjacent valley (ball from position 1 to position 3 in example B in panel 1a). This adjacent valley represents a drastically different state, it can be an instable state, a stable alternative state, or collapse, representing death. When the ball shifts from one valley to another, a so called tipping point is passed (Gunderson, 2000; Scheffer, 2009; van Nes et al., 2016). A tipping point illustrates the idea of a threshold being crossed between the two states and is represented by the peak between the two valleys in the stability landscape (position 2 of the ball in panel 1a). The positive feedback loop is represented by the ball running off the hill to the adjacent contrasting valley (position 3, which is the diseased state in panel 1a).

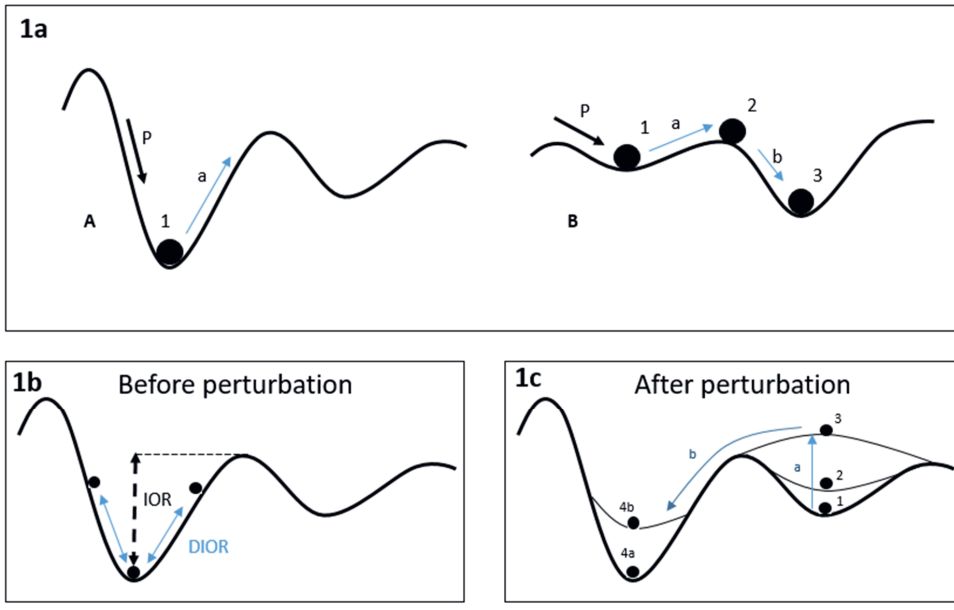

Figure 1. Panel 1a: Two examples of two-dimensional stability landscapes of animal A with high resilience and B with low resilience at a certain timepoint using a single variable. The position of the ball at 1 represents the healthy state (basin of attraction) prior to perturbation (P) and the blue arrow (a) indicates the shift caused by P which brings the ball close to the tipping point (2). The blue arrow (b) represents the positive feedback loop after the tipping point, which brings the ball to the alternative (diseased) state 3. Panel 1b shows that the depth of the valley can be used as an Indicator of Resilience (IOR). The way the ball fluctuates is determined by the shape of the valley and can serve as a Dynamic Indicators of Resilience (DIOR). (D)IOR's can be used to predict if a perturbation can cause the ball to shift to the diseased state. Panel 1c. represents ways to calculate proxies of resilience. A diseased animal is in the alternative state (1) and the recovery process causes the valley to change so that the ball is lifted to position (2) until a new reverse tipping point is reached (3) at which the ball rolls back to the same previous valley (4a) or back to a valley with a different resilience (less deep) (4b). The depth of the valley in the alternative (diseased) state, the time it takes to recover and the state after recovery can be used as proxies of resilience.

Multiple perturbations can also change the state of an animal which brings the ball closer to a tipping point during the fluctuations (position 2 in panel 1a). Once close to the threshold, a relatively small additional disturbance can result in a shift to the unhealthy state (position 3) (Gunderson, 2000; Olde Rikkert et al., 2016).

The tipping point in animals from the healthy to the diseased state can be caused by so called positive feedback loops, which are self-enforcing, caused by intrinsic processes within the animal (van Nes et al., 2016). Because positive feedbacks amplify small initial changes to large ones, they are responsible for the typical intrinsic runaway change that characterises the behaviour of a system when a tipping point is passed. Translated into animal diseases, where often a complex set of disease-causing factors is involved, the peri-parturient disorders in dairy cattle and Porcine Respiratory Disease Complexes in pigs are used as examples in this thesis.

In pigs a tipping point as result of positive feedback loop may occur in the respiratory disease complex (PRDC). The combination of unfavourable environmental circumstances and inadequate management practices with high pathogen load, will require adaptative capacity up to a point that the pigs are no longer able to cope and will become ill. Unfavourable climatic conditions can damage the mucous membranes of the respiratory system which will form a porte d'entrée for PRDC related pathogens, the inadequate climatological conditions in combination with other environmental and management factors can aggravate this process up to a point at which the lungs are severely damaged and recovery is only possible when intervention strategies are applied that often include antibiotics. When a virus such as the porcine reproductive and respiratory syndrome virus (PRRSV), is involved, it is more likely that secondary infections will be aggravated as the PPRSV can modulate the immune system so that the susceptibility to other pathogens is increased (Kimman et al., 2009; Meulenberg, 2000; Thanawongnuwech & Suradhat, 2010; Zhu et al., 2010).

During the transition phase in cows (lasting from three weeks before until three weeks after parturition (Grummer, 1995) the cows face metabolic and physiological changes in preparation for calving and lactation (Huzzey et al., 2005; Urton et al., 2005). In principle, all cows are able to remain healthy during the transition phase. However, during this phase both nutrient and energy demands increase, and these demands are not always adequately met. As a result, dairy cows face the challenge of a negative energy balance (NEB). This makes them prone to develop an unbalanced energy metabolism, disturbed mineral utilization and perturbed immune function (Belaid et al., 2021; LeBlanc et al., 2006; Mulligan & Doherty, 2008). This process may continue up to a point at which the cow can no longer maintain homeostasis and becomes ill, without the capacity to easily recover, hence the tipping point. Illnesses that typically occur post partum are e.g. retained placenta, metritis, mastitis, ketosis, fatty liver, displaced abomasum, ruminal acidosis, milk fever, sub-clinical hypocalcaemia, the downer syndrome (Sordillo & Mavangira, 2014; Wankhade et al., 2017). Recovery of these processes will take time and effort. Conversely, post-partum infections themselves may trigger metabolic disorders, which will further reinforce infections which can cause this positive feedback loop of reinforcing the NEB, which happens when the cow is not resilient enough to deal with the infections themselves. Thus, the onset or appearance of post-partum disorders is not always the same and the pathophysiology is complex, and many organ systems can be involved.

The depth and the slopes of the valley in the stability landscapes establish the animal's resilience, as they determine how difficult it is to change to an unhealthy state. In animals most of the physiological variables fluctuate around their average normal value and these fluctuations are caused by all kinds of minor disturbances that will influence their temporary level (e.g., body temperature is constantly fluctuating around the level of 38.5^{o}C in cows). This can be depicted by the ball rolling from left to right in the bottom of the valley (Figure 1b). The configuration of the valley determines the way the ball fluctuates around the equilibrium (the dynamics of the body temperature over time). Loss of resilience is depicted in the stability landscape as a flatter valley (landscape B in panel 1a) causing the ball to roll more slowly to the bottom when disturbed, thus increasing recovery time after small perturbations. In mathematical terms the rate of change around the equilibrium of a variable decreases. Therefore, the value of the variable becomes more and more like its previous value. This can be calculated as an increase in temporal autocorrelation which is the correlation between the original time series and its lagged version (Dakos et al., 2012; Dakos et al., 2008; Scheffer et al., 2009; Scheffer et al., 2018; Van Nes & Scheffer, 2007). Also, the fluctuations

of a variable will become larger when the valley is shallow thus increasing the variance of the variable's time series. These slower fluctuations of a variable is a phenomenon known in dynamical system theory as 'critical slowing down' (Holling, 1973; Scheffer, 2009; Scheffer et al., 2009; Scheffer et al., 2018; Wissel, 1984). The stability landscape also helps to intuitively understand the concept of critical slowing down and why an increased variance and autocorrelation of a variable are suggested as dynamic indicators of resilience (DIOR's), and this is shown in panel 1b. The indicators do not necessarily need to be dynamic as the depth of the valley itself can also indicate how difficult it is to shift to a contrasting state. In case of animals, a single measurement of a variable could therefore also function as an indicator of resilience (IOR), not being dynamic.

The measurements from wearable electronics and other sources now allow us to analyse the dynamics of physiology and behaviour with high resolution (Scheffer et al., 2018), which makes it possible to follow patterns and dynamics in time. This forms a great opportunity to find these so called dynamic indicators of resilience (DIORs) in humans and animals and they were studied in a variety of cases, but with varying degree of predictive performance for resilience (Gijzel, 2020; Knap & Doeschl-Wilson, 2020; Poppe, 2022; van der Zande et al., 2020; Veerkamp et al., 2013). Although the quantitative aspects of critical slowing down seem very promising for indicating resilience, empirical evidence for the predictive value of DIORs in relation to diseases are still very limited.

To test experimentally if (D)IOR's are predictive of the way an animal responds to a perturbation, i.e. its resilience, they need to be related to the situation after disturbance (Figure 1 Panel 1c), i.e. did the animal end up in the adjacent valley after a perturbation (Figure 1 situation B in panel 1A) or not (Figure 1 situation A in panel 1a). If it landed in the adjacent valley, proxies of resilience can be determined by the depth of the valley reflecting the severity of the disease symptoms, the time to recover and return to the original valley (the recovery process is represented in panel 1c by the flattening valley of the disease state caused by intrinsic recovery processes) and, once returned to the original valley, by the configuration of the valley after recovery (similar, deeper or flatter than the pre-challenge valley). All these aspects that describe the situation after perturbation are proxies of resilience that represent (an aspect of) the resilience of the animal. How they can be measured is described in more detail in the next chapter.

1.3 Proxies of resilience

When we want to study, or influence animal resilience, it is required to translate the concept of resilience into quantifiable measurements that reflect animal resilience and, in this thesis, these are referred to as proxies of resilience. As resilience is related to the responses after perturbation, in general, the outcome of a perturbation is considered to reflect (loss of) resilience. As example how these proxies of resilience can be assessed, the (hypothetic) response of the body temperature of two different animals (A and B) after an infectious challenge is illustrated in Figure 2 (modified according to (Mu et al., 2021; Nakov et al., 2019; Urruty et al., 2016). Time is reflected in the horizontal axis whereas the vertical axis body temperature is depicted. Three properties of the response curve of the body temperature after perturbation can be quantified: Responses in temperature that relate to time (indicated as $tA_{1\text{-}3}$ and $tB_{1\text{-}3}$), to the degree of temperature rise (indicated as iA and iB) or to the capacity to recover to the normal temperature or equilibrium

(indicated by A and B). In general, this level of equilibrium after the recovery can be lower, higher, equal to or outperform the pre-challenge level.

Animal A shows a faster recovery time tA_3 as compared to animal B (tB_3) whereas the time between the moment of infection and peak of temperature is shorter in animal A as compared to animal B ($tA_{1\text{-}2}$ and $tB_{1\text{-}2}$). Animal A also shows a lower maximum temperature iA as compared to animal B (iB) and the temperature level after recovery is back to normal for animal A (A) but slightly higher for animal B (B) for a certain period of time. Thus, when using the variable temperature, animal A shows a faster recovery time, a smaller temperature-rise as compared to animal B, and a recovery to the previous level after infection, rendering animal A as more resilient. The course of response can be measured using other variables, such as for instance, viral load, immunological parameters, production parameters (such as daily milk production and growth), behavioural characteristics, tissue damage or biomarkers related to other physiological mechanisms (Albers et al., 1987; Putz et al., 2019). Not for all variables, it is possible to measure all aspects of the course of response. The time related aspects are only available if frequent or (semi) continuously measurements are possible. Other responses can only be measured once after the challenge, such as pathological or histological findings and therefore only indicate the severity of the response (iA and iB). These measurements will not give insight in recovery patterns (unless multiple subsequent measurements are possible).

As proxy for (loss of) disease resilience often mortality or antibiotic use after infection have been used (Chen et al., 2020), which also are single final measurements that do not include recovery patterns. In addition, other variables that are used as proxies for resilience (mostly in in breeding research) are not specifically or directly related to defined perturbations (which would reflect resilience on that specific perturbation, which can be a disease), but reflect the overall capacity of animals to cope with the continuous environmental challenges they face, thus reflecting generic resilience.

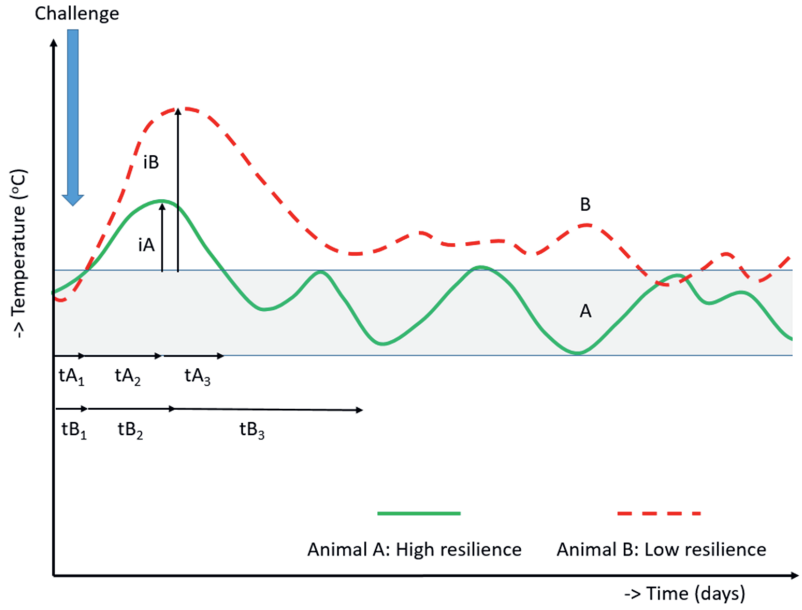

Figure 2. Illustration of the course of response of temperature after infection of two animals A and B with time related properties (tA_{1-3} and tB_{1-3}), the peak in temperature rise (indicated with iA and iB) and level of temperature after recovery (indicated with A and B). The grey band represents the range within which the temperature fluctuates around the equilibrium (homeostasis). It depends on the frequency of measurements and variable whether all the details can be assessed. Modified from (Mu et al., 2021; Nakov et al., 2019).

1.4 Potential influencing factors and indicators of animal resilience

To observe individual variation in resilience, animals can be exposed to a standardised challenge, e.g., an experimental infection or stressors that can induce disease. To investigate possible influencing factors requires the introduction to these external factors before the challenge. The variation in resilience (quantified by the proxies) can be used to examine which external environmental factors can influence animal resilience and which animal factors provide an additional source of variation. External factors are for instance housing conditions, climate, feed and feeding strategy, and examples of animal factors are age, sex, personality, coping strategy or genetic background. Genetic background has been proven to be of relevance for the resilience of animals (Bai & Plastow, 2022; Bengtsson et al., 2022; Harlizius et al., 2020; Misztal, 2017). Breeding for resilience, although considered as important, can be seen as a longer-term strategy and was outside the scope of this thesis. In this thesis the focus to influence resilience were assessed, given the herd or group of animals present at the farm. The animal factors and external factors that were present upon perturbation will determine the (dynamic) state of the animal when it encounters a challenge and is represented as basin of attraction in the stability landscape. The configuration of this basin of attraction (the valley in the stability landscape) can be described by (D)IOR's as explained in chapter 1.2. Therefore, if the state of the individual animals at the moment of challenge is determined with

the (D)IOR's, it can be tested if resilience, (as assessed by the proxies of resilience) can be predicted using the (D)IORs.

1.5 Aim and outline of this thesis

The overall aim of this thesis was to find influencing factors and predictive indicators of resilience in relation to production diseases of livestock animals. To that aim, two disease complexes were chosen in two different species: The respiratory disease complex in young pigs after weaning (chapters 2 and 3) and in dairy cows, the typical transition related disorders that occur after calving (chapters 4 and 5). In the pigs, a co-infection model with Porcine Respiratory Reproductive and Respiratory Syndrome Virus (PRRSV) and *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) was used and disease outcome variables (gross pathology, histology score, rectal temperature, viral load and sickness behaviour) were used as proxies of resilience. Enrichment of the housing conditions prior to challenge was tested as a possible influencing factor of resilience in pigs (Chapter 2). In Chapter 3 variables that were measured once or twice prior to the infection were tested as possible indicators of resilience (IOR's) in pigs.

In dairy cows the transition phase from the dry period to the lactation phase was studied as a challenge often leading to postpartum diseases. The duration and severity of all deficits related to the periparturient diseases after calving were scored and summed to one total deficit score (TDS), which was used as a proxy of resilience. Different patterns of longitudinal behavioural data in cows were calculated during the dry period and tested as predictive (dynamic) indicators of resilience after calving in chapters 4 and 5. In chapter 5 additionally the performance of different predictive models for resilience was evaluated.

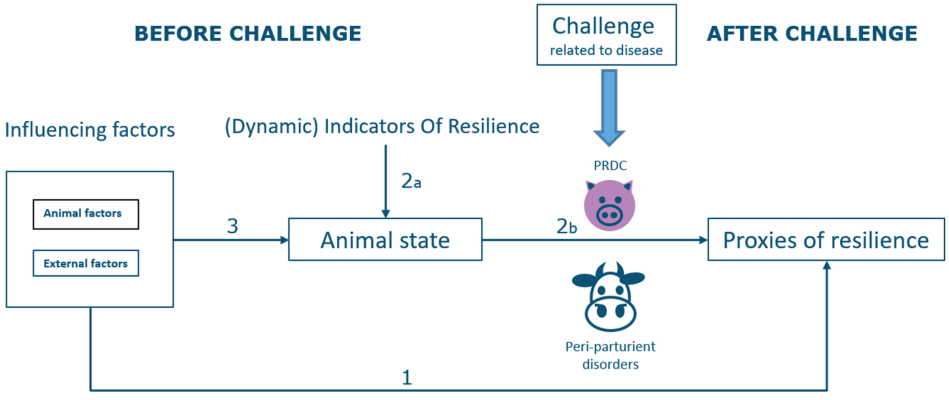

Figure 3. Framework of the experimental set up that was used in this thesis to assess disease resilience of pigs and cows. Resilience was determined after challenge by measures of the severity and duration of the disease, referred to in this thesis as proxies of resilience. Animal based and external factors (environment based) were tested as influencing factors of resilience (1) and (dynamic) indicators of resilience (that reflect the animal state prior to challenge 2a) were tested as predictors of animal resilience (2b). In addition, the effects of influencing factors on the indicators of resilience were tested (3).

CHAPTER 2

2

Enriched housing reduces disease susceptibility to co-infection with porcine reproductive and respiratory syndrome virus (PRRSV) and *Actinobacillus pleuropneumoniae* in young pigs

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Abstract

Until today, anti-microbial drugs have been the therapy of choice to combat bacterial diseases. Resistance against antibiotics is of growing concern in man and animals. Stress, caused by demanding environmental conditions, can reduce immune protection in the host, influencing the onset and outcome of infectious diseases. Therefore psychoneuro-immunological intervention may prove to be a successful approach to diminish the impact of diseases and antibiotics use. This study was designed to investigate the effect of social and environmental enrichment on the impact of disease, referred to as "disease susceptibility", in pigs using a co-infection model of PRRSV and *A. pleuropneumoniae*. Twenty-eight pigs were raised in four pens under barren conditions and twenty-eight other pigs were raised in four pens under enriched conditions. In the enriched pens a combination of established social and environmental enrichment factors were introduced. Two pens of the barren (BH) and two pens of the enriched housed (EH) pigs were infected with PRRSV followed by *A. pleuropneumoniae,* the other two pens in each housing treatment served as control groups. We tested if differences in disease susceptibility in terms of pathological and clinical outcome were related to the different housing regimes and if this was reflected in differences in behavioural and immunological states of the animals. Enriched housed pigs showed a faster clearance of viral PRRSV RNA in blood serum (P=0.014) and histologically 2.8-fold less interstitial pneumonia signs in the lungs (P =0.014). More barren housed than enriched housed pigs developed lesions in the lungs (OR=19.2, P =0.048) and the lesions in the barren housed pigs showed a higher total pathologic tissue damage score (P <0.001) than those in enriched housed pigs. EH pigs showed less stress-related behaviour and differed immunologically and clinically from BH pigs. We conclude that enriched housing management reduces disease susceptibility to co-infection of PRRSV and *A. pleuropneumoniae* in pigs. Enrichment positively influences behavioural state, immunological response and clinical outcome in pigs.

Introduction

Psycho-social stress in man and animal alters disease susceptibility to infectious agents (Biondi & Zannino, 1997; Elenkov & Chrousos, 1999a, 1999b; Jirtle & Skinner, 2007). Social factors, environment and stress are associated with disease susceptibility and are therefore suggested as cofactors in the pathogenesis of infectious diseases (Biondi & Zannino, 1997; Proudfoot & Habing, 2015). Antimicrobial drug therapy has been used against infectious bacterial diseases, but the resistance of bacteria to antibiotics is of increasing concern. Therefore psychoneuro-immunological intervention has been proposed as a potential strategy to diminish the onset and outcome of infectious diseases. However, many uncertainties remain regarding the effectiveness of this strategy (Biondi & Zannino, 1997).

In pigs, the adverse effects of stimulus-poor housing conditions and social stress on behaviour, (re)activity of the hypothalamic-pituitary-adrenal (HPA) axis and mood (pessimistic versus optimistic) are well established (Averós et al., 2010; Beattie et al., 2005; Beattie, O'Connell, Kilpatrick, et al., 2000; Beattie et al., 1995; Bolhuis et al., 2005; Chaloupková, Illmann, Bartoš, et al., 2007; Chaloupková, Illmann, Neuhauserová, et al., 2007; De Jong et al., 2000; Douglas et al., 2012; Elizabeth Bolhuis et al., 2013; Kutzer et al., 2009; Oostindjer et al., 2011; Schrøder-Petersen & Simonsen, 2001; Sneddon et al., 2000; Tönepöhl et al., 2012). Provision of suitable enrichment substrates, such as straw or peat, better meet the behavioural needs of pigs and improve their welfare, but is often associated with disadvantages in costs, labour and hygiene and is incompatible with the generally applied slurry-based manure management (Tuyttens, 2005). Commercially kept pigs are therefore often reared under barren, stimulus-poor housing conditions (Bracke et al., 2013). Studies on the association between the use of natural materials, such as straw, as enrichment substrates and the risk of infectious diseases in pigs are equivocal (Ewald et al., 1994; Roepstorff & Jorsal, 1990; Skjerve et al., 1998; Stege et al., 2001; Tuyttens, 2005). Pigs reared under enriched conditions exhibited fewer days of diarrhoea after weaning and less gastric lesions at slaughter as compared to barren reared pigs (Amory et al., 2006; Bolhuis et al., 2005; Bolhuis et al., 2007; Camerlink et al., 2014; Di Martino et al., 2013; Nielsen & Ingvartsen, 2000; Oostindjer et al., 2010). No direct evidence has been established so far, however, for an effect of housing and management procedures that stimulate natural (social) behaviour in pigs on the development of disease, referred to in this paper as 'disease susceptibility' (Proudfoot & Habing, 2015; Reimert et al., 2014b; Tuyttens, 2005). Therefore, in the current study we compared disease development following co-infection with porcine reproductive and respiratory syndrome virus (PRRSV) and *A. pleuropneumoniae* between pigs reared in environmentally and socially enriched pens and pigs reared in barren, stimulus-poor housing conditions. We hypothesised a reduced disease susceptibility to co-infection, as expressed by reduced clinical signs, in the enriched housed pigs.

We used this model of co-infection with mild virulent pathogens under experimental conditions to obtain measurable variation in clinical symptoms and pathology in the animals. When applied separately, these pathogens do not induce overt clinical signs or pathology, but in combination, probably due to PRRSV's immunosuppressive effect, a higher incidence of secondary bacterial infections occurs (Kimman et al., 2009). Moreover, PRRSV and *A. pleuropneumoniae* are pathogens frequently involved in porcine respiratory disease complex (PRDC) in pigs. PRDC causes

severe health and welfare problems, is difficult to control and due to morbidity and reduced growth rates in surviving animals incurs a significant financial burden to farmers. Although PRRSV vaccines have been developed, none of the current vaccines are able to completely prevent respiratory infection. Moreover, unfortunately current control strategies fail to provide sustainable disease control (Thanawongnuwech & Suradhat, 2010). Enriched housing conditions may possibly contribute to sustainable disease control (Corzo et al., 2010; Kimman et al., 2009; Thanawongnuwech & Suradhat, 2010).

Materials and Methods

The established principles of laboratory animal use and the EU and Dutch laws related to animal experiments were adhered to in this study. The Wageningen University Animal Care and Use Committee (Lelystad Department) approved the experiment under number 2013181.

Experimental design, animals and housing

For the experiment 56 male and female piglets (Topigs 20 line, from Great Yorkshire x Landrace origin) were used (8 litters of 7 piglets). The piglets were offspring of eight multiparous sows obtained from a PRRSV and *A. pleuropneumoniae*-free herd. Sows were inseminated on the same day and the expected parturition day was defined as day 0 for all piglets. From two weeks before parturition onwards, the sows were housed in farrowing crates at research facility A of Wageningen UR Livestock Research, Lelystad, the Netherlands.

The 8 litters were subjected from the first day of life onwards to either barren housing (barren housed: BH) or enriched housing (enriched housed: EH) (Table 1). BH piglets were housed according to current legal requirements for farmed pigs in conventional 5 m^2 barren pens with 100% slatted floor and a 100x45cm solid rubber floor mat. EH piglets were housed in 10m^2 enriched pens with partly slatted (40%) and partly solid (60%) floor.

In the barren pens two chains with blocks were added as enrichment. In the enriched pens rooting substrate was provided which consisted of 1 kg straw, 160 L of moist peat and 180 L of wood shavings. In these pens two jute bags and branches of a broom were added, as well as the same two chains with blocks as in the barren pens. Straw and wood shavings were replenished daily (0.5 kg/day straw, 23 L/day of wood shavings), and on a weekly basis new branch of a wooden broom, new jute bags and 20 L of peat were added to the enriched pens. All pens were cleaned daily by removing faeces and rinsing the slatted floors with water. All materials for all pens, including substrate enrichment and food, were γ-irradiated (9kGy irradiation; Synergy Health Ede BV, the Netherlands). A heating lamp for the piglets was provided in each pen during the first week after birth. On day 3, the piglets received an ear tag, and they were treated with ironject® 20% + B12 (Dopharma, Raamsdonksveer, the Netherlands) according to standard procedures. Tails were kept intact, and the male piglets were not castrated.

All enriched and barren pens had two drinking nozzles, one for the sow and one for the piglets. Sows were fed a standard commercial diet twice a day at 8:00 am and 3:15 pm. The piglets received solid food *ad libitum*, starting at day 3. Lights were on between 6:00 am and 6:00 pm. Temperature was kept at 25°C during the first week after birth and it was decreased by 1°C every week until it reached 22°C, the week before weaning.

From day 13 until weaning, the panels between two adjacent EH pens were removed, allowing piglets from two different EH litters to mingle. Thus, the four individual enriched pens of 10m^2 were temporarily transformed into two pens of 20m^2 to enable early social interaction between EH litters.

On day 17 all piglets were subjected individually to the backtest, to assess their coping strategy (Bolhuis et al., 2003). During the backtest, piglets were held in supine position for 1 minute and the number of struggles, latency to first struggle, number of vocalizations, and latency to the first vocalization were recorded (Luca Melotti et al., 2011).

On day 31, the sows were removed from both barren and enriched pens (i.e., weaning day) and seven piglets per litter were selected. The selection was made taking gender, bodyweight and coping strategy into account in order to obtain piglets with comparable features in each experimental subgroup. The selected piglets were regrouped into eight new groups of seven piglets per group. All piglets were equally mixed and new groups with comparable compositions were formed. The new BH groups included only piglets originating from BH litters and the EH groups included only piglets originating from the EH litters, meaning that housing treatment remained the same as before weaning. The piglets per group were individually tagged using an animal marker for identification.

On day 39, all piglets were transported 4.5 km to facility B of Wageningen UR Livestock Research. Group structure, housing enrichment, pen sizes and access to food and water were kept the same as before transport. Pigs of two barren and two enriched pens were housed in separate High Efficiency Particulate Air (HEPA) filtered animal rooms to prevent pathogen distribution by air. The pigs of the other two barren and the two enriched pens served as negative-controls and were kept in conditions according to their housing conditions in separate pens within one large room in facility B without extra biosafety measures regarding the outgoing air (infected pigs referred to as BHI and EHI pigs, controls as BHC and EHC pigs, Table 1).

Temperature at weaning on day 31 was 28°C and was decreased by 2°C each week until it reached 22°C. It was kept at 22°C from day 42 to day 55.

On day 55 the pigs were euthanised by injecting pentobarbital (Euthasol40%, AST Farma) in the auricular vein, while they were restrained and thereafter exsanguinated.

Table 1. Experimental set up and group names

Housing	Groups before infections	Groups after infections	Number of pigs	Infection	Number of pens
Barren	BH	BHI	14	PRRSV / App	2
Barren	BH	BHC	7	Mock infected (Control)	1
Barren	BH	BHC	7	No infection (Control)	1
Enriched	EH	EHI	14	PRRSV / App	2
Enriched	EH	EHC	7	Mock infected (Control)	1
Enriched	EH	EHC	7	No infection (Control)	1

App: *Actinobacillus pleuropneumoniae*

PRRSV: porcine reproductive and respiratory syndrome virus

BH: Barren Housed, BHI: Barren Housed Infected, BHC: Barren Housed Control

EH: Enriched Housed, EHI: Enriched Housed Infected, EHC: Enriched Housed Control

Behavioural observations and skin lesions

Frequencies of the behaviours listed in the ethogram (Table 2, adapted from (Bolhuis et al., 2006; Camerlink et al., 2012)) were recorded before and after weaning (day 30 and day 32) and before and after transport to facility B (day 38 and day 40). On each observation day, all pens were observed for the duration of 4 ten-minute periods, twice in the morning (between 8:00 and 11:00 am) and twice in the afternoon (between 13:00 and 16:00 am) in an order balanced for housing. Behaviours were averaged per pen and per day and expressed as frequencies per pig per ten minutes. A new bout was scored when the pig stopped the behaviour for more than 2 seconds.

On the same days, skin lesions at the front (head, neck shoulders and front legs), middle (flanks and back) and rear (rump, hind legs, tail) were counted and categorised as a proxy for aggressive behaviour (Simon P. Turner et al., 2006). For each body region, the number and severity of lesions was differentiated using scores from 0 – 4 as follows (modified from (L. Melotti et al., 2011)): **0**: No lesions; **1**: < 5 superficial lesions; **2**: 5-10 superficial lesions or < 5 deep lesions; **3**: 10-15 superficial lesions or 5-10 deep lesions; **4**: > 15 superficial lesions or > 10 deep lesions. Lesion scores were averaged per pen and per day.

Table 2. Ethogram

Behaviour	Description
Social behaviour	Touching or sniffing any body part of a pen mate (frequently head region)
Aggression	Uni- or bilateral fighting by chasing, head knocking (with or without biting) and/or pushing
Manipulate pig	Nibbling, sucking or chewing on any body part of piglet or sow, or belly nosing
Manipulate pen	Nibbling, sucking or chewing on pen components
Playing	Fast running around the pen (galloping), rolling and shaking objects
Mounting	Standing on hind legs with front legs on pen mate

Infection procedure

On day 44 (from here on referred to as infection day 0: ID0), BHI and EHI pigs were inoculated with the mild-virulent European PRRSV serotype 1 strain LV-Ter Huurne. This PRRSV strain was isolated during the 1991 epizootic from a clinical case of PRRS in the Netherlands and was used at the 7th passage on porcine alveolar macrophages (PAMs) (Wensvoort et al., 1991). Pigs were infected intranasally with 1.5 ml inoculum containing 5 $\log_{10}$ 50% tissue culture infectious dose ($TCID_{50}$). This treatment was followed by an aerosol infection at day 52 (ID8) with *A. pleuropneumoniae* serotype 2. The inoculum of *A. pleuropneumoniae* serotype 2 strain 17415 (reference strain) was prepared as described earlier (Velthuis et al., 2002). In short, an aliquot of the reference strain, which was stored at –70°C, was thawed and the suspension was cultured overnight at 37°C under microaerophilic conditions on sheep blood agar plates (SB plates) with nicotinamide adenine dinucleotide (NAD). The next morning, colonies were transferred to fresh SB + NAD plates and cultured for 6 hours. Thereafter colonies were rinsed from the plates and stored in phosphate buffer saline (PBS-13). The number of colony forming units (CFU) in the suspension was determined overnight and the inoculum diluted to achieve a concentration of 9 $\log_{10}$ CFU *A. pleuropneumoniae*/ml suspension. Groups of two to three pigs were simultaneously exposed in a

stainless-steel aerosol chamber (110x 45 x 90 cm). Aerosol was administered using the aerosol nebuliser Aeroneb Pro (EMKA Technologies, Paris, France). An amount of 5 ml of the inoculum suspension was administered during a period of 20 min. The procedures have previously been described (Becker et al., 2012; Hensel et al., 1993).

Half of the BHC and EHC pigs were not inoculated (negative controls) and underwent no extra handlings. The other half of the BHC and EHC pigs underwent the same procedures as the infected animals (mock control) at ID0 and ID8 (Table 1), however instead of PRRSV inoculum, 1.5 ml RPMI medium was used and instead of the *A. pleuropneumoniae* inoculum, 5 ml of PBS was used.

Sickness behaviour

Cameras were placed in all pens in facility B. On day ID-1, ID3, ID6 and ID9, postures and behavioural activities of pigs were scored with 10-min instantaneous scan sampling from 7:00 am until 6:00 pm using the Observer XT 10.0 resulting in 66 scans per pig per day (Noldus Information Technology B.V., Wageningen, the Netherlands). From these observations, both 'lying behaviour' (i.e. proportion of scans lying), 'behavioural activity' (i.e. proportion of scans during which pigs showed active behaviours, such as eating, drinking, social behaviours, manipulation of pen or pen mates and aggression) and 'drinking and eating behaviour' (i.e. proportion of scans during which pigs were (apparently) drinking and eating) were calculated.

Rectal temperature, clinical examination, growth

Rectal temperature was assessed once at ID-2 and from ID0 until ID11 twice per day (first measurement between 8-11 am and second measurement between 3-4 pm, referred to as IDX(1) and IDX(2), respectively). At the days of infections, temperature was measured prior to the infection procedure and exactly 4 hours after the infection procedure took place. Microlife digital thermometers with a resolution of 0.1°C were used. Thermometers were calibrated prior to the experiment.

All piglets were observed and inspected for respiratory symptoms (coughing, breathing problems, sneezing) twice per day (at 9:00 am and 4:00 pm) from ID0 until the end of the experiment (ID11). Body weight of the piglets was measured weekly from the day of parturition until the end of the experiment. Average daily growth was calculated from ID0 to ID8 for the period after PRRSV and from ID8 to ID12 for the period after *A. pleuropneumoniae* infection.

Phenotyping of white blood cells and broncho-alveolar cell populations

Blood samples (serum, EDTA blood) were taken by jugular vein puncture on 7 different days (ID0, ID2, ID4, ID8, ID9, ID10 and ID11). The total count of white blood cells (WBC) and a differential count of lymphocytes, granulocytes and monocytes were performed with a haematology analyser (blood cell counter Sysmex pocH-100iV diff, Kobe, Japan).

Freshly isolated heparinised blood samples (100µl) were used to quantify different phenotypes of WBCs by flow cytometry (FCM). Briefly, the blood cells were incubated with a primary

antibody-mixture of monoclonal antibodies (mAb) against CD3, CD4, CD8 for triple labelling or for single labelling with mAb against CD172a or CD21 (Table 3). The following non-related isotype controls (Southern Biotech) were used for the different Ig classes: IgG1 (clone 15H6), IgG2a (clone HOPC-1), IgG2b (clone A-1) and IgM (clone 11E10).

In the next step, FACS™ Lysing Solution (BD Biosciences) was added for 10 minutes. After washing of cells in PBS supplemented with 1% (v/v) pig serum and 0.1% (w/v) sodium azide, cells were incubated with isotype specific secondary antibodies (goat anti–mouse IgG1-APC, IgG2b-FITC and IgG2a-PE, source Southern Biotech).

For the phenotyping of the alveolar cell population, broncho-alveolar lavage fluid (BALF) was obtained from the right cranial lung lobe during necropsy. A catheter was inserted in the local bronchus and the lobe was flushed with 30 ml PBS and ca. 15 ml inserted PBS was harvested. Cells in the BALF were immediately isolated and preserved as earlier described (Weesendorp et al., 2014). For the phenotypic characterization of intra alveolar lymphocytes, granulocytes and monocyte/macrophages, BALF cells were thawed, transferred to micro titre plates and either triple stained with mAb directed towards CD3, CD4, CD8a to quantify the various lymphocytic phenotypes or double stained with mAb against CD172a in combination with mAb against either CD14 or SLAII or CD163 or TLR4$^+$ or single stained with mAb against porcine granulocytes (Table 3).

Flow cytometer analyses were performed with the fluorescence-activated cell sorter (FACS) flow cytometer (Cyan ADP, Beckman Coulter) and evaluated with the Cyan ADP Summit 4.3 software. Blood cells were first gated on the basis of forward-scatter (FCS) versus sideward scatter (SSC) diagram as described (Nielsen et al., 2003) and then analysed according to their antigen marker profile. Based on the WBC counts in blood by the automated blood cell counter the absolute numbers of the different phenotypes were assessed. Granulocytes were identified in blood by an SSC^{high} CD172a$^+$ profile and in BALF by the use of the granulocyte marker. T-cells were distinguished in the lymphocyte gate by the following marker combinations: naïve/non-activated T-helper cells: CD3$^+$CD4$^+$CD8$^-$, cytotoxic T-cells: CD3$^+$CD4$^-$CD8$^+$, memory/activated T-helper cells: CD3$^+$ CD4$^+$CD8$^+$ and natural killer (NK) cells: CD3$^-$CD4$^-$CD8$^+$ (Nielsen et al., 2003; Shi et al., 2008). Monocytes were identified in blood by low granularity, i.e., SSC^{low}CD172a$^+$. Macrophages in BALF were analysed by their CD172a$^+$ (and CD14) expression in combination with the co-expression and expression levels, of their differentiating markers, i.e., mean fluorescence intensity (MFI). The alveolar cell populations are presented as percentage of the total cell population in the BALF. The macrophage markers are presented as percentage of total cell population in the BALF and as MFI.

Detection of viral RNA in serum

A quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed on RNA isolated from blood serum, sampled at ID0, ID4, ID8, ID10 and ID11. A one-tube qRT-PCR was performed with the Applied Biosystem 7500 Fast System instrument using the Quantitect Probe RT-PCR kit from Qiagen. The reaction mixture (25 µl) contained 0.25 µl of kit-supplied enzyme, 12.5 µl of Quantitect Mix, 15 µM of each primer (Fw: 5'- GAT GAC RTC CGG CAY C -3', Rev: 5'- CAG TTC CTG CGC CTT GAT -3') and 10µM of probe (5'- Fam-TGC AAT CGA TCC AGA CGG CTT-Tamra-3')(Frossard et al., 2012). RT-PCR was performed at 30 min at 50°C and 15 min at 95°C followed by a two-step cycling protocol: 94°C for 20s, and 55°C for 45s for 40 cycles. Analysis was performed

with 7500 Software v2.0.6 (Applied Biosystems). Viral RNA concentration (expressed as median tissue culture infectious dose ($TCID_{50}$) equivalents per ml) of each serum sample was calculated using a standard curve, constructed by extracting RNA from five decimal dilutions of medium spiked with known concentrations of infectious virus (Weesendorp et al., 2013).

Table 3. Antibody panels for fluorescence activated cell sorting (FACS)

	Antigen	Clone	Isotype	Fluorochrome	Labelling	Source of antibody
White blood cells	*Triple staining*					
	CD3	PPT3	IgG1	APC	Secondary[1]	Southern Biotech
	CD4	74-12-4	IgG2b	FITC	Secondary[1]	Southern Biotech
	CD8α	76-2-11	IgG2a	PE	Secondary[1]	Southern Biotech
	Single staining					
	CD172a	74-22-15	IgG2b	FITC	Secondary[1]	VMRD
	CD21	B6-11C9	IgG1	APC	Secondary[1]	Southern Biotech
BALF cells	*Triple staining*					
	CD3	PPT3	IgG1	APC	Secondary[1]	Southern Biotech
	CD4	74-12-4	IgG2b	FITC	Secondary[1]	Southern Biotech
	CD8α	76-2-11	IgG2a	PE	Secondary[1]	Southern Biotech
	Double staining					
	CD172a	74-22-15	IgG2b	PE	Secondary[1]	VMRD
	CD172a	74-22-15	IgG1	PE	Secondary[1]	Beckman Coulter
	CD 14	MIL2	IgG2b	FITC	Primary	BioSource
	SLAII-DR	2E9/13	IgG2b	FITC	Primary	Serotec
	CD163	2a10/11	IgG1	FITC	Primary	Serotec
	TLR4		IgM	FITC	Secondary[1]	Gift by J. Dominguez
	Single staining					
	Granulocytes	2B2	IgG1	FITC		Serotec

[1]For secondary labelling cells were incubated with isotype specific secondary antibodies (goat anti–mouse, source Southern Biotech)

Detection of typical A. pleuropneumoniae lesions and histological assessment of the lungs

After the pigs had been euthanised, special attention was paid during necropsy to patho-morphological changes in the respiratory tract. The type and size of lung lesions were recorded in a lung drawing and the proportions of the affected lung surface was calculated. Necro-haemorrhagic lesions, typical for *A. pleuropneumoniae,* were macroscopically detected and histologically confirmed. Total number of pigs with these lesions, were counted and presented as total number and percentage of pigs with typical *A. pleuropneumoniae* lesions.

For histological assessment of the lungs, 6 tissue samples per pig from predefined locations in the lungs (cranial, cardial and caudal lobe of the left and right lobe) were formalin-fixed, processed and embedded in paraffin. Tissue sections were stained with Haematoxylin-Eosin (HE) and a semi-quantitative, patho-histological assessment of HE stained slides encompassed the extent of pneumonia throughout the predefined locations in the lungs. Patho-histological assessment included

4 features: (1) the presence of focal or diffuse alterations with interstitial or catarrhal pneumonia or atelectasis, (2) the extent of infiltration of alveolar septae with mononuclear cells (3) the extent of infiltration of mononuclear cells in the perivascular/peribronchiolar area and (4) pleuritis. A histological score of 0 to 3 was used to describe the severity of changes per feature (i.e., 0 = no findings, 1 = mild focal manifestation, 2 = moderate, multifocal manifestation or diffuse manifestation, 3 = severe diffuse manifestation). During histological examination, the pathologist was blinded with regard to treatment (Housing and Infection). The scores from all 6 slides per lung were added to obtain an *overall histology score*, which could add up to a maximum of 72 points per pig (6 slides per pig, 4 histological features, and maximum score of 3 per feature). The *total interstitial components score* per pig was also calculated and presented separately as these changes in the lungs are considered to be related to PRRSV infection (Cho & Dee, 2006; Doeschl-Wilson et al., 2009; Meulenberg, 2000; Rossow, 1998). This score could reach a maximum of 18 (6 slides per lung, 1 histological feature, and maximum score of 3).

Re-isolation of A. pleuropneumoniae

For re-isolation of *A. pleuropneumoniae,* two lung tissue samples of predefined lung locations (cranial lobe, caudal dorsal lobe) and samples of bronchial lymph nodes were taken. When a macroscopic visible lung lesion was present at another location than the previous described locations, a third sample was taken. The collected lung and bronchial lymph node tissues were prepared, grinded and plated on SB+NAD plates as described by (Jirawattanapong et al., 2010; Kamp et al., 1987). To confirm that colonies were *A. pleuropneumoniae* serotype 2, dependency for NAD was tested by comparing growth, on SB and SB+NAD plates followed by agglutination with serotype 2-specific hyper-immune rabbit antiserum (Kamp et al., 1987).

Statistical analysis

All statistical analyses were performed with SAS (SAS 9.3, SAS Institute Inc.). The experiment was primarily designed to test the effect of housing management (enriched versus barren) on disease susceptibility. The mock-infected controls were used to make sure that the procedures to infect the pigs alone did not cause any differences. Preliminary statistical analysis (general linear model, GLM) showed indeed no differences in outcome (pathology, rectal temperature, growth and BALF variables) between mock-infected and negative control pigs. These groups were therefore combined and referred to as 'control group'.

For all data, except the occurrence of lung lesions (see below), mixed linear models were used. Behaviour and skin lesions were analysed with pen as observational unit as behaviours and skin lesions of individual pigs within a group are not independent. All other variables were analysed with pig nested in the housing regime as observational unit.

Differences between variables at a specific time point (i.e., behaviour and skin lesions at the day before weaning, growth, histology scores and BALF cells) were analysed with housing and if applicable with infection and the interaction between housing and infection as fixed effect(s) and sow as random effect.

Differences between time dependent variables (i.e., behaviour and skin lesions after weaning, sickness behaviour, viral RNA, WBC, rectal temperature) were analysed with day as repeated effect using the repeated statement of SAS (SAS 9.3, SAS Institute Inc.) with the autoregressive covariance structure. Day, housing and if applicable infection and their interactions were used as fixed effects and sow as random effect.

Effects of housing on behaviour and skin lesions were analysed separately for the day before weaning, because pigs were mixed into other groups after weaning leading to a different group composition for the day after weaning, the day before transport and the day after transport. If needed, variables were square root transformed to obtain normally distributed residuals. In case the interaction effect for a variable was significant, post-hoc testing with Bonferroni adjustment was used.

Differences between BHI and EHI pigs in the occurrence of typical *A. pleuropneumoniae* lung lesions (i.e., lung lesions present or absent) and in occurrence of re-isolated *A. pleuropneumoniae* were analysed with a generalised mixed linear model (GLIMMIX procedure in SAS), with a logit link and binary distribution with housing as fixed effect and sow as random effect. Here, odds ratios (OR) are presented to indicate the extent of the effect.

Results

Behaviour and skin lesions before co-infection

Before weaning, BH pigs showed 2.3 times more oral manipulation of other pigs (i.e., sow and piglets), 2.5 times more mounting and 5.4 times more manipulation of pen fixtures than enriched housed (EH) pigs (Figs 1A, B and D). Aggression (Fig 1C), social behaviour (0.61±0.07 vs. 0.56±0.13 times/pig/10 min, P =0.72) and playing (0.35±0.18 vs. 0.50±0.20 times/pig/10 min, P =0.59) did not differ between BH and EH pigs before weaning.

After weaning, oral manipulation was affected by housing, day and their interaction (Fig 1A). Overall BH pigs showed more oral manipulation as compared to EH pigs. Post hoc analysis revealed that BH pigs showed a 5.6-fold higher frequency of oral manipulation on the day before transport (Fig 1A). Mounting was also affected by housing, with higher frequencies for BH than for EH pigs, and day (Fig 1B). Pen manipulation was affected by housing with higher frequencies for BH than for EH pigs, and day (Fig 1D). Social behaviour was affected by day (P =0.02), but not by housing (BH: 0.19±0.05 vs. EH: 0.15±0.03 times/pig/10 min, P =0.41) or the interaction (P =0.32). Housing (P =0.60) and day (P =0.18) did not significantly affect playing behaviour (BH: 0.18±0.09 vs. EH: 0.13±0.05 times/pig/10 min).

Before weaning, skin lesion scores did not significantly differ between BH and EH pigs (Fig 2). After weaning, skin lesion scores on the front of the body were only affected by day (Fig 2A). BH pigs had more skin lesions on the middle and rear of their bodies after weaning than EH pigs (Figs 2B and C).

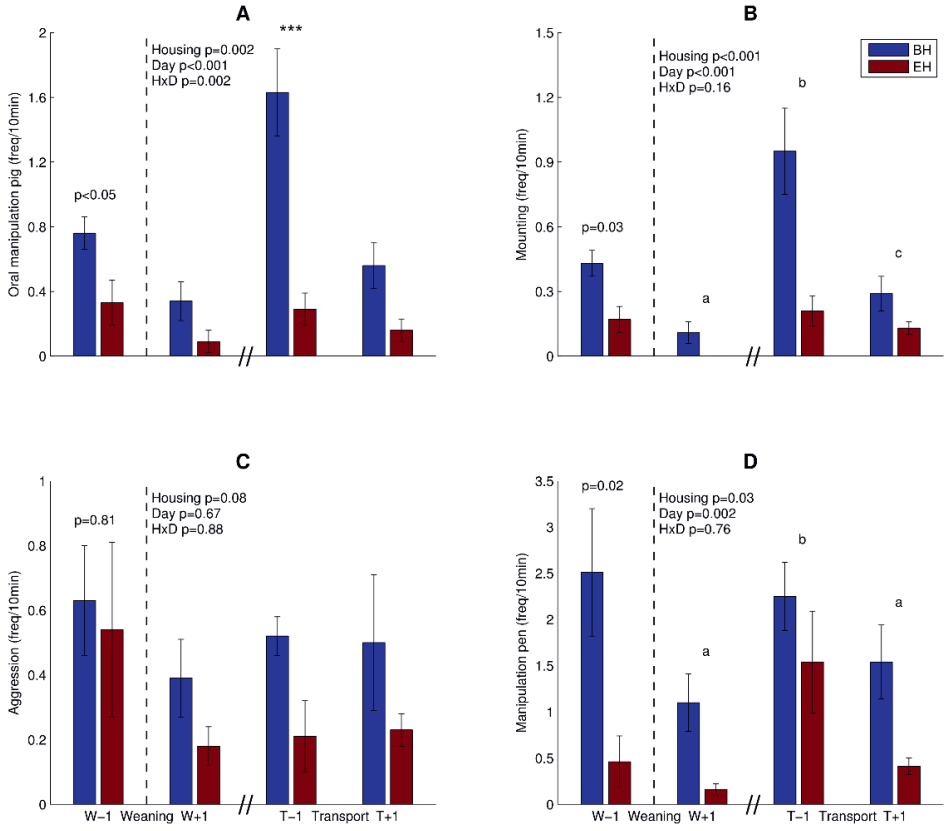

Figure 1. Behaviour of barren housed (BH) and enriched housed (EH) pigs. Behaviours shown as frequencies/pig/10min, mean ± standard error. (A) Oral manipulation. (B) Mounting. (C) Aggression. (D) Manipulation pen. Blue bars: BH pigs, red bars: EH pigs. W-1 and W+1: 1 day before and 1 day after weaning. T-1 and T+1: 1 day before and 1 day after transport. Black dotted vertical line indicates the separate statistical analysis of the day before weaning. HxD: housing-day interaction effect. Day effects are indicated as 'a' - 'c', bars with no common superscript differ significantly ($P<0.05$). (A) Post hoc comparison of housing effect at T-1, ***: $P<0.001$.

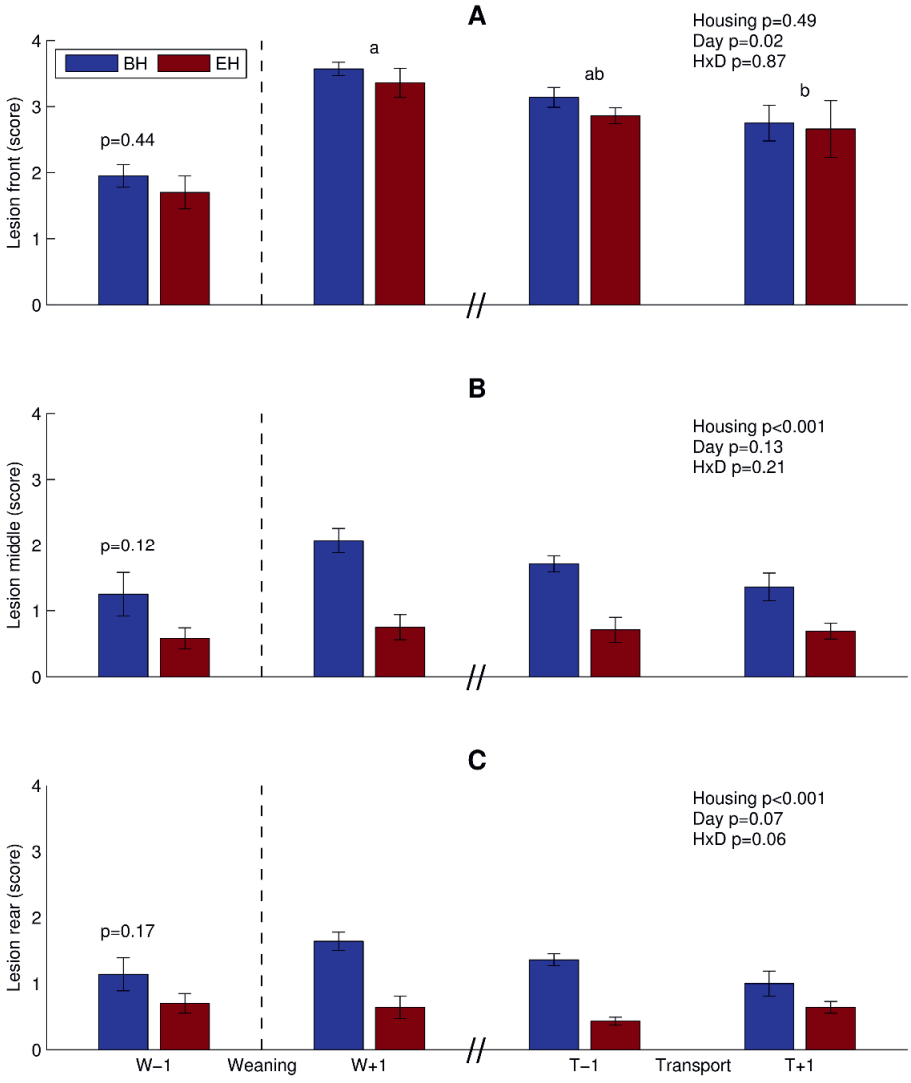

Figure 2. Skin lesion scores (mean of a four-point scale ± standard error) of barren housed (BH) and enriched housed (EH) pigs. (A) Front. (B) Middle. (C) Rear of the body. Blue bars: BH pigs, red bars: EH pigs. W-1 and W+1: 1 day before and 1 day after weaning. T-1 and T+1: 1 day before and 1 day after transport. Black dotted vertical line indicates the separate statistical analysis of the day before weaning. HxD: housing-day interaction effect. Day effects are indicated as 'a' and 'b', bars with no common superscript differ significantly (P<0.05).

Viral RNA in serum

Viral RNA expressed as log10 $TCID_{50}$ eq/ml was affected by day and the housing x day interaction (Fig 3). Post hoc analysis revealed that 4 days after PRRSV infection (ID4) viral RNA amount was equally increased in both BHI and EHI pigs as compared to day ID0, whereas at ID8 viral RNA was significantly lower in EHI pigs as compared to BHI pigs (Fig 3). No viral load differences were found between the two groups at ID10 and ID11 (Fig 3).

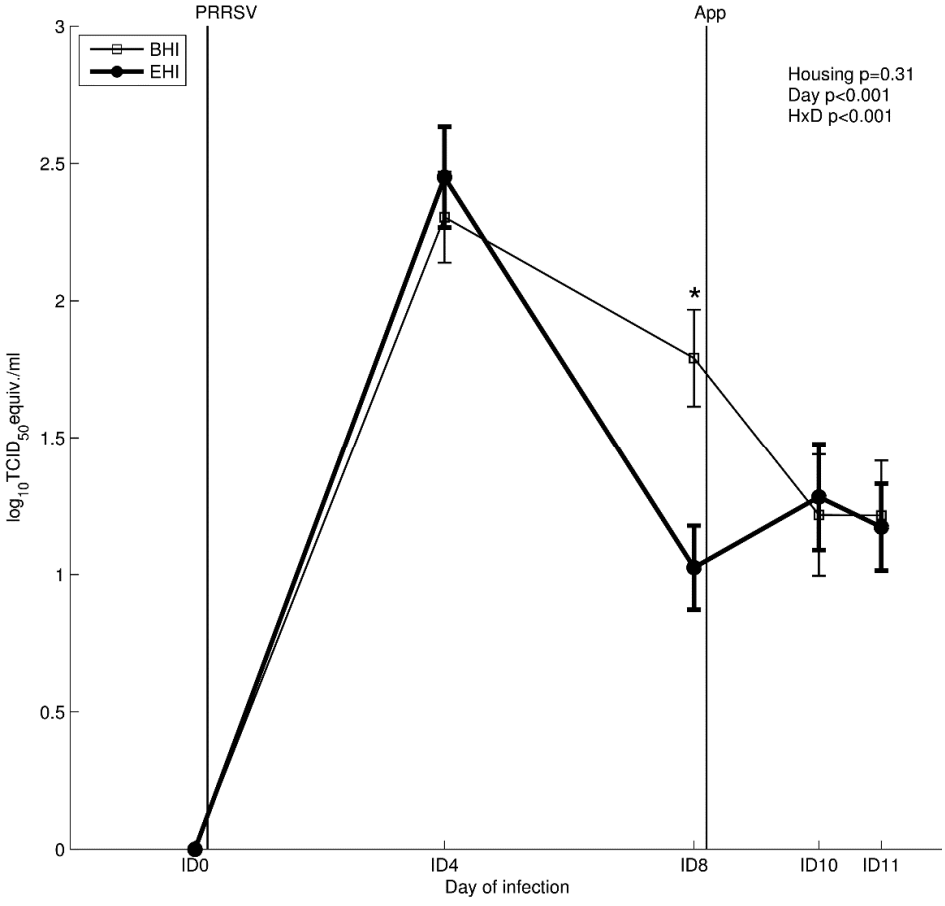

Figure 3. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) of porcine reproductive and respiratory syndrome virus (PRRSV) in serum of barren housed infected (BHI) and enriched housed infected (EHI) pigs (expressed as means of median tissue culture infectious dose ($TCID_{50}$) equivalents per ml ± standard error). Infections at infection day 0 (ID0) with porcine reproductive and respiratory syndrome virus (PRRSV) and infection day 8 (ID8) with *Actinobacillus pleuropneumoniae* are indicated as black vertical lines. HxD: housing-day interaction effect. * Post hoc comparison of housing effect at ID8 ($P<0.05$).

Lung lesions

The most remarkable result was the impact of housing treatment on the number of pigs with typical *A. pleuropneumoniae* lesions. These lesions were characterised by a (multi)focal necro-haemorrhagic pleuro-pneumonia, which was macroscopically observed and histologically confirmed. More BHI pigs (8 out of 14, 57%) than EHI pigs (1 out of 14, 7.1%) developed lesions (OR=19.2, P =0.048). In 5 BHI pigs and 1 EHI pig, *A. pleuropneumoniae* was re-isolated successfully, but this difference was not significant (P>0.10).

To further address the extent of co-infection on lung morphology a broader histological evaluation of the lung samples was performed. In BHI pigs the patho-histological lung tissue score (i.e., the overall histological score) was significantly higher (2.1-fold, Fig 4A), meaning that the extension of altered tissue was larger and that more catarrhal-purulent exudates in alveoli, fibrinous or necro-haemorrhagic changes and fibrinous pleuritis was seen as compared to EHI pigs. Also, interstitial changes (i.e., the total interstitial components score) with increased peribronchiolar and perivascular lympho-monocytic cell infiltration were significantly increased in lungs of BHI pigs compared to EHI pigs (2.8-fold, Fig 4B).

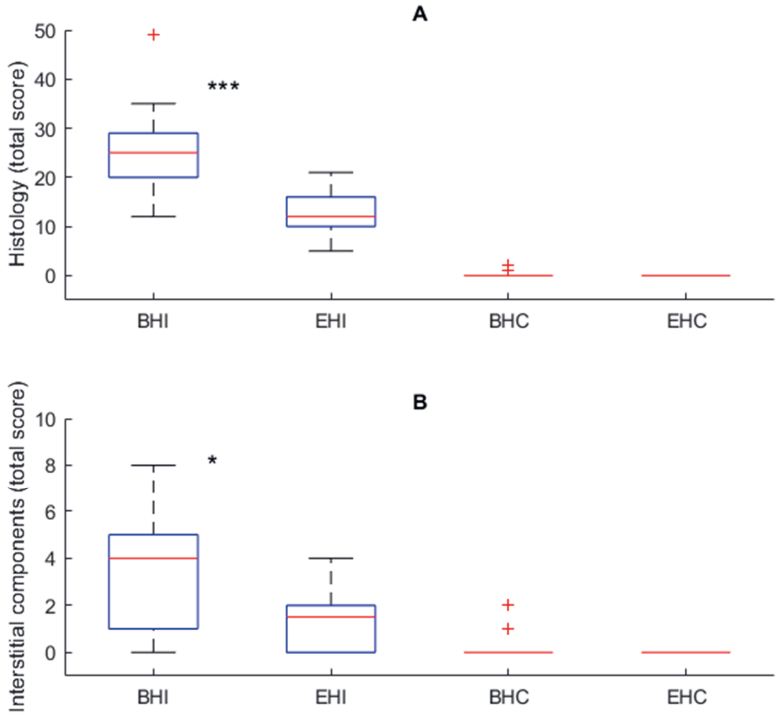

Figure 4. Overall histology (A) and total interstitial component (B) score. The central blue box indicates the lower and upper boundary at the 25% and 75% quantile of the data. The central red line indicates the median of the data. Two vertical lines extending from the central box indicate the remaining data outside the central box that are not regarded as outliers. Outliers are indicated as red crosses. ***: P<0.001; *: P <0.05. Barren housed enriched infected (BHI), enriched housed infected (EHI), barren housed control (BHC), enriched housed control (EHC) pigs.

Sickness behaviour

During daily inspections throughout the experiment, no coughing or breathing problems in any of the groups were detected that would have indicated the occurrence of respiratory problems. Sickness behaviour was addressed by analysing the animals' active, lying and eating and drinking behaviour. An overall housing effect was seen for activity, but not for lying levels or eating and drinking behaviour as shown in Figs 5A-C. A housing-day interaction was seen in activity and lying behaviour, but not for eating and drinking behaviour. A day effect was seen in all behaviours (Figs 5A-C), indicating the difference in behaviour at ID9 as compared to the other days. Post hoc comparisons revealed that BHI pigs were less active and spent more time lying than EHI pigs the day after *A. pleuropneumoniae* infection (ID9) (Figs 5A and B). All pigs spent less time visiting the drinker and feed trough the day after *A. pleuropneumoniae* infection (ID9) as compared to the other three days, as shown in Fig 5C.

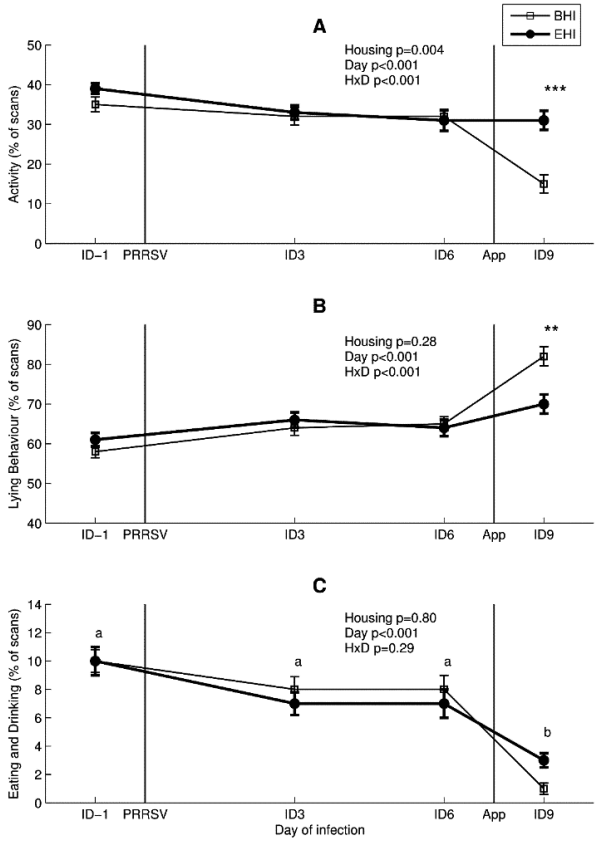

Figure 5. Sickness behaviour (mean (% of scans) ± standard error) of barren housed infected (BHI) and enriched housed infected (EHI) pigs. (A) Activity. (B) Lying behaviour. (C) Eating and drinking. Infections with Porcine reproductive and respiratory syndrome virus (PRRSV) at infection day 0 (ID0) and *Actinobacillus pleuropneumoniae* at infection day 8 (ID8) are indicated as black vertical lines. HxD: housing-day interaction effect. Day effects are indicated as 'a' and 'b', days with no common superscript differ significantly (C) ($P<0.05$). Post hoc comparison at ID9, ***: $P<0.001$ (A), **: $P<0.01$ (B).

Rectal temperature and growth

Housing, day, and their interaction affected rectal temperatures of the infected pigs (Fig 6). Post hoc comparisons showed a rise in rectal temperature (of 0.7 °C on average) after PRRSV infection in both BHI and EHI pigs (average 39.5°C at ID0 to 40.2°C at ID2(2), Fig 6). Average rectal temperature decreased again at ID3 in both groups similarly. After *A. pleuropneumoniae* infection (at ID8(1)) again both BHI and EHI pigs showed a similar peak temperature at 4 hours after the infection (ID8(2)). In BHI pigs, rectal temperature remained raised for 24 hours, whereas in EHI pigs, rectal temperature decreased faster (within 12 hours) (Fig 6). In control groups, overall temperatures were on average 0.16°C higher in BHC pigs as compared to EHC pigs (P =0.002).

BHI and EHI pigs showed a lower growth rate (P <0.001) in the period post infection with PRRSV (BHI: 538.8 ± 48.3 g/day; EHI: 594.9 ± 49.3 g/day) as compared to control groups (BHC: 714.2 ± 33.7 g/day; EHC: 715.3 ± 35.0 g/day). Growth rate in the period after *A. pleuropneumoniae* infection was also lower in the infected pigs (BHI: pigs 371 ± 29.8 g/day vs. BHC: pigs 725 ± 79.4 g/day and EHI pigs: 447.6 ± 43.9 g/day vs. EHC pigs: 775 ± 64.6g/day; P <0.001).

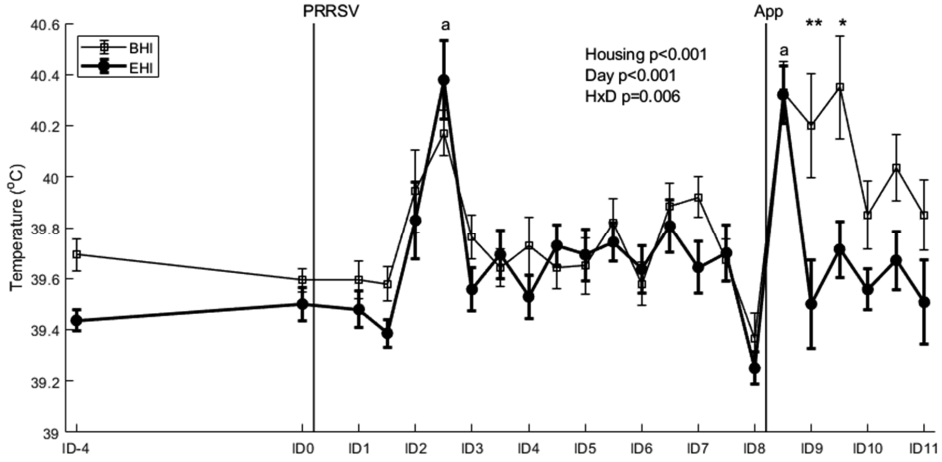

Figure 6. Rectal temperature (mean in °C ± standard error) of barren housed infected (BHI) and enriched housed infected (EHI) pigs. Infections days (ID0 and ID8) are indicated as black vertical lines. HxD: housing-day interaction effect. 'a' denotes higher temperatures on ID2(2) and ID8(2) as compared with other days (both P <0.001). Post hoc comparison at ID9(1) and ID9(2): **: P <0.01 and *: P <0.05.

White blood cell counts

Total white blood cell (WBC) and lymphocyte counts were significantly elevated in serum of EHI pigs as compared to BHI pigs, but this overall housing effect was not observed in the other cell populations (Fig 7, results of memory and naive T-cells not shown). All cell populations showed an overall day effect. For granulocytes, monocytes and memory T-cells an interaction effect between housing and day was also found. Post hoc pairwise comparisons showed that granulocyte counts significantly increased in BHI pigs between ID8 and ID9 (P<0.001, Fig 7C), but not in EHI pigs.

Broncho alveolar cell populations

Housing profoundly affected cellular composition of the BALF in infected pigs. A rise in the percentage of granulocytes (1.7-fold) and T-lymphocytes (6-fold) in lung fluid was measured in both infected groups (BHI and EHI pigs) as compared to their respective control groups (BHC and EHC, Table 4). Especially the relative amount of $CD8^+CD4^-$ (cytotoxic T–cells, 11.5-fold) and $CD4^+CD8^+$ (activated/memory T-cells, 8.7-fold) increased as compared to control pigs, whereas a relative decrease of $CD14^+$ macrophages (1.2-fold) was observed (Table 4). Also, a significant change in the expression (MFI) of the phenotypic markers CD14 (up), CD163 (down) and $TLR4^+$ (down) was measured after infection in both groups (Table 4).

An overall housing effect was seen for $CD172a^+/TLR4^+$ macrophages (P =0.02). Analysis of housing effect on infected and control animals separately showed that BHC pigs had higher percentages of $TLR4^+$ and $CD172a^+/TLR4^+$ macrophages as compared to EHC pigs (Table 4, P=0.02 and P=0.01 respectively).

Table 4. Phenotypic markers of broncho-alveolar lavage fluid (BALF) cell populations

Cell types	Phenotype	BHI	EHI	BHC	EHC	Infection effect (*P*)
		%	%	%	%	
Granulocytes	Granulocytes	2.45±0.54	2.44±0.36	1.42±0.29	1.49±0.22	0.004
Lymphocytes						
T cells,	$CD3^+$	11.87±1.74	11.51±1.08	2.15±0.18	1.78±0.26	<0.001
T-Helper	$CD4^+CD8^-$	2.76±0.48	2.49±0.26	1.21±0.13	0.85±0.12	<0.001
Cytotoxic T	$CD8^+CD4^-$	7.53±1.62	7.43±0.84	0.78±0.08	0.52±0.06	<0.001
Memory T	$CD4^+CD8^+$	0.3±0.07	0.22±0.03	0.04±0.01	0.02±0	<0.001
NK	$CD3^-CD8^+$	0.21±0.04	0.19±0.03	0.08±0.01	0.05±0.01	<0.001
Macrophages	%	%	%	%	%	
	$CD14^+$	67.76±4.21	74.76±2.72	89.38±0.92	88.56±0.97	<0.001
	$CD172a^+$	68.7±4.21	74.96±2.72	88.7±0.92	88.14±0.89	<0.001
	SLA II-DR^+	63.62±5.01	68.89±3.21	88.63±0.88	88.39±0.86	<0.001
	$CD163^+$	56.46±5.97	65.06±2.87	87.34±0.94	86.38±0.95	<0.001
	$TLR4^+$	51.27±4.48	47.75±2.76	62.07±3.48	50.8±2.73	0.05
	$CD172a^+/TLR4^+$	41.83±4.31	37.97±2.15	54.98±6.56	42.74±2.35	0.01
Macrophages	MFI	MFI	MFI	MFI	MFI	
	$CD14^+$	165.22±10.74	172.15±6.38	150.34±6.69	143.67±4.29	0.01
	$CD172a^+$	377.44±41.34	325.22±17.36	400.72±35.06	355.41±13.19	0.30
	SLA II-DR^+	274.74±25.36	263.15±26.23	339.34±47.47	284.96±16.04	0.10
	$CD163^+$	750.32±82.29	775.58±59.62	940.35±66.42	895.18±68.8	0.01
	$TLR4^+$	456.79±51.45	452.3±48.25	738.73±77.88	707.51±62.54	<0.001

Shown as mean percentage and MFI: mean fluorescence intensity ± standard error.

Barren housed infected (BHI). Enriched housed infected (EHI). Barren housed control (BHC). Enriched housed control (EHC).

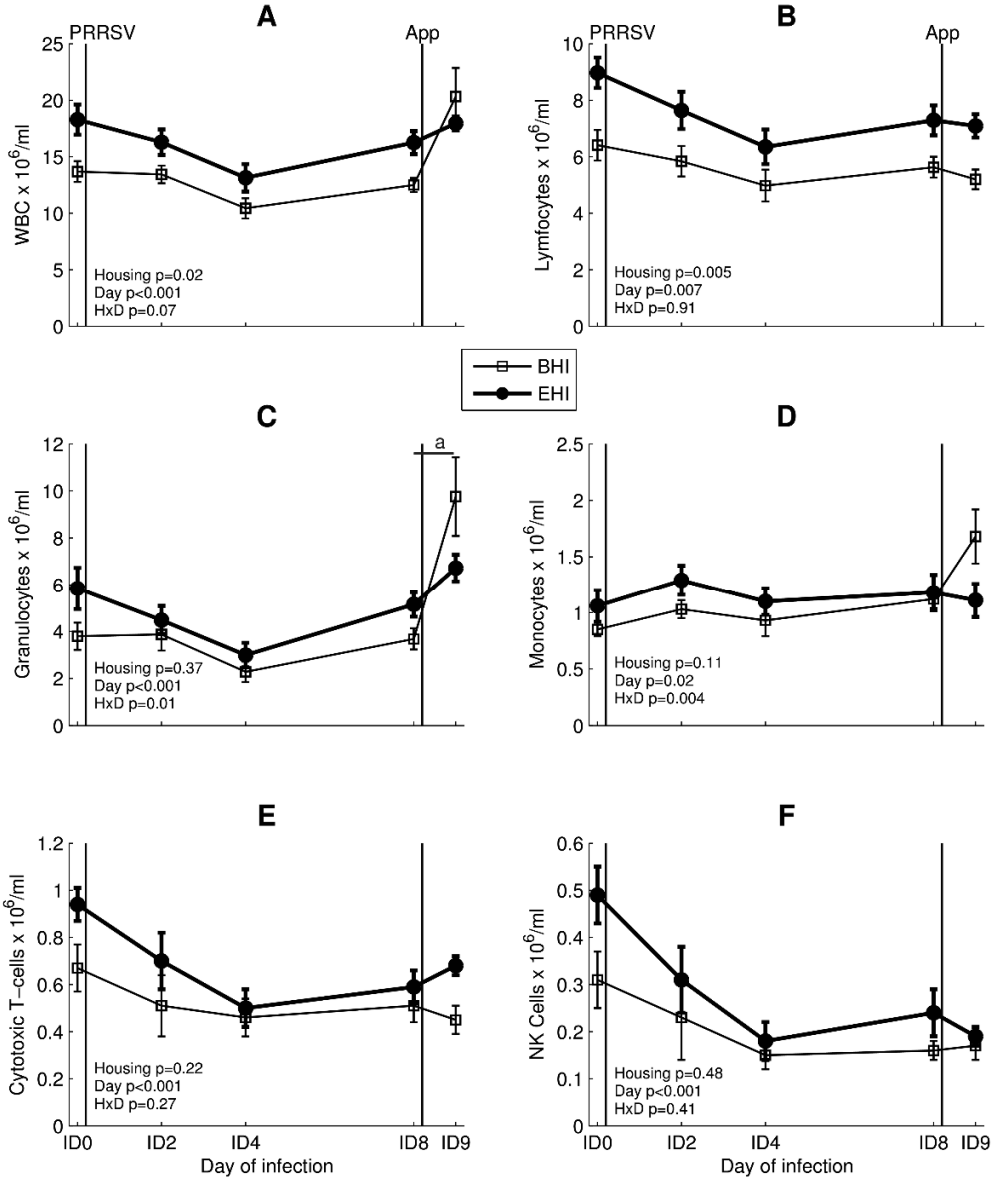

Figure 7. Absolute numbers of blood cells count in barren housed infected (BHI) and enriched housed infected (EHI) pigs (mean ± standard error). (A) White blood cell (WBC). (B) Lymphocytes. (C) Granulocytes. (D) Monocytes. (E) Cytotoxic T-cells. (F) Natural Killer (NK) cells. Moments of infections are represented as black vertical lines. HxD: housing-day interaction. 'a' denotes a significant increase between ID8 and ID9 in BHI pigs (post hoc comparison, $P<0.05$). ID0-9: infection days 0-9. Porcine reproductive and respiratory syndrome virus (PRRSV) and *Actinobacillus pleuropneumoniae* (App).

Discussion

The use of environmental enrichment has repeatedly been proposed to enhance welfare of farm animals (Bolhuis et al., 2005; Elizabeth Bolhuis et al., 2013; Tuyttens, 2005). Our study confirms a decrease of stress related behaviour and decreased skin lesion scores in pigs provided with enrichment materials, indicating improved welfare, and simultaneously shows that enriched housing significantly reduces disease susceptibility to co-infection with PRRSV and *A. pleuropneumoniae*, expressed as a reduction of clinical and histo-pathological signs, of which the eight-fold difference in percentage of pigs with lung lesions was the most remarkable. Most studies on the relation between stress and occurrence of disease symptoms, use stress enhancing conditions, such as heat or cold stress, crowding, forced exercise, social stress, transportation, restraint/immobilization, avoidance learning, isolation or other stressful (often short term) life events (Biondi & Zannino, 1997). In this study we used social and environmental enrichment, applied from birth onwards, to enduringly facilitate the development and display of natural (social) behaviour in contrast to 'conventional husbandry systems conditions' where behavioural needs of pigs are poorly met.

In our experiment we ensured a constant faecal pathogenic load by removing faeces from all pens daily. Also, contamination with other pathogens was excluded as all materials that served as enrichment were replenished daily and were made germ free by γ-irradiation. So, despite the same infectious doses of both pathogens, pigs in the enriched housing regime were better in preventing clinical manifestation of co-infection, resulting in profoundly less pigs with lung lesions and less severe pathologic tissue damage in the lungs, as compared to BHI pigs. The more severe histo-pathological damage in the lungs of BHI pigs also coincided with the more pronounced clinical read outs in these pigs in terms of rectal temperature, sickness behaviour as well as the more pronounced inflammatory reaction of different blood cell counts after *A. pleuropneumoniae* infection. In the BALF the relative increase in granulocyte and lymphocyte populations and decrease in macrophages after infection was similar in both BHI and EHI pigs, indicating that housing did not cause differences in immunological responses within the right cranial lung lobe, in which we did not detect typical *A. pleuropneumoniae* lesions.

Another remarkable finding was the faster viral clearance in EHI pigs as compared to BHI pigs. Both BHI and EHI pigs showed a similar rise of viral RNA in serum 4 days after infection, which is conform other studies in pigs infected with PRRSV serotype 1 LV-Ter Huurne strains (Weesendorp et al., 2013). This indicates that PRRSV infection initially affected both groups similarly. The viral RNA serum curve of BHI pigs followed those earlier described (Weesendorp et al., 2013), whereas EHI pigs were able to reduce viral RNA in serum faster, with a statistically lower level 8 days after PRRSV infection. So, at the moment of *A. pleuropneumoniae* infection BHI pigs still had higher viral serum levels of PRRSV, whereas in EHI pigs it was already reduced. The inflammatory impact of PRRSV in the lungs of BHI pigs was also higher. PRRSV infection in lungs is histologically characterised by septal thickening by macrophages and perivascular cuffing (Doeschl-Wilson et al., 2009; Meulenberg, 2000). BHI pigs showed higher peri-bronchiolar and peri-vascular infiltrates scores as compared to EHI pigs post mortally, representing the more effective containment of PRRSV infection

by the EHI pigs, which did however not result in differences in temperature level or sickness behaviour the period between PRRSV and *A. pleuropneumoniae* infection (ID1- ID8).

PRRSV is supposed to dampen both innate and specific immune responses as infection with PRRSV alters cytokine production of macrophages and monocytes derived dendritic cells, and modifies the expression of molecules involved in antigen presentation (Kimman et al., 2009; Meulenberg, 2000; Rossow, 1998). As a consequence, NK cell activation and mobilization of cells from the acquired arm of the immune system are delayed (Kimman et al., 2009). The faster clearance of PRRSV in EHI pigs could have diminished the impact of the secondary A. *pleuropneumoniae* infection that followed at ID8 in our experiment.

Moreover, we found overall higher levels of WBC as well as higher levels of lymphocytes in EHI pigs as compared to BHI pigs. Deep straw bedding has previously been found to influence variables related to (innate) immunity as well as to stress physiology as compared to relatively barren housing (Reimert et al., 2014b). Differences in immune related variables possibly influence health status and responses to infections in pigs. In contrast to our results, the enriched housed pigs in this previous study had lower WBC than barren housed pigs (Reimert et al., 2014b). A possible explanation for the contrasting outcome as compared to the previous study may relate to differences in starting point and duration of the enrichment provided, the age at which the animals were tested, and the type of enrichment used. In our study, difference in rearing of BH and EH pigs started directly after birth, and included also social enrichment, i.e., mixing with another litter early in life. We do not consider the overall higher WBC level as the result of infection, but we cannot exclude that the EHI pigs have been challenged with environmental microbes, although all enrichment materials had been decontaminated. Possible explanations for our found differences in overall levels and responses after infections of the white blood cell counts remain unknown at this point.

Cytotoxic T-cells and NK cells are considered to play a role in viral clearing (Morgan et al., 2013). It is suggested that enhanced immune responses after PRRSV infection may lead to more severe clinical disease and gross pathology, but also supports an enhanced viral clearance (Gómez-Laguna et al., 2009; Lamontagne et al., 2003; Morgan et al., 2013; Weesendorp et al., 2013). We did however not find differences in clinical findings or immunological responses between the barren housed pigs and enriched housed pigs directly after PRRSV (ID1 until ID8).

The relative decrease of lung macrophages in BALF in both EHI and BHI groups is probably due to influx of lymphocytes and granulocytes but is also in line with the often-proposed destructive effect of PRRSV on lung macrophages. It is suggested that this destructive effect of macrophages would make the lungs more susceptible for secondary infections (Galina et al., 1994).

Barren housed control pigs showed higher percentages of $TLR4^+$ and $CD172a^+/TLR4^+$ macrophages as compared to EHC pigs. The control animals refer to differences in housing regime without infection, or to the situation prior to infection. $TLR4^+$ is an essential part of the LPS (lipopolysaccharide) receptor binding complex (Poltorak et al., 1999). Other components of this complex are CD14 and LPS binding protein (LBP). $TLR4^+$ is stimulated by LPS from gram-negative bacteria and fusion (F) protein from respiratory syncytial virus (RSV) (Lu et al., 2008) and binds to the earlier formed CD14-LPS complex (Van Gucht et al., 2004). Stimulation of $TLR4^+$ can induce potent responses such as sepsis and inflammation induced damage by release of pro inflammatory cytokines. The lower percentage of macrophages with $TLR4^+$ markers in EHC pigs, may be related to a lower sensitivity of alveolar macrophages for LPS in the EH pigs as compared to BH pigs. This may

be of interest when studying the complex pathways that lie behind the different clinical responses to co-infection of the two housing regimes.

Our behavioural observation demonstrate that a combination of rooting substrate, social enrichment and a larger pen size decreases stress related behaviour such as mounting behaviour and oral manipulations directed at pen mates and the pen itself, which is in line with our expectations and as described in earlier studies (Bolhuis et al., 2005; Kamp et al., 1987; Meulenberg, 2000; Oostindjer et al., 2010). Although the occurrence of manipulative activities directed at pen mates is mostly considered to reflect a limitation in performing normal explorative behaviours such as rooting, chewing on substrate or foraging behaviour (Beattie et al., 2005; Bolhuis et al., 2006; Oostindjer et al., 2011), it might also be considered as a strategy to cope with stress (Bolhuis et al., 2005; Elizabeth Bolhuis et al., 2005). Behavioural differences between BH and EH pigs in this study were also indicated by the skin lesion scores. Engagement in reciprocal fighting has previously been found to result in lesions to the anterior third of the body, whilst the receipt of bullying (e.g. unilateral fighting) leads to lesions accruing on the caudal third of the body (S. P. Turner et al., 2006). Overall higher mounting and manipulation of other pigs in barren housed pigs were seen, and higher skin lesions scores especially at the middle and rear of the body after weaning, indicating more bullying (Doeschl-Wilson et al., 2009). The smaller pen size might have inhibited retreat of the bullied pigs (limited possibilities to flee to prevent hierarchy battles), causing more skin lesions in BH pigs, especially at the middle and back. EH pigs in our study might also have developed better social skills, such as a more adequate reaction to threat, more flexibility in using aggression in conflicts and the development of submissive behaviours (Elizabeth Bolhuis et al., 2005) during the pre-weaning mixing period as compared to BH pigs that were confronted with totally new pigs after weaning. Other studies have demonstrated that the behavioural differences between pigs in barren and enriched housing conditions are also reflected in stress physiology measurements, such as HPA-axis (re)activity (Beattie, O'Connell, Kilpatrick, et al., 2000; De Jong et al., 2000; Reimert et al., 2014b), indicating that BH pigs were probably also more (stress) physiologically challenged.

In our study, the enrichment stimulated the EH pigs psychologically differently as compared to the barren housed pigs and diminished (chronic) stress in the animals. Chronic stress in general is considered a potential influencing factor on disease susceptibility, however the complex pathways that mediate the effects of stress on infectious diseases, are not completely understood (Biondi & Zannino, 1997). The better psycho-physiological and immunological state of the EH pigs likely positively affected their immune and inflammatory responses (Dantzer & Kelley, 1989; Khansari et al., 1990; Padgett & Glaser, 2003; Pruett, 2003; Webster Marketon & Glaser, 2008), and in this way, diminished the clinical manifestation. Our results are also in line with the increasing epidemiological evidence in humans and other species that environmentally induced adaptations, occurring at crucial stages of life, can potentially change behaviour, disease susceptibility and survival also known as the 'early origins of the adult disease susceptibility' hypothesis (Gluckman et al., 2008; Jirtle & Skinner, 2007).

In conclusion, enriched rearing leads to a less severe onset and outcome of a PRRSV *A. pleuropneumoniae* co-infection. The enriched housed pigs showed a remarkably reduced impact of infection and were less prone to develop clinical signs of disease. We found more support for implementation of psychoneuro-immunological intervention strategies to reduce the impact of

infectious diseases and by this, reducing antibiotics use. Future research should investigate the possible involved explanatory pathophysiological pathways.

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CHAPTER 3

3

Animal-based factors prior to infection predict histological disease outcome in porcine reproductive and respiratory syndrome virus- and *Actinobacillus pleuropneumoniae*-infected pigs

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Abstract

A large variety in clinical manifestation in individual pigs occurs after infection with pathogens involved in porcine respiratory disease complex (PRDC). Some pigs are less prone to develop respiratory disease symptoms. The variation in clinical impact after infection and the recovery capacity of an individual animal are measures of its resilience. In this paper we examined which ones of a range of animal-based factors (rectal temperature, bodyweight, skin lesion scores, behavior, natural antibody serum levels, serum levels of white blood cells and type of T and granulocyte subsets) when measured prior to infection are related to disease severity. These animal-based factors and the interaction with housing regime of the piglets (conventional or enriched) were modelled using linear regression to predict disease severity using a dataset acquired from a previous study using a well-established experimental co-infection model of porcine respiratory disease virus (PRRSV) and *Actinobacillus pleuropneumoniae.* Both PRRSV and *A. pleuropneumoni*ae are often involved in PRDC. Histological lung lesion score of each animal was used as a measure for PRDC severity after infection. Prior to infection higher serum levels of lymphocytes ($CD3^+$), naïve T helper ($CD3^+CD4^+CD8^-$), $CD8^+$ (as well as relatively higher levels of $CD8^+$), memory T helper ($CD3^+CD4^+CD8^+$) cells and higher *relative* levels of granulocytes ($CD172^a$) related to reduced disease severity in both housing systems. Raised serum concentrations of natural IgM antibodies binding to keyhole limpet hemocyanin (KLH) were also related to reduced disease severity after infection. Increased levels of skin lesions at the central body part (after weaning and before infection) were related to increased disease severity in conventional housing systems only. High resisters showed a lower histological lung lesion score, which appeared unrelated to sex. Body temperature, behavior and growth prior to infections were influenced by housing regime but could not explain the variation in lung lesion scores after infection. Raised basal lymphocyte counts and lower skin lesion scores are related to reduced disease severity independent or dependent of housing system respectively. In conclusion, our study identifies intrinsic animal-based measures using linear regression analysis that predicts resilience to infections in pigs.

Introduction

Porcine respiratory disease complex (PRDC) is an example of a typical polymicrobial production disease that can cause major concerns for animal welfare as well as economic losses in the pig industry worldwide (Opriessnig et al., 2011). Pathogenesis of these multifactorial polymicrobial diseases involves infectious factors of different pathogens (both viral and bacterial) and non-infectious factors. There are a variety of pathogens associated with PRDC, classified as either primary or secondary agents. These pathogens include porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), porcine circovirus type 2 (PCV2), *Mycoplasma hyopneumoniae*, *Pasteurella multocida* and A*ctinobacillus pleuropneumoniae* (Opriessnig et al., 2011). Secondary agents, are opportunist bacteria that will invade the damaged lung following previous infection with a primary agent (often PRRSV) (White, 2011). However, in a highly complex disease situation several primary agents can act together and subsequently to produce a population disease picture that is very difficult to unravel (White, 2011).

Non-infectious factors are important and can play a role in the initiation and disease outcome (Opriessnig et al., 2011) and consist of: (1) environmental factors (2) type of production system and management and (3) pig specific factors (genetics, sex, age, coping strategy, immune status, resilience state of the pig). Resilience state of pigs can be defined as the capacity to withstand perturbations such as infections and can be quantified afterwards by measuring variation of clinical impact and recovery capacity after infection of an individual animal in terms of severity and duration of symptoms (Nakov et al., 2019). Thus, the variation in severity of symptoms between individual pigs caused by infection of these poly-microbials is not only due to the variation of pathogens and their virulence, but pigs kept under similar circumstances will show individual variation in disease manifestation as well (Hansen et al., 2010). The individual variation in clinical outcome or resilience after infection has for some part previously been explained by differences in genetic background, age and also by the immune status (Opriessnig et al., 2006; Rossow, 1998; Thanawongnuwech et al., 1998; Vincent et al., 2006). Especially maternal antibody levels have been shown to be of influence on the clinical manifestation in individual pigs (Reyneveld et al., 2020). Clinical cough symptoms and body temperature at the start of infection have been found to be of significance as indicators for the severity of symptoms after *Escherichia coli* endotoxin and *Pasteurella multocida* challenge in pigs (Halloy et al., 2004). Animal based specific factors such as age, genetic background and sex may be of influence on disease severity, but which other additional animal based factors at time of infections will predict resilience is still largely unknown (Scheffer et al., 2018). How the interaction between animal-based factors and housing or environmental factors prior to or during infections affects disease severity remains to be elucidated. High resilient animals or vulnerable animals can be recognized more precisely when more knowledge is available about the interplay between individual animal-based factors in combination with environmental and social circumstances. This will enable early preventive intervention strategies and or methodological and conceptual tools to improve predictive value of disease outcome in the future (Proudfoot et al., 2012) in complex diseases such as PRDC. Embedding animal-based indicators in model-based approaches can possibly be used in prevention and control strategies at individual operational level (e.g., extra temporary extra supportive attention or changes in diet, housing or climate control to specific animals

or groups), or at strategic management level (e.g., changing management measures, housing condition, breeding strategy).

This study focused on relations between animal-based measures before infection and the severity in terms of pathological presentation of PRDC related lung lesions after infection. A defined set of animal-based measures were tested and consisted of coping strategy, sex, rectal temperature, weight/growth, activity, behavior, skin lesions white blood cell counts (WBC) and phenotypical T and granulocyte differentiation and serum natural antibody (NAb) levels. Interaction with type of housing system was tested (enriched and low stocking density or conventional housing system). A dataset originating from an experiment with 28 pigs was used of a well-established experimental co-infection PRDC model of porcine respiratory disease virus (PRRSV) and *Actinobacillus pleuropneumoniae* (Van Dixhoorn et al., 2016).

Materials and Methods

Animals, experimental design and treatments

A data set of parameters of 28 piglets was used originating from an experiment described earlier (Lu Luo et al., 2017b; Van Dixhoorn et al., 2016). For the experiment, the established principles of laboratory animal use and the EU and Dutch laws related to animal experiments were adhered to in this study. The Wageningen University Animal Care and Use Committee (Lelystad Department) approved the experiment under number 2013181.

The 28 piglets were a selection of the offspring (56 male and female piglets) of 8 multiparous sows obtained from a PRRSV and A. *pleuropneumoniae* free herd. From the first day of life onwards, half of the pigs were housed in 4 conventional 5 m^2 pens (7 pigs per pen) with 100% slatted floor and a 100 × 45 cm solid rubber floor mat (conventional housed pigs: CH pigs). The other half of the pigs were housed in 4 enriched pens (10 m^2 ,7 pigs per pen) with partly slatted (40%) and partly solid (60%) floors (enriched housed pigs: EH pigs). Enrichment consisted of two chains with plastic blocks in both housing conditions. In the enriched pen two jute bags and branches of a broom were provided as well as rooting substrate consisting of straw, peat and wood shavings (see (Van Dixhoorn et al., 2016) for details). Social enrichment was applied to the enriched pens from 13 days of age until weaning, by removing the panels between two adjacent enriched pens until weaning. Each pen was cleaned daily, and enrichment materials and food were γ-irradiated (9 kGy irradiation; Synergy Health Ede BV, the Netherlands). Each pen had two drinking nozzles, one for the sow and one for the piglets. Sows were fed a standard commercial diet twice daily. From 3 days of age, the piglets received solid food ad libitum. Lights in the pens were on between 6:00 a.m. and 6:00 p.m., and the temperature gradually decreased from 25 °C to 22 °C the week before weaning. After weaning at the age of 31 days, 14 piglets were selected per housing treatment. The selection was made taking sex, coping strategy and weight into account in order to obtain piglets with comparable features in each experimental subgroup. All piglets were equally mixed and 4 new groups (2 enriched and 2 conventional) of 7 pigs each were formed, balanced for sex, coping strategy and weight. Coping strategy was assessed at the age of 17 days by performing a backtest (Luca Melotti et al.). During the backtest, piglets were held in supine position for 1 minute and the number of struggles, latency to first struggle, number of vocalizations, and latency to the first vocalization were recorded. Pigs

were classified into "high-resisters" (HR) and "low-resisters" (LR) as described previously by (Bolhuis et al., 2003). This resulted in characterization of each pig by mother (genetic background), pen, sex, coping strategy and housing treatment. These pig specific data were used as experimental variables. Data set details are summarized in Table 1.

Table 1. Data set details - experimental setup

Pig number	Pen	Sex	Coping strategy	Housing
14	2	Male 6	High resisters 2	Conventional
		Female 8	Low resisters 12	
14	2	Male 6	High resisters 3	Enriched
		Female 8	Low resisters 11	

At the age of 44 days CH and EH pigs were intranasally infected with 1.5 ml inoculum containing 5 $\log_{10}$ 50% tissue culture infectious dose (TCID) of the mild-virulent European PRRSV serotype 1 strain LV-Ter Huurne (described in (Van Dixhoorn et al., 2016)). This treatment was followed by an aerosol infection at day 52 (ID8) with *A. pleuropneumoniae* serotype 2. Groups of 2 to 3 pigs were simultaneously exposed in a stainless-steel aerosol chamber (110 x 45 x 90 cm). An amount of 5 ml of the inoculum suspension was administered during a period of 20 min using the aerosol nebulizer Aeroneb Pro (EMKA Technologies, Paris, France). The procedures have previously been described (Hensel et al., 1993). On day 55 the pigs were euthanized by injection of pentobarbital (Euthasol 40%, AST Farma) in the auricular vein while restrained and thereafter exsanguinated, followed by necropsy.

Measurement of disease severity; Histological assessment of the lungs

Typical lung lesions caused by the co-infection are shown in Figure 1A. The histological score of the lungs, obtained during necropsy was used as indication for severity of co-infection. 6 tissue samples per pig from predefined locations in the lungs (cranial, cardial and caudal lobe of the left and right lobe) were formalin-fixed, processed and embedded in paraffin. Tissue sections were stained with Haematoxylin-Eosin (HE) and a semi-quantitative, patho-histological assessment of HE stained slides encompassed the extent of pneumonia throughout the predefined locations in the lungs. Patho-histological assessment included 4 features: (1) the presence of focal or diffuse alterations with interstitial or catarrhal pneumonia or atelectasis, (2) the extent of infiltration of alveolar septae with mononuclear cells (3) the extent of infiltration of mononuclear cells in the perivascular/peribronchiolar area, and (4) pleuritis. Examples of typical histological lesions are shown in Figure 1B-E. A histological score of 0 to 3 was used to describe the severity of changes per feature (i.e., 0 = no findings, 1 = mild focal manifestation, 2 = moderate, multifocal manifestation or diffuse manifestation, 3 = severe diffuse manifestation). During a histological examination, the pathologist was unaware of housing or coping treatment. The scores from all 6 slides per lung were added to obtain an overall histology score, which could add up to a maximum of 72 points per pig (6 slides per pig, 4 histological features, and maximum score of 3 per feature).

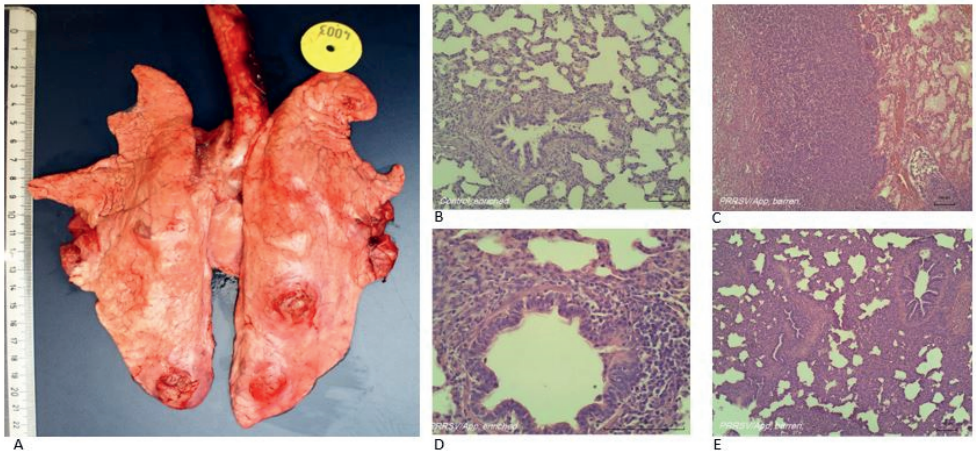

Figure 1. Representative macroscopic (**A**) and histologic (**B-E**) lung lesions caused by co-infection of porcine reproductive and respiratory syndrome virus (PRRSV) and *Actinobacillus pleuropneumoniae*. (**B**) Absence of histological alterations, healthy lungs in non-infected animals. (**C**) Diffuse severe alterations (interstitial or catarrhal pneumonia, infiltrations in alveolar septae and peri-bronchial or perivascular area, pleuritis). (**D**) Close up of (mild) infiltrations of perivascular and peribronchiolar area. (**E**) Diffuse severe alterations (interstitial or catarrhal pneumonia, infiltrations in alveolar septae and peri-bronchial or perivascular area, pleuritis).

Measurements of animal-based factors

Measurements prior to infection were used as possible explanatory predictive variables and consisted of rectal temperature, body weight at different time points, behavioral assessment, skin lesion scores, white blood cell counts and concentrations of serum natural antibodies.

Rectal temperature was measured twice at days 40 and 44 of age prior to infection. Microlife digital thermometers with a resolution of 0.1°C were used. Thermometers were calibrated prior to the experiment. Body weight of the piglets was measured weekly from the day of parturition until the end of the experiment. Difference in body weight between the measurement after weaning at day 44 and before weaning was also calculated.

Frequencies of the behaviors listed in the ethogram (Table 2, adapted from (Camerlink et al., 2012)) were recorded four times, at day 30, 32, 38 and 40. On each day, all pens were observed for 4 ten-minute periods, twice in the morning (between 8:00 and 11:00 am) and twice in the afternoon (between 13:00 and 16:00 am) in an order balanced for housing. Behaviors were expressed as frequencies per pig per ten minutes. A new bout was scored when the pig stopped the behavior for more than 2 seconds. On the same days, skin lesions at the front (head, neck shoulders and front legs), middle (flanks and back) and rear (rump, hind legs, tail) were counted and categorized as a proxy for aggressive behavior (Simon P. Turner et al., 2006). For each body region, the number and severity of lesions was differentiated using scores from 0–4 as follows: 0: No lesions; 1: < 5 superficial lesions; 2: 5–10 superficial lesions or < 5 deep lesions; 3: 10–15 superficial lesions or 5–10 deep lesions; 4: > 15 superficial lesions or > 10 deep lesions. Lesion scores were averaged per day.

Table 2. Ethogram

Behavior	Description
Social behavior	Touching or sniffing any body part of a pen mate
Aggression	Uni- or bilateral fighting by chasing, head knocking (with or without biting) and / or pushing
Manipulate pig	Nibbling, sucking or chewing on any body part of piglet or sow, or belly nosing
Manipulate pen	Nibbling, sucking or chewing on pen components
Playing	Fast running around the pen (galloping), rolling and shaking objects
Mounting	Standing on hind legs with front legs on pen mate

Blood samples taken by jugular vein puncture when piglets were 44 days of age were used to establish total WBC count and phenotyping of WBC (serum, EDTA blood, respectively). A differential count of lymphocytes, granulocytes and monocytes was assessed using a hematology analyzer (blood cell counter Sysmex pocH-100iV diff, Kobe, Japan) as described in (Van Dixhoorn et al., 2016). Freshly isolated heparinized blood samples (100µl) were used to quantify different phenotypes of WBC's followed by FACS analysis (FACS Lysing Solutions BD biosciences) as described in (Van Dixhoorn et al., 2016). NK cells ($CD3^{-}CD4^{+}CD8^{+}$), naive T helper cells ($CD3^{+}CD4^{+}CD8^{-}$), memory T helper cells ($CD3^{+}CD4^{+}CD8^{+}$) and $CD8^{+}$ cells were identified and presented as absolute cell counts. Relative levels of different types of WBC were then calculated as percentage of total WBC. Both absolute cell counts and relative levels of different types of WBC were tested as possible explanatory predictive variables.

Blood samples taken by jugular vein puncture when piglets were 38 and 44 days of age were used to establish levels of Natural antibody (NAb) binding to KLH or phosphoryl-conjugated bovine serum albumin (PC-BSA, Sigma-Aldrich, St. Louis, MO, USA), and natural antibodies binding self-antigen (NAAb) to myelin basic protein (MBP, Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to procedures described by (Lu Luo et al., 2017b). In short, titers of IgM and IgG antibodies binding (KLH, MBP or PC-BSA) were determined by a two-step indirect enzyme-linked immunosorbent assay (ELISA). Each absorbance was expressed relatively to the absorbance of a standard positive control serum sample and antibody titers were expressed as log2 values of dilutions with extinction closest to 50% of Emax, where Emax represents the highest mean extinction of a standard positive serum present on every microtiter plate. Experimental process and timeline are visualized in Figure 2.

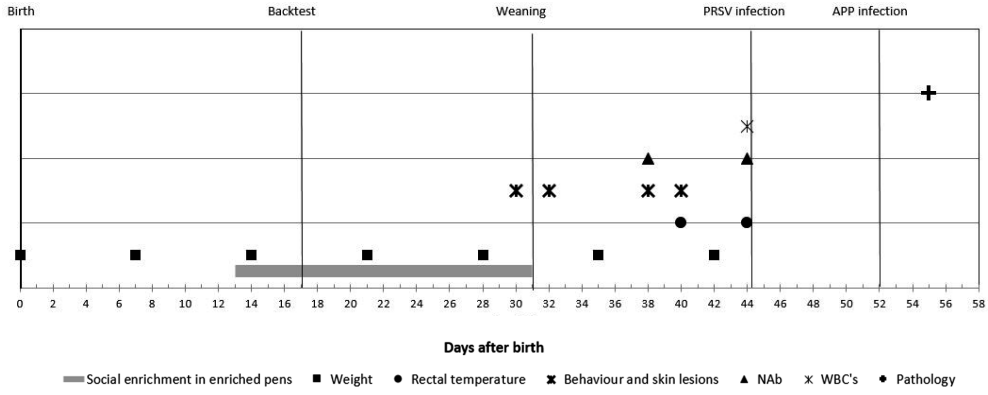

Figure 2. Experimental process and timeline of the measurements: piglets were observed from birth until 55 days of age. Enriched housed pigs experienced social enrichment from day 13 until weaning; coping strategy was assessed at day 17. Piglets were weaned at day 31 and infected with porcine reproductive and respiratory syndrome virus (PRRSV) at day 44 and with *Actinobacillus pleuropneumoniae* (APP) at day 52 and euthanized at day 55. Body weight was measured weekly; rectal temperature was measured at days 40 and 44, behavior and skin lesions were assessed at days 30, 32, 38 and 40. Natural antibodies (NAbs) were measured at days 38 and 44; white blood cell (WBC) at day 44 and histological lung lesion scores were assessed after day 55.

Statistical analysis

The usage of the variables in the statistical models are explained in Table 3 and consisted of four parts. First the effect of the experimental variables (housing, coping, and sex) on the explanatory variables was tested with a linear mixed model. Per explanatory variable we fitted a model with the explanatory variable as the dependent variables, the experimental variables as the independent variables, and pen as a random effect (corresponding to arrows A in Figure 3). Second, we explored the effect of experimental variables on the histology score. This was also done with a linear mixed model that included histology score as the dependent variables and the experimental variables (housing, coping, and sex) as the independent variables and pen as a random effect (corresponding to arrows B in Figure 3).

Table 3. Overview of the used variables and disease outcome

Experimental set up variables	Explanatory variables	Dependent variable clinical severity
Pen (random)	Rectal temperature	Histological score
Coping Strategy	Body weight	
Sex	Behavior	
Housing	Skin lesions	
	WBC	
	NAb	

WBC, white blood cell; Nab, natural antibody.

In the third step we explored the effect of explanatory variables on the histology score (corresponding to arrows C in Figure 3). In this model histology score was used as the dependent variable. Each explanatory variable was analyzed separately as an independent variable on top of a model that contained housing, coping, and pen (random effect). Sex was not included as we did not find a relation between sex and histology score in the previous analysis. Per model we tested for an interaction between housing and the explanatory variable. The interactions between coping and the explanatory variable could not be estimated due to the small number of high resisters. All models were fitted using R package lme4 and p-values were calculated using package lmerTest. In parts 1 and 3 of the analysis results were adjusted for multiple testing. In this adjustment we calculated the false discovery (FDR) rate per variable set with the Benjamini-Hochberg correction.

In the last step we explored if the model using the histology score as the dependent variable could be improved by including more explanatory variables (multi variable approach). Using the base model that included housing, coping, and pen (random effect) explanatory variables were added using forward selection based on improving the Akaike Information Criterion (AIC), to determine which model fitted best. As an additional criterion in this procedure, the sign of an effect size should not change direction when a new variable is included, suggestive of co-linearity of data.

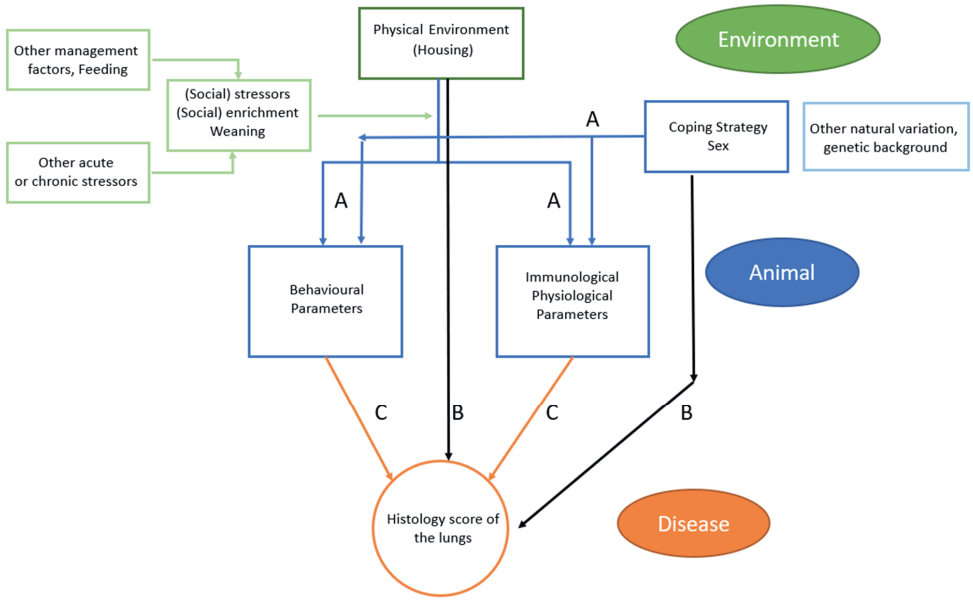

Figure 3. Schematic flowchart of linkage between farm management practices (environment and management factors in green), animal-based measures (in blue) and disease outcome (in orange). Arrows indicate the different relations that were tested: blue arrows (**A**) relate to experimental variables (housing, coping and sex) and explanatory variables; black arrows (**B**) relate to experimental variables (housing coping and sex) and disease outcome; orange arrows (**C**) relate to explanatory variables and disease outcome including housing, coping and interaction housing x explanatory interaction.

Results

Effect of experimental set up variables (housing, coping strategy and sex) on explanatory variables assessed prior to infection

First the effect of housing on explanatory variables that were measured prior to infection was tested (corresponding to arrows A in Figure 3). In conventional housed pigs, temperature 4 days prior to infections was higher, more skin lesions were apparent at the rear of the body after weaning and pigs showed more mounting behavior. Overall absolute WBC and lymphocytes counts were lower in conventional housed pigs prior to infection.

Independent from housing female piglets had higher levels of KLH-binding IgG antibodies at day 38 and lower aggression scores during the complete period prior to infection as compared to the males. The separate analysis of coping strategy effect showed more walking behavior prior to infection by high resisters as compared to the low resisters (Figure 4).

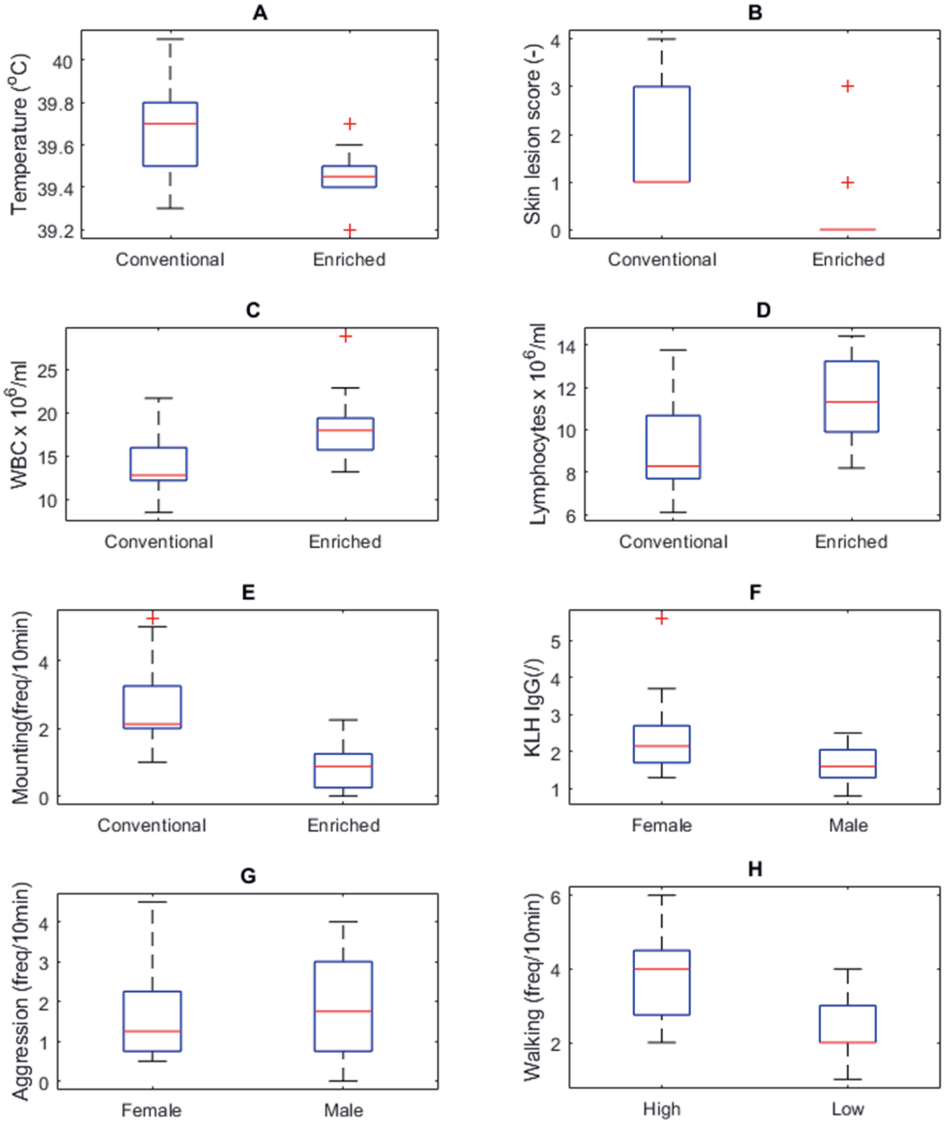

Figure 4. Significant relations between housing (**A-E**), sex (**F-G**) and coping strategy (high and low resisters) (**H**) and explanatory variables. (**A**) Rectal body temperature at day40 ($P<0.01$). (**B**) Skin lesions rear part of the body at day 38 ($P<0.001$). (**C**)White blood cell (WBC) at day 44 ($P<0.05$). (**D**) Lymphocytes at day 44 ($P<0.05$). (**E**) Overall mounting behaviour ($P<0.001$). (**F**) Keyhole limpet hemocyanin (KLH) IgG at day 38 ($P<0.01$). (**G**) Overall aggressive behaviour ($P=0.06$). (**H**) Walking activity ($P<0.01$).

Effects of Experimental variables (Housing, Coping Strategy and Sex) on disease severity

The effect of housing on disease severity (corresponding to arrows B in Figure 3) was also presented earlier (Van Dixhoorn et al., 2016) showing more severe histology scores in conventional housed pigs (Figure 5A, $P<0.05$). An effect of coping strategy was also seen in histology score, where high resisters showed lower histological scores ($P<0.05$, Figure 5B) as compared to the low resisters. An effect of sex on disease severity was not found (Figure 5C).

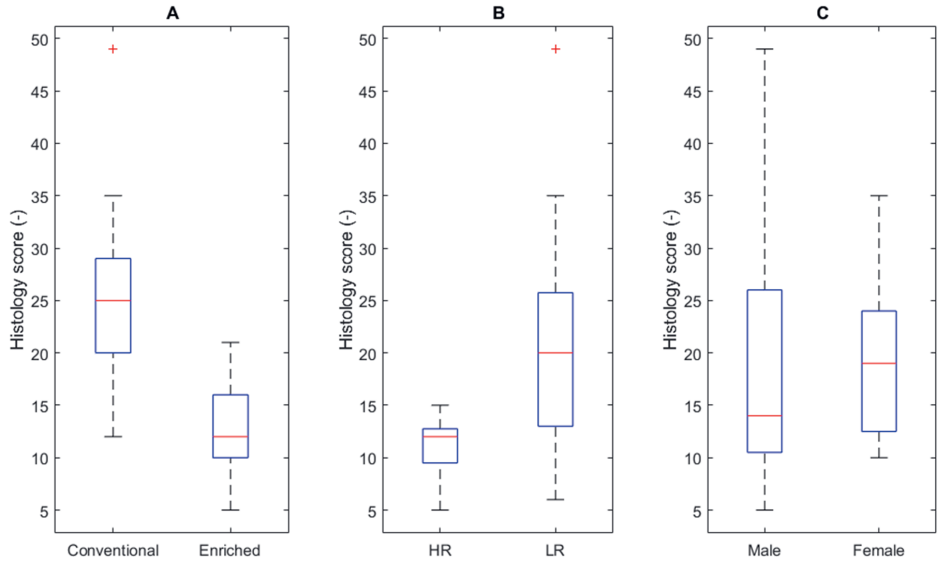

Figure 5. Main effect of experimental variables on histology score. (**A**) Housing ($P<0.05$). (**B**) Coping strategy ($P<0.05$). (**C**) Sex not Significant (NS). HR: High resisters, LR Low resisters.

Effects of explanatory variables on histology score

All explanatory variables were tested to predict histology score as disease severity read out (corresponding to arrows C in Figure 3). The results with histology as response variable and no interaction effect with housing are presented in Table 4 (only the significant explanatory variables with $P<0.05$, and FDR <0.1 are shown). The results with significant interaction effect with housing are shown in Table 5. Temperature and weight variables did not relate to histology scores.

Lower absolute cell counts and relative levels of lymphocytes and T helper cells and lower absolute cell counts of $CD8^+$cells and memory T helper cells prior to infection, were related to higher histology score. This effect was independent of the type of housing. Higher relative level of granulocytes prior to infection was related to higher histology score. No other variables showed a relation with histology score independent of housing regimes.

When the interaction housing x response variable was found significant ($P<0.05$), significance was tested for the two different housing conditions. A lower relative level of $CD8^+$ cells

and lower KLH IgM levels before infections were related to higher histology scores in the lungs in conventional housed pigs only. Higher skin lesion scores after weaning at the center part of the body were related to higher histology scores in the conventional housed pigs (Table 5).

Table 4. Significant explanatory variables for histology score as response variable with no interaction with housing

Explanatory variable (day 44)	Coefficient	*P*-Value	FDR	R2
$CD8^+$ cells	-9.47014	<0.05	0.06	0.07
Lymphocytes	-1.62288	<0.01	0.01	0.11
Memory T helper cells	-88.5637	<0.01	0.01	0.07
Naive T helper cells	-10.786	<0.01	0.01	0.10
Relative granulocytes	38.27377	<0.01	0.03	0.08
Relative Lymphocytes	-48.2953	<0.05	0.03	0.08
Relative naive T helper cells	-244.9	<0.01	0.02	0.08

Variables with FDR < 0.10 are presented in the table. Absolute cell count and relative count as percentage of white blood cells (WBC's) are depicted. FDR, false discovery rate.

Table 5. Interaction effects with type of housing and explanatory variables with histology score as response variable

Explanatory variable	Housing	Coefficient	p-value
Relative $CD8^+$ cells	Conventional	-319.202	<0.01
KLH binding IgM, day 40	Conventional	-6.23362	<0.001
Skin lesions central, day 40	Conventional	7.229481	<0.001

Variables with *P* < 0.05 and FDR < 0.10 are presented in the table. KLH, keyhole limpet hemocyanin; FDR, false discovery rate.

Effects of multi explanatory variables on disease severity

The absolute cell counts, and relative levels of WBC differentiations were tested separately. For the absolute cell counts, the histology score could be explained by the base model with lowest AIC criterion using the absolute level of memory T helper cells, including pen as random effect and coping and housing as fixed effects:

$$histology\ score \sim coping\ +\ (random\ pen)\ +\ housing\ +\ memory\ T\ helper\ cells$$

Then it was tested if AIC was improved by adding other explanatory variables (Table 6). In each step another variable was added to the equation. Adding lymphocytes and $CD8^+$ cells to the equation slightly improved the AIC, but with the addition of naive T helper cells, the AIC was lowest. (Table 6). Then it was tested if the addition of a third variable to the same model could improve the AIC. The inclusion of CD8+ cells to the statistical model changed direction of the sign of the effect and is therefore probably attributed to co-linearity of input variables. Higher histological lung scores were best explained by both lower levels of memory and naive T helper cells. So, the model with lowest AIC was:

$$histology\ score \sim coping\ +\ (random\ pen)\ +\ housing\ +\ memory\ T\ helper\ cells\ +naive\ T\ helper\ cells$$

This same procedure was performed with relative levels of different WBC, resulting in the lowest AIC was reached when naive T helper cells, memory T helper cells and lymphocytes were used in the model. Again, $CD8^+$ cells reduced AIC, but the change in direction of the effect suggests co-linearity (Table 7). Final model with relative numbers of WBC:

$$histology\ score \sim coping\ +\ (random\ pen)\ +\ housing\ +\ memory\ T\ helper\ cells\ +naive\ T\ helper\ cells\ +\ lymphocytes$$

Table 6. Multivariable approach results with absolute numbers of different white blood cells

Base model absolute numbers	Adding	Adding	AIC
Memory T helper cells	-	-	147
Memory T helper cells	Lymphocytes	-	145
Memory T helper cells	CD8+ cells	-	143
Memory T helper cells	Naive T helper cells	-	141
Memory T helper cells	Naive T helper cells	Lymphocytes	141
Memory T helper cells	Naive T helper cells	CD8+ cells	137*

AIC, Akaike information criterion.

*Inclusion of the $CD8^+$ cells changed direction of the sign of the effect.

Table 7. Multivariable approach results with relative numbers of different white blood cells

Base model relative numbers	Adding	Adding	AIC
Naive T helper cells	-	-	143
Naive T helper cells	Lymphocytes	-	137
Naive T helper cells	Memory T cells	-	131
Naive T helper cells	$CD8^+$	-	133*
Naive T helper cells	Granulocytes	Lymphocytes	137
Naive T helper cells	Memory T cells	$CD8^+$ cells	124
Naive T helper cells	Memory T cells	Granulocytes	125

AIC, Akaike information criterion.

*Inclusion of the $CD8^+$ cells changed direction of the sign of the effect.

Discussion

Predicting the individual vulnerability or resilience to disease is one of the main challenges of modern biomedical research. The question if individual behavioral and physiological characteristics can predict this vulnerability to disease has been subject of debate for a long time (Koolhaas, 2008). Developing strategies to accurately predict the expected degree of disease severity, before onset of disease, is a major challenge for farmers and veterinarians. In our study we distinguished three types of non-infectious factors that could affect disease outcome. Environmental conditions (housing), individual animal static parameters (coping strategy and sex) and finally parameters which may vary in time assessed prior to infection (WBC and lymphocytes, NAb, behavior, rectal temperature, skin lesions and body weight). These parameters were considered in this study as basic factors available in farm practice. Even though a limited branch of the adaptive immune system was tested in our study (T cell subsets and granulocytes) many factors of the innate immune system, such as those of the complement cascade and other innate related cytokines and chemokines remain to be examined.

Histology lung scores were used as a PRDC read-out measure in order to accurately determine the level of disease development for each pig.

Higher levels of both memory T helper and naive T helper cells prior to infections were related to lower histology outcome after infection and this appeared independent of the housing regime. Under conventional housing conditions only, lower skin lesion scores at the central part of the body, higher levels of KLH binding IgM antibodies and higher levels of $CD8^+$ cells were related to lower histology score after infection.

The majority of predictive models in (veterinary) medicine explains variability in the target outcome by conditioning on observed risk factors of the physical and social environment alone (Proudfoot et al., 2012). However these studies do not account for latent sources of variability including individual animal based variation (Schulam & Saria, 2016). (Proudfoot et al., 2012) suggests a flow diagram of the potential linkage between farm management practices and disease risk, including influences from both the social and physical environment. (Schulam & Saria, 2016) proposed a hierarchical model for disease severity prediction previously addressing common latent and observed sources of heterogeneity in complex diseases identifying three levels: the population, subpopulation, and individual level. According to (Schulam & Saria, 2016) these three levels and possible interactions may possibly reflect biological variation and disease outcome. Our experimental approach can be compared with the flow diagram as suggested by (Proudfoot et al., 2012), linking individual variation to biological intermediates together with the inclusion of the effect of the physical environment and social stressors. By the provision of the enriched environment to pigs, we improved the possibility for pigs to express their natural behavior in contrast to the conventional housed group. The applied enrichment has proven to reduce stress related behavior in general had been demonstrated (Van Dixhoorn et al., 2016). High stocking densities cause stress by the reduced ability of an individuals to retreat or avoid aggressive behavior from others (Koolhaas et al., 1999). Another interesting finding in our study was that in the conventional housed pigs revealed an increased skin lesion score at the central part of the body after weaning. This was related to more severe histological lung lesions after infection. In most cases during aggressive mutual fights the target of biting is at the front third of the body (Simon P. Turner et al., 2006). Pigs will get skin lesions at the rump when they try to retreat from the fight (Doeschl-Wilson et al., 2009; Prunier et al., 2020). This suggests that the animals in our study that were less able to adequately retreat from the fights and subsequently were more prone to develop more severe lung lesions after infection. This was particularly evident under the conventional housing regime. This relation was not confirmed by the absence of this relation within the enriched house regime and absent om the enriched housing environment. A very low skin lesion score was combined with low histology scores in most animals in this group. Skin lesions at the front or rear of the body were not related to histology score nor were behavioral traits alone. Pre-challenge behavior dynamics (dynamic indicators of resilience, (DIOR's)) were previously not identified as indicators for PRRSV resilience in terms of morbidity or mortality after infection. Changes in activity levels in early stage of infection were suggested as a useful indictor of resilience in the further trajectory of disease development (van der Zande et al., 2020). In this study the authors especially looked at the dynamics of activity of continuous automated measurements but did not include sex or coping strategy in their analysis. Continuous measurements of behavior allow for more insight into level and dynamics of behavior. Our study observations included the distinctions of play, rooting, interactions with pen-mates aggressive behavior or other

stress related behavior which tentatively influences stress levels and may possibly be missed by automation.

Coping strategies are roughly defined as persistent and are correlated with physiological and behavioral responses of animals to a number of stressors (Koolhaas, 2008; Veenema et al., 2003). Animals with reactive coping styles are more pronounced hypothalamic pituitary adrenal (HPA) responders to socials stressors and have been suggested to be at higher risk of infection (Proudfoot et al., 2012). Our study observations confirmed these findings. We showed that the low resisters' coping strategy was associated with a higher clinical impact of disease in both housing regimes. In addition, other personality traits as well as social status within a group could play a role in clinical outcome.

Others found differences when comparing males and females in their physiological response to infection (de Groot et al., 2001; Reimert, Bolhuis, et al., 2014; Rohleder et al., 2001). Our findings in which the sex of the animal appeared not to be predictive of the final histology score after infection, suggests the contrary. Little evidence has been found to link these sex differences and coping style to disease risk (Proudfoot et al., 2012). This may be since in general more males are used in research to exclude hormonal cycle influences in females. There are studies in which male pigs were more susceptible to the development of the multifactorial post-weaning multi-systemic wasting syndrome as compared to female pigs. The authors attributed their findings to castration and associated secondary infections (Opriessnig et al., 2006).

Our study showed differences in levels of IgG NAb binding KLH and activity prior to infection between males and females. However these levels of IgG binding KLH nor activity explained additional variation in diseases outcome themselves. (Reimert, Rodenburg, et al., 2014) previously found that HR enriched housed pigs had a higher KLH-IgG titer when compared to enriched housed LR pigs and that both revealed a higher titer than conventional housed HR and LR pigs. These relations were not confirmed in our study.

In general stressors are thought to have impact on different parts of the immune system (Proudfoot & Habing, 2015). In our study only limited factors of the immune system were evaluated in which housing regime itself indeed increased absolute levels of WBC and lymphocytes. These higher levels were related to reduced disease severity as well. No interaction was observed with housing regime. This suggests that the relation between WBC, lymphocytes and disease severity applies to both enriched housed pigs as well as to conventional housed pigs.

Temperature, body weight or growth are frequently proposed to determine disease outcome. In this study these variables assessed prior to infections were surprisingly not related to severity in clinical outcome after infection. These non-static parameters are probably more of value when measured at the very early stage after infection. Temperature rise, reduced growth rate or diminished activity are typically symptoms of disease. Changes in dynamics or level of these parameters can therefore serve as early warning indicators for disease after infection, but not as predictors prior to infections for disease severity.

We additionally tested if prediction in clinical outcome could be improved when using multivariate models. Different variables represent different biological mechanisms and thus they may contribute on top of each other to improve model accuracy. Indeed, accuracy could be slightly improved when adding thus, confirming that prediction of disease cannot easily be done using single

factors, because complex interactions of external and internal factors exist, and their relations do not always appear to be linear.

We therefore hypothesize that a physiology-based network approach may increase the accuracy of disease severity prediction substantially by including nonlinear relationships. Despite its limited size, this work adds to the body of evidence to explain differences in clinical expression of the polymicrobial diseases observed from pig to pig. These type of challenge studies are useful to establish possible links between external factors, animal-based factors and clinical outcome of disease. At the same time, they have unavoidable limitations with statistical power and the possibility to extrapolate results to commercial settings. Identifying more complex relations will likely require a larger test group. Nevertheless, this work provides a first basis to unravel the complex network of interactions that will enable a quantitative prediction of disease outcomes in pigs. The methodology presented in this paper serves as a blueprint for identification and quantification of similar networks that are encountered in livestock production.

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CHAPTER 4

4

Indicators of resilience during the transition period in dairy cows, a case study

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Abstract

The transition period is a demanding phase in the life of dairy cows. Metabolic and infectious disorders frequently occur in the first weeks after calving. To identify cows that are less able to cope with the transition period, physiological or behavioral signals acquired with sensors might be useful. However, it is not yet clear which combination of signals, and which signal properties are most informative with respect to disease severity after calving. Sensor data on activity and behavior measurements, as well as rumen and ear temperature data from 22 dairy cows were collected during a period starting 2 weeks prior to expected parturition until 6 weeks after parturition. During this period, the health status of each cow was daily clinically scored. A total deficit score (TDS) was calculated based on the clinical assessment, summarizing disease length and intensity for each cow. Different sensor data properties recorded during the period before calving as well as the period after calving were tested as predictor for TDS using univariate analysis of covariance. To select the model with the best combination of signals and signal properties we quantified the prediction accuracy for TDS in a multivariate model. Prediction accuracy for TDS increased when sensors were combined, using static and dynamic signal properties. Statistically, the most optimal linear combination of predictors consisted of average eating time, variance of daily ear temperature, and regularity of daily behavior patterns in the dry period. Our research indicates that a combination of static and dynamic sensor data properties could be used as indicators of cow resilience.

Introduction

The transition period, defined as the period between 3 weeks pre-partum until 3 weeks post-partum (Grummer, 1995), is a demanding period for dairy cows. It makes them vulnerable for the development of metabolic and infectious diseases, especially in the first weeks after calving (Huzzey et al., 2007; Urton et al., 2005). Incidences of common diseases such as milk fever, clinical respiratory diseases, contagious mastitis and clinical parasitism, have been reduced the last 25 years, due to the shift in focus on disease prevention rather than treatment, but incidence of important peri-parturient diseases remains high (LeBlanc et al., 2006). It has been argued that in order to reduce the incidence of periparturient diseases we should look at the farm as an integrated system rather than rely on growing knowledge on pathology and etiology (LeBlanc et al., 2006). Indeed, in particular the existence of complex relationships between peri-parturient diseases and nutritional strategies, housing conditions as well as social and attitudinal factors is believed to hamper effective prevention and control (Mulligan & Doherty, 2008). Therefore, early warning signals for disease and the identification of farm specific risk factors remain key elements for opportune interventions.

Metabolic and inflammatory response measures have been described as early indicators for peri-parturient diseases, providing opportunities for early treatment (Huzzey et al., 2009; Huzzey et al., 2015; Huzzey et al., 2011; Roberts et al., 2012; Seifi et al., 2011; Trevisi et al., 2012). Preferably, indicators are used in farm management even before the onset of disease becomes apparent. For this reason, research should also focus on the detection of cows that are statistically more likely to develop disease, in addition to early disease detection. Detection of these 'cows at risk' would allow for adequate preventive intervention often without the need for medical treatment. There is growing interest in how to use sensor data to detect cows at risk for peri-parturient disorders (Rutten et al., 2017; Weary et al., 2009). Indeed, automatically recorded behaviors have shown to be indicative for risk of disease (Weary et al., 2009). For example, reduced feed intake during the pre-partum transition phase was proven to be an indicator for cows at risk for metritis (Huzzey et al., 2007; Urton et al., 2005). Average levels of rumination, feeding, and other behaviors during the transition period, differ between sick and healthy cows, and were also suggested as early indicators for peri-parturient disorders such as metritis or (sub)clinical ketosis (Calamari et al., 2014; Goldhawk et al., 2009; Huzzey et al., 2007; Urton et al., 2005). Average levels of feeding, rumination and other behaviors can easily be acquired non-invasively, using automated and high-frequent sensor measurements. Moreover, as compared to human observers, sensors are often more sensitive or less biased (Rutten et al., 2017; Weary et al., 2009). Average levels of sensor data output are commonly calculated as representative signal property. When looking only at average levels of sensor-generated time series, useful information on underlying mechanisms may get lost. Especially dynamic properties of time series generated by sensors may contain extra useful information (Peng et al., 2009). Based on these outcomes, we hypothesized that combining sensors and multiple signal properties per sensor can improve prediction of disease length and severity during the transition period. We tested this hypothesis by predicting disease length and severity with different combinations of signal types and properties. To quantify prediction accuracy, we defined the total deficit score (**TDS**), which is a total score of post-partum clinical aberrations, acquired through daily clinical inspection by veterinarians. We calculated prediction errors for TDS based on the dynamic

patterns and averages of high-resolution, high-frequent physiological and behavioral data, recorded in individual cows before calving. We also calculated prediction errors for TDS based on data recorded during the first week after calving.

Material and Methods

The established principles of laboratory animal use and the Dutch laws related to animal experiments were adhered to in this study. The Wageningen University Animal Care and Use Committee (Lelystad Department) approved the experiment under protocol number 2014039.b.

Animals, Housing and Diet

A group of 22 Dutch Holstein-Friesian dairy cows of mixed age (13 primiparous, 9 multiparous) within a Dutch dairy farm situated in the east of the Netherlands was selected for the experiment. The experiment took place between April 14, 2014, and July 26, 2014. During this period 26 cows enrolled in this study based on the expected day of parturition. Experimental period per cow lasted from two weeks prior to expected parturition until six weeks after parturition. Cows were included for analysis when they were scored healthy during the dry period and when a complete clinical dataset of the experimental period was available. Based on these criteria 22 cows were selected. The cows were part of the herd of 180 cows, with an average production of 10040 kg milk (with 4.25% fat and 3.58% protein) per year. The lactating cows, dry cows and pregnant heifers were housed in a freestall barn with cubicles. Bedding material in the cubicles consisted of a layer of ca. 5 cm sawdust on the concrete floor. During the 2 weeks before the expected day of parturition, dry cows and pregnant heifers were kept in one pen with 14 feeding places (with individual head locks), 15 cubicles and one water point. Average group size was 15 cows (range 14-16). On average, one or two cows were introduced, and one or two cows were removed from this dry cow pen every week. When the cows showed signs of parturition, they were moved to an individual straw-bedded maternity pen within the same building. After calving, they were introduced into one of three pens with lactating cows. The three pens with lactating cows were each milked with a Delaval milking robot and were similar with regard to production level. Two of these three pens were of similar size and to both groups six experimental cows each were introduced. In both pens 40-42 cows had access to 60 cubicles and 35 feeding places with individual head locks. The third pen had 43 feeding places with individual head locks and 62 cubicles for 49-51 cows. Ten experimental cows were introduced into this third pen. All lactating cow pens had three drinking places. Number of cows per pen was kept as constant as possible and cows remained in the same group after calving until the next dry period. Group composition was dynamic, as animals were moved between pens before and after the dry period. On average every two weeks one cow was introduced and one cow was removed from lactating cow pens. The cows were fed twice daily a TMR consisting of corn silage, hay silage, with concentrates added (protein and mineral supplement) adjusted to the production level of the group. Dry cows were fed dry cow diet consisting of TMR. Water was available ad libitum. Feed composition was kept constant for the duration of the experimental period.

Clinical Examination and Blood Sampling

A score was calculated based on clinical examination as described by (Hajer et al., 1988) of each cow that was performed daily for the period of 2 weeks before until 6 weeks after parturition. All 22 cows were clinically scored daily during the complete period. During clinical examination the following aspects were scored: heart rate (beats per minute), breathing rate (per minute), rectal temperature (°C), rumination (chews per minute), BCS (Edmonson et al., 1989), locomotion score (according to scoring system of D. Zaaijer, W.D.J. Kremer and J.P.T.M. Noordhuizen in (Hulsen, 2012)), udder condition [per quarter: skin temperature (too warm, too cold, normal), color (red, abnormal, normal), painful during palpation (yes or no), swollen (yes or no), teat condition (flexible, color, painful)], retained placenta (**RP**, was diagnosed, if the placenta was protruding from the vulva 24 h after calving), uterus condition and excretion (size of the uterus by rectal examination, color and smell of vaginal discharge (**VD**) and the amount of pus and mucus), manure consistency (according to scoring system of D. Zaaijer, W.D.J. Kremer and J.P.T.M. Noordhuizen in (Hulsen, 2012)), possible displaced abomasum (**DA**) (by auscultation) and overall condition (healthy or not). Clinical examinations were performed by 3 specialized bovine practitioners and the outcome was not shared with the farmer. The farmer followed his standard strategy to decide to treat a cow or call a veterinarian, if he diagnosed a cow ill, treatments of cows were recorded.

Blood samples were collected every two days from the coccygeal vein into 10 ml sterile serum tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). The blood was centrifuged (1-4 h after collection at room temperature at 1,438 x *g* for 7-10 min) and the serum was separated into several aliquots and stored at -20° C until analysis. Blood values indicative and related to peri-parturient problems were determined in order to corroborate our clinical assessment and consisted of calcium, inorganic phosphorus, albumin, haptoglobin, urea and nonesterified fatty acids (**NEFA**) (Roberts et al., 2012; Seifi et al., 2011; Trevisi et al., 2012). Analyses were performed at a commercial laboratory (GD Animal Health, Deventer, the Netherlands). To evaluate potential farm factors influencing cow performance, a risk analysis and assessment of the quality of housing and management at farm level was performed 6 times during the experimental period using a Dutch farm monitoring tool 'KoeKompas' ('CowCompass', https://zuivelplatform.nl) in which the individual farm is scored on 7 critical success factors, 40 performance indicators and more than 100 management control points. This assessment was performed by a specially trained veterinarian, who was not involved in the clinical examinations and who was unaware of any other results of the current experiment. Based on the assessment, a qualitative report was written by the veterinarian in which the most critical management risks, present at the moment the assessment was performed, were described.

Post-partum TDS

Each aberrant clinical finding or disease related symptom was daily scored as one. Deficits were scored as one, when clinical findings were above or below normal values as described by (Hajer et al., 1988). Disease related symptoms were divided into mastitis related problems (score 1 if one or all of the features were found positive: swollenness, painfulness and or redness), metritis related problems (score 1 if one or all of the features were found positive RP, abnormal VD and or large

uterus), lameness related problems (score 1 if locomotion score was 5, or when diagnosed lame) and other health problems (fever, manure score 1 or 5, dull cows, no rumen activity, not eating, milk fever, treated by farmer). All daily clinically detected deficits were added to one total score (dimensionless) during the 6-week period after calving. This resulted in one total TDS per cow based on clinical findings; blood values were not included in the TDS. By adding daily scores of clinical aberrations, also the length of the disease related problems was taken into account in the TDS. Cows with a low TDS, with few aberrant clinical findings lasting only for a short period of time (fast recovery) were in good health, whereas cows with a high TDS suffered from more health problems and or problems with longer duration (slow recovery), during the 6-week period after calving. We first evaluated whether our TDS was distinctive for impaired health using blood concentrations associated with metabolic stress and other peri-parturient disorders as a reference. For this evaluation, we distinguished three groups: group 1 with scores of TDS <7 (good health); group 2: TDS 7-14 (intermediate health) and group 3: TDS>20 (bad health). These arbitrary thresholds were chosen to obtain similar numbers of cows per group. The area under curve (AUC) of blood values of each individual cow was calculated for two separate periods using the linear trapezoidal method. The following two periods were defined for the TDS evaluation: from 10 days before until 1 day before parturition (ante partum), and from parturition until 36 days after parturition (post-partum). The AUC prior to parturition and after parturition of the different blood values were averaged per TDS group 1,2 and 3 and compared by pairwise comparison. AUC of the period prior to parturition was used to evaluate the health state during the dry period. AUC of the period after parturition was used to evaluate the TDS.

Sensor Data Acquisition

During the 2-week period before calving and 6 weeks after calving, continuous and high-frequent behavioral and body temperature data were obtained with the use of three sensors that previously had been validated (Bewley & Schutz, 2010; Borchers et al., 2016):

1. IceQube sensors (IceRobotics, Edinburgh, UK) for recording activity: per quarter the number of minutes lying and standing (adding up to 15), the number of steps, the number of lying bouts and motion index (a measure of the animal's activity level) (Munksgaard et al., 2006). IceQube sensors were attached at least 2 weeks prior to expected parturition date.
2. SensOor sensors (Agis Automatisering, Harmelen, The Netherlands) for measuring behavior (eating, ruminating and activity level) and ear temperature (Bikker et al., 2014): 60 minutes per hour were divided into number of minutes eating, ruminating, high active, low active and inactive (determined by classifying algorithms, see (Pereira et al., 2018)). Average ear temperature (°C) per hour was recorded. A 3-dimensional accelerometer continuously registered the movements of the cow's ear. SensOor sensors were attached in a later stage of the experiment. Only of 7 cows SensOor data were obtained during the entire 2 weeks before parturition, and of 10 cows during the first week after parturition.
3. BellaAg Bolus sensors (Wynnstay, Powys, UK) for measuring rumen temperature (°C) every 10 minutes (Timsit et al., 2011). BellaAg Bolus sensors were inserted in the rumen at least 2 wk before expected parturition date.

Data Analysis and Statistical Analysis

IceQube data obtained per cow per 15 min were summed to get data at hour level, BellaAg bolus temperature data per cow per 10 min were averaged per hour. Standing time of the IceQube data was excluded from the analysis as this is directly complementary to lying time. Average, variance, autocorrelation (with lag τ=1 hour) and nonperiodicity were calculated per cow for each hourly sensor variable. Sensor variables were obtained during two periods: the dry period, starting 15 days before calving up to and including the day before calving (referred to as long-term) and the period after calving from day 1 up to day 7 after calving (referred to as short-term). For both periods and each sensor variable the average, variance and autocorrelation were calculated over all measurement values. Variance and autocorrelation are indicative for the amount of variation in behavior and other variables, and for the level of consistency or regularity of the behavior. For this research, the so-called nonperiodicity was introduced as a measure for the regularity in daily pattern of the sensor data, and was defined as the mean squared error of the correlogram with a sinusoid with a 24h cycle and an amplitude of 0.25 (Figure 1). The nonperiodicity was calculated over the same two periods. Univariate analysis of covariance was performed with cow as experimental unit, with TDS as dependent variable, and with each sensor-based metric (i.e., average, variance, autocorrelation and nonperiodicity, before and after parturition) as an explanatory variable. Parity was included in the univariate analysis of covariance model as a fixed effect with two levels (i.e., parity 1, parity 2 or higher). Prior to analysis of covariance, we examined the fixed effects of lactating cow pen (pen 1, 2 or 3) on both TDS and sensor-based explanatory variables with ANOVA. Because no significant effects of pen were found, lactating cow pen was not considered in subsequent analyses of covariance. The TDS was log-transformed for the analysis to Log(1+TDS) which led to a less skew distribution and a better model fit. We made a distinction between predictors before calving (long-term predictors) and the predictors after calving (short-term predictors). Statistical significance was assumed if $P \leq 0.05$. Only significant results and tendencies ($P < 0.10$) are shown in the results. Next, a multivariate step was performed using the significant predictors and a selection procedure (best subset selection with the 'regsubsets' function from the R library 'leaps') to obtain a prediction model with an optimal subset of predictors (James et al., 2013). Finally, a leave-one-out cross-validation (**LOOCV)** (James, 2013) was performed. For the LOOCV, each observation is used successively as a validation set with the remaining observations as the training set. The resulting LOOCV estimate is the mean squared error (**MSE**) of the single MSE. The multivariate model was built by applying best subset selection on all significant explanatory variables. Non-periodicity of motion index was excluded, as it is known to be correlated with the number of steps, (De Mol et al., 2013). The results may be influenced by the relative low number of cases as only 7 out of 22 had data of all sensors acquired during the complete measuring period. These 7 cows had a TDS of 0, 6, 10, 14, 21, 72 and 121, which we tentatively considered as a representative distribution of TDS. When only applying the 12 IceQube explanatory variables, all 22 cases could be used. Therefore, an additional analysis was done applying only the IceQube variables based on lying time, number of steps and number of lying bouts as input variables. For the additional analysis again best subset selection was applied with LOOCV using the 'regsubsets' function from the R library 'leaps'. All analyses were performed with the statistical programming language R (R Core Team, 2017).

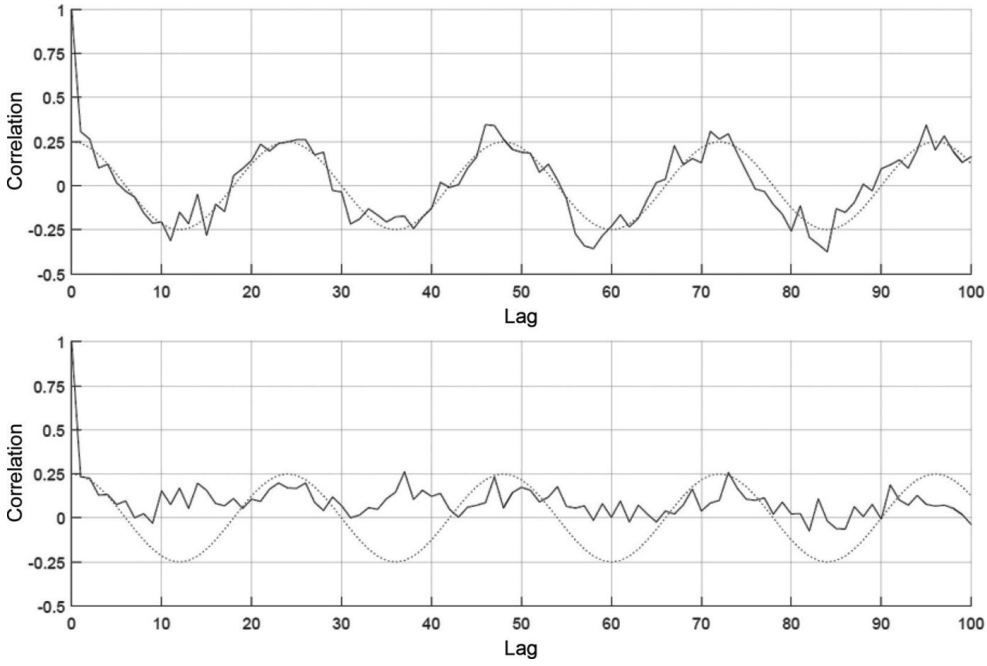

Figure 1. Examples of nonperiodicity, a measure of the regularity in the daily pattern of the sensor data and defined as the mean squared error of the correlogram (black line) with a sinusoid with a 24-h cycle and an amplitude of 0.25 (dotted line). Top: cow 1 with low (0.005) nonperiodicity for eating time; (bottom) cow 2 with high (0.034) nonperiodicity for eating time.

Results

Representativeness of TDS for Disease Severity

Clinical observations were summarized into a TDS per cow after the transition period. TDS values per cow varied between 0 and 121. Six cows had a TDS smaller than 7 (group 1), 7 cows a TDS between 7-14 (group 2) and 9 cows showed a score larger than 20 (group 3). No displaced abomasum was diagnosed, one cow was treated by the farmer for milk fever after calving after calving. This same cow was given analgesics, was systemically treated with antibiotics (treating metritis), and had the highest TDS. Five other cows were treated by the farmer, two cows with analgesics due to parturition problems (one with uterus torsion, one with vaginal injuries), one cow with analgesics and intramuscular oxytetracycline treatment for endometritis and two cows were treated locally between the claws for mortellaro. Mastitis related symptoms were recorded in 11 cows for more than 3 days and 8 cows showed metritis related symptoms for more than 3 days. Lameness was recorded in 7 cows for more than 3 days. General illness was recorded in 4 cows (one cow for 1 day, one cow for 5 days and two cows for 37 and 39 days, respectively). Averages per TDS group for the different blood values are plotted in Figure 2. In the first period, prior to parturition, no

differences were detected between the three groups in any of the blood parameters, which was in line with our clinical findings. The three TDS groups started to diverge in all blood values, except NEFAs and urea after parturition when clinical aberrations started to evolve. In the period after parturition, group 3 showed the slowest recovery in calcium and albumin and an elongated peak in haptoglobin (Figure 2). In the analysis of the period after parturition AUC for calcium in group 1 was significantly higher as compared to group 3 ($P < 0.01$) and tended to be higher as compared to group 2 ($P < 0.10$). For group 2 AUC for calcium tended to be higher as compared to group 3 ($P = 0.06$). AUC for phosphorus in group 3 tended to be lower as compared to group 1 ($P = 0.1$) and 2 ($P < 0.05$). AUC for albumin in group 1 was higher as compared to group 2 ($P < 0.05$) and 3 ($P < 0.001$) and group 2 tended to be higher as compared to 3 ($P = 0.07$). For haptoglobin differences were present between groups 1 and 2 ($P < 0.10$) and between groups 1 and 3 ($P < 0.001$). We also found that an increase in haptoglobin and a decrease in calcium preceded clinical aberrations (results not shown).

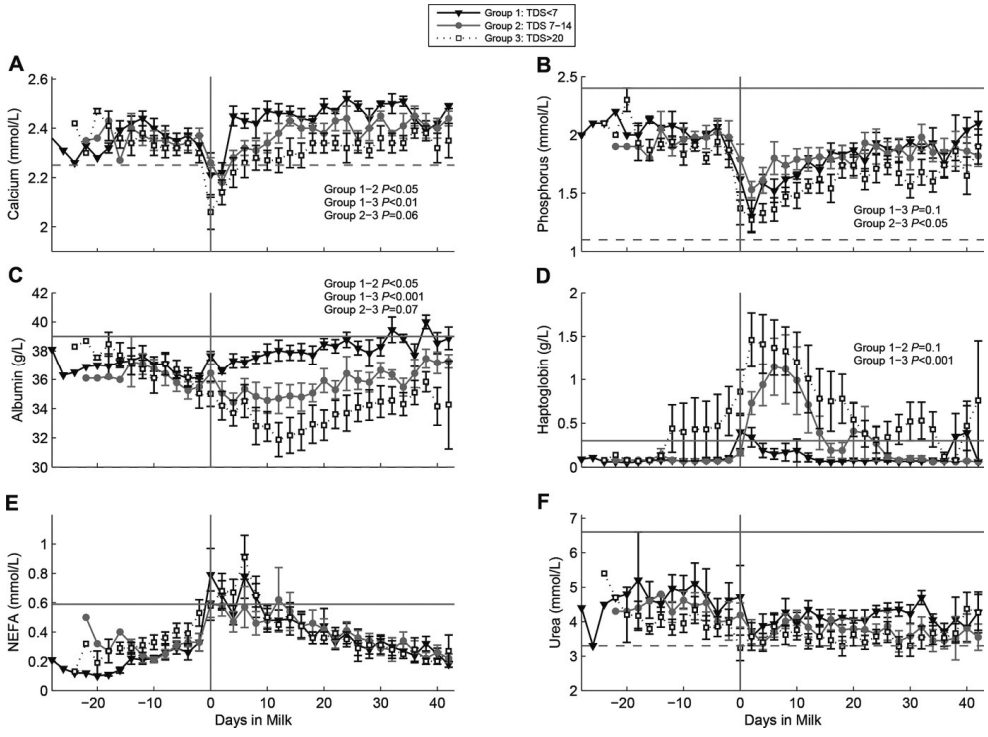

Figure 2. Patterns of serum concentrations (mean per group ± SEM) per total deficit score (TDS) group for (A) calcium (mmol/L), (B) phosphorus (mmol/L), (C) albumin (g/L), (D) haptoglobin (g/L), (E) nonesterified fatty acids (mmol/L), and (F) urea (mmol/L). Black line with downward facing triangle marker: group 1 (TDS <7); gray line with circle marker: group 2 (TDS 7–14); dotted line with square marker: group 3 (TDS >20). Gray vertical line at DIM = 0 indicates the day of parturition. Gray horizontal line indicates upper reference value; gray dotted horizontal line indicates lower reference value. Significant differences of the area under the curve between TDS groups after parturition are indicated in the graphs.

Evaluation of the Risk assessment CowCompass

In the risk assessment analysis ÇowCompass ('KoeKompas'), overstocking (113 feeding places for 165 cows) in combination with insufficient access to important resources such as feed, water and cubicles, was repeatedly identified as important risk factor at the farm where we performed the study.

Long-term predictors (recorded before calving) of post-partum TDS

We first evaluated the sensor data properties that were acquired prior to parturition (long-term) as predictors for TDS after calving. Significant relations were found between Log(1+TDS) and eating time, and between Log(1+TDS) and the variance of ear temperature. Especially nonperiodicity of eating and lying time, number of steps and motion index showed significant relations with Log(1+TDS) (Table 1).

Table 1. Unstandardized Estimates (β) of long-term explanatory variables recorded before calving for Log(1+TDS)[1]

					Unstandardized Coefficients		
Calculation	Sensor	Feature	No.	Intercept	β	SE	*P*-value
Average	SensOor[2]	Inactive (min/h)	7	-0.59	0.09	0.04	0.07
	SensOor	Eating (min/h)	7	3.24	-0.16	0.02	<0.01
	IceQube[3]	Motion index (-)	17	2.53	-0.007	0.004	0.08
Variance	SensOor	Eating (min/h)	7	2.75	-0.009	0.004	0.06
	SensOor	Ear temperature (°C)	7	-0.99	0.79	0.19	<0.05
	IceQube	Steps (no.)	17	1.74	-4×10^{-4}	2×10^{-4}	0.09
Nonperiodicity	SensOor	Eating (min/h)	7	-0.02	63.40	17.60	<0.05
	SensOor	Ear temperature (°C)	7	0.09	7.33	3.04	0.07
	IceQube	Lying (min/15min)	17	0.44	49.81	9.66	<0.001
	IceQube	Steps (no.)	17	0.60	42.20	14.10	<0.05
	IceQube	Motion Index (-)	17	0.50	45.70	15.80	<0.05

[1]TDS = total deficit score.
[2]Agis automatisering (Harmelen, the Netherlands)
[3]Icerobotics (Edinburgh, UK)

The multiple regression model resulted in three long-term predictors: average eating, variance ear temperature and nonperiodicity of number of steps. The training MSE was 0.0279 based on all complete observations (n = 7). Then the MSE was calculated by LOOCV on a multiple linear regression model with the three selected explanatory variables as predictors, this resulted in an average test prediction error (MSE) of 0.188 (TDS). If restricted to one predictor, the MSE increased to 0.0338. The test MSE when using only one predictor decreased to 0.0948.

Best subset selection of the additional analysis applying only the 12 IceQube explanatory variables (based on lying time, number of steps and number of lying bouts as input variables) resulted in four long-term predictors: average number of lying bouts, variance lying time, nonperiodicity lying time and nonperiodicity number of lying bouts with MSE = 0.0551 (MSE = 0.112

if restricted to one predictor). Estimating the MSE by LOOCV resulted in an average MSE of 0.112. The test MSE when using only one predictor increased to 0.140.

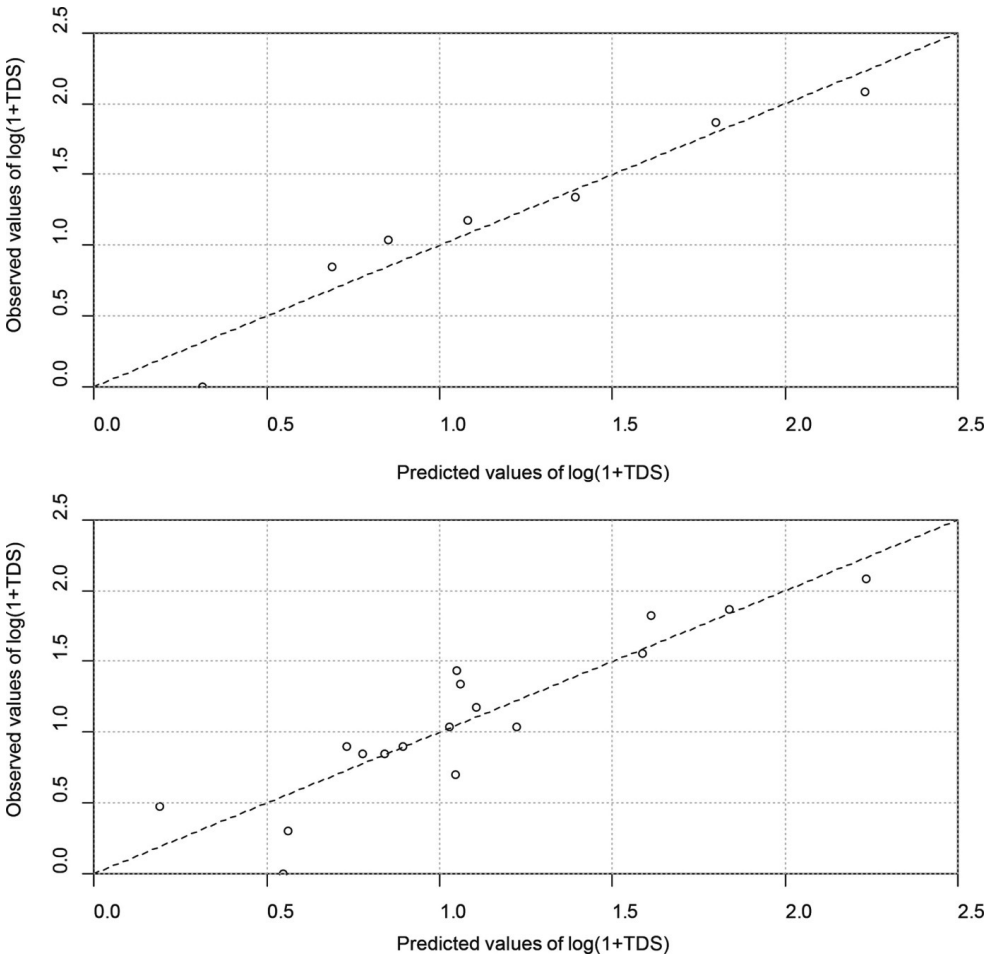

Figure 3. Scatter plots of the observed versus predicted values of log(1 + TDS) found by best subset selection on (top) the 3 most significant variables out of 5 (average eating, variance ear temperature, and nonperiodicity of number of steps) and (bottom) the 4 most significant IceQube (IceRobotics, Edinburgh, UK) variables out of 12 (average number of lying bouts, variance of lying time, nonperiodicity of lying time, and nonperiodicity of lying bouts). TDS = total deficit score.

Short-term predictors (recorded after calving) of post-partum TDS

For the short-term predictors for TDS, we evaluated the sensor data properties that were acquired during the first week after parturition. For nine explanatory variables significant relations between Log(1+TDS) and sensor quantities were found (Table 2). In comparison with the pre-partum

period (long-term predictors), the number of significant explanatory variables was higher for sensor data recorded after calving (short-term predictors).

The multivariate model resulted in a multiple regression model with four short-term predictors: average number of steps, variance number of steps, autocorrelation lying time and nonperiodicity eating. Based on all complete observations (n = 10), the training MSE was 0.0101 (MSE=0.0861 if restricted to one predictor). The MSE calculated by LOOCV on a multiple linear regression model with the four selected short-term explanatory variables as predictors was 0.0367. The test MSE when using only one predictor increased to 0.227.

Table 2. Unstandardized Estimates (β) of short-term explanatory variables recorded after calving for Log(1+TDS)[1]

				Intercept	Unstandardized Coefficients		
Calculation	Sensor	Feature	No.		B	SE	*P*-value
Average	SensOor[2]	Inactive (min/h)	10	0.18	0.05	0.02	0.09
	SensOor	Eating (min/h)	10	1.98	-0.08	0.03	<0.05
	SensOor	Ear temperature (°C)	10	15.97	-0.49	0.14	<0.05
	IceQube[3]	Steps (no.)	18	1.89	-0.01	0.002	<0.001
	IceQube	Motion index (-)	18	1.80	-0.002	$6*10^{-4}$	<0.001
Variance	SensOor	Eating (min/h)	10	1.77	-0.006	0.003	0.08
	BellaAg[4]	Temperature (°C)	18	0.59	1.67	0.93	0.09
	IceQube	Steps (no.)	18	1.32	$-4*10^{-5}$	$1*10^{-5}$	<0.05
	IceQube	Motion index (-)	18	1.28	$-2*10^{-6}$	$8*10^{-7}$	<0.05
Autocorrelation	BellaAg	Temperature (°C)	18	0.01	1.39	0.71	0.07
	IceQube	Lying (min/15min)	18	0.27	1.46	0.65	<0.05
Nonperiodicity	SensOor	Eating (min/15min)	10	-0.33	37.10	10.60	<0.05
	IceQube	Lying (min/15min)	18	0.03	18.50	8.22	<0.05
	IceQube	Steps (no.)	18	0.006	20.60	11.30	0.09

[1]TDS = total deficit score.
[2]Agis automatisering (Harmelen, the Netherlands)
[3]Icerobotics (Edinburgh, UK)
[4]Wynnstay (Powys, UK)

Discussion

Representativeness of TDS for Disease Severity

Slow recovery in calcium and albumin and an elongated peak in haptoglobin after parturition are all indicative for peri-parturient problems (Roberts et al., 2012; Seifi et al., 2011; Trevisi et al., 2012). Among the three groups of cows categorized according to TDS, cows with the highest TDS (group 3) showed the slowest recovery of calcium and albumin, and cows with the lowest TDS (group 1) showed the fasted recovery of calcium and albumin levels after calving. The patterns of the blood constituents over time strongly support the idea that our TDS was representative for the degree and duration of diminished health. The blood results also confirm that prior to parturition, the cows could be classified as healthy. Our TDS was based on clinical examinations only, additional features could be included (for example BHB measured in blood) and possible additional value should be evaluated in future studies.

Average Levels of Single Predictors

Our study identified different statistically significant explanatory variables for TDS before (long-term predictors) and after (short-term predictors) calving. Feeding, ruminating time and DMI are usually reduced prior to parturition and continue to decline after calving when compared to baselines (Schirmann et al., 2013), but this decline is especially larger in sick animals suffering ketosis, metritis, or other health problems (Huzzey et al., 2007; Schirmann et al., 2016; Urton et al., 2005). The decline in feeding, ruminating time and DMI prior to parturition is especially larger in animals suffering ketosis, metritis, or other post parturition problems as previously described by (Huzzey et al., 2007; Schirmann et al., 2016; Urton et al., 2005). Goldhawk et al., (2009) also showed that healthy animals showed higher DMI, visited the feeder more often and spent more time at the feeder during the dry period. The present relations between average signal levels of feeding time and TDS, both before and after calving, are in agreement with previous studies documenting similar relations (Huzzey et al., 2007; Schirmann et al., 2016; Urton et al., 2005). We suggest that explanatory variables recorded after calving (short-term predictors) represent indicators for disease, and that explanatory variables recorded before calving (long-term predictors) represent indicators for the risk to develop disease, as we did not find detectable clinical or hematological aberrations before calving. Our finding that a number of the same statistically significant explanatory variables for TDS were obtained both prior to parturition and after calving (i.e., averages of MI, inactive time, eating time, and variances of eating time and number of steps), could also mean that these variables recorded before calving may relate to the onset of illness before it is clinically or hematologically detectable (Weary et al., 2009).

Dynamic Signal Properties as Single Predictors

We observed considerable added value of using dynamic signal properties (variance, autocorrelation and nonperiodicity) for disease prediction, including variances in ear and rumen temperature, eating time and number of steps, and autocorrelations in rumen temperature and lying time. These dynamic properties indicate how much variation is expressed in the signal of the longitudinally measured behavioral and other variables, and how consistent or regular the behavior is.

The capacity of cows to show consistent and regular diurnal behavior and temperature patterns seemed to be of great value to smoothly undergo the transition phase. To quantitatively express this characteristic, we introduced the so-called nonperiodicity as a measure for the consistency and regularity of (daily) patterns. A consistent and regular diurnal behavior or temperature pattern is equivalent with a low non-periodicity during the dry period, and vice versa. Thus, cows with a low TDS showed regular behavioral patterns, or low nonperiodicities during the dry period, reflecting stable diurnal rhythms of these behaviors, whereas cows with serious health disorders after parturition exhibited the opposite picture. In mammals, daily rhythms in behavior and physiology are programmed by a hierarchical interconnection of biological clocks located throughout brain and body, known as the circadian system (Jennifer A. Evans & Alec J. Davidson, 2013). Mounting evidence indicates that disruption of circadian regulation or misalignment in mammals is associated with a wide variety of adverse health consequences (Jennifer A. Evans & Alec J. Davidson, 2013) or, conversely, the integrated circadian timekeeping system enhances health, wellness and

longevity (Davidson et al., 2006; Gillette, 2013). The role of circadian disruption is difficult to assess in disease disorders, as the influence of potential confounding factors is difficult to unravel. What we do know is that disturbed behavior patterns frequently coincide with accelerated disease progression and severity (Gillette, 2013). This might be one of the reasons why non-periodicity was associated with disease severity in the current study.

Relation between (Dynamic) Indicators and TDS within Farm Context

Circadian misalignment, sleep deprivation and exposure to light at night are thought to be primary sources of disruption in different species (especially human shift workers) contributing to adverse health effects (J. A. Evans & A. J. Davidson, 2013). External factors causing variation between individual circadian patterns within a herd of cows may be related to competition, overstocking, lack of synchronization possibilities due to housing facilities or ambiguity in the ranking order, due to the frequent change in herd composition (Collings et al., 2011; Cooper et al., 2007; Witaifi et al., 2018). When overstocking leads to deprivation of lying time, a rescheduling of the daily time budget may occur, to recover the lost lying time (Cooper et al., 2007). This may explain the variation in disturbed circadian patterns that we found in the current study, although recently, no relations were found between behavioral circadian patterns and stocking density (Wang et al., 2016). Group size, grouping strategy and group feeding behavior may have a large impact on the competition between animals for feed, feed intake and (resting) space (Grant & Albright, 1995). Aggressive interactions at the feed bunk or avoiding aggressive interactions have previously been found to be related to the development of metritis after calving (Huzzey et al., 2007). In addition to this, physiology is influenced by overstocking (Huzzey et al., 2012). Competition itself is also perceived differently depending on the social rank degree of the cow (Sundrum, 2015). Under high stocking densities, the behavior of certain cows, possibly the more submissive ones (Huzzey et al., 2006), may have been compromised to the extent that diurnal behavior patterns were disturbed and the intake of (high quality) feed was inadequate (Huzzey et al., 2006). Notably, during the risk assessment analysis CowCompas ('KoeKompas'), overstocking in combination with insufficient access to important resources such as feed, water and cubicles, was identified as an important risk factor at the farm where we performed the study. Thus, we suggest that cows with regular circadian patterns and low nonperiodicities were more successful in coping with the competitive situation at the farm where our experiment took place than cows with irregular circadian rhythms and high nonperiodicities. Such differences between cows, in turn, may have ultimately led to differences in TDS after calving. If we define resilience in animals as the capacity to cope with perturbations in their environment and return rapidly to their pre-challenge status (Colditz & Hine, 2016), cows with regular circadian patterns could be considered as animals showing high resilience on this farm. Future research is necessary to address the specific hypothesis that avoiding risk factors such as overstocking or insufficient access to important resources (e.g., feed or water) during the transition period in dairy cows leads to a decrease in nonperiodicity of behavioral and physiological measures of all cows before calving, and a concomitant decrease in TDS after calving.

Dynamic Indicators of Resilience

Changing dynamics, expressed by increasing autocorrelation and variance have been proposed as indicators for resilience in a variety of complex dynamic systems (Scheffer et al., 2009). Resilience in general is also defined as the capacity of a system to absorb disturbance and reorganize while undergoing change so as to still retain essentially the same function, structure and feedbacks, and therefore identity (Ge et al., 2016). When the capacity to adjust, fails, the system will collapse or shift to a different state or equilibrium, also referred to as tipping point (Scheffer et al., 2009). Once the threshold is passed, intrinsic processes in the system form a positive feedback loop, leading to a shift in state towards an alternative equilibrium (van Nes et al., 2016). Close to a tipping point, the state dynamics become increasingly slow in recovering from small perturbations, a phenomenon known in dynamical systems theory as 'critical slowing down' (Scheffer et al., 2009). As a consequence of this slowing down, its dynamics in a stochastic environment are characterized by increasing standard deviation or variance and increasing correlation between subsequent states (Scheffer et al., 2009). We defined resilience in dairy cows as the capability to stay healthy during and after the demanding challenge of transition by adapting the definition of resilience in farm animals as proposed by Colditz and Hine (2016). When the cow is not able to adequately reorganize, critical slowing down may be clinically revealed as peri-parturient disorder. The self-propelled accelerating change (positive feedback loop) causing a tipping point as described by (van Nes et al., 2016) also occurs in cows that cannot adequately handle the transition phase. The ability to self-recover is lost in this case and intervention with medications is needed to support the animal to recover. The positive feedback loop can be initiated at the moment when the gap between nutrient intake and demand becomes too large. Metabolic disorders will appear and may lead to further health problems, such as infections through dysfunctional inflammatory responses and oxidative stress (Sundrum, 2015). This will reduce DMI, reinforcing the metabolic stress, which will further increase the dysfunctional inflammatory responses and, hence, the positive feedback loop is in progress. Conversely, post-partum infections can also start the positive feedback loop. Collectively, the present findings show that dynamic properties of sensor data are of great additional value and seem to fit theoretical concepts of resilience. We suggest that dynamic indicators of physiological parameters such as increased non-periodicity, variance and autocorrelations in (ear and rumen) temperature, are signs of an upcoming tipping point between a healthy state and a clinically detectable disease state, and may represent 'dynamic indicators for individual resilience' of cows.

Multivariate Analysis versus Single Predictor Model

Combining average eating time, variance in ear temperature and nonperiodicity of number of steps exposed the lowest prediction error for TDS. A possible reason why best subset selection results in a model with multiple predictors, representing static as well as dynamic signal properties, might be because such a combination of predictors better reflects the numerous factors and interconnected risk factors that are involved in the complex pathophysiology of postpartum diseases (Sundrum, 2015). However, given the low numbers of observations in the present experiment, our findings have to be treated with care. Prediction accuracy for TDS increased when static as well as dynamic signal properties of IceQube sensors were used, but decreased when all sensors were combined, using only 7 cows in the analysis. Perhaps the multivariate approach explored here, might

lead to novel ways of studying the complexity of adaptive processes in cows during the transition period.

Conclusions

This case study presents statistical evidence that static and dynamic aspects of continuously recorded high-resolution physiological and behavioral measures prior to parturition can predict the disease severity in cows during the early lactation period. Being able to timely identify cows at risk for the development of disease after calving would be extremely helpful, because it would allow the farmer to anticipate potential health problems, and to take remedial action if required. Statistically, the most optimal linear combination of predictors to predict disease severity during the early lactation period, consisted of low average eating time, nonperiodicity in number of steps (indicative for disturbance of daily patterns) and variance in ear temperature. Our results suggest that this combination of quantitative variables derived from sensor data are promising indicators for disease severity or resilience during the transition period. Follow-up studies with more cows and on different farms are required to extend and confirm the present findings.

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CHAPTER 5

5

Behavioral patterns as indicators of resilience after parturition in dairy cows

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Abstract

During the transition phase dairy cows are susceptible to develop post-partum diseases. Cows that stay healthy or recover rapidly can be considered to be more resilient in comparison to those that develop post-partum diseases. An indication of loss of resilience will allow for early intervention with preventive and supportive measures before the onset of disease. We investigated which quantitative behavioral characteristics during the dry period could be used as indicators of reduced resilience after calving, using noninvasive Smarttag Neck and Smarttag Leg sensors in dairy cows. We followed 180 cows during two weeks before until 6 weeks after parturition at 4 farms in the Netherlands. Serving as proxy for loss of resilience, as defined by the duration and severity of disease, a clinical assessment was performed twice weekly and blood samples were taken in the first and fifth week after parturition. For each cow, clinical and serum value deviations were aggregated into a Total Deficit Score (TDS total). We also calculated TDS values relating to inflammation, locomotion or metabolic problems which were further divided into macro-mineral and liver related deviations. Smarttag Neck and Leg sensors provided continuous behavioral activity signals of which we calculated the average, variance and autocorrelation during the dry period. Diurnal patterns in the behavioral activity signals were derived by Fast Fourier Transformation (FFT) and the calculation of the non-periodicity. To select significant predictors of resilience, we first performed a univariate analysis with TDS as dependent variable and the behavioral characteristics that were measured during the dry period, as potential predictors with cow as experimental unit. We included parity-group as fixed effect and farm as random effect. Next, we performed multivariable analysis with only significant predictors, followed by a variable selection procedure to obtain a final linear mixed model with an optimal subset of predictors with parity-group as fixed effect and farm as random effect. The Total TDS was best predicted by average inactive time, non-periodicity ruminating, non-periodicity of bouts standing up and FFT stand still. Average inactive time was negatively correlated with average eating time and these two predictors could be exchanged with only little difference in model performance. Our best performing model predicted Total TDS at a cut-off level of 60 points, with a sensitivity of 79.5% and a specificity of 73.2% with a positive predicted value of 0.69 and a negative predicted value of 0.83. The models to predict the other TDS categories showed a lower predictive performance as compared to the TDS Total model, which could be related to the limited sample size and therefore low occurrence of problems within a specific TDS category. Furthermore, more resilient dairy cows are characterized by high averages of eating time with high regularity in rumination and low averages of inactive time. They reveal high regularity in standing time and transitions from lying to standing, in the dry period. These behaviors can be used as indicators of resilience and allow for preventive intervention during the dry period in vulnerable dairy cattle. However, further examination is still required to find clues for adequate intervention strategies.

(**Key words**: transition period, resilience, post-partum disease, sensor data)

Introduction

Around the time of calving 30 to 50% of dairy cows are affected by some form of post-partum disorder (LeBlanc, 2010; Wisnieski et al., 2019). During this transition phase, dairy cows face metabolic and physiological changes in preparation for calving and milk production. Failure to adequately adapt and cope can lead to metabolic stress which increases the risk for post-partum disorders (Belaid et al., 2021; LeBlanc et al., 2006). As a consequence, post-partum disorders may occur, including ketosis, a fatty liver, digestive issues, macro-mineral imbalance and inflammatory complications, or more frequently, a combination of these (Sordillo & Mavangira, 2014; Sundrum, 2015; Wankhade et al., 2017).

Resilience is defined for animals as the capacity to remain healthy, or respond minimally and recover rapidly in response to challenges (Friggens, 2022; Ge et al., 2016; Scheffer et al., 2018; Wright et al., 2019). These challenges might be intrinsically driven, such as pregnancy, parturition, or milk production, or may be generated externally by factors such as pathogens, stress due to e.g., regrouping, overcrowding, inadequate housing or farm management. During the transition phase of dairy cows, both internal and external challenges can occur simultaneously. Cows that do not develop post-partum disorders or recover rapidly can be considered as highly resilient animals. The variation in clinical manifestation of impaired health in terms of severity, duration and recovery of post-partum disease can therefore be used as a measure or deviation of resilience during the periparturient period in the life of a dairy cow (Van Dixhoorn et al., 2018). An indication of a cow's lack of resilience before the lactation period even starts, may allow for early intervention with preventive or supportive measures.

At present, it remains debatable which indicators, or combinations thereof, reflect the capacity to adequately cope with the transition period. Metabolic stress biomarkers and other indicators associated with oxidative stress or inflammation have been described as predictors of post-partum disease, but may require invasive blood sampling (Huzzey et al., 2011; LeBlanc, 2010; Ospina et al., 2010; Wisnieski et al., 2019). In addition, the time of measurement might influence the outcome, and a multitude of sampling timepoints are required to assess dynamic patterns. When using non-invasive accelerometers in dairy cows, specific behavioral activity signals can be obtained recording their lying, walking, standing, eating or even ruminating motions. Moreover, activity patterns that span multiple days, as well as deviations from these patterns, can be quantified. Variance and autocorrelation **(AC)** can be calculated from the longitudinal data and the data can also be converted into individual spectral components which provide frequency information about the respective signals. Variance, autocorrelation, the skewness of deviations, the slope of a reaction norm and circadian rhythm patterns derived from behavioral measurements, have previously been proposed as potential Dynamic Indicators Of Resilience **(DIOR)** in individual animals (Tom V. L. Berghof et al., 2019; Poppe, Mulder, et al., 2022; Scheffer et al., 2018; van der Zande et al., 2020; Van Dixhoorn et al., 2018).

In this paper we aimed to investigate if behavioral characteristics during the dry period could be used as indicators of resilience using noninvasive Smarttag Neck and Smarttag Leg sensors in dairy cows. We tested the hypothesis that behavioral activity signals measured in the dry period can be used as predictors for disease severity after calving. We designed a statistical model and evaluated

various potential sensor-based behavioral variables that were measured during the dry period as predictors for decreased resilience in terms of disease severity and duration after parturition. Subsequently, we tested the reliability of the detection method in terms of true and false positive rates.

Materials and Methods

The established principles of laboratory animal use and Dutch laws related to animal experiments were adhered to in this study. The Wageningen University Animal Care and Use Committee (Lelystad Department) approved the experiment under protocol number AVD401002016749 with samples size of 170 cows based on a prediction of a total deficit score (TDS) value with sensor data.

Animals, Housing and Diet

The present study was conducted between July 2017 and September 2018 at four commercial dairy farms located in the Netherlands. A total of 180 Holstein-Friesian dairy cows were monitored from 2 weeks prior to expected parturition until 6 weeks after parturition. Cows enrolled in the study once based on the expected day of parturition. Cows were used in the analysis when they showed no clinical signs of illness prior to parturition and when a complete dataset until 6 weeks after calving was available, resulting in 173 cows (37 primiparous, 43 parity 2, 38 parity 3 and 55 parity 4 and higher). At all four farms dry and lactating cows were housed in cubicles in the same building in which also a straw bedded maternity pen was present. Cows were moved to the maternity pen with the first signs of parturition, and they stayed there until 1-3 days after calving. Thereafter they were introduced into pens with lactating cows. Thus, group size and composition changed due to (re)introduction, but cows remained in the same group after calving until the next dry period. Post-partum cows were milked twice daily, and water was provided ad libitum on all farms.

Farm 1 had 125 cows with an average production of 9050 kg milk per year (with 4.35% fat and 3.71% protein). Cows were milked twice daily at 7:00 AM and 6:15 PM and fed Total Mixed Ration **(TMR)** once per day at 9:30 AM and this was pushed to the feeding fence at 8:30 PM and 10:15 PM. Feed residues were removed from the fed bunk before each new TMR delivery. Lights were on at 6:30 AM and off at 11:00 PM.

Farm 2 had 100 cows with an average production of 9430 kg milk per year (with 4.35% fat and 3.55% protein). Cows were milked twice daily at 6:00 AM and 5:45 PM and fed TMR once per day at 8:30 AM and this was pushed to the feeding fence at 5:30 PM. Feed residues were removed from the fed bunk before each new TMR delivery. Lights were on at 5:15 AM and off at 10:15 PM.

Farm 3 had 75 cows with an average production of 8900 kg milk per year (with 4.53% fat and 3.52% protein). Cows were milked twice daily at 5:45 AM and 5.00 PM and fed TMR once per day at 1:30 PM and this was pushed to the feeding fence at 5:40 AM, 4:55 PM, 8:00 PM and 10:30 PM. Feed residues were removed from the fed bunk before each new TMR delivery. Lights were on at 5:45 AM and nightlights being switched on at 10:30 PM.

Farm 4 had 150 cows with an average production of 8900 kg milk per year (with 4.34% fat and 3.59% protein). Cows were milked twice daily at 5:30 AM and 4:30 PM and fed TMR once per day at 4:30 PM and this was pushed to the feeding fence at 8:30 AM and 10:30 PM. Feed residues were

removed from the fed bunk before each new TMR delivery. Nightlights were switched on based on a dusk sensor.

Clinical Examination and Blood Sampling

Cows were scored clinically by four veterinarians twice weekly until 6 weeks after parturition. The veterinarians scored 19 different clinical signs (Table 1) of the cows, and they estimated overall condition according to measurements and cut-off values as earlier described by Hajer et al. (2011) in which clinical examination according to a fixed format is described as well as normal and deviating clinical values per organ system. The following aspects were scored: heart rate (beats per minute), breathing rate (breaths per minute), rectal temperature (°C), rumination (chews per minute), Body Condition Score **(BCS**) according to Edmonson et al. (1989), and locomotion score and lameness according to Hulsen (2012). Udder condition was scored per quarter in terms of skin temperature (too warm, too cold or normal), color (red, abnormal, normal), painful during palpation (yes or no), swollen (yes or no) and teat condition was scored in terms of flexibility (yes or no), color (red, normal, abnormal) and painful during palpation (yes or no). Retained placenta was scored if it was protruding from the vulva after more than 24 hours after calving. Uterus condition and excretion were scored by rectal palpation, the size was estimated and assigned as normal or abnormal according to the expected involution. The color and smell of vaginal discharge and the amount of mucus or pus was estimated. The consistency and digestion of the manure was scored according to Hulsen (2012). Other specific clinical diagnoses consisted of hypocalcemia, or a displaced abomasum, confirmed by auscultation. Inter-observer variation between the trained veterinarians was verified every 4 months. The veterinarians were blinded to scores of other veterinarians and the individual cow treatments. Blood samples were collected from the coccygeal vein into 10 mL sterile serum tubes (Vacutainer, Becton Dickinson, Franklin Lakes NJ) in the first (1.8 ± 1.2 d) and fifth week (29.8 ± 1.6 d) after calving. Samples were submitted to the routine veterinary laboratory of Royal GD (Deventer, the Netherlands). This laboratory performed all analyses and works according to a quality management system meeting NEN-EN-ISO 9001:2015 requirements and Clinical-chemical parameters were assessed using UniCel® DxC 600 Synchron® Clinical System (Beckman Coulter). Test procedures for all parameters (except for calcium, magnesium, IL6 and haptoglobin) were NEN-EN-ISO/IEC 17025:2017 accredited by the Dutch Accreditation Council (2017). Colorimetric methods were used to analyze serum calcium, phosphorus (ammonium-molybdate method), magnesium, total bilirubin, (dimethylsulphoxide method), haptoglobin, total protein **(TP)** (Biuret method) and albumin concentrations (Bromocresol Green method). The globulins were calculated by subtraction: TP minus albumins (g/L). Enzymatic methods were used to analyze serum urea (urease method), non-esterified fatty acids **(NEFA)** and β-hydroxybutyric acid **(BHBA)** concentrations. Aspartate aminotransferase **(AST)** and gamma-glutamyl transferase **(GGT)** concentrations were analyzed using enzymatic methods according to the International Federation of Clinical Chemistry (IFCC) reference procedures for the measurement of catalytic activity concentrations of enzymes at 37 °C. Interleukin-6 concentrations in serum were analyzed using an AlphaLISA Bovine IL-6 Detection Kit (PerkinElmer, Inc., Waltham, MA, USA) following the kit's instructions. The inter-assay coefficient of variation (CV%) was below 10% for all methods.

Post-partum TDS

As measure for disease severity was calculated as previously described by Van Dixhoorn et al. (2018) and was referred to as Total Deficit Score **(TDS).** Briefly, all aberrant clinical findings were used to calculate TDS. Table 1 lists which clinical values were assigned to metabolic stress, inflammation and locomotion. This resulted in 4 different TDS scores: TDS Total, TDS Inflammation, TDS Locomotion and TDS Metabolic. In addition, serum values contributed to TDS when they were below or above specific thresholds (Table 2). Based on specific serum values, TDS Metabolic was sub-divided into TDS scores related to liver function (TDS Liver) and macro-mineral shortage (TDS Macro-minerals). The points assigned to the different TDS categories are shown in Table 1 and 2. Suboptimal clinical findings were counted as one point of the TDS (dimensionless) per sampling moment during the 6 weeks period after calving. When the veterinarian diagnosed a specific disease (retained placenta (**RP**), metritis, mastitis, lameness, displaced abomasum (**DA**), respiratory infection, milk fever, diarrhea), the specific diagnosis was reported, and two points were assigned to the respective TDS. In addition, related treatments received two points. Each corresponding deviating serum value at the two sampling moments (week 1 and 5 after parturition) received 6 TDS points. As a consequence, healthy cows showed low TDS values in contrast to cows with high TDS values, suffering more health-related issues during the 6-week study period.

Cut-off values for serum value parameters were based on the upper and or lower limit of the reference intervals for the corresponding parameters as provided by veterinary laboratory of Royal GD (Deventer, the Netherlands), except for BHBA, NEFA, and calcium. The cut-off value for BHBA was chosen based on the threshold for subclinical ketosis (Duffield et al., 2009), whereas the for NEFA was chosen based on the threshold for an increased risk of early lactating culling, and clinical diseases (Ospina et al., 2013; Ospina et al., 2010; Roberts et al., 2012). The cut-off value for calcium was chosen based on the thresholds for clinical hypocalcemia as described by Kimura et al. (2006) and Martinez et al. (2012).

Table 1. Overview of the clinical observations that were assessed twice per week by trained veterinarians on 4 selected farms (in total, 180 cows enrolled in the study from 2 wk before expected parturition day until 6 wk after parturition)[1]

Clinical observation	Points	TDS
Ears cold	1 if yes	Inflammation, Metabolic, Total
Secretion from nose visible	1 if yes	Inflammation, Total
Jugular pulse visible above mid neck region	1 if yes	Inflammation, Total
Rectal Temperature	1 if T >39.2 2 if T >40	Inflammation, Total
Breathing abnormal (> 30 / min)	1 if yes	Inflammation, Total
Body Condition Score (BCS)[2]	1 if difference between BCS in dry period >1	Metabolic, Total
Rumen visible when standing behind cow	1 if no	Metabolic, Total
Rumen fill weak	1 if yes	Metabolic, Total
Rumen score[2]	0 if score 3-5; 2 if score =0; 1 if score = 1	Metabolic, Total
Udder edema palpable	1 if yes	Total
Udder score per quarter		
Firm LF, RF, LB, RB[3]	0.5 if yes per quarter	Inflammation, Total
Red LF, RF, LB, RB[3]	0.5 if yes per quarter	Inflammation, Total
Abnormal uterus fill, excreta	1 if yes	Inflammation, Total
Manure score[2]	2 if score =1, 1 if score is 2 and 5, 0 if score is 3 to4	Metabolic, Total
Abnormal digestion visible in manure	1 if yes	Metabolic, Total
Locomotion Score[2]	0 if score 1 or 2; 1 if score = 3-5	Locomotion, Total
Lame LB, LF, RB, RF[3]	1 if yes per leg	Locomotion, Total
Cow is diagnosed with a disease	2 if yes	assigned to specific TDS depending on disease
Treatment	2 if yes	assigned to specific TDS depending on disease

[1]The values assigned to the total deficit score (TDS) categories per deviating clinical observation, are presented. The TDS categories consisted of total, inflammation, locomotion, and metabolic. Each suboptimal clinical value (according to the references values) was scored with 0.5, 1, or 2 points.

[2]References used for interpretation of observations and score systems for BCS, manure score, locomotion, and rumen score according to Royal GD, Deventer, the Netherlands (Gezondheidsdienst, Hajer et al., 2011).

[3]LF: left front, RF: right front, LB: left back, RB: right back. Diseases that could be diagnosed were mastitis, metritis, displaced abomasum, milk fever, lameness, respiratory disease, diarrhea.

Table 2. Overview of the serum parameters that were taken from 180 cows on 4 farms in wk 1 and 5 after calving; the cutoff values per serum parameter are specified for wk 1 and wk 5 separately, and the total deficit score (TDS) categories to which the points were assigned are given[1]

Parameter[2]	Unit	TDS	Week 1	Week 5
Total protein	g/L	Inf, Total	>85	>85
Total protein	g/L	Met, Total	<55	<55
Albumin	g/L	Met, Total	<31	<31
Urea	mmol/L	Met, Total	<3.3	<3.3
Urea	mmol/L	Met, Total	>6.6	>6.6
NEFA[3]	mmol/L	Met, Total	>0.8	>0.4
BHBA[4]	mmol/L	Met, Total	>1.2	>1.2
Calcium	mmol/L	Macro, Met, Total	<2.00 (day 0–1) <2.20 (day 2–7)	<2.20
Magnesium	mmol/L	Macro, Met, Total	<0.78	<0.78
Phosphorus	mmol/L	Macro, Met, Total	<0.9	<1.1
AST[5]	IU/L	Liver, Met, Total	>115	>115
GGT[6]	IU/L	Liver, Met, Total	>34	>34
Total Bilirubin	µmol/L	Liver, Met, Total	>7	>7
Haptoglobin	g/L	Inf, Total	>0.6	>0.3
IL-6	ng/mL	Inf, Total	>10	-
Globulins (TP[7]-albumin)	g/L	Inf, Total	>49 and TP<85 and albumin >31	>49 and TP<85 and albumin >31

[1]The TDS categories consisted of TDS total, TDS inflammation (Inf), and TDS metabolic (Met), with TDS metabolic subdivided into TDS scores related to liver function (Liver) and macro-mineral shortage (Macro). Per sampling point (wk 1 and wk 5), a level exceeding the values as indicated in the last 2 columns counted as 6 points in the TDS.

[2]Cut-off values of serum metabolites parameters were based on the upper and or lower limit of the reference intervals for the corresponding parameters as provided by veterinary laboratory of Royal GD (Deventer, the Netherlands; Gezondheidsdienst voor Dieren, 2023), except for BHBA, NEFA, and calcium. The cutoff value for BHBA was chosen based on the threshold for subclinical ketosis (Duffield et al., 2009), whereas the cutoff value for NEFA was chosen based on the threshold for an increased risk of early-lactation culling and clinical diseases (Ospina et al., 2010, 2013; Roberts et al., 2012). The threshold for calcium was based on Kimura et al. (2006) and Martinez et al. (2012). The average DIM of blood sample collection were 1.8 ± 1.2 d and 29.8 ± 1.6 d for the sampling time points at the first and fifth weeks, respectively.

[3]NEFA = nonesterified fatty acids.

[4]BHBA = β-hydroxybutyric acid.

[5]AST = aspartate aminotransferase.

[6]GGT = gamma-glutamyl transferase.

[7]TP = total protein.

Predictive behavioral variables

Behavioral activity data were obtained during the two weeks prior to calving using the Smarttag Neck and Leg sensors manufactured by Nedap N.V. (Groenlo, The Netherlands). The sensors were previously validated for accuracy by Borchers et al. (2021). The neck sensor provided four activity features per cow: eating, ruminating, inactive or active (time spent (min/h), Table 3). We aggregated the basic data into hourly data by aggregating the minutes per clock hour. In addition, the neck sensor provided an overall activity level per 15 minutes which was registered as a dimensionless measure provided by the manufacturer. Overall activity level was aggregated in an activity level (dimensionless) per hour. The leg sensor recorded three behaviors per cow per 15 minutes (Table 3): lying, standing still and walking. These behavioral durations were expressed in full minutes. During each 15-minute time segment, the leg sensor provided a count of steps and a count of transitions from lying to standing or walking. We computed this basic data into hourly data by aggregating the four hourly time slots with starting times at each clock hour. The leg sensor revealed the partition or count per cow per hour as follows: time spent lying, standing still, walking, not lying (standing still + walking) (min/h), counts of steps, and counts of transitions from lying to standing (bouts standing up).

Next, we calculated average, variance, autocorrelation (with lag τ = 1 hour), non-periodicity and fast Fourier transformation (FFT) for each variable using the hourly data per cow for the dry period, starting 15 days prior to calving up to and including the day before calving. The variance describes the distribution of the data around the average and the autocorrelation describes the correlation (at lag 1) between successive values of hourly data of the sensor variable, thus the similarity between successive values. Non-periodicity was defined as the mean squared difference of a correlogram with a sinusoid with a 24-h cycle and an amplitude of 0.25, where the correlogram is a plot of the autocorrelation for a range of time lags as visualized by Van Dixhoorn et al. (2018). The lags were based on hourly intervals with the exception of active time and the count of bouts standing up where a 6-h period was used instead as hourly data was often zero. Thus, the non-periodicity was used as a measure for the regularity in daily pattern of the sensor data. The Fourier analysis was used as the conversion from the time domain to a representation in the frequency domain. This was done to identify prominent frequencies that may be present in the sensor data, because cows have a daily pattern in their eating and lying behavior. Patterns in the sensor data will then become visible as frequencies with high peaks. This conversion from the time to the frequency domain was done using the FFT algorithm (Chatfield & Xing, 2019). For our application, Fast Fourier Transform was defined as the sum of the peak heights at 1, 2, 3 and 4 in the amplitude spectrum of the variable determined with a Fast Fourier Transformation. This was interpreted as a measure of the regularity of behavior occurring once, twice, three or four times per day. Hence, the outcome of FFT indicated the extent to which cows display circadian (i.e., once every 24 h) to ultradian rhythms (several cycles of behavior within a day).

Table 3: Predictive behavioral explanations of the measurements recorded by the Smart Tag neck and Smart Tag leg sensors (Nedap (N.V.)

Sensor	Measurement	Explanation
Neck	Eating time	Time spent eating, min/h
	Ruminating time	Time spent ruminating, min/h
	Active time	Time spent active, min/h
	Inactive time	Time spent inactive, min/h
	Activity level	Overall activity level per 15 minutes
Leg	Count of steps	Count of steps per 15 minutes
	Count of bouts standing up	Count of transitions from lying to standing per 15 minutes
	Lying time	Time spent lying, min/h
	Walking time	Time spent walking, min/h
	Standing still time	Time spent standing still, min/h

Statistical Analysis

Descriptive statistics (range, average, median and standard error of the mean (**SEM**)) were calculated for the different TDS categories per farm and per parity group. Differences in the average of TDS level per TDS category between farms and parity groups were tested using a general linear model. Correlation coefficients **(r)** were calculated between the different TDS categories as well as between all behavioral variables. For statistical analysis R was used (RCoreTeam, 2020), with the packages: lmerTest, emmeans, MuMin, ROCR and car (Kuznetsova et al., 2017, Russell V. Lenth, 2021, Kamil Barton, 2020, Sing et al., 2005, Fox and Sanford Weisberg, 2019). Due to the large number of behavioral predictors, we narrowed down the number of these variables first by a univariate analysis. Cow was the experimental unit and the different TDS categories (TDS Total, TDS Inflammation, TDS Locomotion, TDS Metabolic, TDS Liver, TDS Macro-minerals) were analyzed as dependent variables with parity-group as fixed effect and farm as random effect. Three parity groups were chosen: group 1 with first parity only, group 2 with parity 2 and 3 and group 3 with parities 4 and higher. The TDS was transformed to ln (TDS + 0.5) for the analysis in order to comply with the model assumptions. If $P \leq 0.2$ in the univariate analysis, the predictive behavioral variables were selected for the multivariable approach. For the multivariable approach we used the selected predictors as described above and applied backward selection to obtain optimal subset of predictors for a linear mixed model with parity group as fixed effect and farm as random effect. All models were built according to the following formula in which the linear mixed model assumptions were met:

$$Ln\,(TDS\ +\ 0.5) \sim Parity\ group\ +\ (activity\ descriptors)\ +\ random\,(Farm)$$

First, we investigated models with only Smarttag Leg or only Smarttag Neck variables. Subsequently, combinations of Smarttag Neck and Leg variables were included in the selection of variables. We retrained three candidate models per TDS based on backward selection. Additionally, an all-possible subset selection procedure was performed to test if additional candidate models were proposed among the best performing models based on Akaike Information Criterion **(AIC)**. Of all candidate models resulting from the backward selection and all possible subset selection procedures, the marginal R^2 (variance explained by the fixed effects) and the conditional R^2 (including random farm effect, variance explained by the entire model) as described by Barton (2020) were calculated. A small difference between marginal and conditional R^2 indicates that the extra degree of variation in the TDS values by type of farm is small. Best performing models with the highest marginal and conditional R^2 as well as smallest difference between marginal and conditional R^2 were then selected and the predictive behavioral variables of these best performing models per TDS were calculated. In this paper we aimed to elucidate animal related indicators of resilience that were farm independent and models were not further examined when differences between marginal and conditional R^2 were large.

Next, the best performing models were further evaluated to select the final model. The variables in the models were tested for significance and co-linearity was tested by calculating the Variance Inflation Factor **(VIF)** as earlier described by Fox and Monette (1992). When the VIF was higher than 5, marginal and conditional R^2 were investigated for models where one of the correlating variables was left out. The final models per TDS included significant variables only, met all linear mixed model assumptions and were best in comparison with the other candidate models and in terms of having little difference between marginal and conditional R^2. Subsequently we investigated if interaction of the variables with parity group was significant ($P < 0.05$). Models with and without the significant interactions were compared and tested for robustness and predictive performance. Robustness was tested with a 10-fold cross validation. The Root Mean Squared Error **(RMSE)** was calculated, and the estimated coefficients were monitored. We split the data randomly into 10 equally sized parts and used 90% of the data to train the model and continued to test it on the remaining 10% of the data.

We calculated the sum of squares to get insight in the contribution of each variable to the explained variation in the final model and created Receiver Operating Characteristic **(ROC)** curves of the final models to estimate sensitivity and specificity for the optimal cut off values per TDS. These optimal cut-off values were chosen based on highest Area Under Curve **(AUC)** and positive and negative predictive values were assessed from the ROC curves per model.

Results

TDS distribution and descriptive statistics

Our final data set included 173 cows, of which 136 were multiparous and 37 were primiparous. The average days in milk (**DIM**) of blood sample collection were 1.8 ± 1.2 d and 29.8 ± 1.6 d for the first- and fifth-week sampling timepoints, respectively. Summary statistics of the TDS values of all cows studied are shown in Table 4. The TDS values per farm are visualized with boxplots in Figure

1. The Total TDS, TDS inflammation and TDS Locomotion were affected by farm ($P < 0.05$). The descriptive statistics and pairwise differences are added in Table S 1.1 in the supplementary materials. Farm three had remarkably high TDS Locomotion compared to the other three farms. TDS Metabolic, TDS Liver and TDS Macro minerals did not differ between farms.

The TDS values per parity group are visualized with boxplots in Figure 2. For all TDS categories, except for TDS Macro-minerals, an effect of parity group was seen. Post hoc comparison showed that TDS Total and TDS Locomotion values were higher for cows in the parity 4 and higher group as compared to the younger parity groups ($P < 0.05$). The parity 4 and higher group had higher TDS Metabolic and TDS Liver values than parity 1 cows, with the group cows of parity 2-3 in between ($P < 0.05$). For TDS Inflammation, values were higher for the parity 4 and higher group as compared with the parity 2-3 group, with the first parity group in between ($P < 0.05$, Figure 2). The descriptive statistics and pairwise differences are included in Table S 1.2 in the supplementary materials.

Table 4: Descriptive statistics per Total Disease Score (TDS) category of 173 cows at 4 farms; TDS was calculated by summing aberrant clinical findings and deviating serum values that were assessed during 6 wk after calving[1].

TDS	Range	Median	Average	SEM
TDS Total	12- 172	55	60	32
TDS Inflammation	03 - 61	21	23	13
TDS Locomotion	00 - 74	8	12	13
TDS Metabolic	03 - 114	20	24	15
TDS Liver	00 - 84	12	14	12
TDS Macro-minerals	00 - 84	15	17	12

[1]The range, median, average and standard error of the mean (SEM) are given. The TDS Total includes TDS Inflammation, TDS Locomotion and TDS Metabolic. TDS Metabolic includes TDS Macro-minerals and TDS Liver.

Individual TDS Total calculations are shown in Figure 3. A gradual linear increased TDS value was observed until TDS Total reached a level of 75 points (140 cows with TDS < 75). Above 75 (36 cows), TDS Total values increased exponentially from 75 with a maximum TDS of 179 (Figure 1). The TDS inflammation values ranged from 3 to 61 with an average of ± 13 (SD).

Significant correlation coefficients ($P < 0.05$) were found between all different TDS categories and TDS Total (TDS Total with TDS Inflammation: 0.82, with TDS Locomotion: 0.68, with TDS Metabolic: 0.74, with TDS Liver: 0.66 and with TDS Macro-minerals: 0.66). Significant correlations were seen between TDS Liver, TDS Macro-minerals and TDS Metabolic (all three with an $r > 0.80$). Between TDS Locomotion and the other TDS categories r was low (< 0.20) and not significant except for the correlation with TDS Inflammation (r of 0.47). A significant correlation was found between TDS Inflammation and TDS Metabolic (r of 0.40).

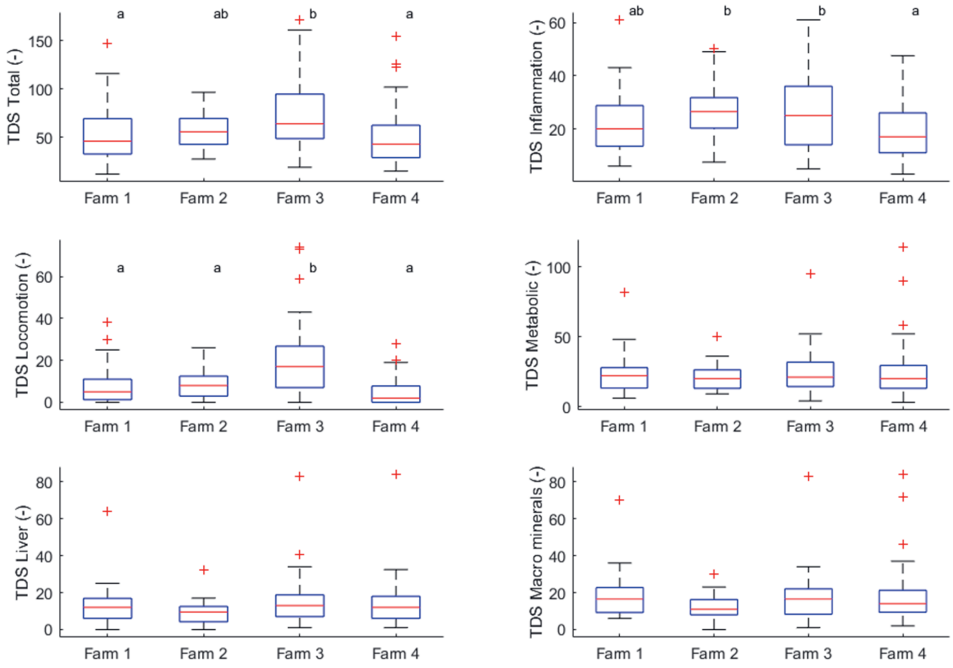

Figure 1. Boxplots of the observed TDS values per farm. From top left to bottom right, the TDS Total, TDS Inflammation, TDS Locomotion, TDS Metabolic, TDS Liver and TDS Macro-minerals are shown. The number of cows per farm were 19 for farm 1, 20 for farm 2, 75 for farm 3 and 59 for farm 4. The bottom and top of each box are the 25th and 75th percentiles, respectively. The distance between the bottom and top of each box is the interquartile range. The red line in the middle of each box is the median. The outliers are marked as red + sign and are the values that are more than 1.5 times the interquartile range away from the bottom or top of the box. The whiskers go from the end of the interquartile range to the furthest observation (minimum and maximum value). An overall farm effect was seen for TDS Total, TDS Inflammation and TDS locomotion, but not for TDS Metabolic, TDS Liver and TDS Macro-minerals. Letters a and b indicate significant difference ($P < 0.05$), with a being the lower value as compared to b.

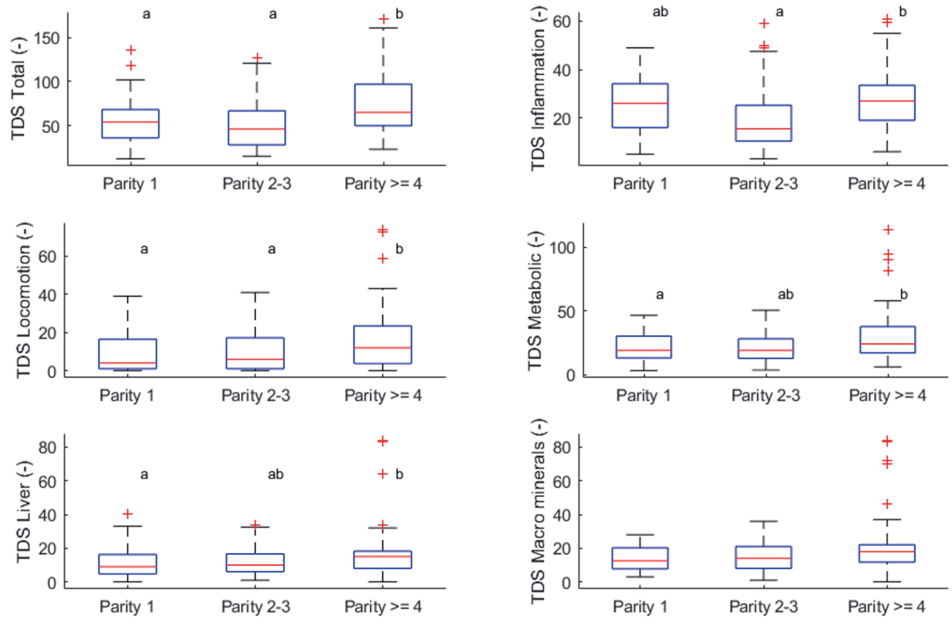

Figure 2. Boxplots of the observed TDS values per parity group. From top left to bottom right, the TDS Total, TDS Inflammation, TDS Locomotion, TDS Metabolic, TDS Liver and TDS Macro-minerals are shown. The number of cows per parity group: 37 with parity 1, 81 with parity 2 or 3 and 55 with parity 4 or higher. The bottom and top of each box are the 25th and 75th percentiles, respectively. The distance between the bottom and top of each box is the interquartile range. The red line in the middle of each box is the median. The outliers are marked as red + sign and are the values that are more than 1.5 times the interquartile range away from the bottom or top of the box. The whiskers go from the end of the interquartile range to the furthest observation (minimum and maximum value). An overall parity-group effect was seen for TDS Total, TDS Inflammation and TDS locomotion, TDS Metabolic, TDS Liver, but not for TDS Macro-minerals. Letters a and b indicate significant difference (P<0.05), with a being the lower value as compared to b.

Correlations between behavioral predictive variables

Significant correlations coefficients (P < 0.05) above 0.6 were not found for the non-periodicities of inactive time and count of standing up and FFT calculations of inactive, standing still and eating time. All calculations of walking time and count of steps were highly correlated. The AC of steps and walking were positively correlated with FFT count of steps and walking and in addition, non-periodicities for walking time and count of steps were negatively correlated with FFT of these variables. Non-periodicity and FFT calculation of rumination were also correlated. A significant correlation was found between average time ruminating and eating with variance of eating time and a negative correlation was found between these three variables and average of inactive time.

Average of eating time was also positively correlated with average of count of steps and walking time and in addition, AC eating time was also correlated with AC walking time and count of steps which was also the case for the non-periodicities of walking time and count of steps and eating. Non-periodicity of eating and walking were also correlated. The non-periodicities of eating and walking

for two cows are visualized in Figure 4. One cow with high non-periodicities and one cow with low non periodicities for eating and walking are presented. Variance of lying time was correlated with variance of standing still time, and autocorrelation of lying time with autocorrelation standing still time.

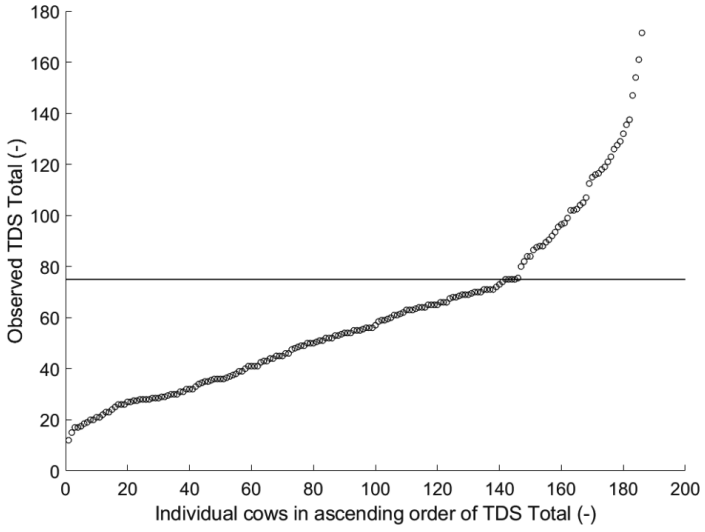

Figure 3. Observed Total Deficit Score (TDS) values per cow in which all clinically detected deficits and deviating serum values are combined into one TDS Total value which is dimensionless indicated as (-). On the x axis, the cows are plotted in ascending TDS Total order, on the y-axis the TDS Total value of each cow is given. The horizontal line indicates the TDS value of 75 at which TDS value changes from a gradual increase to an exponential increase of TDS value.

Selected behavioral variables

The selection of single predictive behavioral variables per sensor recorded before calving are shown in Tables 5a-b. Predictors with $P \leq 0.2$ were included in the multivariable steps. The direction of the effect is indicated per variable as positive (higher value of the variable relates to a higher TDS) or negative (higher value of the variable relates to a lower TDS). For TDS Total, 10 variables were selected, for TDS Inflammation 7, TDS Metabolic 13, TDS Locomotion 11, TDS Liver 24 and for TDS Macro-minerals 12. For TDS Inflammation, only variables measured with the Smarttag Neck sensor were selected. Averages for eating, active and inactive time, activity scores, and count of steps, lying, walking and standing still time were all included in the multivariable approach. Averages of scores for ruminating and bouts standing up were excluded. Autocorrelations of all measurements except for active time were included. Variances of behaviors were all included except for inactive time and bouts standing up. Most FFT and non-periodicity calculations were included except non-periodicity of eating, lying and standing still. Autocorrelation calculations were always positively related to TDS scores except for AC ruminating. Non-periodicity of bouts standing up had a positive effect on TDS Liver but negative on TDS Total and TDS Locomotion.

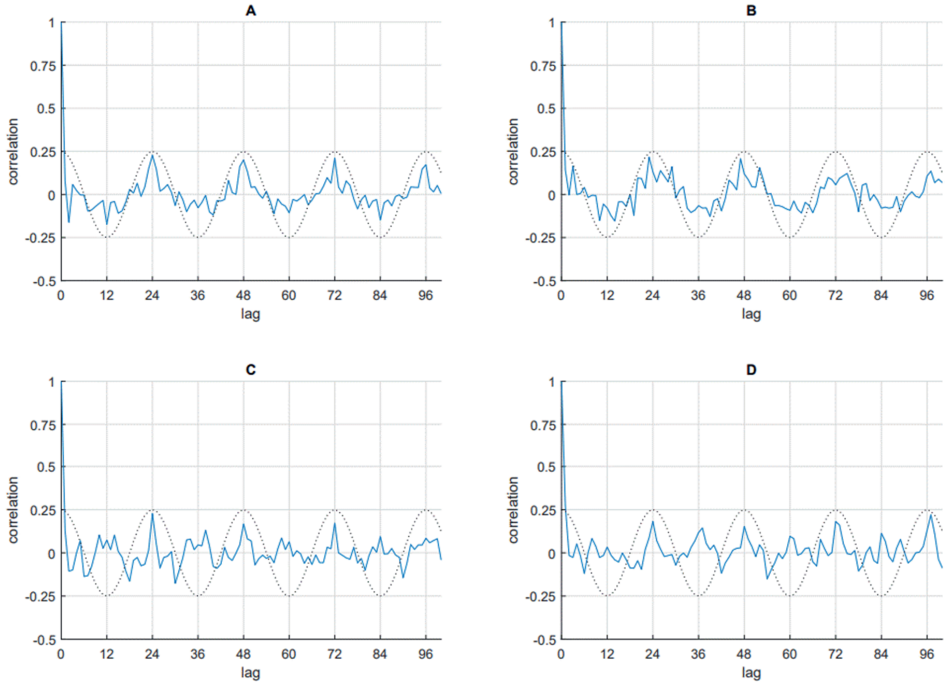

Figure 4. Visualization of the nonperiodicity of eating time and walking time as metric of the regularity in the daily pattern of two different cows. Correlograms are made of hourly data assessed during the dry period (from 14 days before parturition until parturition). Eating time and walking time correlograms of cow nr 506 (second parity) are depicted in panels A and B and of cow nr 23 (first parity) in panels C and D. The correlogram of eating time assessed with Smarttag Neck sensor is depicted on the left (panels A and C), the correlogram of walking time assessed with the Smarttag Leg sensor is depicted on the right (panels B and D). Non periodicity is the calculation of the mean squared error (MSE) of the correlogram (blue line) with a sinusoid with a 24-h cycle and an amplitude of 0.25 (dotted line). Cow nr 506 showed more regularity (low non-periodicity) with an MSE for eating time of 0.0173 (panel A) and walking time of 0.0158 (panel B) as compared to cow nr 23 with an MSE for eating time of 0.0361 (panel C) and walking time of 0.0412 (panel D).

Table 5a. Overview of the selection of the single predictive behavioral activity variables; sensor data were recorded during the 14 d before calving using Smart Tag neck sensors (Nedap N.V.) that were attached to 173 cows at 4 different farms[1]

Sensor	Measurement	Calculation	Direction	TDS
Neck	Eating	Average	Negative	Inflammation
		Autocorrelation	Positive	Locomotion, Metabolic, Total
		Variance	Negative	Inflammation
		FFT	Positive	Locomotion
	Ruminating	Autocorrelation	Negative	Inflammation
		Variance	Negative	Metabolic, Macro
		Non-periodicity	Positive	Inflammation, Metabolic, Macro-minerals, Total
		FFT	Negative	Inflammation, Macro-minerals, Liver
	Active time	Average	Negative	Inflammation, Locomotion
		Variance	Negative	Locomotion, Total
		Non-periodicity	Negative	Liver
		FFT	Positive	Liver
	Inactive time	Average	Positive	Inflammation
		Autocorrelation	Positive	Liver, Total
		Non-periodicity	Negative	Metabolic, Macro-minerals, Liver
		FFT	Positive	Metabolic
	Activity score	Average	Negative	Locomotion
		Autocorrelation	Positive	Metabolic, Macro-minerals, Liver
		Variance	Negative	Locomotion, Total
		Non-periodicity	Negative	Liver
		FFT	Positive	Liver

[1]Average, variance, autocorrelation, nonperiodicity, and fast Fourier transformation (FFT) were calculated per activity measurement and related to total deficit score (TDS) categories (total, locomotion, and metabolic, with metabolic subdivided into TDS scores for liver and macro-minerals), and that were assessed during 6 wk after calving. Only predictors with $P \leq 0.20$ in the univariate step to predict a TDS category are shown. The direction (positive or negative) indicates how the predictive variable increases or decreases TDS, respectively.

Table 5b. Overview of the selection of the single predictive behavioral activity variables; sensor data were recorded during the 14 d before calving using Smart Tag leg sensors (Nedap N.V.) that were attached to 173 cows at 4 different farms[1]

Sensor	Measurement	Calculation	Direction	TDS
Leg	Count of steps	Average	Positive	Liver
		Autocorrelation	Positive	Metabolic, Macro-minerals, Liver, Total
		Variance	Positive	Liver
		Non-periodicity	Negative	Locomotion, Metabolic, Macro-minerals
		FFT	Positive	Locomotion
	Bouts standing up	Autocorrelation	Positive	Liver
		Non-periodicity	Negative	Locomotion, Total
		Non-periodicity	Positive	Liver
		FFT	Positive	Liver
	Lying	Average	Negative	Metabolic, Macro-minerals, Liver
		Autocorrelation	Positive	Macro, Liver
		Variance	Positive	Metabolic, Liver
		FFT	Positive	Liver
	Walking	Average	Positive	Liver
		Autocorrelation	Positive	Metabolic, Macro-minerals, Liver, Total
		Variance	Positive	Liver
		Non-periodicity	Negative	Locomotion
		FFT	Positive	Locomotion
	Standing still	Average	Positive	Metabolic, Macro-minerals, Liver
		Autocorrelation	Positive	Macro-minerals, Liver
		Variance	Positive	Metabolic, Liver, Total
		FFT	Negative	Total

[1]Average, variance, autocorrelation, nonperiodicity, and fast Fourier transformation (FFT) were calculated per activity measurement and related to total deficit score (TDS) categories (total, locomotion, and metabolic, with metabolic subdivided into TDS scores for liver and macro-minerals), and that were assessed during 6 wk after calving. Only predictors with $P \leq 0.20$ in the univariate step to predict a TDS category are shown. The direction (positive or negative) indicates how the predictive variable increases or decreases TDS, respectively.

Multivariable regression results per TDS

TDS Total model. The backward and all possible subset selection procedures identified four best candidate models based on best AIC, R^2 marginal and R^2 conditional calculations. Assumptions for the mixed models were met and VIF of the models were low, so no effect of collinearity was expected. The predictive behavioral variables of the four candidate models with highest R^2 values are shown in Table 6. Two models included only Smarttag Neck variables (models 1 and 3, Table 6), one model included only Smarttag Leg variables (model 2, Table 6) and two models included both Smarttag Neck and Leg variables (models 4 and 4a, Table 6). The following four behavioral predictors were present: average minutes inactive, non-periodicity of frequency of standing up and FFT standing still. The positive effects of average minutes inactive per day indicate that the more inactive the cows, the higher the TDS value. The positive effects of the non-periodicities of ruminating and frequency of standing up bouts on TDS Total indicate that reduced regularity results in increased TDS Total. The negative relation of TDS Total with FFT of minutes standing indicates that high regularity is associated with low TDS Total. High regularity in standing still behavior relates to a low TDS Total.

Model 4 had lowest AIC, highest R^2 and smallest difference between R^2 marginal and R^2 conditional and was the final model. Model 4a was similar to model 4 but also included the parity-FFT standing still interaction. For models 4 and 4a the cross validation was performed, and ROC curves were calculated. Variation in RMSE after 10-fold cross validation was limited, but somewhat larger in the model with the interaction as compared to model without the interaction. Variation calculated for the estimate coefficients in the model was limited for all estimates in both models, indicating good model stability independent of input. R^2 marginal and R^2 conditional improved to 0.37 and 0.37 in model 4a as compared to model 4 (R^2 marginal and R^2 conditional of 0.33 and 0.35, respectively).

Table 6. Predictors and model performance of the candidate models to predict total deficit score (TDS) total using a data set of 173 cows originating from 4 farms[1]

Sensor	Predictors	Model[2]							Effect on TDS Total
		1	2	3	4	4 Ssq	4a	4a Ssq	
	Parity	Y	Y	Y	Y	3.44	Y	0.44	
Smarttag Neck	Average min inactive	Y	N	Y	Y	1.26	Y	1.78	Positive
Smarttag Neck	Non-periodicity ruminating	N	N	Y	N		N		Positive
Smarttag Leg	Non-periodicity of bouts standing ups	N	Y	N	Y	1.19	Y	1.23	Positive
Smarttag Leg	FFT stand still	N	Y	N	Y	1.31	Y	1.43	Negative
	Parity x FFT stand still	N	N	N	N		Y	0.63	
Model sum of squares						7.20		5.51	
Model performance									
R^2 marginal		0.26	0.29	0.26		0.33		0.37	
R^2 conditional		0.30	0.33	0.31		0.35		0.37	
Variance components residuals						0.18		0.18	
Variance component farm						0.006		0.002	
AUC						0.78		0.80	
Sensitivity %						79.5		79.5	
Specificity %						63.9		73.2	

[1]The effect of the behavioral predictors on TDS is indicated as positive (a higher value of the predictor increases TDS) or negative (a higher value of the predictor reduces TDS). The behavioral predictors were calculated from data that were measured during the 14 d before calving using Smart Tag neck and leg sensors (Nedap N.V.).
[2]Included predictors per model are indicated as Y = yes, predictor is present in the model, or N = no, predictor is not present in the model.
Ssq = sum of squares of the effects for models 4 and 4a.
Fast Fourier transformation (FFT). Area under curve (AUC).

We calculated the AUC of the ROC curves for both models 4 and 4a (Table 6, 0.78 and 0.80 respectively, with an optimal threshold of TDS of 60 (lnTDS of 4.1)). For model 4 this corresponded to a sensitivity of 79.5% and a specificity of 63.9% with a positive predicted value of 0.62 and a negative predicted value 0.81. For model 4a this corresponded to a sensitivity of 79.5% and specificity of 73.2% with a positive predicted value of 0.69 and a negative predicted value 0.83. The parity x FFT standing still interaction improved the model, rendering model 4a best performing and its equation is added in the supplementary materials. The sum of squares of the effects, the model

sum of squares as well as the variance components for the random effects for models 4 and 4a are included in Table 6. The transformed values from the model prediction for model 4a were back-transformed to a TDS value and plotted against the original TDS value in Figure 5.

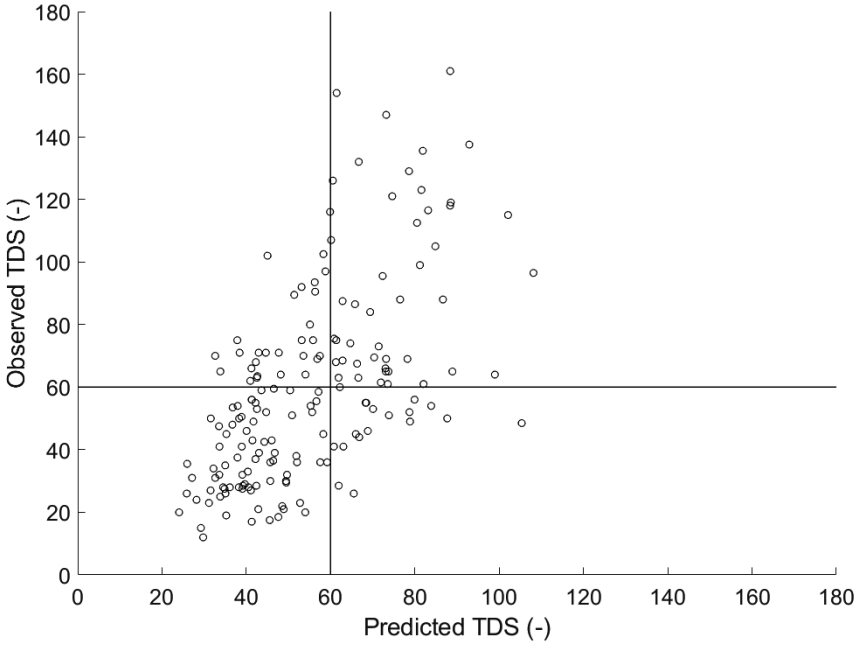

Figure 5. Scatter plot of the observed versus predicted values of Total Deficit Score (TDS) of 173 cows, using the variables average minutes inactive, non-periodicity ruminating, non-periodicity of bouts standing ups, FFT stand still and the interaction parity x FFT stand still. The transformed values from the model prediction were back-transformed to a TDS value and plotted against the original TDS value. The model is based on the transformation of ln (TDS + 0.5). This figure visualizes what the model means in terms of actual TDS value at cut off value of 60. (FFT = Fast Fourier Transformation calculation).

TDS Inflammation model. The backward selection procedure and all possible subset procedures identified 2 candidate models with only Smarttag Neck variables as selected indicators after evaluation. For these two models the assumptions required for mixed models were met. The predictive behavioral variables of these two candidate models are shown in Table 7. The VIF of the model with two variables (model 2 in Table 7) was low, so no collinearity was expected. Only two behavioral predictors were present in the models: average minutes eating and non-periodicity ruminating. The negative relation of average minutes eating indicates that eating more in the dry period results in lower TDS Inflammation. The positive relation of non-periodicity ruminating indicates that the more regular the behavior of ruminating (low non-periodicity), the lower the TDS Inflammation value will be. The variable non-periodicity ruminating was not significant, hence model 1 was chosen as the final model (Table 7). The interaction of minutes eating, and parity was not significant and was therefore not included in the model. The AUC was 0.78 with an optimal threshold

of TDS Inflammation of 22 (lnTDS of 3.11) which corresponded to a sensitivity of 79.7% and a specificity of 67.0% with a positive predicted value of 0.68 and a negative predicted value of 0.79. We calculated the sum of squares of the effects, and these were 5.47 for parity and 3.67 for the average minutes eating (Table 7). The equation for the final model (model 1) is added in the supplementary materials.

Table 7. Predictors and model performance of the candidate models to predict total deficit score (TDS) inflammation using a data set of 173 cows originating from 4 farms[1]

Sensors	Predictors	Models			Effect on TDS Inflammation
		1	1 Ssq	2	
	Parity	Y	5.47	Y	
Smarttag Neck	Average min eating	Y	3.67	Y	Negative
Smarttag Neck	Non-periodicity ruminating	N		Y	Positive
Model sum of squares			9.14		
Model performance					
	R^2 marginal		0.20	0.19	
	R^2 conditional		0.23	0.23	
	AUC		0.78		
	Sensitivity %		79.7		
	Specificity %		67.0		

[1]Included predictors per model are indicated as Y = yes, predictor is present in the model, or n = no, predictor is not present in the model. The effect of the behavioral predictors on TDS is indicated as positive (a higher value of the predictor increases TDS) or negative (a higher value of the predictor reduces TDS). The behavioral predictors were calculated from data that were measured during the 14 d before calving using Smart Tag neck and leg sensors (Nedap N.V.).

Sum of squares of the effects for model 1 (Ssq).

Area under curve (AUC).

TDS Metabolic model. The backward and all possible subset selection procedures identified 3 candidate models based on best AIC, R^2 marginal and R^2 conditional calculations. The predictive behavioral variables of the candidate models 1, 2 and 3 are shown in Table 8. These models included both Smarttag Neck- and Leg variables. The following behavioral predictors were present: non-periodicity inactive time, non-periodicity ruminating, non-periodicity of count of steps, AC activity, AC count of steps, FFT inactive time, variance lying time and variance stand still, and are shown in Table 8. Non-periodicities of inactive time, of ruminating and of count of steps influenced TDS in a positive direction, indicating that the higher the non-periodicity (the less regular in the respective behavior), the higher TDS Metabolic values will be and the more regularity in lying behavior, the lower the TDS Metabolic will be.

Assumptions for the mixed models for TDS Metabolic were met except for collinearity in model 3. Variance of lying and variance of standing still were highly correlated, leading to a high VIF in model 3. Therefore, we left out one of the two correlated variables and tested the model performance. No difference was found in model performance where we left out 'variance lying' or 'variance stand still', indicating that these variables can be exchanged. We therefore only show the results of model that included 'variance lying' only (model 4). Marginal and conditional R^2 both decreased to 0.12 in model 4 as compared to model 3 with marginal and conditional R^2 of 0.15. We tested the significance of the parity x variance lying interaction leading to model 4a which was similar to model 4 but with the significant parity x variance lying interaction and the marginal and conditional R^2 increased to 0.18 and 0.18, respectively (model 4a).

For models 3, 4, and 4a, the cross validation was performed, and ROC curves were made. Variation in RMSE after 10-fold cross validation was limited in all tested models as was the variation calculated for all estimated coefficients, indicating good model stability independent of input. We calculated the AUC of the ROC curves with an optimal threshold of TDS Metabolic of 25 (lnTDS Metabolic of 3.24). AUC as well as the sensitivity and specificity were higher in model 4 with an AUC of 0.67, a sensitivity of 80%, a specificity of 49% with a positive predicted value of 0.44 and a negative predicted value of 0.83, as compared to the model 3 with an AUC of 0.54, a sensitivity of 80%, a specificity of 24% with a positive predicted value of 0.35 and a negative predicted value of 0.70. Including the parity x variance lying interaction in the model improved predicative performance with an AUC of 0.72, a sensitivity of 80%, a specificity of 57% with a positive predicted value of 0.48 and a negative predicted value of 0.85 in model 4a, rendering model 4a best performing and its equation is added in the supplementary materials. We calculated the sum of squares of the effects, and these were added in Table 8.

Table 8. Predictors and model performance of the candidate models to predict total deficit score (TDS) metabolic using a data set of 173 cows originating from 4 farms[1]

Sensor	Predictors	Models						Effect on TDS Metabolic
		1	2	3	4	4a	4a Ssq[c]	
	Parity	Y	Y	Y	Y	Y	2.68	
Smarttag Neck	Non-periodicity inactive	Y	Y	N	N	N		Positive
Smarttag Neck	Non-periodicity ruminating	Y	Y	N	N	N		Positive
Smarttag Leg	Non-periodicity n steps	Y	Y	N	N	N		Positive
Smarttag Leg	AC activity	N	Y	Y	Y	Y	1.82	Negative
Smarttag Leg	AC count of steps	N	N	Y	Y	Y	1.08	Positive
Smarttag Neck	FFT inactive	N	N	Y	Y	Y	1.26	Negative
Smarttag Leg	Variance lying	N	N	Y	Y	Y	1.18	Positive
Smarttag Leg	Variance stand still	N	N	Y	N	N		Negative
	Parity x variance lying	N	N	N	N	Y	3.30	
Model sum of squares							11.32	
Model performance								
	R^2 marginal	0.09	0.10	0.15	0.12		0.18	
	R^2 conditional	0.09	0.10	0.15	0.12		0.18	
	AUC			0.54	0.67		0.72	
	Sensitivity %			80	80		80	
	Specificity %			24	49		57	

[1]Included predictors per model are indicated as Y = yes, predictor is present in the model, or n = no, predictor is not present in the model. The effect of the behavioral predictors on TDS is indicated as positive (a higher value of the predictor increases TDS) or negative (a higher value of the predictor reduces TDS). The behavioral predictors were calculated from data that were measured during the 14 d before calving using Smart Tag neck and leg sensors (Nedap N.V.).

Sum of squares of the effects for model 4a (Ssq).

Autocorrelation (AC).

Fast Fourier transformation (FFT)

Area under curve (AUC).

TDS Liver model. All possible subset selection was not possible with too many available input variables when both Smarttag Neck and Leg sensor variables were included. The predictive behavioral variables of the best performing candidate models are shown in Table 9. Variance of minutes eating was present in both models which included Smarttag Neck variables. Variance in minutes standing still and AC of minutes lying were present in both models which included Smarttag Leg variables. Non-periodicity of frequency of standing up was included in the model with only Smarttag Leg variables. The negative effects of variance of minutes eating and standing still per day indicate that the more variance the cows show in eating and standing still behaviour, the lower the TDS Liver. High non-periodicity of bouts standing ups reflects low regularity, which was related to higher TDS Liver. This was also the case for high autocorrelation of lying time related to high TDS Liver score. The VIF was low for the models 1, 2 and 3, so no risk for collinearity existed. The other assumptions for the mixed models for TDS Liver were also met.

Model 3 performed best yielding the lowest AIC, highest R^2, and smallest difference between R^2 marginal and R^2 conditional (Table 9). Model 3a was similar to model 3 but also included the parity x variance minutes eating interaction. For models 3 and 3a, the cross validation was performed, and ROC curves were calculated. Variation in RMSE after 10-fold cross validation was limited, but somewhat larger in the model including the interaction as compared to the model without the interaction. Variation calculated for the estimate coefficients in the model was limited for all estimates in both models, indicating good model stability independent of input. The marginal and conditional R^2 both improved to 0.20 and 0.20, respectively in model 3a as compared to model 3 with a marginal and conditional R^2 of 0.16 and 0.16, respectively.

We calculated the AUC of the ROC curves for models 3 and 3a (Table 9) and these were 0.68 and 0.70, respectively with an optimal threshold of TDS of 11 (lnTDS of 2.44). For model 3 this corresponded to a sensitivity of 79.5% and a specificity of 41.5%, with a positive predicted value of 0.59 and a negative predicted value of 0.65. For model 3a this corresponded to a sensitivity of 79.5% and specificity of 47.6%, with a positive predicted value of 0.62 and a negative predicted value of 0.68. Including the parity x variance minutes eating interaction improved the model, making model 3a the best performing model. We calculated the sum of squares of the effects, and these were added in Table 9. The equation for model 3a is provided in the supplementary materials.

Table 9. Predictors and model performance of the candidate models to predict total deficit score (TDS) liver using a data set of 173 cows originating from 4 farms[1]

Sensor	Predictors	Models					Effect on TDS Liver
		1	2	3	3a	3a Ssq	
	Parity	Y	Y	Y	Y	2.22	
Smarttag Neck	Variance min eating	Y	N	Y	Y	6.09	Negative
Smarttag Leg	Variance min stand still	N	Y	Y	Y	1.20	Negative
Smarttag Leg	AC min lying	N	Y	Y	Y	2.40	Positive
Smarttag Leg	Non-periodicity number of bouts standing up	N	Y	N	N		Positive
	Parity x variance min eating	N	N	N	Y	3.78	
Model sum of squares						15.69	
Model performance							
R^2 marginal		0.13	0.14	0.16		0.20	
R^2 conditional		0.13	0.14	0.16		0.20	
AUC				0.68		0.70	
Sensitivity %				79.5		79.5	
Specificity %				41.5		47.6	

[1]Included predictors per model are indicated as Y = yes, predictor is present in the model, or n = no, predictor is not present in the model. The effect of the behavioral predictors on TDS is indicated as positive (a higher value of the predictor increases TDS) or negative (a higher value of the predictor reduces TDS). The behavioral predictors were calculated from data that were measured during the 14 d before calving using Smart Tag neck and leg sensors (Nedap N.V.).

Sum of squares of the effects for model 3a (Ssq).

Autocorrelation (AC).

Area under curve (AUC).

TDS Locomotion model. For all candidate models, R^2 marginal with values between 0.16 and 0.18 and an R^2 conditional with values between 0.32 and0.33 were found. The candidate models that included FFT walking and FFT count of steps showed a high VIF, indicating collinearity. Apart from the variables FFT walking and FFT count of steps, the candidate models included different combinations of non-periodicities of walking, count of steps and count of standing up. Non-periodicity of count of steps and walking were also highly correlated, resulting in a high VIF. All locomotion models showed a low R^2 marginal with values between 0.13 and 0.18 as compared to R^2 conditional with values between 0.32 and 0.38 indicating a large farm effect on TDS Locomotion and were not further evaluated. The candidate models for the prediction of TDS Locomotion and their performance are provided in the supplementary materials (Table 3.1 in supplement 3).

TDS Macro-minerals. Three candidate models for TDS Macro-minerals included Smarttag Neck variables only and 4 models included Smarttag Leg variables only. Three models were identical to the TDS Metabolic models but the explained variance for TDS Macro-minerals was lower with an R^2 marginal and R^2 conditional of ≤ 0.10. Due to the low explained variance, no further analysis of these models was investigated. The candidate models for the prediction of TDS Macro-minerals and their performance are provided in the supplementary materials (Table 3.2 in supplement 3).

Discussion

With this study we investigated which behavioral characteristics during the dry period could be used as indicators of resilience using noninvasive Smarttag Neck and Smarttag Leg sensors in dairy cows. We tested if behavioral activity signals and patterns measured in the dry period could be used as predictors for a total disease severity score and for scores related to specific diseases after calving. The data showed that cows with reduced resilience have higher average of inactive time, and lower regularity in bouts standing up and in time standing still during the dry period. As the variables of inactive time, eating and ruminating were highly correlated, more resilient cows are more active, eat and ruminate more, with a larger variation during the day. These resilient cows reveal distinct active periods and alternate this with resting periods in regular diurnal patterns in contrast to the more vulnerable cows. These behavioral variables may therefore serve as indicators of a cow's resilience and their daily activity patterns as dynamic indicators of resilience (DIOR), as earlier described by Scheffer et al (2018) and Van Dixhoorn et al. (2018). It can be noted that the combination of both Smarttag Leg and Neck behavioral predictors increased model performance.

A remarkable aspect of this study was the gradual linear increase of TDS Total points reaching a value of 75 points, followed by an exponential increase up to 172 points. This trajectory of points of TDS Total values might reflect the 'tipping point hypothesis' indicating that complex dynamic systems can absorb disturbances and continue to function up to a certain tipping point at which the ability to self-recover or absorb disturbances is lost (van Nes et al., 2016). Once this tipping point is surpassed, intrinsic processes inside the system form a positive feedback loop, leading to an alternative state (van Nes et al., 2016). The hypothesis that cows are characterized by a self-propelled accelerating change (positive feedback loop) when they cannot adequately adapt to all requirements during the transition phase, leading to postpartum disease (as tipping point) was previously proposed (Van Dixhoorn et al., 2018). This phenomenon of a self-accelerating positive

feedback loop in cows can be initiated when physiological mechanisms are no longer able to effectively re-organize and adjust to all requirements of the transition phase. This can be caused by the gap between nutrient intake and demand, resulting in metabolic disorders, inducing other health issues, such as infections (Esposito et al., 2014; Sundrum, 2015; Trevisi et al., 2012). A subsequent reduced nutrient intake reinforces hampered metabolism potentially affecting health status at other physiological sites (Mulligan and Doherty, 2008, Sundrum, 2015). This positive feedback loop can also be initiated by other disorders, such as lameness, difficult parturition or cesarean section, retained placenta, reduced feed intake (due to e.g., overcrowding) or when resources, like nutrients or resting and feeding areas, simply are limited. These destructive feedback loops were similarly described as the result of intertwined components of metabolic stress of altered nutrient metabolism, dysfunctional inflammatory responses and oxidative stress (Sordillo & Mavangira, 2014). This justifies the use of a Total TDS in our study approach in which all post-partum problems are included, signifying that post-partum related disorders should be approached as a complex disorder. Comorbidity after parturition in our study was confirmed by the high correlation coefficients between TDS Metabolic and TDS Inflammation. These two TDS categories were calculated by the sum of independent points derived from their respective clinical and serum values. However, the sample size and consequently low incidence of diseases in our study could have driven the correlation between TDS Metabolic and TDS Inflammation. TDS Locomotion correlated less with the other TDS categories with the exception of TDS Inflammation. Locomotion problems are typically less related to the transition phase in contrast to metabolic and inflammation disorders (Daros et al., 2020). However, locomotion and cow comfort problems may cause discomfort and pain and give rise to reduced feed intake, intensifying post-partum diseases (Daros et al., 2020; LeBlanc et al., 2006).

In line with our study, Wisnieski et al. (2019) also showed that prediction performance of models for combinations of early lactation diseases was better in comparison to a single disease approach when using biomarkers. The biomarkers related to inflammation, oxidative and nutrient stress in their study were assessed at dry off, occurring approximately 48 d prior to parturition (Wisnieski et al., 2019). They identified candidate models for each metabolic stress component (nutrient metabolism, oxidative stress and inflammation) and for the combined model including all stress components. Prediction of specific post-partum diseases can thus be performed when variables are used relating directly to the particular disorder. The behavioral patterns that we used as predictors of resilience are not direct indicators of metabolic stress and inflammatory issues. However, specific behavior, reduced eating behavior for example, may lead to or reinforce metabolic stress, rendering cows vulnerable to other post-partum diseases as well. In addition, the variables were collected non-invasively and thus easier to implement on a commercial setting. While they may not be as accurate as blood measurements, they are more practical.

Prepartum behavior has been used by others to detect cows at risk of post-partum diseases (Belaid et al., 2021). In that study behavior was described as time spent at the feed bunk (min/day), frequency of meals (n/day), step count (n/day), count of lying bouts per day and lying time. Decreased eating time, increased lying and decreased active time measured prepartum (described in min/day) were previously associated with post-partum related disorders (Cattaneo et al., 2020; Hendriks et al., 2022; Hut et al., 2021; Kaufman et al., 2016; Menichetti et al., 2020; Piñeiro et al., 2019a, 2019b), which is in line with our study. In addition more lying bouts, fewer meals and fewer steps taken, were seen in cows with metritis or ketosis after calving (Belaid et al., 2021). Reduced

rumination time was also previously found as a predictor for early detection of metritis, albeit not as adequate predictor for somatic cell counts (Cocco et al., 2021). Stangaferro et al. (2016abc) used a combination of rumination and activity to timely detect post-partum diseases. All these studies focused on daily averages or total time spent on behaviors. Variance and autocorrelation of daily step count has also been calculated and tested as indicator trait for resilience by Poppe et al. (2022). They showed that mean and autocorrelation as well as mean negative residuals were candidates for resilience indicators based on heritability and genetic associations with health, fertility and BCS. Heritability and genetic associations of the non-periodicities and FFT calculations might as well serve as new traits for cow resilience.

The farm effect in the prediction of locomotion related problems was larger as compared to that in the other TDS prediction models. This suggests that farm specific housing and management factors play a relatively large part in the development of locomotion-related problems. This makes it more difficult to predict locomotion-related problems in the dry period based on sensor data variables in the dry period alone. Sensor variables, however, have been proven to be of value to actually diagnose lameness (Rutten et al., 2013). In our study we focused on farm-independent prediction of loss of resilience. Risk factors at farm level are probably more indicative for the propensity to develop locomotion problems.

Even with the low explained variance, the final TDS Total model had an acceptable predictive ability, when using a cut off value of Total TDS of 60, which makes it possible to identify cows at risk for post-partum diseases with sensor data alone during the dry period. With regards to the contribution of the individual effects to the total explained variance, it appeared that the of sum of squares was more less comparable, which indicates equal importance in the final Total TDS model. The variance component for the farm effect was smaller in the model without interaction, which suggests that some variation was captured in the interaction term. In the TDS Inflammation and TDS Metabolic model the sum of squares of the parity effect was larger as compared to the other effects. This indicates that the contribution of behavioral variables in these models was limited. In the TDS Liver model, the contribution of the variance of minutes eating was relatively high as compared to the other effects. To include the standard deviation of eating time in monitoring models to detect cows with ketosis was previously suggested to be of value by González et al. (2008). However, the results of the specific TDS categories should be interpreted with caution due to the low incidence of problems within a specific TDS category.

The predictive capacity that we found for the TDS Total is comparable to models using biomarkers as predictors for metabolic stress components (Wisnieski et al., 2019) and other predictions or early detection of diseases using sensor-data only (Urton et al., 2005, Stangaferro et al., 2016abc, Belaid et al., 2021). Indeed, combinations of metabolic components increase predictive ability (up to a sensitivity of 88.2% and specificity of 87% (Wisnieski et al., 2019)), which is superior to the predictive performance of our models. In our study, the use of non-invasive sensors is beneficial, in contrast to models that require invasive blood sampling.

The approach used in this research however has several limitations. We were only able to reasonably predict Total TDS, with rather low conditional and marginal R^2. These values were even lower in the other models for predicting specific TDS categories and therefore we were not able to differentiate between specific diseases. This means that with our research approach, we were only able to detect cows at risk for some kind of post-partum disease, without knowing the exact

underlying primary cause. This will still require further individual examination to find clues for early intervention strategies that might prevent or reduce post-partum diseases.

The low predictive value of sensor data for specific TDS categories could be related to the low number of disease cases per category, likely resulting in insufficient statistical power. Belaid et al. (2021) were able to identify metritis, DA, and ketosis, but not mastitis and RP, using differences in feeding behaviour, but included a larger number of cows in their study (489 multiparous cows). The low sample size in our study is an important limitation of our study. Therefore, more research with larger sample sizes and more cases per disease category are needed to draw conclusions on the predictive value of sensor data for TDS related to specific diseases. This would also allow for a better assessment of the merit of using a Total TDS score.

In the study of Belaid et al. (2021), the diseases diagnosed, with the exception of ketosis, were based on clinical cases, and the cows that were not diagnosed as diseased could have included subclinical cases. The merit of using the additive scoring system to calculate the TDS values, including blood values, is that it includes subtle changes in health status. More subtle deficits could relate to subclinical issues and when they are present for a longer period, this will lead to a higher TDS value. These subtle changes can be missed when diseases are assessed as absence or presence and not as a build-up score such as our TDS, inclusive of the severity and duration of the deviations in health status. A binary evaluation of a specific disease might miss subclinical issues. An option could be to lower the cut off values in models with a binary outcome, which will increase the risk of false positives, reducing specificity of the models (Wisnieski et al., 2019). The lower specificity will identify a relatively large number of false positives, which may possibly lead to unessential interventions.

Models predicting cows at risk with low specificity might be more of value to detect shifts in the predicted percentage of cows at risk within the herd. An increased percentage of cows at risk requires preventive measures at herd level, which will be beneficial for the health of all cows. However, over-treating cows, may have negative effects to the economics and production efficiency (Salar et al., 2017). Intervention aims to reduce the percentage of cows at risk instead of the prevention of diseases in individual cows. In addition, these models could be used to evaluate management measures that are intended to increase overall cow resilience within the herd. Effective interventions should result in a lower percentage of cows at risk within the herd.

By combining the sensor data using a general linear model, a prediction accuracy for TDS Total of 73.2% was achieved with a cut-off value of 60 with 20.5% false positives and 26.8% of false negatives. Still, a large number of false positives and false negatives will be assigned. This cut-off is 15 points below the value of 75 which was indicated as a tipping point, as described above. The question is whether this margin of 15 points is sufficiently chosen to interfere and turn the tide at the individual cow level or if a lower cut-off value is necessary, increasing the number of false positives. Some additional care for cows at a predicted TDS level of 50 could be beneficial. The question remains if it is possible to increase predictive performance for loss of resilience in individual cows when using sensor data alone.

The uncertainty of unknown events influencing the outcome during the timeframe between measurement (sensor data acquisition) and the manifestation of diseases will limit predictive performance in general. To assess cows at risk before diseases occur often while the animal is still healthy, is more challenging as compared to diagnosing diseases at the moment of occurrence as disease specific symptoms or other disease specific values are not present before the onset of

disease. When the disease is already present, it is too late for prevention, leaving treatment as the only solution left. Our predictive models of resilience in cows allow for timely implementation of interventions to prevent disease development after calving, although the exact nature of effective intervention strategies remains to be determined. The behavioral patterns that are observed in cows at risk may provide clues for management adjustments, which may include improvement of environmental and housing factors to improve cow-comfort, nutrition or dry cow treatments (LeBlanc et al., 2006).

Conclusions

The risk to develop some kind of post-partum disease can be predicted when using sensor data alone during the dry period when all clinical aberrations are integrated into one score. More resilient dairy cows eat more, are more active, and show high regularity in rumination, standing time and transitions from lying to standing as compared to vulnerable cows. These behaviors can be used as indicators of resilience and may allow for preventive intervention during the dry period in dairy cattle. However, additional examination of the cows at risk is still required to find clues for adequate intervention strategies. With our research strategy, the scores for specific disease categories could not be predicted accurately using sensor data, which could be related to the low number of cases per category.

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Supplemental Material

SUPPLEMENT 1 Descriptive statistics of TDS values per farm and parity group

Table S 1.1 Descriptive statistics of the TDS values per farm, corresponding to Figure 1. Number of cows per farm were 19, 20, 75 and 59 for farms 1 to 4 respectively. The range, median, average and standard error of the mean (SEM) are given. Differences in average between farms were tested. An overall farm effect was seen for TDS Total, TDS Inflammation and TDS locomotion, but not for TDS Metabolic, TDS Liver and TDS Macro-minerals. Letters a and b indicate significant difference ($P < 0.05$), with a being the lower value as compared to b.

TDS	Farm	Range	Median	Average		SEM
Total	1	135	46	55.32	a	7.50
	2	69	56	57.60	ab	4.30
	3	153	64	70.25	b	3.95
	4	139	43	49.93	a	3.67
Inflammation	1	55	20	22.66	ab	3.02
	2	43	27	26.55	b	2.41
	3	56	25	26.11	b	1.61
	4	45	17	18.98	a	1.38
Locomotion	1	38	5	9.21	a	2.60
	2	26	8	9.10	a	1.75
	3	74	17	19.28	b	1.75
	4	28	2	5.23	a	0.88
Metabolic	1	76	22	24.66		4.08
	2	41	20	21.05		2.35
	3	91	21	24.27		1.57
	4	111	20	24.34		2.46
Liver	1	64	12	14.55		3.20
	2	32	10	9.65		1.58
	3	82	13	14.47		1.31
	4	83	12	14.20		1.88
Macro-minerals	1	64	17	19.29		3.40
	2	30	11	12.05		1.59
	3	82	17	16.67		1.25
	4	82	14	17.55		1.84

Table S 1.2 Descriptive statistics of the TDS values per parity group corresponding to Figure 2. The number of cows per parity group were 37 with parity 1, 81 with parity 2 or 3 and 55 with parity 4 or higher. The range, median, average and standard error of the mean (SEM) are given. Differences in average between parity groups were tested. An overall parity group effect was seen for all TDS categories except for TDS Macro-minerals. Letters a and b indicate significant difference between parity groups ($P < 0.05$), with a being the lower value as compared to b.

TDS	Parity Group	Range	Median	Average		SEM
Total	1	124	54	56.50	a	4.28
	2 - 3	113	46	50.24	a	3.03
	≥ 4	149	65	76.03	b	4.66
Inflammation	1	44	26	25.22	ab	1.83
	2 - 3	56	16	19.30	a	1.39
	≥ 4	55	27	27.63	b	1.71
Locomotion	1	39	4	8.78	a	1.69
	2 - 3	41	6	10.40	a	1.26
	≥ 4	74	12	16.92	b	2.11
Metabolic	1	44	19	21.20	a	1.79
	2 - 3	47	19	20.51	ab	1.20
	≥ 4	108	24	30.25	b	2.77
Liver	1	41	9	11.47	a	1.49
	2 - 3	33	10	11.77	ab	0.84
	≥ 4	84	15	18.06	b	2.29
Macro-minerals	1	25	13	13.74		1.17
	2 - 3	35	14	14.80		0.94
	≥ 4	84	18	21.11		2.23

Supplement 2: Equation with all coefficients for the best performing models

TDS Total model

Final model4a:

$$TDS\ Total \sim Parity\ +\ average\ min\ inactive\ +\ non-periodicity\ bouts\ standing\ up\ +\ FFT\ stand\ still + (parity\ -\ FFT\ stand\ still)\ +\ Farm$$

Table S2.1. TDS Total Model estimates per covariate for the final model (4a). Parity τ is the factor that is added or subtracted from the intercept, slopes of the covariates (cov) are given per parity group. cov 1: average min inactive, cov 2: non periodicity bouts standing up, cov 3: FFT stand still.

	intercept	Parity τ	Slope cov1	Slope cov2	Slope cov3
Parity_1	3.747567	0.0000000	0.0303651	4.395993	-0.9649214
Parity 2-3	3.747567	-0.5264976	0.0303651	4.395993	-0.3551448
Parity 4 and more	3.747567	-0.4564559	0.0303651	4.395993	0.0508739

For Parity = 1 the fixed part of the regression equation is

$$TDS\ Total\ =\ 3.748\ +\ 0.03\ *\ average\ minutes\ inactive\ +\ 4.396\ *\ non-periodicity\ bouts\ standing\ up - 0.965\ *\ FFT\ minutes\ stand\ still$$

For Parity = 2-3 the fixed part of the regression equation is

$$lnTDS\ Total\ =\ 3.221\ +\ 0.03\ *\ Average\ minutes\ Inactive\ +\ 4.396\ *\ non-periodicity\ bouts\ standing\ up - 0.355\ *\ FFT\ minutes\ stand\ still$$

For Parity = 4 or more the fixed part of the regression equation is

$$lnTDS\ Total\ =\ 3.291\ +\ 0.03\ *\ Average\ minutes\ Inactive\ +\ 4.396\ *\ nonperiodicity\ bouts\ standing\ up\ 0.051 + *\ FFT\ minutes\ stand\ still$$

TDS Inflammation model

Final model 1:

$$TDS\ inflammation \sim Parity\ +\ average\ min\ eating\ +\ Farm$$

Table S2.2. TDS Inflammation Model estimates per covariate for the final model (1). Parity τ is the factor that is added or subtracted from the intercept, slopes of the covariate (cov) are given per parity group. cov 1: average min eating.

	int	Parity τ	Slope cov1
Parity_1	4.099856	0.000000	-0.0569074
Parity 2-3	4.099856	-0.417899	-0.0569074
Parity 4 and more	4.099856	-0.092969	-0.0569074

For Parity = 1 the fixed part of the regression equation is:

$$lnTDS\ Inflammation\ =\ 4.1\ -0.057\ *\ average\ min\ eating$$

For Parity = 2-3 the fixed part of the regression equation is

$$lnTDS\ Inflammation\ =\ 3.682\ -0.057\ *\ average\ min\ eating$$

For Parity = 4 or more the fixed part of the regression equation is

$$lnTDS\ Inflammation\ =\ 4.007\ -0.057\ *\ average\ min\ eating$$

TDS Metabolic model 4a

Final model 4a:

$$TDS\ metabolic \sim Parity + Autocorrelation\ of\ Activity + FFT\ min\ Inactive + Variance\ min\ Lying + Autocorrelation\ of\ number\ of\ steps + (parity - Variance\ min\ Lying) + Farm$$

Table S2.3. TDS Metabolic Model estimates per covariate for the final model (4a). Parity τ is the factor that is added or subtracted from the intercept, slopes of the covariates (cov) are given per parity group. cov 1: Autocorrelation activity, cov 2: FFT min Inactive, cov 3: Variance min Lying, cov 4: Autocorrelation of number of steps.

	int	Parity τ	Slope cov1	Slope cov2	Slope cov3	Slope cov4
Parity 1	5.079027	0.000000	-0.8543349	-0.5441989	-0.0042703	0.0045598
Parity 2-3	5.079027	-2.181163	-0.8543349	-0.5441989	0.0002787	0.0045598
Parity 4 and more	5.079027	-1.897137	-0.8543349	-0.5441989	0.0002895	0.0045598

For Parity = 1 the fixed part of the regression equation is

$$Log\_TDS\_met = 5.079 + -0.854 * AC\_NeckAct + -0.544\ FFTNeckmInact + -0.004\ VarLegmLie + 0.005 * AC\_LegnStep$$

For Parity = 2-3 the fixed part of the regression equation is

$$Log\_TDS\_met = 2.898 + -0.854 * AC\_NeckAct + -0.544\ FFTNeckmInact + 0\ VarLegmLie + 0.005 * AC\_LegnStep$$

For Parity = 4 or more the fixed part of the regression equation is

$$Log\_TDS\_met = 3.182 + -0.854 * AC\_NeckAct + -0.544\ FFTNeckmInact + 0\ VarLegmLie + 0.005 * AC\_LegnStep$$

TDS Liver model

Final model 3a

$$TDS\ liver \sim Parity + variance\ min\ eating + variance\ min\ standing + autocorrelation\ min\ lying + (Parity - variance\ min\ eating) + Farm$$

Table S2.4. TDS Liver Model estimates per covariate for the final model (3a). Parity τ is the factor that is added or subtracted from the intercept, slopes of the covariates (cov) are given per parity group. cov 1: variance min eating, cov 2: variance min standing, cov 3: autocorrelation min lying.

	int	Parity τ	Slope cov1	Slope cov2	Slope cov3
Parity_1	4.666467	0.000000	-0.0069001	-0.0016397	1.115327
Parity 2-3	4.666467	-1.413864	-0.0013555	-0.0016397	1.115327
Parity 4 and more	4.666467	-1.028062	-0.0016307	-0.0016397	1.115327

For Parity = 1 the fixed part of the regression equation is

$$lnTDS\ Liver = 4.666 + -0.007 * variance\ min\ eating + -0.002 * variance\ min\ standing + 1.115 * autocorrelation\ min\ lying$$

For Parity = 2-3 the fixed part of the regression equation is

$$lnTDS\ Liver = 3.253 + -0.001 * variance\ min\ eating + -0.002 * variance\ min\ standing + 1.115 * autocorrelation\ min\ lying$$

For Parity = 4 or more the fixed part of the regression equation is

$$lnTDS\ liver = 3.638 + -0.002 * variance\ min\ eating + -0.002 * variance\ min\ standing + 1.115 * autocorrelation\ min\ lying$$

Supplement 3: Candidate models for TDS Locomotion and TDS macro-minerals

TDS Locomotion

Table S3.1. Candidate models for TDS Locomotion with Smart Necktag sensor variables or Smart Legtag variables alone and with both Smart Neck- and Legtag variables combined. Marginal (R^2M) and conditional R^2 (R^2C) are given. Model selection based on backward or all possible subset selection (APSS). In grey model with highest marginal and conditional R^2.

Sensor	Model	TDS Locomotion	R^2 M	R^2 C
Necktag	Backward	-	-	-
	APSS	Autocorrelation min Eat* + FFT min Eat	0.13	0.35
Legtag	Backward	FFT number of steps* + FFT min walk* + (parity-FFT min walk)+(parity–FFT number of steps)	0.20	0.38
	APSS	FFT min walk* + FFT number of steps* + non-periodicity min walk + non-periodicity number of steps + non-periodicity bouts standing up	0.17	0.32
Neck- and Legtag	Backward	Same as Leg only	-	-
	APSS	Autocorrelation min Eat + FFT min walk* + FFT number of steps * + FFT min Eat + non-periodicity number of steps + non-periodicity bouts standing up + non-periodicity min walk	0.18	0.33

* Indicate variable is significant ($P < 0.05$)

TDS Macro-minerals

Table S3.2. Candidate models for TDS Macro-minerals with Smart Necktag sensor variables or Smart Legtag variables alone and with both Smart Neck- and Legtag variables combined. Marginal (R^2M) and conditional R^2 (R^2C) are given. Model selection based on backward or all possible subset selection (APSS).

Sensor	Model	TDS Macro-minerals	R^2 M	R^2 C
Necktag	Backward	-	-	-
	APSS	Non-periodicity of min inactive + non periodicity min ruminating	0.06	0.06
Legtag	Backward	Variance min lying* + Variance min standing*	0.10	0.10
	APSS	Autocorrelation min lying + autocorrelation min stand still + non -periodicity number of steps	0.06	0.06
Neck- and Legtag	Backward	Same as Leg only	-	-
	APSS	Not possible, too many variables	-	-

* Indicate significant variable ($P < 0.05$)

SUPPLEMENT 4 Percentage of cows with serum values outside the reference values

Table S 4.1 Overview of the serum parameters that were taken from 180 cows on 4 farms in week 1 and 5 after calving. The cut off values per serum parameter are specified for week 1 and week 5 separately and the Total Deficit Score (TDS) categories to which the points were assigned to are given. The percentage of cows indicate the fraction of cows with serum values above or below the thresholds per timepoint. The TDS categories consisted of TDS Total, Inflammation and Metabolic with TDS Metabolic sub-divided into TDS scores related to liver function (TDS Liver) and macro-mineral shortage (TDS Macro-minerals). Per sampling point (week 1 and week 5), a level exceeding the values as indicated in the columns with cut-off values, counted as 6 points in the TDS.

Parameter	Unit	TDS	Cut-off at week 1	% of cows in week 1	Cut-off at week 5	% of cows in week 5
Total protein	g/L	Inf[1], Total	>85	2	>85	13
Total protein	g/L	Met[2], Total	<55	1	<55	0
Albumin	g/L	Met, Total	<31	2	<31	3
Urea	mmol/L	Met, Total	<3.3	33	<3.3	20
Urea	mmol/L	Met, Total	>6.6	4	>6.6	2
NEFA[4]	mmol/L	Met, Total	>0.8	13	>0.4	21
BHBA	mmol/L	Met, Total	>1.2	4	>1.2	15
Calcium	mmol/L	Macro[3], Met, Total	<2.00 (day 0–1) <2.20 (day 2–7)	53	<2.20	14
Magnesium	mmol/L	Macro, Met, Total	<0.78	13	<0.78	5
Phosphorus	mmol/L	Macro, Met, Total	<0.9	10	<1.1	1
AST[6]	IU/L	Liver, Met, Total	>115	8	>115	3
GGT[7]	IU/L	Liver, Met, Total	>34	2	>34	8
Total Bilirubin	µmol/L	Liver, Met, Total	>7	8	>7	0
Haptoglobin	g/L	Inf, Total	>0.6	22	>0.3	22
Interleukin-6	ng/mL	Inf, Total	>10	14	-	-
Globulins (TP[8]-albumin)	g/L	Inf, Total	>49 and TP <85 and albumin >31	2	>49 and TP <85 and albumin >31	3

[1]Inf: TDS Inflammation; Met[2]: TDS Metabolic; Macro[3]: TDS Macro-minerals; NEFA[4]: non-esterified fatty acids , BHBA[5]: β-hydroxybutyric acid, AST[6]: aspartate aminotransferase , GGT[7]: gamma-glutamyl transferase, TP[8]: Total Protein. Cut-off values of serum metabolites parameters were based on the upper and or lower limit of the reference intervals for the corresponding parameters as provided by veterinary laboratory of Royal GD (Deventer, the Netherlands), except for BHBA, NEFA, and calcium. The cut-off value for BHBA was chosen based on the threshold for subclinical ketosis (Duffield et al., 2009), whereas the cut-off value for NEFA was chosen based on the threshold for an increased risk of early lactating culling, and clinical diseases (Ospina et al., 2010, Roberts et al., 2012, Ospina et al., 2013). The threshold for calcium was based on Kimura et al. (2006) and Martinez et al. (2012). The average DIM of blood sample collection were 1.8 ± 1.2 d and 29.8 ± 1.6 d for the first- and fifth-week sampling timepoints respectively.

CHAPTER 6

6

General discussion

The overall aim of this thesis was to find influencing factors and possible predictive indicators of resilience to typical multifactorial production related diseases in livestock animals. This thesis shows that resilience can be improved by providing social and environmental enrichment to pigs, as shown in Chapter 2. In addition, it was found that (dynamic) indicators of resilience, i.e. (D)IORs, can predict before a challenge how resilient animals are to that challenge (Chapters 3, 4 and 5). Moreover, a combination of (D)IORs could better predict resilience as compared to a single predictor (Chapter 3, 4 and 5). It was shown that animal factors such as coping strategy in pigs (Chapter 3) and parity in cows (Chapter 5) explained additional variation in resilience, and therefore could be taken into account in prediction models of resilience.

The general discussion consists of three parts, in the first part I will discuss the proxies used to assess resilience to challenges, the influencing factors and the (D)IORs. In the second part I will discuss the utility of the concept of dynamical system theories of resilience and tipping points in relation to animal health and disease. In the third part I will integrate the proxies, influencing factors and indicators of resilience into a mathematical model that is based on system and control theory. Such as model could serve as a basis for a decision support system. I will end the discussion with some concluding remarks.

6.1 Influencing factors and predictive indicators of (proxies of) resilience

6.1.1. Proxies of resilience after challenges

The proxies of resilience were used as gold standard to evaluate whether variables prior to challenge could serve as predictive indicators of resilience (IORs or DIORs). The proxies of resilience that were used in chapters 2 and 3 were signs of clinical disease in pigs following the co-infection model of Porcine Respiratory Reproductive and Respiratory Syndrome Virus (PRRSV) and *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*). The proxies of resilience used were the severity of the clinical symptoms and the duration of the clinical deviations. Of course, many other measurements of disease severity or duration can serve as proxy of resilience. Examples are the pathogen load, the recovery time, the height of response peaks, the area under the curve or the level after recovery of all kind of physiological variables (Luo et al., 2022; Parois et al., 2022b; van der Zande et al., 2020). In chapters 4 and 5 total deficit score (TDS) was used that included the severity as well as the duration of the clinical findings and blood serum values after parturition in dairy cows. The idea behind this TDS system was that by the combination of severity and duration of different clinical responses, more facets of resilience were included into one semi-continuous score as compared to a single response, thus better reflecting total disease load as also was suggested by (Friggens et al., 2022).

Instead of using the responses after challenge, as done in this thesis, morbidity, or other variables that can be dichotomised are used as proxies of resilience. The use of dichotomous read outs simplifies the statistical analysis and leads to easy interpretation and presentation of results (Altman & Royston, 2006). However, at the same time much information is lost, and the statistical power to detect a relation between a possible DIOR and disease outcome is reduced, which means that more animals are needed to find significant differences as compared to proxies expressed on a (semi) continuous scale (Altman & Royston, 2006), such as the TDS value and the histology score as used in this thesis. In addition, dichotomous proxies will underestimate the extent of variation between animals in resilience, as these are dichotomously indicated as resilient or non-resilient. Individuals close to but on opposite sides of the cutting point are then characterised as being very different rather than very similar (Altman & Royston, 2006).

In this thesis we used proxies of resilience specifically related to two disease challenges. This means that the variation in resilience that was found applies to these challenges specifically and may not reflect the generic resilience of the animals under study. Proxies used to indicate generic resilience reflect the consequences of accumulative challenges during the lifetime of an animal, such as accumulation of cortisol in hair, slaughter findings (Parois et al., 2022b), whole life performance (Luo et al., 2022), longevity or generic morbidity (Poppe, Veerkamp, et al., 2022). At this moment it remains unknown whether and how resilience upon a specific challenge is related to the resilience to another challenge. Future research should examine whether resilience to a particular challenge is related to resilience to other challenges, and also predicts the generic resilience of animals.

6.1.2. Influencing factors of resilience

External factors

In chapter 2 we studied the impact of housing conditions, as an external factor, on resilience of pigs to an experimental PRRSV- *A. pleuropneumoniae* co-infection. We showed that enriched housing conditions reduced the pathological and clinical responses, which were used as proxies of resilience, to the co-infection. Environmental enrichment is defined as a modification to the environment of captive animals that improves their biological functioning (Newberry, 1995). In chapter 2 the enrichment consisted of enlargement of the pen (twice the size of a conventional pen) with a specific area with extra rooting and play material. Additionally, the piglets were co-mingled prior to weaning, mimicking the natural situation, which is assumed to facilitate the development of social skills and thereby reduce social stress at weaning (Jensen & Redbo, 1987; Ko et al., 2020).

The positive effects of enriched housing on the welfare of pigs have been demonstrated extensively, for instance through its effectiveness to reduce undesirable behaviours such as tail biting, aggression and stereotypies at different stages of the life of the pig (Beattie, O'Connell, & Moss, 2000; Bracke et al., 2006; Godyń et al., 2019; M. Studnitz et al., 2007; Tuyttens, 2005; van de Weerd & Day, 2009; Van De Weerd et al., 2003). Recently the effects of various types of environmental enrichment on resilience in pigs have been studied by others (Luo et al., 2022; Parois et al., 2022a, 2022b), including the effect of co-mingling only (Gavaud et al., 2023). The challenges that were applied in these studies were transport, a challenge with Lipopolysaccharide (LPS), heat stress and/or isolation from conspecifics (Luo et al., 2022; Parois et al., 2022a, 2022b). These studies indicated that enrichment benefits resilience of pigs under a variety of challenges. Although an LPS challenge is considered to mimic the response to infection, even though no replication of a pathogen occurs (de Groot et al., 2007; Zielen et al., 2015), none of the studies so far studied resilience to an experimental infection as was done in this thesis. We showed, for the first time, that enriched housing in pigs increased resilience to a PRRSV- *A. pleuropneumoniae* co-infection. These findings were confirmed by a follow up study (de Bruijn et al.). In this latter study we applied the enrichment during different life stages, either from birth onwards or starting after weaning. In line with the results of Chapter 2, clinical findings and pathology indicated improved resilience in the pigs kept under enriched conditions from birth onwards as compared to the conventionally housed pigs. The pigs that were provided enrichment after weaning also had less severe clinical responses than the conventionally housed pigs, but the effect was smaller compared to the pigs that were provided enrichment from birth onwards. This suggests that enrichment is profitable for resilience in all stages of life, but especially when provided as early as possible.

Which physiological and immunological changes exactly underlie the effects of enrichment on resilience is unknown. Several studies have shown that pigs kept under contrasting housing conditions differ in development and responses of the immune system, the hypothalamic–pituitary–adrenal axis (HPA-axis) and/or the development of the microbiome (Beattie, O'Connell, Kilpatrick, et al., 2000; De Jong et al., 2000; Heimbürge et al., 2019; Kudielka & Kirschbaum, 2005). Such pre-challenge differences may affect the response to a disease challenge and may therefore be linked to disease resilience. Higher levels of white blood cell counts and lymphocytes were found in enriched housed pigs in this thesis and by (Gavaud et al., 2023), while (Wen et al., 2021) reported lower levels of granulocytes and monocytes in the enriched housed groups as compared to the conventional

housed pigs only. Luo et al. (2017b) found significantly lower levels of natural (auto-)antibodies (N(A)Abs) before infection in the enriched housed pigs of our Chapter 2 experiment (Lu Luo et al., 2017a) although in previous studies higher NA(A)bs titres were found for enriched housed pigs (L. Luo et al., 2017; Reimert et al., 2014b). So, several studies showed changes in immune development as a result of enriched housing. Why the measurements of variables of the immune system between contrasting housing conditions (with or without enrichment) vary, is difficult to explain, but can be related to differences in the experimental set up, e.g., the age of the animals, time points of measurement, and applied enrichment material. Despite some unequivocal effects of enrichment on the immune system, it can be concluded that that enriched housing affects immune (re)activity, which may influence the resilience of animals upon a disease challenge (Biondi & Zannino, 1997; de Groot et al., 2001; Glaser & Kiecolt-Glaser, 2005; Mason, 1991; Padgett & Glaser, 2003; Proudfoot & Habing, 2015; Proudfoot et al., 2012; Salak-Johnson & McGlone, 2007; Webster Marketon & Glaser, 2008).

It is possible that the effect of enrichment is related to its influence on maturation and development of the gut microbiome, which in its turn is known to affect immune development (Fouhse et al., 2016; Martin et al., 2010; Niederwerder, 2017). We have shown that the maturation of gut microbiota was accelerated in pigs kept under enriched conditions (similar to those in chapters 2 and 3), as compared to conventionally housed pigs (Wen et al., 2021). This contrast between housing conditions could be related to ingestion of rooting materials in enriched housed pigs and/or a contrast in HPA axis activity, as conventionally housed pigs may suffer from chronic stress due to the limited possibilities to develop and display important behaviours (Wiley et al., 2017). Thus, an effect on intestinal microbiome development could have contributed to an increased disease resilience in enriched housed pigs, but this remains speculative.

In general, based on chapter 2 and work of others, it can be concluded that resilience of pigs can be improved by providing living conditions that better meet their needs. However, the exact working mechanisms and the interactions between living conditions, HPA axis, immune system, and microbiome in their effects on resilience needs to be elucidated by future studies.

The application of enrichment material has been questioned, because it could be a source of pathogens and mycotoxins and may contain sharp objects such as iron wires, nails or splinters from wood (Nordkvist & Häggblom, 2014; Tenbrink et al., 2020; Wagner et al., 2018). High risk materials should therefore be ruled out as enrichment material, such as maize products due to the high risk of mycotoxin contamination and peat as it frequently contains mycobacteria (Tenbrink et al., 2020; Wagner et al., 2018). Furthermore, provision of straw or other bedding materials may be labour costly, it may not fit in the current slurry system, and it may have a negative trade off on greenhouse gas and ammonia emissions (Tuyttens, 2005). However, if the materials are processed and stored on the same criteria as feed, the biosecurity risk and risks of mycotoxin contamination were judged as limited by others (Schollenbruch et al., 2021; Tenbrink et al., 2020; Wagner et al., 2018). The arguments they raised were firstly that most of the organic materials are seldom a source of pathogenic bacteria, that might cause infection or pose a risk to animals (Wagner et al., 2018). Second argument was that growth rate of possible hazardous bacterial species is limited, due to competition with competitive harmless bacteria such as *Lactobacillus sp*. which is one of many commensal epiphytes on plants like straw (Schollenbruch et al., 2021). As third argument, we could add the accelerated maturation of gut microbiota as a result of ingestion of enrichment materials

(Wen et al., 2021), which may shorten the vulnerable period in the process of maturation. During the maturation process the gut microbiota is unstable and more prone to dysbiosis, which can evolve into a diseased state (St-Pierre et al., 2023). So, including the increased disease resilience, the benefits of enrichment materials may outweigh the possible biosecurity risk. Additionally, alternatives for manure management, such as belt-based housing, can handle the enrichment material and have proven to reduce emissions of NH3 and CH4 substantially (De Vries et al., 2013; Koger et al., 2014).

The provision of environmental enrichment with natural materials such as straw and wood shavings is less prominent of benefit for cows as compared to pigs (Mandel et al., 2016; Tuyttens, 2005). The provision of these kinds of materials in cattle is more related to the comfort in the lying area of cows, being soft, nonabrasive, clean, and dry and does not seem to fulfil other behavioural needs (Fregonesi, Veira, et al., 2007; Tuyttens, 2005). Enrichment for cows might be access to pasture, which offers improved opportunities for cows to exhibit natural behaviour such as grazing and more synchronised herd behaviour (Arnott et al., 2017; Crump et al., 2019; Nielsen et al., 2023). On pasture, cows walk for longer distances than when housed indoors, which more closely aligns with their natural locomotion behaviour (Nielsen et al., 2023). Additionally, on pasture, cattle can use trees or other objects, if available, for grooming, which is a behavioural need. In this thesis these kinds of external factors that might influence resilience in cattle were, however, not investigated.

The indicators of resilience found in this thesis (that will be further discussed in the next section 6.1.3) suggested some possible influencing factors for cows A long time spent eating, was an important indicator of high resilience during the transition phase. The cows were considered healthy in the dry period, so a lower feeding time as indicator of resilience was probably not the result of an underlying (disease) problem at the moment of measurement. A high amount of dry matter intake is essential to meet the nutritional needs of high producing dairy cows (Sundrum, 2015). Thus, management that ensures that all cows in the herd can spend enough time eating, could have a positive effect on their resilience. Other influencing factors to increase eating time, and thus possibly resilience, in cows, could be group size, grouping strategy, and stocking density as these factors may have considerable impact on the competition between animals for feed and space, feeding time and thus feed intake (Grant & Albright, 1995). Management factors that support or undermine regularity in daily rhythms may also be influencing factors of resilience, as regularity in behaviour was found to be an important indicator of increased resilience (Chapters 4 and 5). Examples of management factors that may cause misalignment with the natural circadian rhythm are e.g. irregular feeding and milking patterns, a suboptimal resting area, high stocking density or an inadequate light–dark cycle (McCabe et al., 2021; Van Erp et al., 2020). Indeed, minimizing circadian disruptions, was suggested to support health by others, but the exact mechanism between disease resilience and (disturbed) regularity or circadian rhythm remains to be examined (McCabe et al., 2021).

In general, the results of this thesis and studies by others, suggest that housing and management factors that better meet the natural behavioural and physiological needs of pigs and cows, can be considered as important factors to enhance their resilience for diseases. More specifically, disease resilience in pigs was enhanced by providing the opportunity to express highly motivated behaviours of rooting and chewing by offering rooting substrate (Merete Studnitz et al., 2007) and by a reduction of social stress through facilitating the development of social skills. The results of chapters 4 and 5 gave some clues that resilience may be enhanced by avoiding high

stocking densities (Collings et al., 2011; de Groot et al., 2001; Fregonesi, Tucker, et al., 2007; Huzzey et al., 2006; Huzzey et al., 2012; Turner et al., 2000), alignment of management to daily patterns (De Jong et al., 2000; Wagner et al., 2021; Witaifi et al., 2018), meeting the nutritional needs (Sundrum, 2015) or providing the opportunity for grazing behaviour (Arnott et al., 2017; Crump et al., 2019), but the effect of the suggested factors on resilience should be verified in future studies.

Animal factors

Variation in resilience is also the result of the characteristics of individual animals within the same group or herd. Animal factors that have been mentioned to influence responses upon challenges are e.g. breed, sex, coping style, age and social status within a group (Proudfoot et al., 2012). We therefore additionally tested whether animal factors such as sex or personality in pigs and parity in cows could explain a part of the variation that was seen in the proxies of resilience.

In humans as well as pigs, vulnerability for specific diseases can vary between males and females (Rohleder et al., 2001). (Opriessnig et al., 2006) found male pigs to be more prone to the development of the multifactorial post-weaning multi-systemic wasting syndrome, but attributed these findings to the castration and associated secondary infections. Others also found sex differences in physiological responses to an immune challenge (de Groot et al., 2001; Reimert et al., 2014a; Reimert, Rodenburg, et al., 2014), but also in these studies, castrated males were used. In chapter 3 we found no differences in proxies of resilience between females and entire males, but it should be noted that the sample size was limited.

The pigs with a pro-active coping style in our study appeared to be more resilient to the disease challenge than the pigs with the reactive or passive coping style, as shown in chapter 3. This suggests that animals with passive coping styles may be at higher risk for infections. However again due to the limited sample size, these findings should be interpreted with caution. In addition to that, the effect of coping style to challenges may also depend on housing condition and group composition in terms of percentage of active and passive copers (Bolhuis et al., 2003; Hessing et al., 1993). Although these findings are scientifically interesting and can explain additional variation in the proxies of resilience, this knowledge is difficult to apply in operational management as the coping style of individuals at farms is not known.

In cows, parity was taken into account in the prediction of resilience and indeed differences were found between the parity groups. Cows with parity 4 and higher had higher TDS scores than the younger cows, which was in line with expectations (Jamali et al., 2018). This means that the need for preventive measures might differ per parity group. Social rank within the herd or coping style could also be of influence, especially when resources are limited, but this was not further examined here.

Genetic background has also been suggested as influencing animal factor for resilience and breeding for resilience is therefore often considered as important strategy to reduce the impact of different kinds of disturbances (Bengtsson et al., 2022; T. V. L. Berghof, H. Bovenhuis, et al., 2019; T. V. L. Berghof, M. Poppe, et al., 2019; Harlizius et al., 2020; Hermesch et al., 2015). However, Kalisch et al. (2017) emphasised that resilience should not be considered as a trait, a stable personality profile or a specific genotype, as resilience strongly depends on early life experiences and the current situation, thus stressing the effect of external factors on animal resilience. This indicates

that genetic background for resilience in livestock is relevant, but only of limited value if the effects of management in terms of (early) life experience and the encouragement of favourable conditions, which aim to enhance resilience are neglected.

So, apart from external influencing factors, characteristics of the animals can influence resilience. The animal factors of resilience in this thesis differ from the external influencing factors in the sense that some external factors can be actively changed by the farmers, whereas the animal factors are given, but can help the farmer to adjust the management to animal specific needs.

6.1.3. Indicators of resilience

Resilience itself can only be defined ex post facto, meaning by measuring the responses after challenges, in this thesis referred to as proxies of resilience (Kalisch et al 2017). The term '(dynamic) indicators of resilience' ((D)IORs) is meant for measurements that reflect the state of the animal prior to the challenge of interest and are predictive of (the proxies of) resilience. Thus, (D)IORs provide an estimation of the animal's resilience without the need to determine the proxies of resilience themselves. Note that the term (dynamic) indicator of resilience is sometimes used by others for variables that are measured after the challenge of interest, thus reflecting the response (Putz et al., 2019; van der Zande et al., 2020). A clear distinction between (D)IORs (measured prior to challenge) or proxies of resilience (measured post challenge) can improve collective understanding.

In chapter 4, variables that were measured after calving were referred to as 'short-term predictors' in contrast to the variables that were measured before calving which we referred to as 'long-term predictors'. The latter are considered (D)IORs, whereas we argued that the short-term predictors represent indicators for the (early) onset of post-partum diseases, but are no (D)IORs, as these indicators were collected after the start of the challenge that makes cows vulnerable to develop illness, i.e. the transition from calving to the lactation phase (Grummer, 1993; Huzzey et al., 2011; Ingvartsen et al., 2003). The difference between short term predictors and (D)IORs is that the indicators of resilience can be determined prior to the challenge, when the animals still are assumed to be healthy. This provides the opportunity to take preventive measures. The short-term predictors have additional value, as a curative treatment can be implemented at an earlier stage.

(D)IORs can be divided into IORs taken from single measurements, IORs as the average values of multiple measurements and DIORs as calculations of the dynamic patterns of multiple measurements.

IORs from single measurements

In chapter 3 we showed that pigs with higher levels of Memory T helper cells, Naïve T helper cells, $CD8^+$ cells and N(A)Abs prior to challenge were more resilient to co-infection with PRRSv and *A. pleuropneumoniae*, suggesting that the immune status of these pigs was more advantageous as compared to pigs with lower levels of these immune variables. Using the (baseline) immune status pre-challenge as possible predictor for the disease response after this challenge (i.e., as an IOR) is not common. Unlike the results of chapter 3, other studies find little evidence of the predictive value of immune parameters measured prior to challenge for disease resilience (Bai et al., 2020; Zekarias et al., 2002).

Higher levels of N(A)ABs prior to co-infection were related to a better disease resilience (chapter 3). Other studies also found that the NAb levels in blood could serve as IOR to polymicrobial disease in piglets (Chen et al., 2020). High NAb levels, especially IgM binding Keyhole limpet hemocyanin (KLH), were also related to higher survival in commercial laying hens which were not specifically challenged (Star et al., 2007; Wondmeneh et al., 2015). Additionally, higher levels of NAb tended to be associated with greater disease resilience in pigs, based on lower mortality, fewer parenteral antibiotic treatments, higher average daily growth and lower day-to-day fluctuations in feed intake (Chen et al., 2020). Also higher Nab levels in chickens increased their resistance to avian pathogenic Escherichia coli (APEC) infection (T. V. L. Berghof, H. Bovenhuis, et al., 2019).

In this thesis immunological variables were not tested in cows, but others found increased blood levels of immunity reactants as IOR, to identify less resilient cows (e.g. IL6, IL-10, TNF, Haptoglobin, serum amyloid A, albumin) when measured prepartum, but white blood cell counts were not found to be of predictive value (Huzzey et al., 2009; Huzzey et al., 2011; Islam et al., 2013; Roberts et al., 2012; Wisnieski et al., 2019; Zhang et al., 2018). We collected blood samples in the dry period of the cows originating from the experiment that was described in chapter 5 to determine a number of variables. Cows with low levels of magnesium and elevated levels of total protein and haptoglobin had an increased risk for postpartum diseases thus these blood variables can also serve as IOR (unpublished results, de Bruijn et al, paper in preparation).

Variables measured in blood are often only measured once, because of the invasiveness of the sampling procedure, which is demanding for the animals. A single measurement, including variables measured in blood, as IOR is easy to use, but the main disadvantage of single measurements is that the values and thus predictive performance may be influenced by the moment of sampling, and might not reflect a representative value, especially when the variables fluctuate over the day. More frequent measurements give a better estimation of the pre-challenge, baseline value, and might increase predictive performance.

Average value of multiple measurements as IOR

As most physiological variables fluctuate across time, the calculation of an average value of measurements at multiple timepoints may provide a better representation of the average value of the variable of interest as compared to a single measurement. The average values of rectal, rumen and ear temperature were tested in both pigs and cows as potential IORs, but none of these were predictive of disease resilience (chapters 3 and 4). We did find an overall higher baseline level in the conventionally housed pigs as compared to the enriched housed pigs, which is in line with previous experiments (De Jong et al., 1998). This might be related to a more pronounced stress reaction to the temperature measurement procedure in the conventionally housed pigs as compared to the enriched housed pigs, as stress can affect the body temperature level (Ekkel, 1996). Other suggested chronic stress due to blunted circadian rhythm and a higher metabolic rate as explanation for the higher baseline body temperature levels of pig kept under barren, conventional housing conditions (De Jong et al., 1998; Parois et al., 2022b).

In cows the temperature was measured using a sensor in the ear and rumen (chapter 4). The average values of the temperature were not a found as IOR in cows, but a lower ear temperature measured in the first week post-partum was related to a higher TDS. This is as expected as ear temperature in cows is often lower during some postpartum health disorders such as milk fever

(Stevenson, 2022; Venjakob et al., 2016). The average rumen temperature was not predictive of the TDS when measured prior to calving nor after calving.

High average eating time and a low average inactive time per day were found as IORs for post-partum diseases in cows (chapters 4 and 5), in line with other studies ((Belaid et al., 2021; Calamari et al., 2014; Huzzey et al., 2007; Piñeiro et al., 2019b; Weary et al., 2009). The average level of activity provides global insight in the time budget of animals. The high milk production levels in dairy cows require large amounts of dry matter intake (DMI), making cows with decreased eating time and thus less DMI more prone to metabolic diseases and thus less resilient. The behaviour of animals of a herd is influenced by their living conditions and the farm management (van Dixhoorn et al.) submitted for publication). Differences in behaviour between individuals within a herd may be caused by hierarchical position within the herd, which becomes more evident under higher stocking densities as was explained in section 6.1.2. Our findings show that this individual variation in behaviour within a herd also provides information about their resilience.

Bodyweight or growth pre-challenge were not found as indicators of resilience in pigs, and in cows, the bodyweight was not measured. In pigs we only tested the average growth around weaning as indicator of resilience with the assumption that less resilient pigs may have had a more pronounced growth slowdown around weaning as compared to more resilient pigs. This was based on the assumption that resilience to the challenge of weaning is related to resilience for a co-infection, but this relation was not found. In cows, BCS was also not found to be a good predictor for post-partum disease by others (Heuer et al., 1999; Roche et al., 2015; Wisnieski et al., 2019). Thus, although fast growth or other high average levels of production are often considered to reflect good health, the growth in pigs was not found to predict disease resilience in this thesis. The average level of production will be reduced in most cases during illness, and being healthy can be seen as a prerequisite for good production, but the relation between production levels and resilience seems to be more complex. The current high production levels of livestock are demanding for the animals and require perfect alignment with the individual needs. The pressure to produce at the lowest cost and with the highest efficiency will increase the probability for misalignment with the needs of the animal, which may enlarge the risk for reduced resilience in farm animals.

The average values of multiple measurements as IORs may give a better representation of the level of the variable of interest as compared to a single measurement and may therefore better predict resilience. However, we only found the average level of eating and lying time as relevant IORs in cows. We used a linear model to test the relation between IORs and resilience. This suggests that, in case of significant relationship, the higher or the lower the average value of the IOR, the better resilience. For some variables, however, an optimum level exists, where a higher or lower level could both reflect a less favourable state (e.g. lying activity) (Piñeiro et al., 2019a).

DIORs from longitudinal measurements

As most physiological and behavioural variables fluctuate across time, the dynamics of these fluctuations were also proposed as possible DIORs (Scheffer et al., 2009 & 2018). The dynamics of variables may better reflect how animals cope with their living environment and how they react to small disturbances, also known as micro-recoveries, as was explained in the introduction. To quantify the fluctuations in variables we calculated the variance, the lag-1 autocorrelation, the non-periodicity and a measure that was derived from a fast fourier transformation (FFT) analysis. The variance

indicates how far the values are spread around the mean value across a given time period and the lag-1 autocorrelation determines the correlation between successive values of a signal (Dixhoorn et al submitted for publication). For the hourly data the lag-1 corresponds to the correlation between successive hours. The non-periodicity, as reflection of the daily rhythm with a 24h cycle, was introduced in chapters 4 and 5 a measure derived from the FFT was additionally introduced in chapter 5.

The variance and autocorrelation of a number of behavioural variables were found as possible DIOR in the univariate analysis. In line with the theory of critical slowing down, a higher variance of steps, walking, lying, and ear temperature was related to a lower resilience, but this was not the case for a higher variance of eating, ruminating and activity, which were related to a better resilience. Reduced resilience was predicted, as hypothesised, by increased autocorrelation in all variables measured, except for ruminating which predicted TDS in the opposite direction. When calculated after calving, a higher variance and higher autocorrelation of rumen temperature were related to a higher TDS. Given the generic character of critical slowing down theory, one may assume that it can be used as predictor, using any kind of time series (Gijzel, 2020). This is based on the assumption that the variable of interest is regulated around an equilibrium, having a setpoint towards which it will return to maintain homeostasis. In case of ear temperature, the value is regulated around a preferred level and the higher variance as indicator for increased resilience may be a confirmation of the theory of critical slowing down. In case of the activity data, with the chosen timescale (on an hourly basis), an equilibrium seems to be absent. Activity patterns within a day are not regulated around a certain equilibrium, which makes that the concept of critical slowing down as predictor of loss of resilience may not be applicable to these kinds of data. The variance and autocorrelation of the activity data nevertheless give insight in how the activity is distributed over the day, which can be related to the physiologic state of the animal (van Dixhoorn et al.) submitted for publication). So, the DIORs that were found for activity data did not reflect a critical slowing down but were probably based on the favourable distribution of activity over the day. This might also explain why a higher variance or autocorrelation of activity was sometimes an indicator for increased resilience and sometimes for reduced resilience.

The daily patterns in behaviour and ear temperature during the dry period, described by the non-periodicity and FFT measures, were found as important DIORs in dairy cows, with the more strict patterns being predictive of better resilience to post-partum diseases, as was described in chapters 4 and 5. All cows in our experiments were assumed to be healthy during the dry period, and were submitted to the same management within each farm. So, one option as explanation for this finding is, that the availability of feeding places and lying areas was limited due to high stocking density, leading to competition for these facilities which might have forced some of the cows within a herd into irregularity and as a consequence lowered their resilience. Another option is that some of the cows, although considered healthy according to our clinical examination, had nevertheless an underlying subclinical health problem or experienced stress, which caused the irregularity in their behaviour. Both options might explain the predictive value of the regularity for post-partum diseases.

The scan sampling that was performed to assess behaviour in pigs (chapter 2 and 3) did not allow to calculate dynamical aspects of their behaviour. In general, in pigs the continuous automated monitoring of activity of individual animals is more difficult as compared to cows when using sensors that are attached to the animal, as these can be damaged by pen mates. However, van der Zande

et al. (2020) used accelerometers in ear tags and visual imaging of pigs and tested whether variance, autocorrelation and skewness of activity measurements could serve as DIORs following a PRRS modified live virus vaccination. Their DIORs (prior to the challenge) were not predictive for their proxies, but the dynamical aspects of activity that were calculated post challenge were related to morbidity and mortality which were defined as proxies of resilience (van der Zande et al., 2020). As an alternative for the dichotomous proxies of resilience (morbidity and mortality), a (semi)-continuous proxy of resilience could be used that will capture the variation between the animals in their response to a challenge. This might be a better way to test predictive performance of a (D)IOR, as was discussed in chapter 6.1.1.

Instead of the average value of production-related variables, others tested the fluctuations around the production levels, independent of the level of production, as DIOR. In pigs, laying hens, and tilapia fish body weight fluctuations were calculated as the natural logarithm-transformed variance (ln(variance)) of deviations, the skewness and the lag-one autocorrelation of deviations of longitudinal body weight data (T. V. L. Berghof, H. Bovenhuis, et al., 2019; Mengistu et al., 2022). In cows fluctuations in milk yield around the expected lactation curve were suggested as DIORs (calculated as the natural log transformed variance (lnvar) and lag 1 autocorrelation) as relations were found between the production-related DIORs and other phenotypic health traits such as udder health and longevity in cows.(Poppe, 2022; Poppe et al., 2020). Interestingly, they showed that cows with lower fluctuations in milk yield (that were proposed as the more resilient cows) had higher dry matter intake than cows with higher fluctuations at the same milk yield. Therefore, (Poppe, 2022) suggested that focus on feed efficiency may be detrimental for resilience. So based on other research, the production-related DIORs seem to be more promising indicators for diseases resilience than the average levels of production only.

With these sections I gave some empirical proof for a number IORs and DIORS, representing the static and dynamic values of variables in relation to diseases resilience in pigs and cows although further validation of these (D)IORs is recommended.

Recommendations for prediction of disease resilience using (D)IORs

To implement (D)IORs as predictors for resilience in farm management, their practical use, their predictive performance and other concerns require some extra contemplation. The (D)IORs that we calculated were based on data that were obtained during the last two weeks before parturition in cows and a few days prior to infection in pigs. The DIORs can indicate that action is required to enhance resilience. However maybe an indication of reduced resilience should be available at an earlier stage, to enable effective adjustments that can prevent diseases. In cows, it is suggested that preparation and prevention should already start in or even before the dry period preceding the lactation, so 14 days prior to calving may be too late (Daros et al., 2022). Starting earlier with data collection before a challenge, allows (D)IOR calculations at an earlier stage, and would be a way to further evaluate and validate the (D)IORs (van Dixhoorn et al., 2023). The use of sensor data additionally allows to follow animals individually and detect individual deviations in DIORs.

When the time series of the measured variable are not stationary, an equilibrium is lacking in the data, whereas the presence of an equilibrium was the assumption for the theory of critical slowing down as was discussed in the previous section (Dakos et al., 2015). The data should then

first be processed so that possible trends or nonstationary aspects are removed (also known as detrending the timeseries) and then the variance or autocorrelation can be calculated (Dakos et al., 2012). To calculate the non-periodicity is not a detrending procedure, but with a similar effect, because the diurnal pattern was subtracted from the original data. The outcome is informative in itself as was shown in chapters 4 and 5, but an additional step could consist of the calculation of the variance and lag-1 autocorrelation of the calculated differences between the sinusoid and the actual values at the successive timepoints. Then the variance and autocorrelation of the regularity pattern could be tested as DIORs. Another option is to use daily averages of behavioural data instead of hourly data. Daily averages will be more comparable over time, with a preferred daily average level. However, when using daily averages in the critical slowing down calculations, the fluctuations within a day will be missed. In addition, it would also require much longer measurements as at least 50 datapoints in a timeseries seem to be needed for appropriate calculation of critical slowing down indicators (Gijzel, 2020).

Other variables or calculations might also serve as (D)IOR. As additional variable the synchrony of one animal in relation to the group or herd is suggested. Synchrony is considered as important in group living animals such as cows and pigs (ref). Separation or management that interferes with this synchronicity might induce stress and therefore could influence the resilience of the animals (Arey, 1999; Proudfoot & Habing, 2015).

As additional calculation, others tested whether increased skewness of the data, or increased cross-correlation between data from different physiological subsystems could be used as indicators for reduced resilience (Gijzel, 2020; Scheffer et al., 2018). Also the loss of complexity of biologic signals as indicator of impaired ability to adapt to physiologic stress, thus reduced resilience, was described (Goldberger et al., 2002; Lipsitz & Goldberger, 1992). Loss of complexity of the stochastic behaviour of physiological variables can be captured using chaos theory and entropy (Gleick, 1987; Goldberger et al., 1990; Goldberger & West, 1992). In addition, a method was described that used the absolute daily sensor data in combination with a random forest algorithm for the prediction of lifetime resilience classification (Ouweltjes et al., 2019; Ouweltjes et al., 2021). These calculations could also be applied to our data and tested in future studies for their applicability as (D)IOR. This shows that there is a lot to further explore in the development and use of (D)IORs in farm animals in relation to disease resilience.

Finally, we also showed in chapters 3, 4 and 5 that combining multiple (D)IORs that relate to different behavioural and physiological systems, improved the prediction of the proxies of resilience as compared to single (D)IORs only. Since resilience should be understood as the outcome of a dynamic process after a challenge or stressor it is indeed relatively unlikely that a single measure can predict the resilience outcome (Gijzel, 2020). Each (D)IOR will individually capture another facet of resilience and combining these indicators will probably improve the identification of resilient animals as more aspects are then taken into account.

6.2 The utility of the concept of resilience and tipping points in production disease of livestock animals

In this thesis the resilience of animals to diseases such as Porcine Respiratory Disease complex (PRDC) in pigs and periparturient disorders in dairy cows was assessed. With regards to the progress of multifactorial diseases, evidence is growing that the shift from a healthy to a diseased state is not always smooth, but can also be more abrupt, like a tipping point that illustrates the shift from a healthy state to an alternative diseased state (Chen et al., 2012). As explained in the introduction, a positive feedback loop can accelerate processes that are involved in the pathogenesis of these diseases and the accelerating processes are suggested to serve as driving force of the phenomenon of tipping points (van Nes et al., 2016). These processes were illustrated with the stability landscapes in Figure 1 in the introduction. The transition from a healthy to a diseased state can also be illustrated by a graph with the influencing factors on the x-axis and with clinical values on the y-axis. The clinical values are used in veterinary practice to diagnose diseases (Figure 4). This figure illustrates that a healthy state (light green bar), determined by normal reference values, can be maintained under a broad range of circumstances, such as for instance, a body temperature range of cows between 38°C and 39°C. At a certain point with an additional challenge (illustrated with the yellow lightning), it can no longer be maintained at the normal level and will get ill, illustrated by the shift to another level (that lies within the light orange bar in figure 4). The new equilibrium can be higher (e.g., fever after infection) or lower (e.g., drop in white blood cell counts or other blood values). The shift from a healthy to a diseased state is indicated by the red arrow 1. Whether this shift appears suddenly or gradually was not tested in this thesis although Figure 3 in chapter 5 suggested the occurrence of a tipping point after a TDS value of 75 points in cows. It often occurs that the recovery of illness requires time and management involvement. The recovery process can be facilitated by an improvement of environmental conditions, so that the animals are comfortable, by extra attention to the provision of feed for the increased energy need and, or by additional medical treatments (red arrow 2 and 3). The figure thus illustrates that external factors can influence resilience so that a tipping point is more or less likely to occur (illustrated by the green arrow from dark to light). We found that influencing factors that better meet the need of animals can cause a shift away from the tipping point from B to A (from light green to dark green). The same challenge (indicated by the yellow lightning) may not induce a tipping point in situation A, whereas a tipping point will occur in situation B. In addition, we found indicators ((D)IORs) that can distinguish between animals that are in state A or state B (in Figure 4). The (D)IORs can be added to the figure with an additional x-axis on the top of the figure.

The absence or presence of specific symptoms, pathogens, or biomarkers have long been available to decide if an animal is ill (orange bar) or healthy (green bar) (Hajer et al., 1988). This thesis and other recent papers have provided indicators of resilience that allow to additionally distinguish between high and low resilient animals. Although these animals are considered healthy according to the normal reference values, they can be more or less prone to develop diseases. The (D)IORs thus allow us to quantify the capacity to remain healthy before the onset of disease, which is a phase prior to the early state of a disease.

The transition from health to disease may also be a more gradual process without a tipping point. In that case in terms of a stability landscape, the ball does not shift to a different valley, but the valley itself changes by becoming flatter. A gradual cure will deepen the valley again. This is illustrated in Figure 5. The figure also illustrates that the indicators of resilience similarly can differentiate between are more (A) and less (B) resilient state and that the influencing factors of resilience also apply to diseases in case the disease process is more gradual.

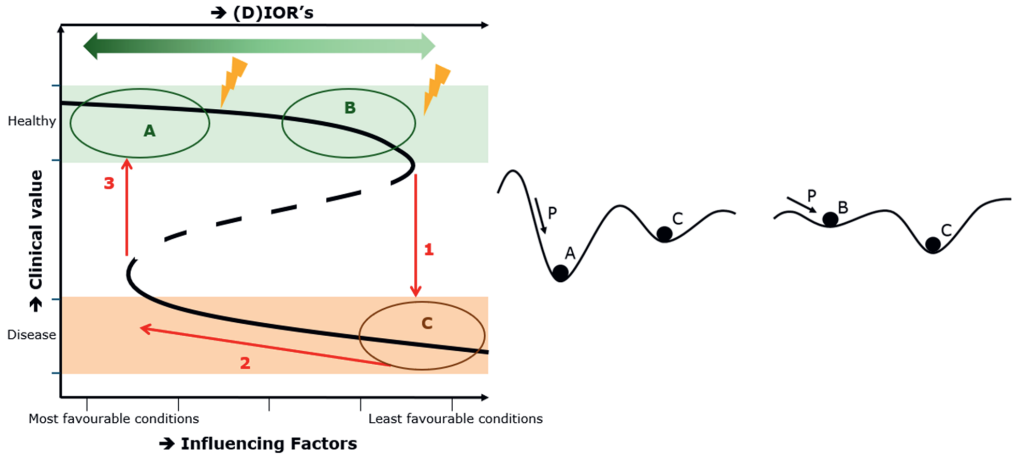

Figure 4. Graphic representation of the binary differentiation between the healthy (green bar) and diseased states (orange bar) of individual animals. The black line in the figure indicates a variable that represents the state of the animal under varying conditions. The binary differentiation between the healthy (A and B) and diseased states (C) can be assessed by (clinical) diagnostic values (e.g., body temperature, blood cell count, heart rate). Depending on the influencing factors (on x-axis below) and additional disturbances (yellow lightning) a healthy, high resilient state (A) might shift towards a healthy, low resilient state (B), indicated by the green arrow to a point that the healthy state suddenly shifts towards an unhealthy state (C), indicated by the red arrow 1. The resilience state is predicted with (D)IORs at the x-axis on top, so that high resilient state A can be distinguished from low resilient state B. Recovery back to the healthy state requires time, improvement of environmental conditions and / or additional treatments indicated by the red arrow 2 until a point that the reverse tipping point is reached to the healthy state again (red arrow 3). The letters A, B and C in the stability landscapes in the right panel correspond with those in the left panel.

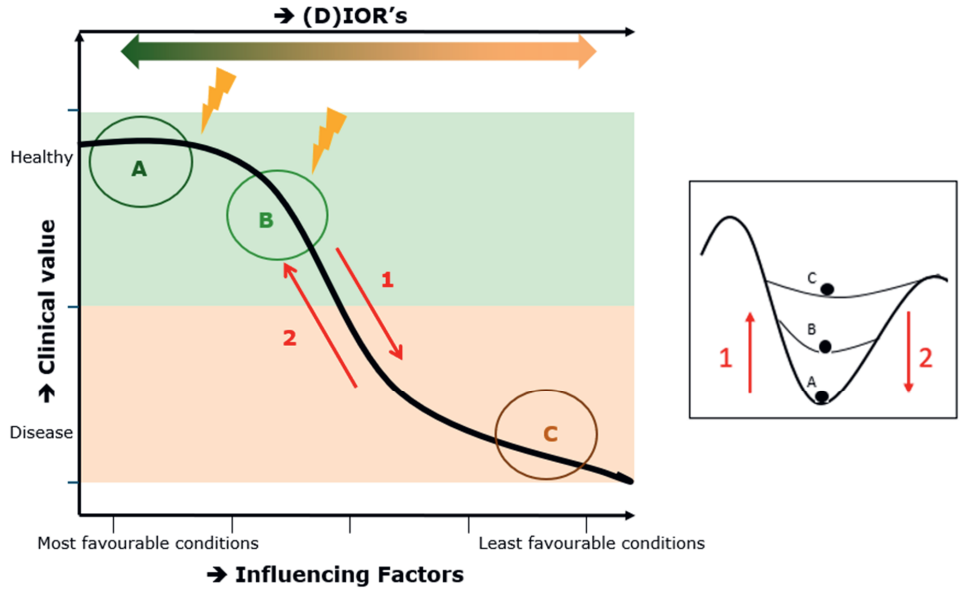

Figure 5. The left panel illustrates a gradual shift from healthy (green) to a diseased state (orange), without a tipping point. Still high resilient state (A) can be distinguished from low resilient state (B) and a diseased state (C). The stability landscape of this situation is shown in the right panel where the valley gradually becomes more and more shallow (red arrow 1) until it is determined as diseased (C). The recovery process proceeds also gradually (indicated by the red arrow 2) until the animal is healthy again (A or B).

6.3 Systems theory perspective – closing the management loop

The measured (D)IORs provide an estimation of the animal's resilience without the need to measure the proxies of resilience themselves. This can be formalised into a concept for a predictive model. Such a model could in turn be used for automation and automated decision support in farm management with the focus on animal resilience. The first steps for such a decision support model were made in chapters 3, 4 and 5 in which possible models were suggested that predict resilience using (D)IORs prior to challenges. In chapter 3, a visualization of the relations between the components of resilience was given (including environmental factors, animal factors, behavioural, immunological and physiological variables and disease severity). In chapters 4 and 5 a multivariable prediction model for resilience in cows was developed and the predictive performance of this model was tested. In this chapter I speculate on a possible conceptual approach for a decision support model with the focus on animal resilience, which is visualised in Figure 6.

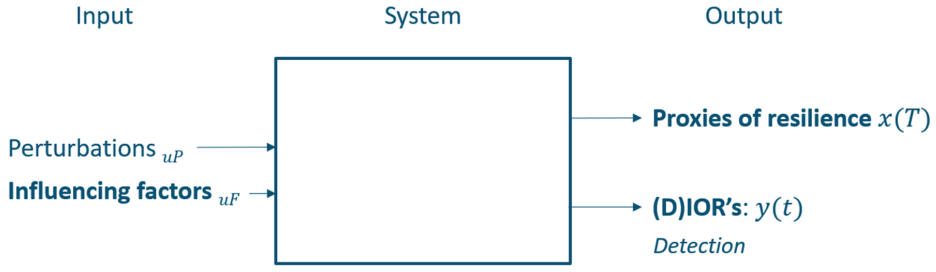

Figure 6. Illustration of the systematic structure that visualises the inputs and outputs of a possible decision support model for animal resilience. To train the model both the (D)IORs prior to challenge and the proxies of resilience after challenge should be monitored. If the (D)IORs give a good representation of the expected proxies of resilience, with adequate sensitivity and specificity, they can be used on farm as measured system output.

The input of the model consists of the animal based and external influencing factors of animal resilience (u_F) and the perturbations or challenges (u_P), which were the disease challenges in this thesis. The outputs of the model are (1) the proxies of resilience $x(T)$ where timepoint T is a timepoint after challenge and (2) the (D)IORs $y(t)$ at timepoints t being timepoints prior to challenge. The DIORs can be followed in time using the appropriate detection methods (which the use of sensors, blood value measurements, camera techniques or other measuring method). The resilience components, being the proxies and indicators of resilience, and their quantitative and dynamics aspects can be described in terms of mathematical equations, inspired by control systems perspectives in precision farming and systems and control theory (van Mourik et al., 2021).

The dynamic state and the measured output of the animal before a specific challenge, can be described in the following dynamic systems form.

$$\frac{dx(t)}{dt} = f(x(t), uF(t), uP(t), t) \quad [1]$$

$$y = g(x(t), uF(t), t) \quad [2]$$

In equation [1] x is a vector describing the animal state of interest, f is a function that describes the dynamics of the animal's state, as function of state x, inputs (uF and uP), and time (or stage in life) (t). In equation [2], g relates the state (x) to the observed output y given the input (uF) at timepoint (t) before challenge. This equation [2] can for example be an equation that is based on the (dynamic) indicators of resilience as described in chapters 4 and 5 predicting the TDS in cows. If x^* is an equilibrium, the function f in equation [1] describes the dynamics of the animal state at timepoint (t) with a value that represents the equilibrium point (the ball at the bottom of the valley) in the stability landscape, as described in Figure 1 in the introduction of this thesis).

Equations [3] and [4] represent the linearization of f and g in x* and u* (as reference input) with matrices A, B, C and D. The shape of a single valley (in the stability landscape) could be analysed through the local linear approximation of the landscape (y in equation [4]), that is influenced by the influencing factors (DuF), and the state of the animal Cx. Cx is the transformation of the possible states of vector x. The matrices provide information on (1) whether the animal state is observable (is it possible to reconstruct the current animal state with the chosen sensing equipment?), (2) whether the states are controllable (i.e. can the state be locally steered by the influencing factors (uF) towards any desired value?), or whether the equilibrium is locally stable (i.e. are there small perturbations possible without the system going to another state?). Matrix A is composed of animal factors like sex, personality, genetic background, and matrix B describes the influence of environmental and other input factors uF on the stability landscape. Matrices C and D are similar matrices as A & B, but with different properties.

$$\frac{dx(t)}{dt} = Ax + BuF \quad [3]$$

$$y = Cx + DuF \quad [4]$$

The input upon perturbation consists of the input caused by a combination of the perturbation uP and the uF. The uP can be seen as the equivalent of the black arrow in the stability landscape. So, the influence of a perturbation (uP) and the shape of the valley uF and on the dynamics of x could be related to the response of the animal after perturbation and described by equation [5]. The final state can then be predicted using equation [6].

$$\frac{dx(t)}{dt} = Ax + B_f uF + B_p uP \quad [5]$$

$$x(T) = x0 + \int_0^T (Ax + B_f uF + B_p uP dt) \quad [6]$$

So, the animal state after perturbation is influenced by the landscape properties ($B_f\, uF$), the perturbation properties ($B_p\, uP$) and the animal factors Ax. The (D)IOR's (y) can be used (with sensing techniques) that give an approximation of the predicted $x(T)$. This leads to the following structure as visualised in Figure 7.

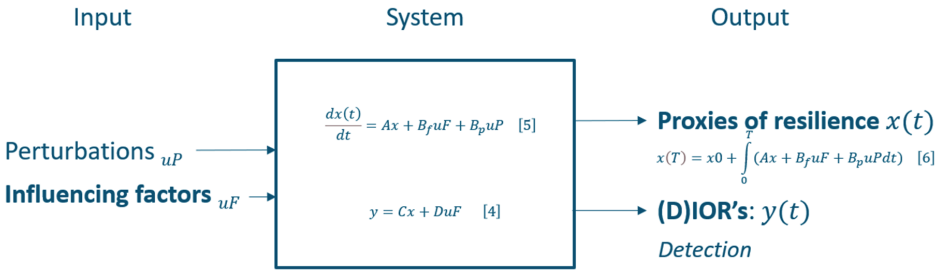

Figure 7. Illustration of the systematic structure and mathematical equations that visualises the inputs and outputs of a possible decision support model for animal resilience.

Next, I suggest adding a 'decision and control element' to the structure which will close the management loop, as is illustrated in Figure 8. The Influencing factors (uF) will change into uCo including the management action. One of the major assumptions of this model is the existence of (local) linear relationships. Perhaps a nonlinear model might fit better. However, this might complicate the analysis for observability, stability and controllability.

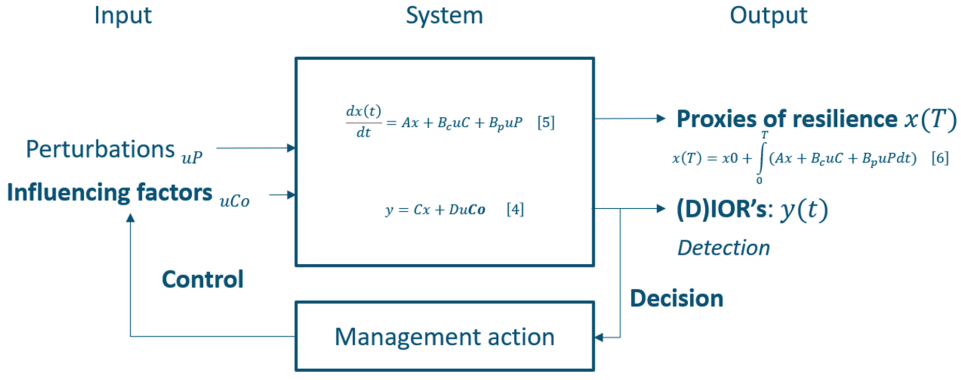

Figure 8. Illustration of a systematic structure for a decision support model and mathematical equations that includes management actions.

Methods from the system and control technique have already proven usable in other areas of agriculture (van Mourik et al., 2021). The suggestion for this decision support model can be seen as a prelude to develop a structured approach to manage farm animal resilience, and calls for transdisciplinary collaboration and follow-up research.

6.4 Concluding remarks

This thesis focused on the identification of influencing factors and predictive indicators of individual animal resilience in relation to multifactorial diseases which are common in current livestock farming systems. We made a distinction between proxies of resilience, predictive indicators of resilience and influencing factors of resilience. Clear indicators and influencing factors can be used in farm management to support and/or improve the resilience of the animals. With improved resilience, the damage of disease challenges will be less severe.

We showed that resilience can be quantified in addition to the diagnosis of being ill or not, with the use of (D)IORs. The (D)IORs that were identified, consisted of static (immunological variables) in pigs and dynamic (behavioural and physiological) variables in cows. The DIORs are the most promising to adequately predict disease resilience, as the dynamics of the variables better reflect how animals cope with their living environment in general and how they react to small disturbances. This appears to forecast how they will react to larger disturbances. The (D)IORs identified in these studies, still need further development and validation before being actually applicable on farms. Future development of wearables, video imaging, other sensor technologies, machine learning and artificial intelligence will facilitate the acquisition of DIORs from all kinds of behavioural and physiological processes. Additionally, a combination of multiple DIORs, may improve the prediction accuracy of disease resilience.

Enrichment was found to be an important influencing factor to improve the resilience in pigs. Enrichment materials (especially organic materials) and biosecurity protocols are often seen as incompatible, but from a resilience perspective I suggest that the benefits of enrichment in terms of disease resilience may outweigh the risks. Apart from the enriched housing regime in pigs, other findings in this thesis support the idea that providing a living environment that more closely meets the needs of the animals will increase disease resilience, such as the alignment of management to the circadian rhythm and to meet the nutritional needs.

Strategies that are meant to increase resilience acknowledge challenges as an inherent part of the system and aim to reduce the negative effects or support a quick recovery. However, in some cases, the strategies that aim to diminish (the risk of) the challenge, may have conflicting effects on measures that promote the resilience of the animals (e.g., biosecurity versus the benefits of enrichment material). The challenge for future animal farming systems lies in integrating strategies that aim to prevent diseases, promote health and increase resilience, all in one.

This thesis provides evidence that animal disease resilience can be influenced and predicted which may be used in farm management. I suggest paying more attention to the resilience of animals as it provides more information to the dichotomous classification of illness. Furthermore, when resilience is improved, the damage of disease challenge will be less severe. Therefore, I recommend shifting focus from production efficiency towards an animal centred focus that aims to enhance animal resilience in balance with livestock productivity in a sustainable fashion.

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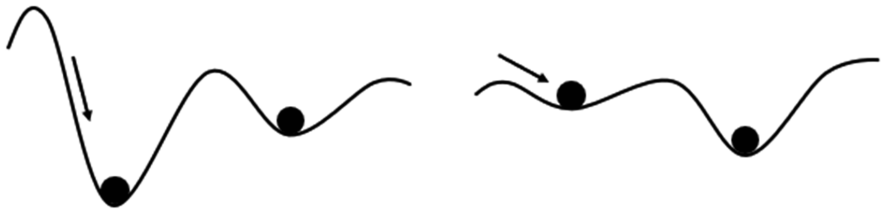
Appendices



Summary

Despite animal health protocols and biosecurity measures, the incidence of diseases in livestock remains high. In combination with the urgency to reduce the use of chemicals including antimicrobials, new strategies are needed to ensure farm animal health. More focus on disease resilience is suggested as an additional strategy to improve animal health. Disease resilience can be described as the ability of animals to be minimally affected by disease challenges and if affected, to recover quickly. At this moment knowledge is limited on how to assess and influence disease resilience in farm animals. Therefore, we studied possible ways to influence disease resilience. In addition, we investigated whether indicators measured pre-challenge can help to predict the resilience of animals.

As a model to study disease resilience in farm animals, the porcine respiratory disease complex in pigs, and in dairy cows, post-partum diseases were used. Resilience can only be assessed after a challenge, measuring proxies of resilience that indicate the severity or duration of clinical or pathological symptoms, the recovery time, or the level of performance after recovery. In this thesis the proxies of resilience were assessed following disease challenge in pigs and after calving in cows, by measuring the intensity and duration of disease related symptoms. In addition, we examined whether a combination of environmental and social enrichment could influence disease resilience in pigs. Subsequently we tested whether individual animal factors could explain variation in resilience between animals. In cows we tested whether static and dynamic physiological and or behavioural indicators measured prior to the challenges could predict resilience, as assessed by the severity and duration of disease symptoms, i.e., the proxies of resilience.

Chapters 2 and 3 focused on disease resilience in pigs. In chapter 2, the influence of social and environmental enrichment on disease resilience was tested following a Porcine Respiratory Reproductive and Respiratory Syndrome Virus (PRRSV) and *Actinobacillus pleuropneumoniae* co-infection challenge. After the challenge, clinical symptoms, the severity of lung lesions, sickness behaviour, rectal temperature and viral clearance were measured as proxies of resilience. We showed that enriched housed pigs had a faster clearance of PRRSV RNA. Moreover, they were less likely to develop lung lesions after infection and the pathological tissue damage score of the lungs was reduced as compared to the pigs that were not exposed to the enrichment. We also found differences in physiological and immune responses that might explain the differences in disease outcome between pigs from the enriched and non-enriched conditions. So, evidence was provided that disease resilience can be improved by creating living conditions for pigs that better meet their behavioural needs. In chapter 3 we showed that a number of variables measured prior to the co-infection challenge predicted the variation in disease outcome in individual pigs and therefore could serve as indicators of resilience (IORs). Pigs with better disease resilience had higher blood levels of lymphocytes prior to the co-infection challenge, more specifically, higher levels of naïve T helper cells, memory T cells, cytotoxic T cells and T helper cells as well as higher relative levels of granulocytes and natural

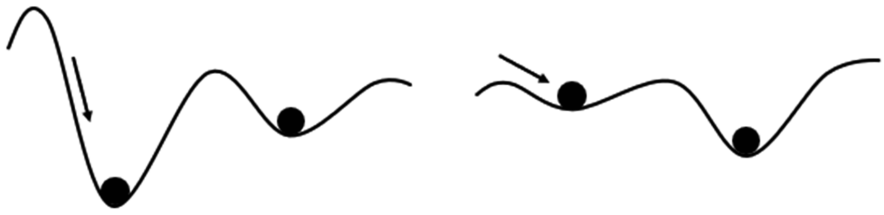
(auto)antibodies. Body temperature and growth prior to infection did not explain variation in the disease outcome. Furthermore, pigs with a more proactive coping strategy showed lower lung lesion scores after challenge as compared to pigs with a more reactive coping strategy.

Chapters 4 and 5 focused on resilience of dairy cows after the transition from the dry period to the lactation phase. To assess individual variation in disease outcome, a Total Deficit Score (TDS) was developed that was composed of all clinical deviations that were collected during the first six weeks after calving, including duration and intensity of clinical symptoms. Prior to parturition, physiological and behavioural activity data were collected using wearable neck and leg sensors. From the sensor data, the average value, variance, autocorrelation and non-periodicity (chapter 4) were calculated and tested as indicators of resilience. The non-periodicity is a measure that gives insight in the diurnal pattern of a variable. In chapter 5 the calculation of a measure derived from the Fast Fourier Transformation was added, including patterns occurring more frequently than once or twice per day. We showed that a higher average level of eating time, a lower level of inactive time, a lower variance in ear temperature and more regularity in daily behaviour including eating, ruminating and (in)activity in the dry period were predictors for increased resilience. Multivariable models that included combinations of IORs and DIORs increased prediction performance for post-partum disease resilience in dairy cows. In chapter 5, the sensitivity and specificity of a number of candidate prediction models were calculated. The best performing models included the averages of eating time and time being inactive, the regularity in rumination, number of steps and lying bouts, and the variance in ear temperature.

In chapter 6, the general discussion, I first discussed the results from this thesis with respect to possible influencing factors and indicators of resilience. The most important findings is, that animal disease resilience can be improved when animal specific needs are better met. The (im)possibilities of the (D)IORS were discussed and some suggestions for further development were given. The most important conclusion is that with a combination of static and dynamic indicators, resilience can be predicted best. In the second section I discussed the utility of resilience and tipping points for livestock production diseases from a more conceptual point of view. Here it was visualised and discussed that indicators of resilience allow quantification of resilience in addition to the dichotomous differentiation between being ill or not. So, even if animals are considered healthy, according to normal reference values, they vary in how prone they are to develop diseases. These (D)IORs thus allow us to quantify the capacity to remain healthy before the onset of disease. In third section I proposed a method to operationalise animal disease resilience using a systems and control perspective. In a prototype decision support model, the relationships are embedded between the proxies, indicators and influencing factors of resilience. The suggestion for this decision support model can be seen as a prelude to develop a structured approach to manage farm animal resilience and calls for trans-disciplinary collaboration and follow-up research.

With this thesis I provided evidence that disease resilience can be influenced and predicted using two production related disease models in pigs and dairy cows. The findings in

this thesis can help to assess and improve animal disease resilience on farms. To improve disease resilience, it seems most crucial to better meet animal-specific needs.



Nederlandse Samenvatting

Ondanks allerlei diergezondheidsprotocollen en bio-security maatregelen blijft de incidentie van ziekten bij landbouwhuisdieren vrij hoog. In combinatie met de noodzaak om het antibiotica en ander medicijngebruik te verminderen, zijn er nieuwe strategieën nodig om de gezondheid van landbouwhuisdieren te waarborgen. Meer aandacht voor de resilience ('veerkracht') van dieren voor ziekten wordt in dit proefschrift als aanvullende strategie gesuggereerd om de diergezondheid te verbeteren en de impact van dierziekten te verminderen. Resilience voor ziekten kan worden omschreven als het vermogen van dieren om met verschillende verstoringen om te kunnen gaan, waardoor ze gezond kunnen blijven of, indien er toch sprake is van ziekte, snel kunnen herstellen. Op dit moment is de kennis beperkt over hoe resilience voor ziekten bij landbouwhuisdieren gemeten en beïnvloed kan worden. In dit proefschrift onderzochten we of resilience voor ziekten beïnvloed kon worden door varkens onder verrijkte omstandigheden te houden. Daarnaast werd bij varkens en koeien onderzocht of indicatoren die vóór de ziekte worden gemeten, kunnen helpen om de resilience van dieren voor ziekten te voorspellen.

Om de resilience voor ziekten te bestuderen, werd bij varkens een model gebruikt van meerdere ziektekiemen die longproblemen veroorzaken. Bij melkkoeien werd de overgangsperiode van de droogstand naar de lactatie gebruikt. Deze periode rond het afkalven is veeleisend voor koeien met een verhoogde kans op het ontstaan van verschillende gezondheidsproblemen. Resilience kan gemeten worden door de reactie op een verstoring te beoordelen. De ernst en de duur van klinische en pathologische symptomen en de hersteltijd kunnen fungeren als maat voor resilience. In dit proefschrift werd de veerkracht bepaald na een ziekte-challenge bij varkens en na het kalven bij koeien, door het meten van de intensiteit en duur van ziekte gerelateerde symptomen. Daarnaast onderzochten we of een combinatie van omgevingsfactoren en sociale verrijking de resilience bij varkens kon beïnvloeden. Vervolgens testten we of individuele dierfactoren de variatie in resilience tussen dieren konden verklaren. Bij koeien testten we of statische en dynamische fysiologische en gedragsindicatoren, gemeten voorafgaand aan het afkalven, de resilience konden voorspellen.

In de hoofdstukken 2 en 3 werd het varkens onderzoek beschreven. In hoofdstuk 2 werd de invloed van zowel sociale als omgevingsverrijking op de resilience van ziekte onderzocht na een co-infectie met het Porcine Respiratory Reproductive and Respiratory Syndrome Virus (PRRSV) en de bacterie *Actinobacillus pleuropneumoniae*. De klinische symptomen, de ernst van longlaesies, het ziektegedrag en de rectale temperatuur werd gemeten. Ook werd onderzocht hoe snel het virusgehalte in het bloed afnam. De verrijkt gehuisveste varkens hadden een kleinere kans om longlaesies te ontwikkelen en de pathologische weefselschade in de longen was minder ernstig in vergelijking met varkens die deze verrijking niet kregen. Ook daalde het virusgehalte in het bloed sneller bij de verrijkt gehouden varkens. We vonden ook verschillen in fysiologische en immunologische reacties tussen varkens die wel of geen verrijking hadden gekregen, wat de verschillen in ziekte-uitkomsten zou kunnen verklaren. Met dit

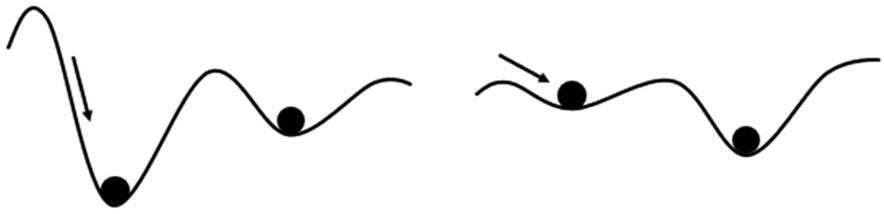
onderzoek is bewijs geleverd dat de resilience voor ziekte bij varkens kan worden verbeterd door sociale- en omgevingsverrijking aan te bieden. In hoofdstuk 3 lieten we vervolgens zien dat een aantal variabelen, welke gemeten waren voorafgaand aan de co-infectie, de variatie in de ziekte-uitkomsten bij individuele varkens konden voorspellen. Deze variabelen kunnen daarom dienen als indicatoren van resilience (IOR's). Varkens met een betere resilience voor ziekte hadden voorafgaand aan de co-infectie een hoger gehalte aan lymfocyten in het bloed. Ze hadden meer naïeve T-helpercellen, memory T-cellen, cytotoxische T-cellen en T-helpercellen evenals een hogere relatieve hoeveelheid granulocyten en natuurlijke antilichamen in het bloed. Lichaamstemperatuur en groei voorafgaand aan de infectie verklaarden de variatie in het ziekteresultaat niet. Bovendien waren varkens met een meer proactieve coping-strategie meer resilient, omdat ze minder ernstige longlaesiescores hadden na de infectie in vergelijking met varkens met een reactieve coping-strategie.

In de hoofdstukken 4 en 5 werd de resilience van melkkoeien bepaald na het afkalven, omdat dan de meest gezondheidsproblemen optreden. Om de individuele variatie in de mate en duur van voorkomende ziekten te beoordelen, werd een Total Deficit Score (TDS) ontwikkeld die was samengesteld uit alle klinische afwijkingen tijdens de eerste zes weken na het afkalven. Hierdoor werden de duur en intensiteit van alle klinische symptomen samengevoegd in één score. Voorafgaand aan het afkalfmoment werd een aantal fysiologische en gedragskenmerken verzameld met behulp van sensoren. Van deze sensorgegevens werden de gemiddelde waarde, de variantie, de autocorrelatie en een zogenaamde non-periodiciteit berekend en vervolgens werd getest of ze als indicatoren van resilience konden dienen. De non-periodiciteit is een maat die inzicht geeft in het dagelijks patroon van een gemeten variabele. In hoofdstuk 5 werd nog een extra berekening toegevoegd die is afgeleid van de Fast Fourier Transformatie. Deze maat houdt ook rekening met patronen die vaker dan één of twee keer per dag voorkomen. De berekeningen die een dynamisch aspect van de data weergeven worden dynamische indicatoren van resilience genoemd (DIORs). We toonden in deze hoofdstukken aan dat een hogere gemiddelde eet-tijd en een kortere inactieve tijd per dag, een lagere variantie in temperatuur gemeten aan het oor en meer regelmaat in het dagelijkse gedrag zoals eten, herkauwen en (in)activiteit tijdens de droogstand, goede voorspellers waren voor een betere resilience. Multivariabele modellen met combinaties van verschillende voorspellers verhoogden de voorspelkracht voor ziekte resilience na het afkalven. In hoofdstuk 5 werden de gevoeligheid en specificiteit van een aantal kandidaat-voorspellingsmodellen berekend. De best presterende modellen bevatten de gemiddelde eet- en inactieve tijd, de regelmaat in de patronen van herkauwen, het aantal stappen en ligbeurten en de variantie van de oor-temperatuur.

In hoofdstuk 6, de algemene discussie, besprak ik eerst de resultaten van dit proefschrift met betrekking tot mogelijke beïnvloedende factoren en indicatoren van resilience. De belangrijkste bevinding is dat de resilience van dierziekten kan worden verbeterd als er beter aan dier specifieke behoeften wordt voldaan. De indicatoren (IORs en DIORs) werden besproken en er werden enkele suggesties gegeven voor verdere ontwikkeling. De belangrijkste conclusie is dat resilience het best voorspeld kan worden met een combinatie van statische en dynamische

indicatoren. Vervolgens besprak ik het nut om resilience bij landbouwhuisdieren toe te passen vanuit een meer conceptueel oogpunt. Aan de hand van een visualisatie heb ik besproken dat resilience indicatoren een kwantitatieve voorspelling van resilience mogelijk maken, welke gebruikt kunnen worden naast het onderscheid tussen 'wel of niet ziek zijn'. Dit laat zien dat dieren die volgens normale referentiewaarden als gezond worden beschouwd, kunnen verschillen in hoe kwetsbaar ze zijn voor het ontwikkelen van ziekten. Met behulp van de (D)IORs kunnen we de capaciteit om gezond te blijven kwantificeren. In het laatste deel van de discussie heb ik een methode voorgesteld om de veerkracht van dierziekten te operationaliseren vanuit een systeem- en regelperspectief, zodat dit op bedrijven gebruikt kan worden. In het voorgestelde prototype model zijn de relaties weergegeven tussen de proxies, de indicatoren en mogelijke beïnvloedende factoren van resilience. Dit prototype model kan worden gezien als een opmaat voor de ontwikkeling van een meer gestructureerde aanpak om de resilience van landbouwhuisdieren te stimuleren en vraagt om trans disciplinaire samenwerking en vervolgonderzoek.

Met dit proefschrift heb ik aan de hand van de twee casussen, bewijs geleverd dat resilience voor ziekten beïnvloed en voorspeld kan worden. De bevindingen in dit proefschrift kunnen helpen om de resilience van dieren te beoordelen en te verbeteren. Op dit moment lijkt het cruciaal om meer tegemoet te komen aan dier-specifieke behoeften wanneer we de resilience van onze landbouwhuisdieren voor ziekten willen verhogen.



Dankwoord

In 2009 deelde Sierk Spoelstra, destijds afdelingshoofd van systeeminnovaties, op een vrijdagmiddag zijn inzichten over het boek van Marten Scheffer, getiteld "Critical Transitions in Nature and Society". Hierin beschrijft Marten dat complex dynamische systemen, ondanks hun intrinsieke vermogen om een bepaalde toestand te handhaven, onder invloed van externe omstandigheden kunnen omslaan naar een ongewenste toestand, bekend als 'Tipping Point'. Het vermogen om de gewenste toestand te behouden, of snel naar toe terug te keren, is de veerkracht van het systeem en het blijkt dat er universele indicatoren bestaan die de nabijheid van zo'n Tipping Point voorspellen. Sierk opperde: "Zou het niet kunnen zijn dat de overgang van gezond naar ziek bij dieren, inclusief de mens, ook zo'n tipping point is? En als dat het geval is, zijn er dan indicatoren die kunnen voorspellen in welke mate dieren ziek kunnen worden?" Dit idee vormde de basis voor het onderzoek in dit proefschrift, dat daar, die middag werd geïnspireerd.

Nu, veertien jaar, zeven onderzoeksprojecten, vijf experimenten en (bijna) 11 artikelen later, ligt er nu eindelijk dit boekje. Het zal overduidelijk zijn dat ik immense dankbaarheid voel, zowel in het algemeen als voor iedereen in het bijzonder die op welke manier dan ook heeft bijgedragen aan het tot stand komen, de uitvoering en de afronding van dit traject. Dank, Norbert Stockhofe-Zurwieden en Bas Kemp als mede-initiators van dit alles.

In het bijzonder wil ik mijn (co)promotoren Annemarie Rebel, Liesbeth Bolhuis, Kees van Reenen en Simon van Mourik bedanken, jullie waren er vanaf het begin bij en een grote bron van inspiratie en steun geweest. Kees, onze samenwerking is uitgegroeid tot een dierbare vriendschap. Jouw moed, doorzettingsvermogen en wetenschappelijke motor zijn bewonderenswaardig. Het zal jou helpen om door het aankomende traject, alias 'het parallelle universum', te navigeren. Rudi de Mol, Joop van der Werf, Dennis te Beest, Sabine Schnabel en Johan van Riel, jullie waren mijn data bewaarders, bewerkers, rekenwonders en statistische basis. Dank: VKON, Michiel Vreriks en Marije Thybaut, voor de perfecte organisatie van de complexe veerkracht proeven, Joost de Veer en Gerrit Hegen voor het veerkracht denken en veterinaire inbreng. Alle coauteurs en dierverzorgers op de Edelhert-, Runder- en Houtribweg, en Dirk Anjema voor het mogelijk maken van het onmogelijke.

Dank aan Gertia en Mieneke, mijn zussen en steun en toeverlaat. Het is verdrietig dat onze ouders recentelijk zijn overleden. We realiseren ons hoe papa en mama voortleven in ons, en welke fundamenten ze hebben gelegd om ons te vormen tot wie we zijn. Wat zouden ze trots zijn geweest op ons alle drie. Dank Ier dat je mijn paranymf bent en voor al je gesprekken, energie en humor. Tot slot dank Damesch'90 voor al het kant noch wal gebeuren.

Helaas hebben we ook afscheid moeten nemen van Geert André, hij stond aan het begin van dit project en ontwikkelde een dynamisch model voor hartslagvariabiliteit dat zijn tijd ver vooruit was en Else Wijnhof, zij verzorgde zowel de foto als het ontwerp van de omslag. ✿

Lieve Peter, ons leven kan je samenvatten als complex dynamisch systeem, waarbij jij mijn basin of attraction bent en onze kinderen Jente, Sigrid, Diederik, Ragnar, Quirijn en Louis ons leven verrijken. Jullie vormen mijn veerkracht! Lieve Jente, Sigrid en Diederik, ook al ben ik trots op het proefschrift, het meest trots ben ik op jullie. Ik hoop als voorbeeld voor jullie te mogen zijn door te laten zien dat als je ergens in gelooft, met de juiste steun en begeleiding, het meer dan de moeite waard is ervoor te gaan, ook al duurt het traject wat langer.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Rik Borgerink
Mieneke van Dixhoorn
Joop van der Werf
Iris Schreuder
Sierk Spoelstra
Marlies Immink
Daan en Femke
Brigitte de Bruijn
Arnold van Zoelen
Quirijn
Eveline de Pont
Inonge Reimert
Karianne Lagendaal
Brenda Dambrink
Lies en Martin Jonsson
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Rudie Lammers
Egbert van Nes
Jan ten Nap
Tjeerd Kimm
Michiel V
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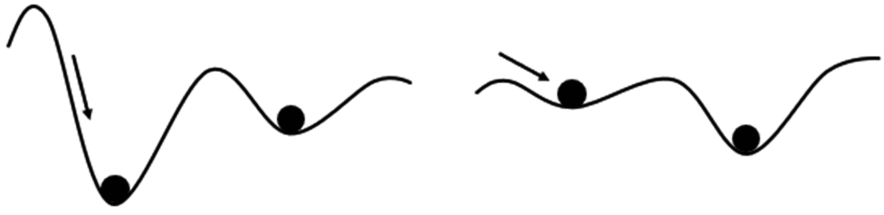
Versteeg
Annemieke Mintjes
Thybaut
Marien Gerritzen
Kluivers
Beatrijs van der Griendt
Jan Anne Kuipers

A



About the author

Ingrid van Dixhoorn was born on August the 7th, 1970 in Wageningen the Netherlands. She completed her pre-university education at the Marnix College in Ede and the Palmcrantz-Skolan in Östersund, Sweden.

Ingrid continued her education at the faculty of Veterinary Medicine in Utrecht and graduated in 1998. She started her career at the department of anatomy, followed by the department of ruminant health, obstetrics, and reproduction at the Faculty of Veterinary Medicine in Utrecht. Between 2002 and 2007 she worked as a bovine practitioner in Leerdam and Beilen, the Netherlands.

She currently works as a senior scientific researcher at Wageningen Livestock Research at the department of animal health and welfare. Her research focusses on the application of resilience thinking to livestock production systems at different system levels and the use of (sensor) data to predict, manage and improve animal health and welfare.

List of publications

This thesis

Van Dixhoorn, I. D. E., Reimert, I., Middelkoop, J., Bolhuis, J. E., Wisselink, H. J., Koerkamp, P. W. G. G., Kemp, B., & Stockhofe-Zurwieden, N. (2016). Enriched housing reduces disease susceptibility to co-infection with porcine reproductive and respiratory virus (PRRSV) and actinobacillus pleuropneumoniae (A. Pleuropneumoniae) in young pigs. PLoS One, 11(9). https://doi.org/10.1371/journal.pone.0161832

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de Bruijn, B. G. C., **van Dixhoorn, I. D. E**., Bolhuis, J. E., Kluivers, M., Stockhofe-Zurwieden, N., & Rebel, J. M. J. Environmentally enriched housing conditions reduce disease severity in pigs after co-infection with Porcine Reproductive and Respiratory virus (PRRSV) and Actinobacillus pleuropneumoniae, with stronger enrichment effects when applied from birth than from weaning. In preparation for submission.

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PhD portfolio

The Basic Package (3 ECTS[1])	year
WIAS Introduction Course	2013
Course on philosophy of science and ethics	2014

Disciplinary Competences (10.5 ECTS)	
Methodical approach to engineering design	2007
Use of Laboratory Animals	2011
Basic statistics	2012
Statistics time series analysis and forecasting	2012
Bayesian statistics	2012
Ultrasound theory and practical use in the abdominal cavity	2013
Haematology refresher course	2014
Neonatology and paediatrics in cats and dogs	2014
Resilience of living systems	2018
Systematic reviews of animal studies	2020

Professional Competences (4.9 ECTS)	
Kenneth Smith basic course, Expert in Sales	2011
Writing for academic publication	2012
Waarderend auditing TNO management consultants	2014
WIAS Course The Final Touch: Writing the General Introduction and Discussion	2021

Societal Relevance (ECTS 2.6)	
Organisation of the course on ultrasound use of the abdominal cavity	2016
Symposium Resilience, January 19th Bathmen the Netherlands	2017
Membership College van deskundigen KoeKompas	2012-2017

Teaching competences (maximised on 6 ECTS)	
Supervising 2 BSc, 6 MSc Students and 1 internship	2008-2023
Wageningen Academy: Verlenging levensduur van melkvee	2014
Farm technology course: behavioural patterns as indicators for resilience	2016-2017
Global One Health Summer course: August 28, 2020: healthy and resilient livestock	2020

Presentation Skills (Maximised on 4 ECTS)	
World Buiatrics Congress, Budapest Hungary, July 6-11, oral	2008
DSI European user group meeting, Berlin Germany, March 21-22, oral	2013
ECPLF, Leuven Belgium, September 10-12, oral	2013
WAFL, Clermont-Ferrand France, September 3-5, poster	2014
DSI European user group meeting, Leeds England, January 29-30, oral	2015
Production Disease in farm animals, Wageningen, the Netherlands, June 20-23, oral	2016
WIAS science day, Wageningen, the Netherlands February 6, oral	2017
AgEng, Wageningen the Netherlands July 8-12, oral	2018
Anco symposium, Vienna Austria, November 7, oral	2019
WIAS science day, Wageningen the Netherlands, April 28-29, oral	2021
EAAP, Lyon France, August 26-31, oral	2023

[1]European Credit Transfer and Accumulation System (ECTS). One EQTS credit equals a study load of 28 hours.

Colophon

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