University of Antwerp
Faculty of Pharmaceutical, Biomedical and Veterinary Sciences

Faculty of Pharmaceutical, Biomedical and Veterinary Sciences
Department of Veterinary Sciences

Saliva as a biological matrix to assess piglet's welfare

PhD thesis submitted for the degree of Doctor in Veterinary Sciences at the University of Antwerp to be defended by Sara Prims

Supervisors: Prof. dr. Chris Van Ginneken, Prof. dr. Xaveer Van Ostade, Prof. dr. Christophe Casteleyn

Antwerp, 2024

Cover lay-out: Natacha Hoevenaegel, Nieuwe Mediadienst, University of Antwerp
Cover images: Julia (6), Gust (3)

Composition of the PhD Examination Committee

Supervisors

Prof. dr. Chris Van Ginneken
Laboratory of Comparative Perinatal Development, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Wilrijk, Belgium

Prof. dr. Xaveer Van Ostade
Protein science, proteomics and epigenetic signaling (PPES), Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Wilrijk, Belgium

Prof. dr. Christophe Casteleyn
Laboratory of Comparative Perinatal Development, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Wilrijk, Belgium

Members of Internal Committee

Prof. dr. Jean-Pierre Timmermans
Laboratory of cell biology and histology, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Wilrijk, Belgium

Prof. dr. Dirk Adriaensen, emeritus
Laboratory of cell biology and histology, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Wilrijk, Belgium

Prof. dr. Peter Delputte
Laboratory for Microbiology, Parasitology and Hygiene (LMPH), Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Wilrijk, Belgium

Members of External Committee

Prof. dr. Ana-Maria Gutiérrez Montes
BioVetMed Research Group, Department of Animal Medicine and Surgery, Veterinary School, CEIR Campus Mare Nostrum (CMN), University of Murcia, Murcia, Spain

Prof. dr. Friederike Ebner
Infection Pathogenesis, Technical University of Munich, Freising, Germany

Table of contents

List of abbreviations

11β-HSD1	11β-hydroxysteroid-dehydrogenase type 1
11β-HSD2	11β-hydroxysteroid-dehydrogenase type 2
2D-LC	Two-dimensional liquid chromatography
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
a.m.	*Ante meridiem,* before midday
ACN	Acetonitrile
ACTH	Adrenocorticotropic hormone
ADG	Average daily gain
AGC	Automatic gain control
APP	Acute phase protein
AUC	Area under the curve
AVP	Arginine-vasopressin
BLAST	Basic Local Alignment Search Tool
CAs	Catecholamines
CD	Cluster of differentiation
CN	Cranial nerve
CO_2	Carbon dioxide
CRH	Corticotropin-releasing hormone
d	Days
Da	Dalton
DNA	Deoxyribonucleic acid
E. coli	Escherichia coli
E.g.	*Exempli gratiā,* for example
ELISA	Enzyme-linked immunosorbent assay
FA	Formic acid
FDR	False discovery rate
GCs	Glucocorticoids
GH	Growth hormone
h	Hours
H&E	Haematoxylin and eosin
H_2O	Hydrogen dioxide
H_2S	Hydrogen sulphide
HCD	High energy collision activated dissociation
HCl	Hydrochloride
HPA	Hypothalamic-pituitary-adrenal

HPG	Hypothalamic-pituitary-gonadal
HPLC	High-performance liquid chromatography
i.c.	*In casu,* in this case
I.V.	Intravenous
IEF	Isoelectric focusing
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IL	Interleukin
iTRAQ	Isobaric tags for relative and absolute quantification
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
LC	Liquid chromatography
LPS	Lipopolysaccharides
m/z	Mass/charge
MALDI	Matrix-assisted laser desorption/ionization
min	Minutes
MMTS	Methyl methanethiosulfonate
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular weight
NH_3	Ammonia
NPY	Neuropeptide Y
p.m.	*Post meridiem,* after midday
PAS	Periodic Acid-Schiff
PCV2	Porcine circovirus type 2
pI	Isoelectric point
PLUNC	Palate lung and nasal epithelium clone
POMC	Pro-opiomelanocortin
ppm	Parts per million
PRM	Parallel reaction monitoring
PRRSV	Porcine reproductive and respiratory syndrome virus
PVN	Paraventricular nucleus
RNA	Ribonucleic acid
RP	Reversed phase
s	Seconds
S/N	Signal-to-noise
SAM	Sympathetic adrenomedullary
SCX	Strong cation exchange
SD	Standard deviation
SDS	Sodium dodecyl sulphate

SGLT-1	Sodium-glucose linked transporter 1
sIgA	Secretory immunoglobulin A
SO_2	Sulphur dioxide
SPE	Solid phase extraction
sPRM	Scheduled parallel reaction monitoring
TCEP	Tris-(2-carboxyethyl) phosphine
TEA	Total esterase activity
TEAB	Triethylammonium bicarbonate
TMT	Tandem Mass Tag
TNF	Tumour necrosing factor
TOF	Time of flight
TR-IFMA	Time-resolved immunofluorometric assay
ρ	Spearman's rank correlation coefficient

Chapter I - General introduction

I.1 Preface

Pigs in farm settings are inevitably exposed to several stressors, such as regrouping [1-3], castration [4], and road transport [5]. When a stressor exceeds a certain threshold in duration and/or magnitude, the body's homeostasis is disturbed. The equilibrium can be re-established by physiological and behavioural adaptive responses and/or by removing the stressor. However, failure to generate sufficient adaptive responses leads to chronic stress, implying compromised animal welfare and lower profit due to a suppressed immune system (e.g., [6, 7]), reduced zootechnical performance such as reduced daily weight gain and feed conversion (e.g., [3, 8]), and disturbed breeding performance (e.g., [9, 10]). It has been proven that the effect of different stressors is generally cumulative, meaning that one stressor leads to less severe consequences than two, which in their turn are less severe than three [3]. Therefore, identifying possible stress at the farm, acute or chronic, can provide helpful information for the farmer. A fast, easy, and reliable tool to monitor stress will aid this mission. This tool can also help to evaluate different management strategies and interventions in agricultural research. Thirdly, easy tools to assess stress in pigs can be valuable to evaluate welfare on the farm, promote good practices and elicit transparency to the consumer (Fig. 1). Numerous methods to assess pig's welfare exist, unfortunately, all with their own limitations. As a result, no definitive set of parameters or indicators of animal welfare exists. Therefore, the goal of the studies described in this doctoral thesis was to explore saliva as a source for potential biomarkers to assess chronic stress in piglets.

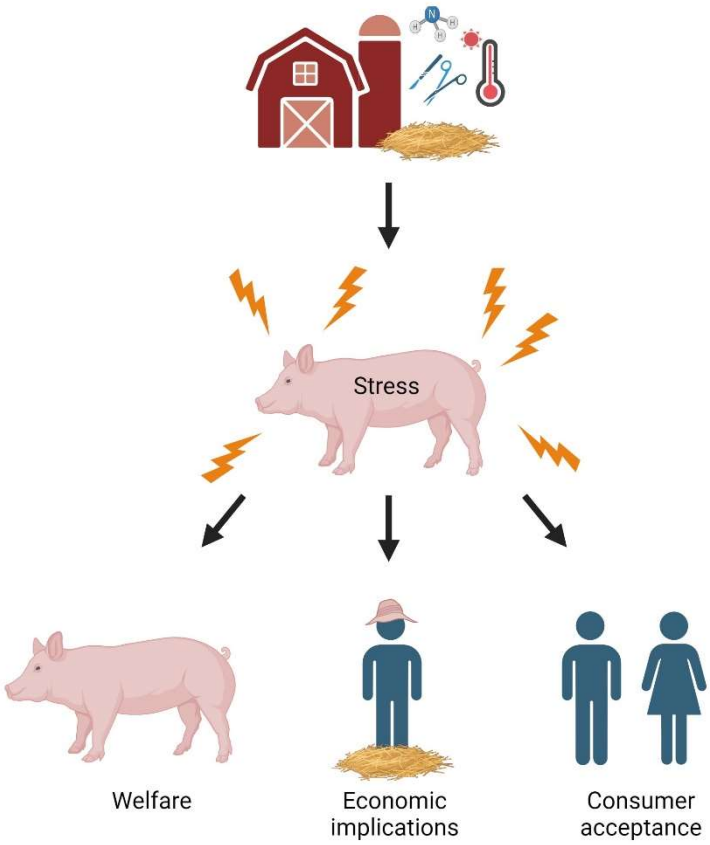


Figure 1. Importance of identifying and eliminating stress. *Stress does not only affect animal welfare, additional to the economic consequences for the farmer, consumer acceptance depends more and more on this factor as well. Created with BioRender.com.*

I.2 History of "*stress*"

The term "stress" was borrowed from physics, alluding to forces applied on a material, and was first used outside the domain of physics by Hans Selye in the 1930s to describe the "*non-specific response of the body to any demand upon it*" [11, 12]. While, according to many dictionaries, stress derives from the Latin *stingere*, meaning "to bind" or "draw tight" [13]. The term "stress" is relatively new, but the concept of stress has a long and complex history (for reviews, see e.g., [13-15]), going back to circa (c.) 500 BC when Heraclitus said that a natural condition does not equal an unchanged state but rather the capacity to undergo and cope with constant changes. Later, Empedocles (c. 450 BC) suggested that all matter consists of different elements and that the survival of living organisms relies on the balance and harmony of these. At the same time, Hippocrates stated that health resulted from of a harmonious balance of all elements and that disturbing forces that could lead to disease are

of natural sources and should therefore be counterbalanced naturally. In the 17th century, Thomas Sydenham described that the individual's adaptive response to restore the balance could result in direct pathological changes [14]. William Osler (19th century) expanded this theory by describing the idea that the body's response to stress could have long-term consequences by suggesting that the typical heart diseased patient were hard-working, ambitious men [16]. Later, the idea that the word *stress* could also be used to refer to the inner state instead of only indicating physical strain or injury slowly emerged. Claude Bernard (19th century) described the harmony of elements as a dynamic internal physiological equilibrium, "le milieu intérieur" [17], while Walter Cannon introduced the term "homeostasis" and broadened the term to both the physical and emotional state [18]. Cannon was the first to describe increased adrenal secretion in animals subjected to stress and called it the "fight or flight" response [19] which brought attention to the involvement of the sympathetic adrenomedullary (SAM) system in the stress response. Hans Selye, sometimes referred to as the father of stress research [12], proposed the "General Adaptation Syndrome", a theory of coping with stressors that involves three stages. The initial alarm reaction, similar to the "fight or flight reaction", is followed by an adaptation phase with resistance to the stressor, and eventually, a stage of exhaustion and organismic death [20]. The last phase results in, for example, enlargement of the adrenal glands, atrophy of lymphoid tissue in the thymus, spleen, and lymph nodes, and bleeding gastrointestinal ulcers [21, 22].

Stress can, therefore, be seen as a physical or emotional (or metabolic or immunological) force that threatens the body's homeostasis. The subject will attempt to re-establish this homeostasis through both physiological and behavioural adaptive responses. According to Chrousos and Gold, any stressor that exceeds a certain intensity, duration, or severity threshold could lead to a diseased state [14].

I.3 The modern concept of stress

An individual will experience stress when there is a sense of threat to its homeostasis. The specific responses to these threats will, to some degree, depend on the character of the threat that is perceived [27, 28]. In other words, there is no such thing as "the" stress response. Different types of stressors will result in different responses. Making an interpretation of these

responses even more challenging is that the individual's perceptions of the stressors and the ability to cope with these will vary between individuals [23, 24].

I.3.1 Stress responses

Stress responses are initiated when a subject experiences an acute sense of threat. The involved mechanisms modulate physiological and behavioural adaptations to restore the body's homeostasis. First, a fast, almost reflexive response involves activating the sympathetic nervous system, initiating the so-called "fight-or-flight" through the SAM response. This is followed by a slower, hormone-driven, amplified, and protracted secondary response through the hypothalamic-pituitary-adrenal (HPA) axis (Fig. 2) (e.g., [25]).

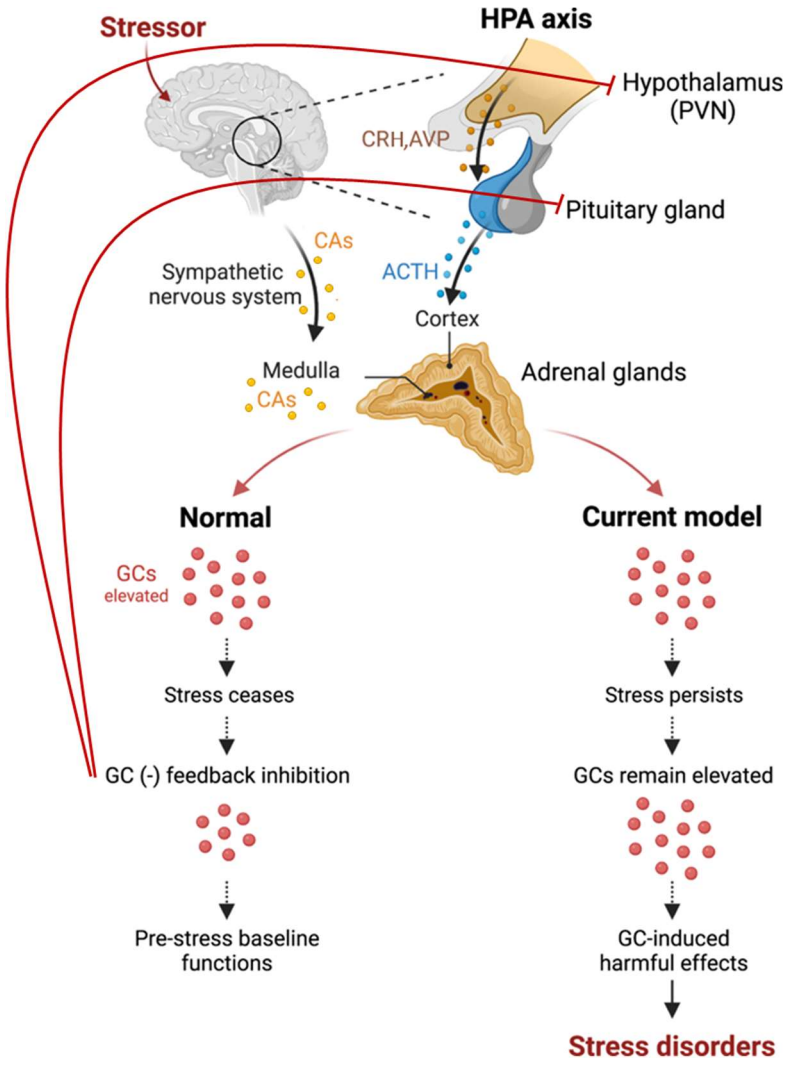


***Figure 2**. **Schematic overview of neuroendocrine stress responses.** Left: A normal, healthy response to an acute stressor, after which pre-stress baseline levels are re-established once the stress ceases. The negative feedback system of glucocorticoids inhibits the release of hormones both at the level of the hypothalamus and at the pituitary glands. Right: The current*

glucocorticoid model of direct stress effects suggests that repetitively elevated glucocorticoids to stressors lead to stress-related disorders. PVN: paraventricular nucleus of hypothalamus, CRH: corticotropin-releasing hormone, AVP: arginine-vasopressin, ACTH: adrenocorticotropic hormone, CAs: catecholamines, GCs: glucocorticoids. Adapted from [25].

A stressor activates the SAM axis, leading to the secretion of catecholamines by the adrenal glands. These catecholamines, epinephrine and norepinephrine, are secreted into the bloodstream and induce a rapid physiological adaptation to increase alertness, vigilance, and focussed attention to aid the subject in making a strategic decision at the start of a stressful challenge (Fig. 3). The physiological changes entail cardiovascular changes and metabolic actions leading to increased blood glucose through glycogenolysis and gluconeogenesis, lipolysis, increased oxygen consumption, and thermogenesis (e.g., [26, 27]). When the stressor has been dealt with, the physiological and adaptive responses are counteracted by activating the parasympathetic nervous system.

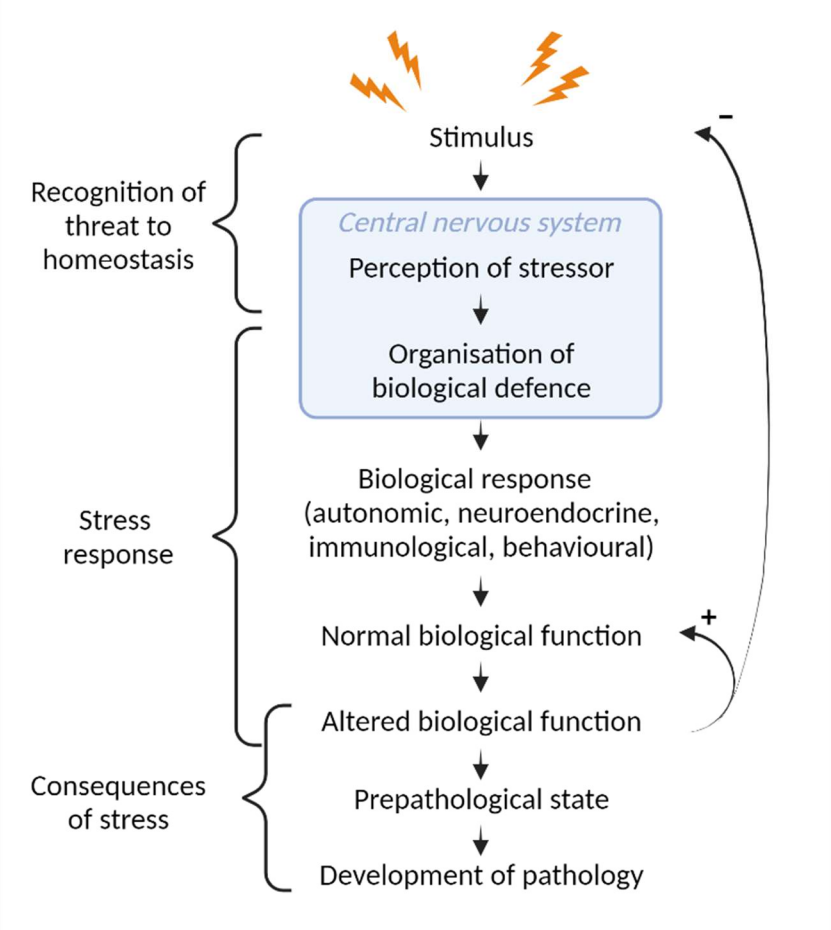


Figure 3. Overview of the biological responses and consequences of stress. *Adapted from [28] with BioRender.com*

Simultaneously, the stressor activates a slower response through modulation of the HPA axis. This hormonal response system is present in humans, pigs, other mammals, and even birds [29]. Upon stimulation, neurons in the paraventricular nucleus (PVN) of the hypothalamus release the corticotropin-releasing hormone (CRH) and other secretagogues such as arginine-vasopressin (AVP). The former hormone is transported through the hypophyseal portal circulation to the anterior pituitary gland (adenohypophysis) where it initiates the cleavage of pro-opiomelanocortin (POMC) into adrenocorticotropin (ACTH), β-endorphin, and other peptides, and their subsequent release from the anterior pituitary gland into the bloodstream. The ACTH signal is carried through the peripheral circulation to the adrenal glands, where it triggers the release of glucocorticoids and adrenal androgens from the adrenal cortex (e.g., [25, 30]). Glucocorticoid receptors are present in almost all tissues of the body. Therefore, cortisol can affect nearly every organ system. Glucocorticoids can have catabolic, lipogenic, immunosuppressive, and anti-reproductive effects and may influence the cardiovascular system and behaviour [31]. The most studied glucocorticoid in relation to stress is cortisol, probably due to its widespread regulatory influences (e.g., [32, 33]).

Circulating glucocorticoids suppress the secretion of CRH from the hypothalamus and directly inhibit ACTH secretion from the pituitary gland. Additional feedback loops include the inhibitory effects of ACTH, β-endorphin and CRH itself on the hypothalamic CRH neurons [34]. This negative feedback mechanism limits the duration of the body's exposure to the effects of these glucocorticoids.

It might be clear that the activation of the sympathetic nervous system and the HPA axis in response to a stressor results in the release of a variety of hormones, neuropeptides, and neurotransmitters. Bidirectional communication between the nervous, endocrine, and immune systems through the shared use of these ligands and receptors leads to a broad spectrum of biological and behavioural consequences.

I.3.2 Acute vs. chronic stress

Not all stress is bad. Like Hans Selye stated: "Stress is the Spice of Life; the absence of stress is death". Acute stress is essential for our survival and not harmful in its essence. Only when the animal fails to restore its homeostasis, we speak of chronic stress that may result in stress symptoms with a pathological character. This can result from of a single major traumatic acute

stressful event or could be induced by the duration or magnitude of repeated single or multiple stressors (e.g., [35]).

While acute “fight-or-flight“ responses help the individual respond and adapt to an acute stressor, this adaptation mechanism is useless for chronic stress. Similarly, elevated glucocorticoid levels may be effective in the acute phase but are noxious when they persist over a long period. Chronic stress can lead to hyperreactivity of the adrenal cortex and result in an exaggerated ACTH response to new acute stressors [36]. Eventually, chronic stress will affect the body in many ways, short-term, and long-term (e.g., [3, 6-10]).

I.3.3 Stress and animal welfare

Stress and welfare are inevitably connected. However, as mentioned before, a mild acute stressor is not necessarily bad if the biological cost of the stress response is met by the body’s reserves. When this point is crossed, thus when the animal fails to restore its homeostasis due to inadequate, inappropriate, or excess activation of the compensatory system, one speaks of welfare-threatening stress [37]. This type of harmful stress can affect the immune system (e.g., [6, 7]), reduce zootechnical performance (e.g., [3, 8]) and disturb breeding performance (e.g., [9, 10]). As a consequence, one could say that if an animal is healthy and performing well, it is faring well. However, in this definition of welfare the animal’s feelings are not recognised. Therefore, another view on animal welfare states that only if an animal also feels well, it is faring well. Lastly, one could also argue that an animal will feel most comfortable if it is free to express natural and normal behaviour [38].

One often used definition of Animal welfare includes these three concepts. According to the Farm Animal Welfare Council (FAWC)[39], an animal is in a good state of welfare when there is:

- Freedom from thirst, hunger and malnutrition
- Freedom from thermal and physical discomfort
- Freedom from pain, injury and disease
- Freedom from fear and distress
- Freedom to express normal behaviour.

This definition implies that we know how to guarantee these freedoms. The freedom of thirst is relatively easy to fulfil, supplying unlimited water access. However, is it as easy to guarantee

freedom of fear? This suggests that we know what causes fear in animals, and that this is the same for all individual animals. Or would it be more accurate to identify fear? Or even better, the absence of fear? Welfare scientists are challenged to find methods to identify both good and hampered animal welfare.

I.4 How can we monitor chronic stress?

As mentioned, stress is perceived when a stimulus threatens the body's homeostasis. As a result, the subject will attempt to re-establish its homeostasis through both physiological and behavioural adaptive responses. As such, stress can be monitored by focusing on different parts that comprise these stress responses (Fig. 4): 1) causal indicators: these include indicators that cause stress, such as high temperatures; 2) biological response indicators: these are indicators that measure the physiological responses, the most well-known are cortisol and catecholamines, but also immunological and behavioural responses; 3) consequence indicators: such indicators are specific for the consequences of these physiological responses, which are altered biological functions or (pre-)pathological processes [40]. There is already a wide range of possible indicators for chronic stress available, however, as will become clear from the following overview, each technique has its limitations. The complexity of chronic stress, which is characterized by the diverse responses to different stressors, the individual coping mechanisms, the add-on effect of different stressors, previous exposure to stressful events, etc. contributes to the variability of responses and consequences, making identification of chronic stress challenging.

I.4.1 Causal indicators

A significant task of welfare scientists is identifying possible stressors to eliminate or prevent these in farm settings. Many different stressors have been identified, of which physical and mental stressors are probably the most challenging to identify, followed by immunological, metabolic, and toxic stressors.

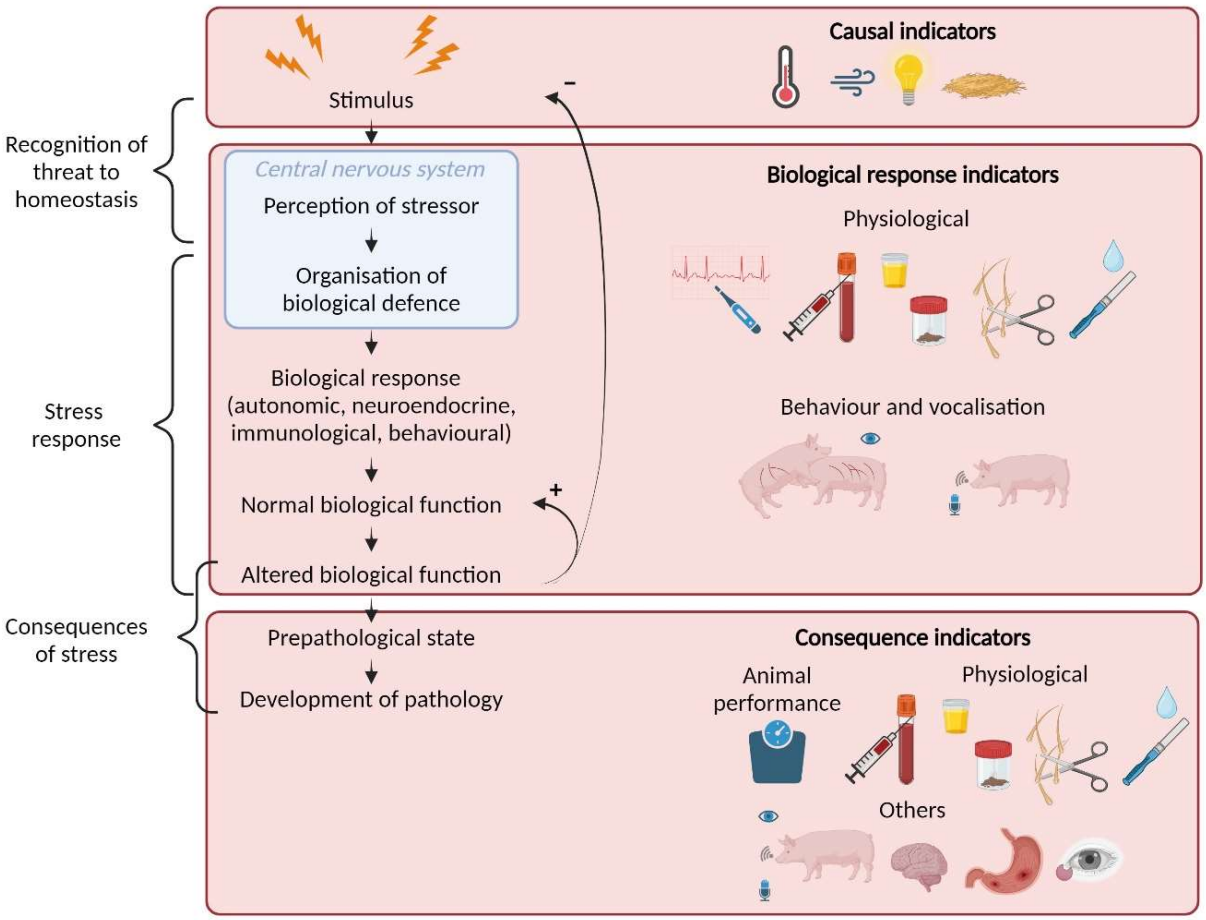


Figure 4. Overview of different types of indicators for stress that were described in this introduction. *Causal indicators - factors that will most probably cause stress for the pig - such as, inappropriate temperatures or lighting, draft, and deprivation of cage enrichment. Biological response indicators - the direct biological response to stress exposure – reflected as a physiological response such as, an increased heart rate and body temperature, but also altered constitution of biological samples such as blood, urine, faecal samples, hair and saliva, as well as altered behaviour and vocalisation. Consequence indicators - reflection of the long term consequences of stress - such as an affected animal performance, altered physiological responses that can be detected in biological samples such as blood, urine, faecal samples, hair and saliva but also altered behaviour and vocalisation, impaired cognitive capacity, increased prevalence of gastric ulcers or chromodacryorrhea. Created with BioRender.com*

I.4.1.1 *Physical/environmental stressors*

In intensive pig farming, many physical factors should be controlled as much as possible to minimize their effect on animal welfare and in consequence profitability. A legislative

framework (2008/120/EC)[1] is set around these parameters in order to protect pigs in agriculture as much as possible. Heat stress is a well-known environmental stressor (e.g., [6, 41]). However, equally important is cold stress (e.g., [42, 43]). Pigs are, to some extent, capable of adapting to deviations from the ideal ambient temperature. However, the ambient temperature should be as close as possible to the thermal neutrality for the age of the pigs (Table 1). When the ambient temperature is too low, feed conversion ratios will increase in an animal's attempt to maintain its body temperature. Young piglets are especially sensitive to hypothermia since they are born without brown fat. Additionally, heat loss per unit of body weight is inversely related to body size resulting in piglets being more at risk (e.g., [44]). A draft will contribute to the aversive effect of cold, lowering the perceived temperature even more. In contrast, when the temperature is too high, feed intake will reduce, resulting in lower weight gain. Too high humidity will lower the animal's ability to lose heat, increasing the perceived temperature (e.g., [6, 41]). Temperatures too extreme, either too hot or too cold, will lead to the animal's death.

Sometimes, in hot or cold areas/seasons, maintaining this optimum is challenging, especially with global warming affecting the climate. Another challenging thermal problem is the gap between the optimal temperature for sows, which is around 20°C, and that of her piglets, which is 10 to 15°C higher. Other factors like light exposure and sound intensity could also influence the animal's wellbeing [45]. Measuring these causal environmental factors is not that difficult. In addition, air quality is also of great importance to the animal's sense of wellbeing (Table 1). However, assessing this parameter is more challenging (e.g., review [46]). The maximally allowed concentrations of ammonia (NH_3), carbon dioxide (CO_2), hydrogen sulphide (H_2S), and dust, the most studied air pollutants, are set by the European government. Exceeding ammonia levels released from manure are known to influence animal growth performance, feed consumption, health, immune responses and trigger aversive behaviour. Hydrogen sulphide, released in bubbles from liquid manure, is toxic in low concentrations but lethal in high concentrations. Dust, from a.o. food, skin, and faeces, are built up by particles

[1] See: EUR-Lex Access to European Union law https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32008L0120 (Accessed 28 October 2023).

of different sizes, which in their turn will affect the animal's respiratory system in different ways. More recently, interest in investigating the effect of air pollutants like sulphur dioxide (SO_2), silo gasses and even odour on the animal's health has risen. However, the methods for measuring and the concentration calculations of these air pollutants still vary greatly, affecting interpretation and comparability (e.g., review [34]).

Table 1. Overview of different factors that, by the European law, should be controlled at the farm since these affect the pig's sense of wellbeing. *(Adapted from 2008/120/EC)*[2].

Physical/environmental factors		
Temperature		Optimal temperature (°C)
	New-borns	35 - 37
	Piglets	28 - 32
	Weaners	25 - 27
	Growers	20
	Finishers	18 - 20
	Sows	18 - 23
	Boars	16 - 18
	Be aware of high fluctuations in temperature	
	Additional compounds to take into account:	
	Humidity (relative)	50 - 80%
	Draft	Max. 0.20 m/s (finishers); max 0.15 m/s (piglets)
Air quality		Concentration per m^3 air
	NH_3	Max. 10 ppm
	CO_2	Max. 2000 - 3000 ppm (min. ventilation)
	H_2S	Max. 5 ppm
	Dust	< 2.4 mg/m^3
Light intensity	Min. 40 lux Min. 8 h/day Daylight	
Noise	Max. 85 dB Avoid sudden noise	
Psychosocial factors		
Floor space	Weight class	Free floor space (m^2/animal)
	< 10 kg	0.15
	10 - 20 kg	0.20
	20 - 30 kg	0.30
	30 - 50 kg	0.40
	50 - 85 kg	0.55
	85 - 110 kg	0.65
	> 110 kg	1.00
Cage enrichment	Permanent access to material that can be investigated and manipulated E.g. straw, hay, wood, sawdust, …	

[2] See: EUR-Lex Access to European Union law https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32008L0120 (Accessed 28 October 2023).

Mixing	Mixing should be avoided as much as possible If mixing needs to be performed, this should be done as young as possible, preferably before the end of the first week after weaning
Deprivation of the mother	No piglets shall be weaned before the age of 28 days. However, piglets may be weaned up to 7 days earlier to specialised housings.
Other factors	
Hunger	Feed at least once a day All animals in the same room must be fed at the same time
Thirst	Sufficient access to water

I.4.1.2 *Psychosocial stressors*

The most common cause of social stress in pig farming is regrouping and mixing of unfamiliar animals. This happens in various stages of the productive cycle and can start as soon as hours after birth during cross-fostering. Groups of pigs have a social hierarchy that is usually established through fights. Such social stress can therefore be acute, immediately after regrouping (e.g., [47]), or chronic, when the animals are socially subordinate or isolated, or when the dominant pig is constantly challenged to maintain its dominant position [48], or as a result of repeated social regrouping (e.g., [2]).

In modern, intensive pig industry, supernumerary piglets are a big challenge. Litter sizes have increased and the number of live-born-piglets within one litter exceeds the average number of functional teats in the sow. Therefore, from birth onwards, competition between littermates starts. Several management strategies have been developed to assist in rearing these supernumerary piglets, often involving another stressor in the form of short or early maternal deprivation as during split suckling or artificial rearing (e.g., [49, 50]).

When the piglet has overcome these struggles early in life, weaning is probably the next most stressful event due to regrouping, a new environment, a new type of feed, and deprivation of the mother (e.g., [51]). Overcrowding and restricted floor space could contribute to this social stress, forcing more social interactions and implementing more movement restrictions (e.g., [52, 53]). Research nowadays is focussing on making these transition as smooth as possible. However, quantifying the effect of these interventions on the piglet's wellbeing is not that easy.

Luckily, some interventions have proven to be effective to improve animal welfare, like the presence of straw bedding and cage enrichment (e.g., [8, 54]). Because pigs have an intrinsic

urge to explore, it is obliged to provide cage enrichment. Suppressing this need will lead to reduced welfare (e.g., [54, 55]). Additionally, anticipation of physical aversive events, such as restraint, isolation, pain, noise, can lead to fear and mental stress [56]. However, if this adverse event is embedded in the daily routine and becomes predictable, it is experienced as less stressful than whether the event will occur randomly during the day [57]. The lack of predictability of the environment could lead to mental stress for the pig [58].

The problem with these psychosocial stressors is that different pigs can experience the same stressful situation differently. Coping strategies, previous experiences, character, presence of other stressors, can affect the biological responses and long-term consequences. This is probably one of the major drawbacks of causal indicators.

I.4.1.3 *Other stressors*

Immune-related stress, evidently is the stress that infection (viruses, bacteria and others), vaccines and inflammation imposes on the animal. Most studied are Escherichia coli lipopolysaccharides (*E. coli* LPS), porcine reproductive and respiratory syndrome virus (PRRSV) and *Salmonella* challenges [40].

On the other hand, metabolic stress is the result of food and/or water restriction or deprivation (e.g., [59]). Metabolic stress can appear in intensive farming when animals are subjected to restricted feeding [59]. Overcrowding and mixing could also result in metabolic stress because submissive individuals could have less or even no access to food [60]. Although immunological and metabolic stressors can influence welfare of the pigs, these types of stressors are beyond the scope of this dissertation.

It might be clear that pigs are exposed to several inevitable but also many avoidable stressors in their lives. Identifying whether welfare at the farm is compromised is important to alarm the farmer that action is needed. Also, with having the consumer in mind, welfare-levels at the farm should be easily verified and transparent. Additionally, easy stress-monitoring tools could facilitate research that focusses on overcoming the hurdles associated with modern pig farming and hyperprolific sows. The most intense and critical moment is clearly the first weeks

of the pig's life. Therefore, the focus of this doctoral thesis is on developing suitable methods to identify psychosocial chronic stress in young pigs.

I.4.2 Biological response indicators

While causal indicators are a separate category, some parameters or biological samples used to identify the biological response to stress could also reflect the consequences of these prolonged stressors. Therefore, this section on indicators is further divided into solely biological response indicators, indicators for biological responses and its consequences, and pure consequence indicators.

I.4.2.1 *Physiological assessment*

I.4.2.1.1 Heart rate, respiratory rate and body temperature

Heart rate, respiration rate and body temperature are often monitored as a sign of distress due to inappropriate high temperatures (e.g., [61]), but also during acute stress as signs of catecholamine release (e.g., [62]).

I.4.3 Biological response and consequence indicators

I.4.3.1 *Physiological assessment*

I.4.3.1.1 Blood analysis

Blood is a well-studied and diverse biological fluid containing a range of biomarkers. Circulating neuroendocrine biomarkers in blood have been investigated in pig's stress research context. The most studied is cortisol, but also cortisone, ACTH and (nor)adrenaline concentrations have been assessed, as do levels of serotonin and tryptophan, dopamine, progesterone, prolactin, and estradiol (e.g., [63]). Cortisone is the inactive form of cortisol, that is formed by the enzyme 11β-hydroxysteroid-dehydrogenase type 2 (11β-HSD2), which is an enzyme that is mainly situated in the kidney, distal colon, sweat and salivary glands, placenta and vascular walls (Fig. 5). Cortisone can be converted back into cortisol by 11β-hydroxysteroid-dehydrogenase type 1 (11β-HSD1), an enzyme mainly present in the liver but also in lungs, adipose tissue, brain, and gonads (e.g., [64]). It is thought that cortisone could be used as a proxy for the amount of circulating cortisol that is insensitive to an acute rise of cortisol due to an acute stressor. Therefore, cortisone concentrations could better reflect the chronic perception of stress. However, the conversion rate of cortisol into cortisone by 11β-

HSD2 could be hampered by the effect of stress because lower levels of 11β-HSD2-activity have been reported in humans with higher levels of prolonged perceived stress [65].

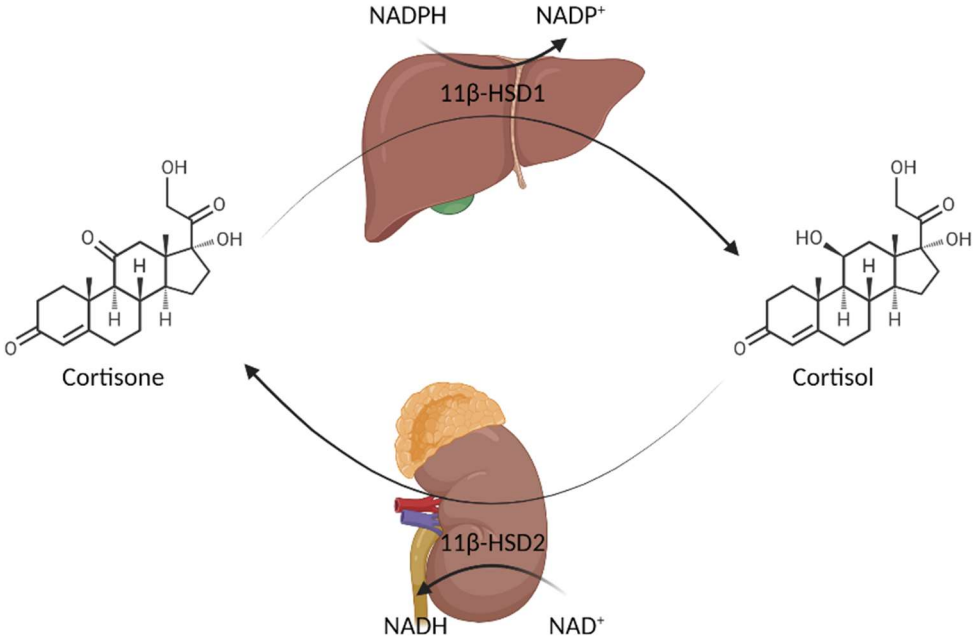


Figure 5. Interconversion of cortisol and cortisone*. Two isotypes of 11β-hydroxysteroid-dehydrogenase (11β-HSD) are able to convert the active cortisol into the inactive cortisone and back. Created with Biorender.com.*

While a short, acute stressor may boost the immune system, chronic or repeated stress stimuli may have the opposite effect. For example, piglets that were shortly isolated at the moment of weaning appeared to be less susceptible to an infectious challenge with *Staphylococcus aureus* [66]. In contrast, pigs that are repeatedly socially isolated presented lower values of circulating tumour necrosing factor (TNF) and a lower CD4+/CD8+ ratio in blood [67]. Cortisol is known to have anti-inflammatory and immunosuppressive effects, particularly via the inhibition of pro-inflammatory cytokines (e.g., interleukin (IL)-1, IL-2, IL-6, IL-8, IL-12 and TNF-α), and suppression of T-cells. These immunological biomarkers (leukocyte counts and cytokine levels) [47, 67-69] next to immunoglobulins (Ig's) and acute phase proteins (APPs) were monitored in porcine blood after several types of stress (e.g., [5, 70]). Other circulating blood compounds like glucose, lactate, cholesterol, urea nitrogen, insulin, and non-esterified fatty acids can be tested since their levels correlate to the metabolic consequences of stress [52, 71].

Although blood analysis can be useful to study the consequences of different stressors and to unravel mechanisms behind these responses, it has some limitations. Blood sampling not only requires animal restraint, sometimes with snaring, but is associated with pain. (Mock) blood sampling is even sometimes used to induce acute stress in pigs [72]. Blood sampling can therefore affect physiological blood biomarkers, possibly masking the effect of other stressors and will affect welfare.

I.4.3.1.2 Urine and faeces analysis

Some hormones are excreted via urine. Therefore, many stress-related hormones, like cortisol, testosterone and catecholamines can be detected and analysed in the context of stress research. To correct for variation due to hydration differences, determined hormone levels should be normalised for creatinine levels (e.g., [73, 74]). However, urine collection involves some challenges. Metabolic cages are, not an option since these imply social isolation, while catheterization or suprapubic bladder aspiration are invasive procedures that affect welfare [75]. Especially on-farm collection poses problems. Spontaneously excreted urine can be easily to collect in tethered sows. This is, however, more difficult in free-ranging animals. A beaker attached to a stick or handle is sometimes used to avoid approaching the animal. Alternatively, for piglets, urination can be triggered by placing the feed into water [73]. Unfortunately, this technique again requires manipulation.

Faecal samples contain relevant concentrations of corticosteroids and their metabolites. Circulating hormones are integrated over a certain period. As a result, this concentration reflects a cumulative secretion of hormones over a more extended period. Therefore, this sample is unsuitable for detecting physiological responses to acute stressors [76]. There is a 48-hour delay between the rise of circulating cortisol and faecal secretion [77]. As for urine, individual collection in tethered sows is more feasible than in loose-housed animals.

However, sampling and storage must be performed precisely since hormones, especially in urine and faecal samples, are sensitive to bacterial degradation [78].

I.4.3.1.3 Hair

As mentioned before, cortisol is often used as a biomarker to assess stress, as it is released upon activation of the HPA axis [79]. However, interpreting cortisol concentrations in biological fluids, such as saliva or blood, has certain constraints, since they are influenced by various factors including a circadian rhythm [32]. Furthermore, cortisol concentrations might rise in response to an acute stressor, or physical activity, and therefore present only a snapshot of an animal's physiological state [33]. Since cortisol concentrations in hair accumulate over time [80, 81], this parameter might be a better indicator for chronic stress than salivary cortisol concentrations.

Hair analysis has been performed already for nearly 50 years [82, 83]. The earliest use of hair samples was in the context of drug abuse [82, 84] and environmental trace element pollutants [83]. Later, hormone concentrations in hair were analysed in humans as a tool for doping control [85, 86]. Cortisol concentrations in hair related to social status and stress were first documented in animals in 2001 in the rock hyrax [80]. Since then, research on hair cortisol analysis to assess a subject's welfare state has grown exponentially.

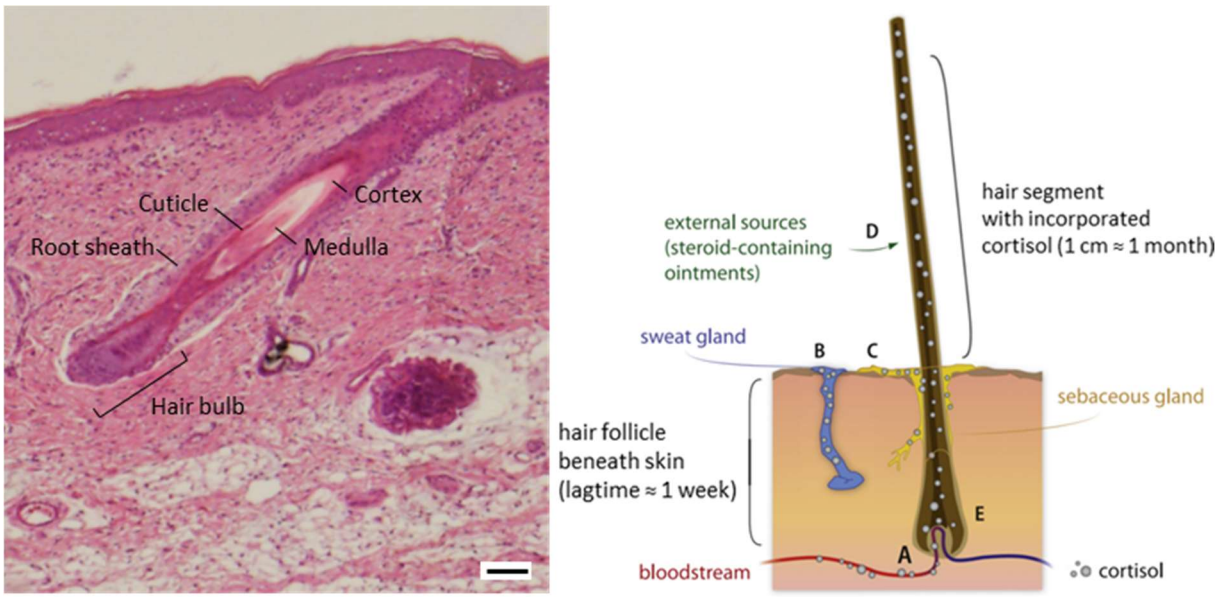


Figure 6. Hair - histology and cortisol incorporation. Left. *Histological section of a porcine hair follicle, visualising the hair bulb and root sheets, and the three layers of the hair shaft. The inner medulla is surrounded by the cortex, which is covered by a single layer comprising the cuticle. Haematoxylin and eosin (H&E) staining. Scale bar = 100 µm.* ***Right.*** *Different mechanisms of cortisol incorporation into hair, through passive diffusion from blood (A), sweat*

(B) and/or sebum (C) as well as from external sources (D), or through locally produced cortisol (E). (Right figure adapted from [87]).

Hair is produced in hair follicles where matrix cells in the hair bulb divide extensively and are pushed upwards. The exact mechanism on how substances, like cortisol, are incorporated into the hair is still under debate (reviews include e.g., [87-89]). The multi-compartment model by Henderson suggests several routes [90]. The first, with probably the largest contribution, is through passive diffusion from the bloodstream into the medulla of the hair shaft (Fig. 6). The unbound cortisol fraction from the bloodstream is incorporated into the growing hair during the anagen phase. However, hair grows in three phases, i.e., the active growth phase (anagen), the transition phase (catagen), and the resting phase (telogen). Consequently, not all hairs are in the same phase and have incorporated cortisol from the same period. The second route is through the diffusion of cortisol in body secretions such as sweat and sebum into the hair. In this way, cortisol can also be incorporated after the hair has been formed. Thirdly, however of less relevance in pigs, cortisol can be integrated into the grown hair due to diffusion from external contamination sources like cortisol-containing creams (reviews include e.g., [87-89]). Finally, a fourth option has been discovered, i.e., the local production of cortisol in the skin. Different skin compartments, like hair follicles, present the equivalent of an HPA axis. Isolated, cultured human hair follicles stimulated with CRH showed increased immunoreactivity to ACTH and secreted substantial levels of cortisol in the culture medium. As a result, even without endocrine, neural, or vascular systemic connections, hair follicles directly respond to CRH stimulation, including synthesis and secretion of cortisol and activation of neuroendocrine feedback loops [91]. This local secretion mechanism may explain regional differences in cortisol concentration throughout the body.

Although cortisol is the most studied incorporated hair compound in relation to stress, other hormones are similarly incorporated in hair and are gaining interest (e.g., [92, 93]).

I.4.3.1.4 Saliva

Saliva is an easily accessible body fluid that is widely accepted as a potential medium to assess an individual's health status (e.g., [94]). Using saliva has multiple advantages. It can be collected non-invasively, stress-free does not require qualified personnel, and several samples can be taken during a short time period. Like in the previous described biological samples,

cortisol is by far the most studied hormone in saliva but the reliability of only using this biomarker to assess stress is debatable (e.g., [40]).

I.4.3.2 *Behaviour and vocalisation*

An emotional stimulus directs behaviour and indicates the underlying emotion's intensity and valence. Therefore, assessing behaviour can provide helpful insight into the animal's emotional state and, consequently, their welfare state. A positive emotional state will contribute to a good welfare, while a negative emotional state will reduce this.

Behaviour can be assessed in two different ways. Either via monitoring specific behaviour and behavioural patterns or the pig can be subjected to a behavioural test (reviewed by e.g., [95]).

I.4.3.2.1 Behaviour and behavioural patterns

Stress in behavioural sciences is regarded as the perception of a threat that results in anxiety, discomfort, and emotional tension. To identify stress, behaviour that reflects these emotions should be monitored. Excessive aggression could be a sign of stress, and therefore, fighting is often monitored by recording the duration or frequency of fights (e.g., [73, 96, 97]). However, aggression can be portrayed in more subtle ways as well. For example, nudging could be driven by aggression or lack of cage enrichment, but it could also be a sign of playfulness. Sometimes stress can cause abnormal behaviour such as stereotypical behaviour, which is a repetitive and invariant behaviour pattern with no apparent purpose (e.g., [98]), for example tail and ear biting (e.g., [99]), sham chewing (e.g., [100]), frequent defecation and urination (e.g., [101]), restlessness (e.g., [102]), or reduction in exploration behaviour (e.g., [101]). As mentioned before, reduction in eating and drinking behaviour can be caused by stress and is therefore often used in the ethogram. Other types of behaviour like belly nosing, nosing other parts (tail, ear, anogenital area) of pen mates, nosing towards the floor, the wall or the enrichment material, or mounting behaviour (e.g., [97]) are often monitored in stress-related research.

Additionally, instead of monitoring fighting behaviour, the physical results of these fights, thus the lesions, could be assessed through various scores and protocols (e.g., [53]). However, only severe aggressive encounters would give lesions and therefore not all levels of aggression are

reflected by this parameter. Consequently, lesion scores can only be a good indicator of aggression in a herd if larger groups are observed, and preferably complemented with other indicative data [103].

Identifying stress and compromised welfare by focusing on negative emotions is one option. However, more and more research focuses on signs of positive emotions. An indicator of a positive state is, for example, playing, as this usually occurs when all other needs have been fulfilled and when there is no sense of threat. Social play is any reciprocal contact between two pigs that lasts at least five seconds. This can be a.o. play-fighting, pushing, lifting, gentle nudging, and play-biting (e.g,. [104]). However, defining play and distinguishing this behaviour from conflict behaviour is challenging. Additionally, not all social play is a sign of positive emotions. For example, this play behaviour can also be used to establish dominant hierarchical relationships and may escalate to actual conflict. It was also reported that social play may be increased during periods of social stress to reduce social tension (e.g., [105]).

Solitary play and object play are less dubious indicators of a positive state of emotion in pigs. Solitary play includes waving of the head, scampering, jumping, pawing, pivoting, hopping, and running around with a rapid change of direction (e.g., [97]), while object play includes shaking or carrying an object (e.g., [45]. Another possible indicator that may reflect a positive mood is an anticipatory behaviour associated with the release of dopamine and endorphins (e.g., [106]). However, only subtle differences have been found between anticipation of positive and negative emotions, which makes interpretation difficult.

Assessing behaviour can be executed via direct observations (e.g., [107]) or video recordings (e.g., [48]). Usually, scan sampling is performed, meaning that all selected animals are observed for a certain period with a set interval (e.g., [107]). The downside is that this process is labour intensive and sensitive to inter-observer variation. Automatizing this process by implementing machine vision and social analysis software packages overcomes these limitations. However, the analysis should be able to discriminate between play-fights and agonistically driven fights. Additionally, like in humans, behaviour can be contagious. Sometimes, behaviour is adapted from a distressed animal, while the underlying sense of threat for the other pig is not present (e.g., [105]).

I.4.3.2.2 Vocalisation

Although linked to behaviour, vocalisation could be classified as a different type of indicator for stress responses. Although less studied than porcine behaviour, sound characteristics like intensity, frequency, duration, and tone have been found to vary among different, distinct vocal responses in pigs. These vocal characteristics make it possible to discriminate between several physical types of distress like pain, thirst, hunger, and extreme temperatures [108, 109] or mental stress [108]. However, factors such as age and sex affect vocalisation [109]. Thus, more research is needed to correctly interpret vocalisation as a stress indicator.

I.4.3.2.3 Behavioural tests

The majority of behavioural tests that are used for pigs nowadays are designed to identify fear and anxiety. These are emotions that could be driven by stress. These behavioural tests are mostly modified versions of tests used to study behaviour of rodents such as the Open Field Test, Elevated Plus maze, Novel Object Test, Human Approach Test, Open door/Emergency Test, and Light and Dark Test. These tests are validated for rodents, but critical reviews indicate that this does not hold for porcine behaviour in their current form [101, 110, 111].

I.4.4 Consequence indicators

I.4.4.1 *Animal performance*

Mapping zootechnical performance information from pigs can indicate the pigs' wellbeing. The most used parameter is calculating weight gain over a certain period (e.g., [73, 112, 113]) and, to a smaller extent, also feed intake (e.g., [73, 113]) and feed conversion rate (e.g., [113]). As previously mentioned, one of the costly consequences of chronic stress to the farmer is reduced growth since it is known that the average daily gain (ADG) of pigs is affected by stress. This reduction in weight gain can be the cause of reduced feed intake. Some stressors, like inappropriate temperature, could result in lethargy and a reduced feed intake (e.g., [3]). However, the stress response systems can also interact with the appetite-satiety centres of the CNS. An acute rise of CRH upon activation of the HPA axis may lead to suppressing appetite [114, 115]. Several studies have indicated that the relationship between stress and food intake can be negative or positive. This means that both increased and decreased feeding behaviour has been observed [116]. The outcome of stress-associated alterations in feed intake depends on many factors, such as the severity of stress and the level of stress hormones, as previously

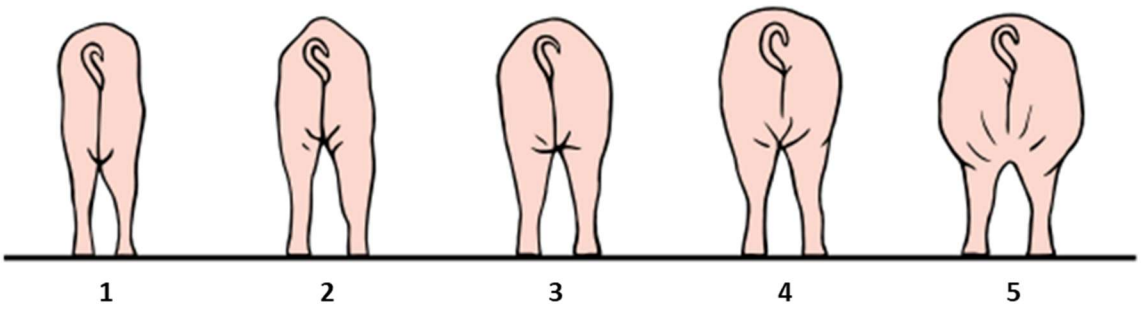
described, but also on levels of feeding-related neuropeptides such as neuropeptide Y (NPY) [117]. Suppression of NPY, a potent orexigenic peptide, could be involved in the anorectic effect of stress [118].

On the other hand, stress can also increase energy expenditure and alter feed efficiency. For example, the reduction in ADG in pigs with limited floor space was not always driven by a lower feed intake (e.g., [119]). As mentioned previously, stress may promote muscle catabolism, that, combined with reduced feed intake, will lead to a negative energy balance (e.g., [120, 121]). Additionally, chronic social stress appears to affect glucose uptake by the ileal sodium-glucose linked transporter 1 (SGLT-1), resulting in reduced body weight gain and feed efficiency [113].

Lastly, stress can have inhibitory effects on the growth hormone/insulin-like growth factor axis. Long term activation of the HPA axis leads to suppressed secretion of growth hormone (GH) and insulin-like growth factor-1 (IGF-1)(e.g., [122]). This occurs either directly through the inhibitory effects of glucocorticoids or indirectly through CRH that promotes the secretion of GH-suppressing somatostatin (e.g., [123]).

Besides weighing the animals and/or tracking feed intake, one could opt for an alternative to have an idea on whether the animal is nourished optimally by applying the body condition score (Fig. 7)(e.g., [124]). Although these animal performance indicators can be an indication of thermal, social or other types of stress, this can also be a sign of sickness or the quality or palatability of food.

Chronic stress induces a downregulation of the pro-inflammatory cytokines at the level of the gut [113]. Additionally, intestinal transepithelial permeability appears to be affected by chronic social stress [113, 126]. When the normal gut barrier function is affected, the individual becomes much more susceptible for infections. Associated with this, some important enteric pathogens can even adhere better to the intestinal mucosa through upregulation of circulating neuroendocrine modulators, such as catecholamines and ACTH [127].



1 = Emaciated sow with very prominent backbone
2 = Thin sow with prominent backbone
3 = Ideal condition during lactation and at weaning. Backbone just palpable.
4 = Slightly overweight. Backbone not palpable.
5 = Over fat. Rotund body.

Figure 7. Body condition scoring. *Adapted from [125].*

Table 2. *Overview of different stress responses and its consequences when triggered by either, mild, intense or extreme stress perception. Adapted from [126].*

Physiological system	Physiological mediator	Normal state	Mild and/or acute stress	Intense and/or prolonged stress	Extreme intense and/or very prolonged stress
Hypothalamic-pituitary-adrenal Axis	Glucocorticoids ACTH	Seasonal life-history needs a. Energetic needs b. Behavioural needs c. Preparative needs	Inhibit immune system Energy mobilization Change behaviour Inhibit reproduction Inhibit growth	Immunosuppression Diabetes Muscle breakdown Reproductive suppression Decreased survival	Energy dysregulation Water balance failure Catecholamine insufficiency Decreased survival
Sympathetic nervous system	Heart rate Heart rate variability Blood pressure	Life-history energy needs	Fight-or-flight Energy mobilization	Hypertension Myocardial infarction Muscle breakdown	Hypotension Lethargy Decreased survival
Behaviour	Foraging/feeding Locomotion Migration Conspecific aggression	Life-history changes: a. Energy needs b. Energy availability c. Predator presence d. Mate access	Fleeing behaviour Freezing behaviour Increase/decrease foraging Increase food intake Increase vigilance Conspecific fighting	Tonic immobility Obesity Anxiety Fear Violence	
Immune system	Prostaglandin T-cell activation Antibody titters Cytokines	Seasonal ability to fight infection	Mobilization of immune system	Autoimmune Immunosuppression	Immune failure
Central nervous system	Neurogenesis Dendritic arborization Neurotransmitter concentrations Cytokines	Life-history changes in neural networks Learning and memory	Increase neurotransmission (titters or receptors) Increase learning and memory	Neuronal atrophy/death Depression Decrease learning and memory	Post traumatic stress disorder

I.4.4.2 *Other parameters*

I.4.4.2.1 Tear staining

Tear staining, chromodacryorrhea or so-called bloody tears are caused by porphyrin-pigmented secretion from the Harderian gland [127, 128]. This gland is located adjacent to the eye and is present in most land vertebrates but absent in, for example, humans [129]. It has been reported that the size of these red or rusty stains correlated with the number of aggressive interactions between unfamiliar weaned pigs in tiny cages that lacked enrichment [130]. Social isolation in combination with either a sterile environment or regrouping leads to more prominent stains that increase in size when the stressor continues [131]. However, when comparing different age groups, it was observed that younger animals showed lower scores [99]. Whether this was because piglets experienced less stress than older animals or whether older animals had better-developed Harderian glands has yet to be investigated. Thus this parameter is less suitable for piglets. Lastly, these tear stains can be a sign of disease or poor air quality, indicating high levels of dust and ammonia [132, 133].

I.4.4.2.2 *Post-mortem* analysis

The first reports on the correlation between stressors and gastric ulcers were reported in the 30's by Hans Seyle [11]. *Post-mortem* analysis by evaluating hyperkeratosis and ulcer formation in the stomach can indicate the degree of lifetime stress exposure (e.g., [24, 134-136]. This is performed using different scoring methods including four, five, or six severity categories [134-136]. The lower the score, the healthier the stomach (*pars oesophagea*). The higher score is given to a stomach with extended hyperkeratosis including ulcerations.

The fact that this is a *post-mortem* analysis is a limitation for many stress-related studies, but it can be a helpful screening tool at, for example, slaughterhouses. Additionally, mainly severe, unpredictable, and uncontrollable stress leads to stomach damage [24, 102], making this method less sensitive to more subtle forms of stress. Associated with this, passive copers appear to express more stomach damage than active copers. Passive copers react to stress conditions with an increased parasympathetic nervous activity that may specifically influence the stomach wall. In contrast, active copers react predominantly with a sympathetic nervous activity that affects the heart and blood vessels and less the stomach [135]. Although they

were exposed to the same stressors, the influence on the stomach wall appeared noncomparable in both types of copers, making interpretation more difficult.

Additionally, often straw is given to increase welfare. This can directly affect the stomach wall due to its structural properties when the straw is eaten. Straw may reduce the exposure of the sensitive *pars oesophagea* to acidic stomach contents (e.g., [137]). This could mask the adverse effects of other stressors. Additionally, gastric ulceration can be influenced by feed characteristics such as feed particle size, pelleted feed, and ad libitum feeding (e.g., [138, 139]) or by an infection with *Helicobacter* spp. (e.g., [140]), hampering the sensitivity of this indicator.

I.5 Why further investigate saliva?

It might be clear that the option to study both the biological stress response and the long term (pre)pathological consequences is of added value. The relatively easy collection of saliva that is non-invasive is, in the context of animal welfare, equally important. This sample requires limited manipulation after collection before it can be analysed benefiting labour cost and time to results. For these reasons, we opted to further explore saliva as a tool to identify chronic stress in pigs.

I.6 What is saliva?

Saliva has several essential functions including clearing substances from the mouth, maintaining tooth mineralization, buffering of pH, facilitating wound healing, neutralising harmful dietary components, influencing the oral microbiome, and protecting, lubricating, and hydrating oral mucosal surfaces (reviewed by [141, 142]).

In pigs, saliva is mainly produced by three major paired salivary glands, i.e., the parotid gland, the mandibular gland, and the sublingual gland (Fig. 8). The minor labial, lingual, and buccal glands secrete smaller amounts of saliva. Gland-specific saliva is secreted in the oral cavity and mixed with gingival crevicular fluid, buccal cells, and microorganisms [141].

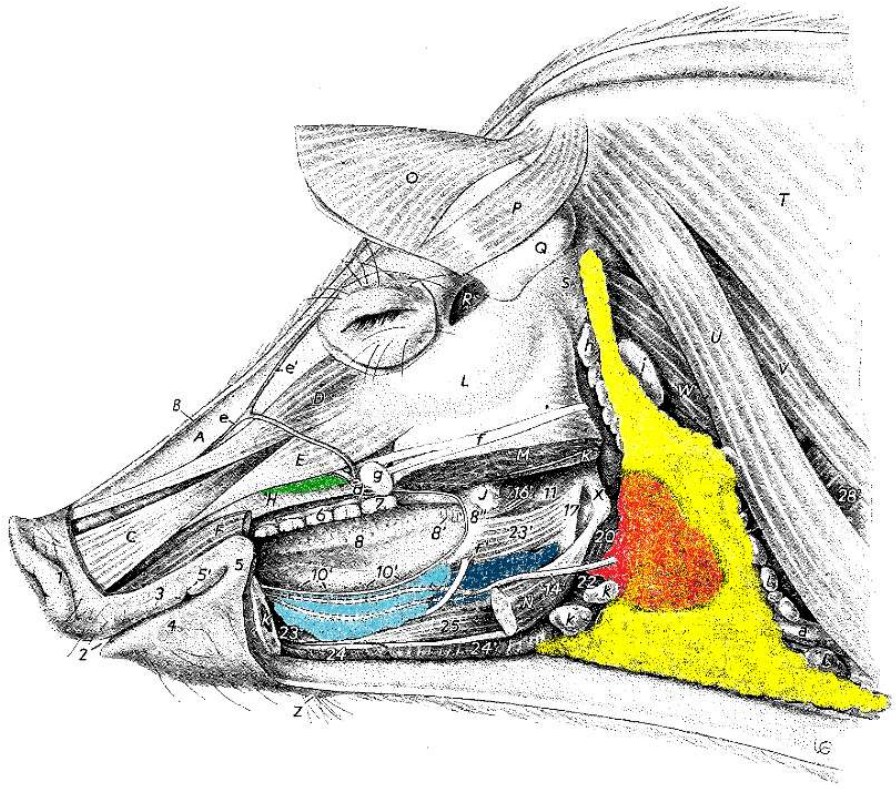


Figure 8. *Topography of the three major paired salivary glands, i.e., the parotid (yellow), mandibular (red) and sublingual (blue) glands. The latter is composed of a monostomatic (dark blue) and polystomatic (light blue) part. The buccal glands are depicted in green (Adapted from [143]).*

Salivary glands are composed of different acini, a collection of saliva-secreting epithelial cells (Fig. 9). These acinar cells produce and store secretory proteins in large granules. Depending on the type of proteins produced, they are categorised as serous or mucous of nature. The parotid gland consists of serous secreting cells that secrete watery saliva containing high levels of a.o. α-amylase [141, 144]. The mandibular gland is a mixed gland as it comprises both serous and mucous cells. The latter produce mucus, which is a viscoelastic secretion high in a.o. mucins, glycoproteins, and immunoglobulins. The sublingual glands are also categorised as mixed glands although these contain a higher ratio of mucin-producing acini compared to the mandibular gland.

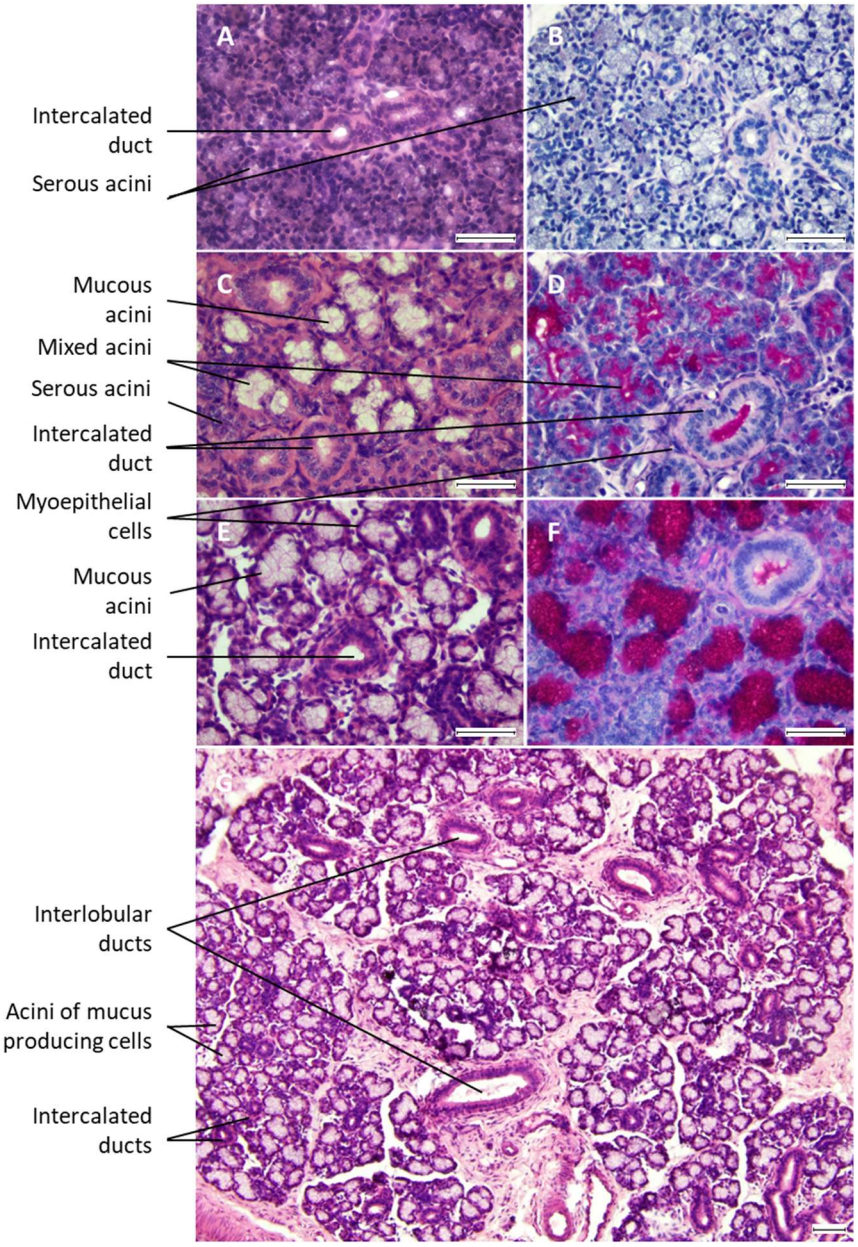


Figure 9. Histological sections of salivary glands of a 28-day old piglet. A. *Parotid gland with serous acini and intercalated duct (H&E staining). Scale bar = 100 µm.* ***B.*** *Parotid gland stained with the Periodic Acid-Schiff (PAS) stain that reacts with polysaccharides like mucins. No pink reaction is observable in the histological section of the parotid gland.* ***C.*** *Both serous, mucous as mixed acini are observable in the porcine mandibular gland (H&E staining).* ***D.*** *Mucins stained PAS-positive in the acini and in the lumen of the intercalated duct of the mandibular gland. Myoepithelial cells are visible around the duct.* ***E.*** *Mainly mucous acini are visible in the sublingual gland of pigs. Notice the flattened nucleus of the myoepithelial cells surrounding the acini (H&E staining).* ***F.*** *Acini stained deep pink using the PAS-stain, as does the saliva present in the lumen of the intercalated duct of the sublingual gland.* ***G.*** *Overview of a sublingual gland that mainly consists of mucous acini. Intercalated ducts between the acini collect saliva from the individual acini. Different glandular lobules are separated by connective tissue.*

The minor salivary glands are predominantly mucus-producing, although some, the von Ebner glands, which are situated adjacent to the circumvallate papillae of the tongue, secrete serous saliva [141, 145]. Each acinus comprises one layer of cuboidal cells surrounding a lumen into which the saliva is secreted. Saliva travels through the intercalated ducts that depart from each acinus. Myoepithelial cells surround these acini and intercalated ducts, giving these structures contractile support. Further, saliva gathers through the striated ducts into the interlobular ducts that combine in one central duct for each gland [141]. The parotid gland's saliva exits through the parotid papilla opposite the third or fourth upper cheek premolar. The saliva that is excreted through the ipsilateral sublingual caruncle derives from the mandibular and lingual glands [143].

I.6.1 Secretion

The secretion of saliva from the major salivary glands is stimulated in a reflex-like response by the detection of tastants by taste buds on the tongue and activating mechanoreceptors in the periodontal ligament and mucosae (Fig. 10) [146]. Additionally, the sensation of cold and pungent or cooling substances can evoke salivary secretion [147, 148]. Smells associated with food can trigger salivation from the mandibular and sublingual glands but not from the parotid gland [149]. In contrast, suppression of salivation resulting in a dry mouth is known to arise during fear and anxiety. Since these emotions modulate salivary secretion, higher neural structures are involved. Sympathetic nerve stimulation evokes a protein-rich secretion, whereas parasympathetic stimulation produces a large saliva volume. It might be clear that salivary glands are controlled by many brain loci and have the potential of sympathetic-parasympathetic interaction and are therefore capable of secreting very different salivary profiles as response [150].

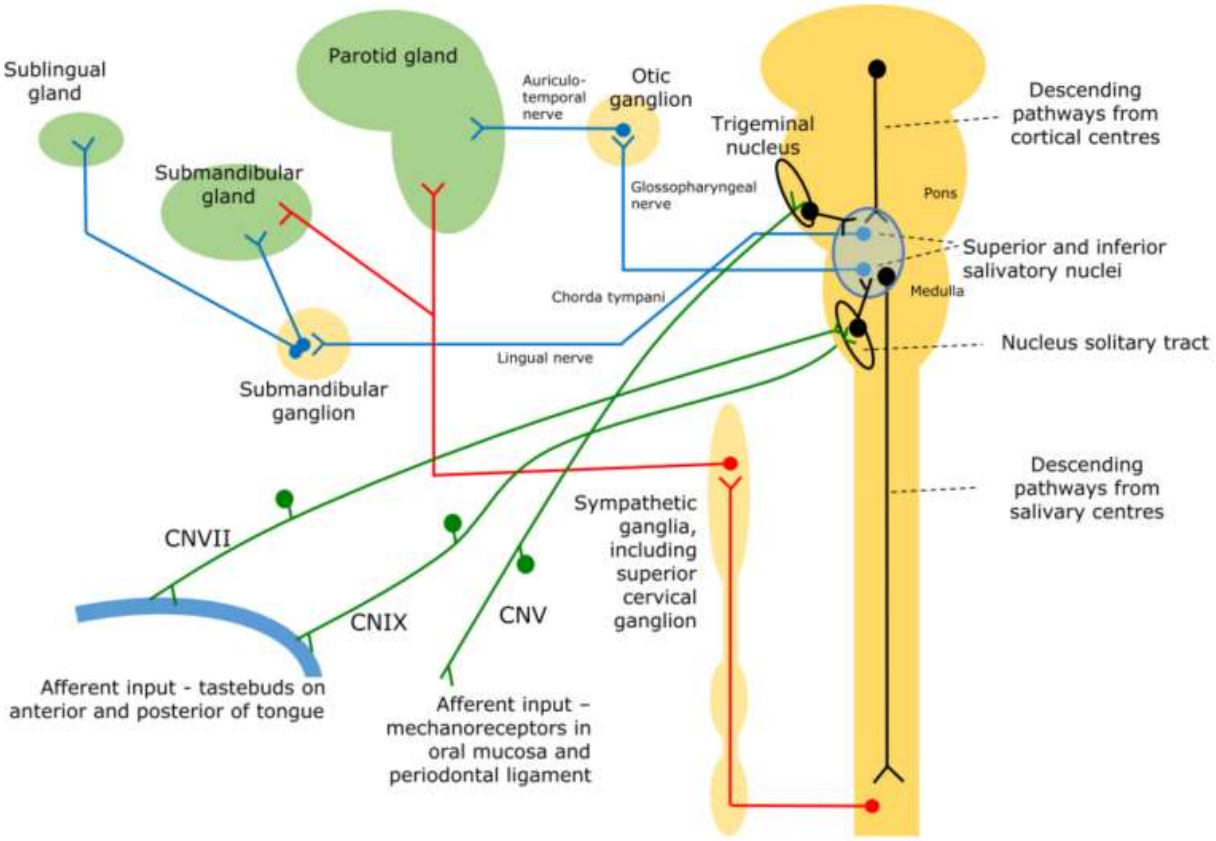


Figure 10. Salivary secretion pathways. *Saliva secretion is controlled by both the parasympathetic and sympathetic nervous system. The salivary reflex starts with detecting tastants by tastebuds and mechanoreceptors on the tongue. In parallel, chewing mechanism is detected by mechanoreceptors in the periodontal ligament around the teeth. Signals in afferent sensory nerves (green) are transmitted to the salivary centres through nerves within the central nervous system (black) and influence nerve-mediated signals to the salivary glands via efferent parasympathetic nerves (blue) and sympathetic efferent nerves (red) that arise from the thoracic spinal cord. Cranial nerve (CN) IX, glossopharyngeal nerve; CN V, trigeminal nerve; CN VII, facial nerve (figure from [141]).*

I.6.2 Content

Saliva consists for 99% of water, complemented with a broad spectrum of electrolytes, hormones, lipids, nucleic acid-containing molecules, micro-organisms, proteins, and peptides [141, 151-153]. These can function as salivary biomarkers to indicate normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.

Electrolytes enter the saliva via osmotic gradients (e.g., [141]). The electrolyte concentrations of swine saliva were first studied in 1966 [154], analysing sodium, potassium, calcium, and phosphate. Later, chloride and bicarbonate were also determined in porcine saliva [155]. The

latter can neutralise acid and acts as a buffer. At the same time, most of the other electrolytes are a.o. responsible for tooth protection or taste perception (e.g., [156]). The profile of electrolytes in porcine saliva is similar to that of humans in composition and in concentration ranges under normal physiological conditions [157, 158].

Saliva contains nucleic acid-containing molecules, a.o. through cells such as oral epithelium cells and leukocytes, but also microorganisms. Analysing salivary deoxyribonucleic acid (DNA) can be challenging due to the high portion of bacterial DNA. However, despite this challenge, salivary DNA methylation studies have been performed in humans in the context of many conditions such as cancer, air pollution, smoking, psychiatric and lifestyle-related diseases. Additionally, cell-free saliva also contains DNA, specifically cell-free DNA, or in some cases circulating tumour DNA. Higher levels of cell-free DNA have been found in relation to psychosocial stress. Like DNA, different types of ribonucleic acid (RNA), including messenger RNA (mRNA) and cell-free mRNA, and various types of noncoding RNA, such as microRNA, piwi-interacting RNA, and circular RNA, are detectable in saliva (review [159]). Exosomes could be responsible for the contribution of mRNA and microRNAs [160]. Although increasingly studied in humans, investigations on nucleic acid-containing molecules in porcine saliva remain scares.

Understudied compared to other components of saliva, both in humans (e.g., [161-163]) as in pigs (e.g., [164-167]), remains the salivary metabolome. The metabolome is a signature for physiological states and includes low-molecular-weight compounds such as peptides, lipids, carbohydrates, amino acids, vitamins, and minerals. Metabolites analysed in saliva are, for example, butyrate and 2HOvalerate, formate, malonate, and propionate [165].

The salivary proteome is intensively investigated in humans, and the number of proteins identified largely exceeds the 3000 (e.g., [168-170]). In comparison, the fraction of porcine salivary protein identifications is merely a snippet [72, 171-180]. This diverse repertoire of salivary proteins consists of enzymes, such as α-amylase, lysozymes and carbonic anhydrase [177, 181], glycoproteins such as mucins [182], immunoglobulins such as IgA [183], and many others. As mentioned before, some salivary proteins can either be locally produced in the salivary glands or derived from the bloodstream (approximately 27%) through diffusion,

filtration, or active transport. Of these proteins, a wide variety of hormones are detectable in saliva. Some hormones are lipophilic and can cross the oral and salivary gland epithelium freely. Of these hormones, mainly steroid hormones, such as cortisol, progesterone, and testosterone, the free unconjugated fraction is well quantifiable in saliva (e.g., [184]). Other conjugated hormones, such as dihydroepiandrosterone sulfate, diffuse through tight junctions between the epithelial cells, while others, such as leptin, are locally produced in salivary glands. As a result, their detection and quantification is less reliable [185, 186].

In porcine research, the focus has been mostly on sex hormones associated with stress and inflammation [176, 184]. Not locally produced, but deriving from the bloodstream, is a range of pheromones such as androsterone, androstanol, and oxytocin that are secreted through saliva [187].

The microbiome of pigs has been studied recently. The predominant orders were lactobacillales, clostridiales, and corynebacteriales [188].

I.6.2.1 *Salivary biomarkers for stress*

To evaluate stress, biomarkers that reflect the pathophysiological responses to stress can be monitored. Various biomarkers representing the different body systems are involved. The SAM axis response is represented by α-amylase [22] and chromogranin A [23]. The HPA axis response, which can be monitored by glucocorticoids, is predominantly studied through cortisol [24]. The hypothalamic-pituitary-gonadal axis response (HPG) can be monitored through testosterone [25], salivary lipocalin, prolactin inducible protein [177] and odorant-binding protein [26]. Finally, the immune system can be evaluated by APPs (serum amyloid A [27], or albumin [189]), IgA [19], IgM [177], lipocalin-1 [28], protein S100-A8, protein S100-A9, protein S100-A12 [177] and IL-18 [29]. Alternatively, total esterase activity (TEA) or the abundance of some individual enzymes with esterase activity, like carbonic anhydrase IV, has proven to be increased in situations of pain and discomfort [30, 31]. A similar profile is found for haemoglobin, while the adenosine deaminase concentration and activity was reduced [177]. These markers all respond to an acute stressor. To monitor chronic stress, cortisol (e.g., [24]), chromogranin A [32, 33], and serum amyloid A [34] are suggested. However, these biomarkers are also affected by acute stress and could mask the presence of chronic stress.

Chapter II - Brief introduction to proteomics

The reasons for opting for proteomic approaches to analyse saliva are multiple. One main advantage is that an untargeted approach can be used facilitating hypothesis-free testing. The whole proteome can be studied at once and differences in salivary protein abundances between treatment groups are highlighted. Using this method, up- or downregulation of unexpected proteins can also be identified. On the other hand, targeted approaches can be used in which the (relative) abundance of specific proteins are analysed. One of the main advantages is that it avoids the use of antibodies. The pig is not a model organism for research, and, although the use of pigs as animal models is gaining interest, the availability of antibodies or antibody-based assays specific for porcine proteins is more scares compared to human or other more used animal models like mice. Although often high homologies between the amino acid sequences of porcine and human proteins are observed, that does not always guarantees success. Therefore, we opted for both untargeted and targeted proteomic approaches to study the porcine salivary proteome. What follows is a brief introduction to proteomics with a focus on one untargeted approach, being isobaric Tags for Relative and Absolute Quantification (iTRAQ), and one targeted approach, being parallel reaction monitoring (PRM), that were used to analyse porcine saliva in the research chapters.

II.1.1 Separation

Porcine saliva contains a complex mixture of proteins. To identify as much proteins as possible, the peptide and/or proteins need to be separated and fractionated first. This separation can be performed in a gel using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) or with multidimensional liquid chromatography (LC).

During 2D-PAGE, proteins are separated by isoelectric focusing (IEF) in the first dimension and by sodium dodecyl sulphate (SDS)-PAGE in the second dimension [190]. Afterwards, target spots/proteins within the gel are digested, and peptides are characterised using MS-techniques. However, this separation technique has some drawbacks, like low reproducibility, and difficulties to identify low-abundance proteins, small or very large proteins, membrane proteins or proteins with extreme isoelectric point (pI) values. Separation using LC largely

overcomes these hurdles. Intact proteins in a complex mixture can be fractionated (e.g., [191, 192]), however in that case every fraction must be processed resulting in an exponentially increasing workload. Therefore, proteins are usually first digested enzymatically, and the resulting peptides are then separated (e.g., [192, 193]). In the majority of cases, the protease Trypsin is used, which specifically cleaves after basic amino acids lysine and arginine. This workflow facilitates automatization since the final separation step of the peptide digest can be connected online to the identification step by mass spectrometry (MS). During LC, analytes in a mobile phase are separated based on their physicochemical properties by interaction to varying degrees to a solid stationary phase (i.c. resin in column). These interactions can be based on a.o. size, charge or hydrophobicity, depending on the composition of both the mobile and solid phase. As a result, several LC-separation techniques are available, but the most used combination is that of strong cation exchange (SCX) chromatography, based on charge, in combination with reversed phase (RP) chromatography, based on hydrophobicity (e.g., [192, 194]). During SCX-LC, peptides are positively charged by lowering the pH of the solution (Fig. 11). The positively charged peptides bind to the negatively charged resin of the columns. Bound analytes are eluted from the column by applying either a salt gradient or, less frequently used, a pH gradient. Peptides will also elute with a pH gradient when the mobile phase reaches their pI, neutralising the charge of these peptides (e.g., [24]). This LC-separation can be performed under pressure, resulting in lower elution times and higher resolutions, and is referred to as high-performance LC or HPLC.

During RP-LC, analytes are separated based on their hydrophobicity by which the interaction between the peptides and the stationary phase (e.g., a long hydrophobic carbonyl chain such as C18H37) is controlled by gradually lowering the polarity of the mobile phase, often using increasing concentrations of acetonitrile (ACN) (e.g., [192]).

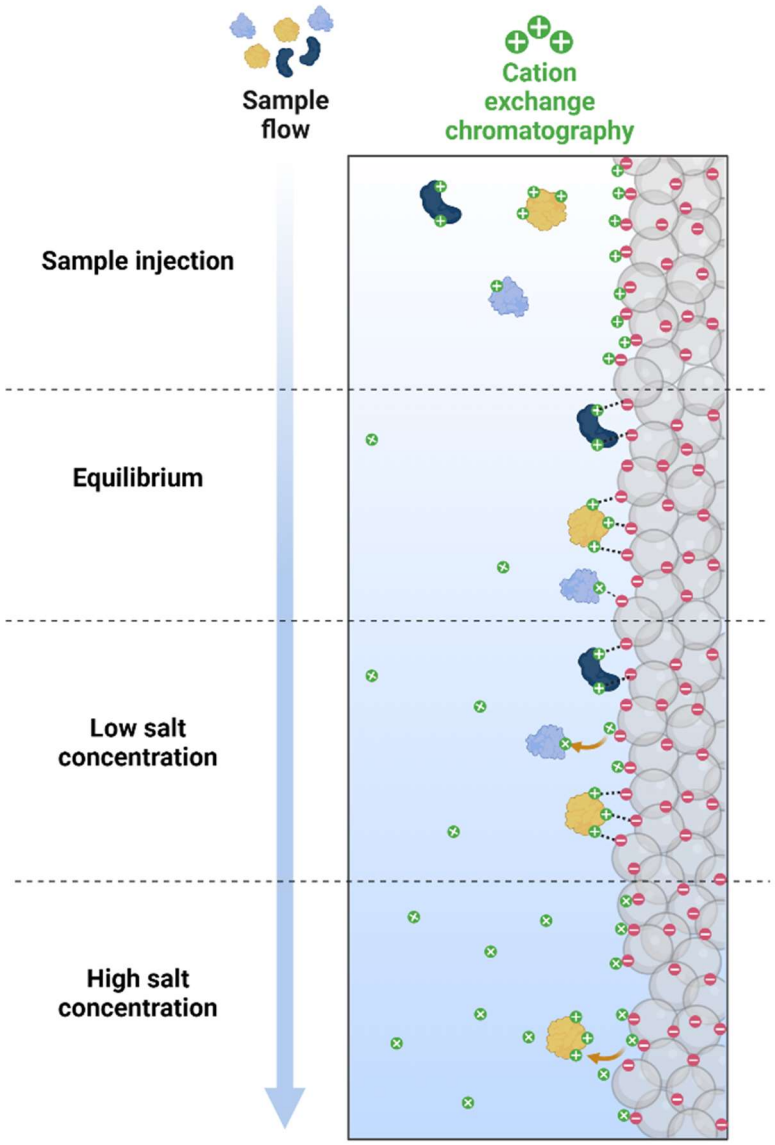


Figure 11. Graphical overview of cation exchange chromatography. *Molecules with different positive charges, in a liquid phase, are loaded into a column packed with a negatively charged resin to which they bind. When the salt concentration in the mobile phase increases, single charged peptides will elute first, followed by double-charged peptides with higher salt concentration and so on, until all peptides have eluted. Created with BioRender.com.*

II.1.2 Characterisation

When the complex sample is separated and fragmented, the resulting, less complex fractions are further analysed, and peptides are characterised using MS. As mentioned before, “online” connections between the LC-column and the mass spectrometer ensure a continuous flow of separated peptides into the MS. There, analytes are ionised, allowing to identify their respective charge and mass (mass/charge or m/z ratio). Therefore, MS instruments always consist of the following components: an ion source, which converts the analytes into ions; a

mass analyser, which sorts the ions according to their m/z ratio; and a detector, which measures the abundance of each detectable ion. There are different methods of ionisation, mass analysis, and detection, resulting in a variety of MS techniques (e.g., [192, 193, 195]).

A more complex form of MS is tandem-MS or MS/MS. With this technique, the analyte undergoes two MS rounds with a fragmentation step in between. The peptides are first analysed and their m/z and/or abundance and/or elution time is recorded. Afterwards, specific peptides (called precursor ions) are selected and fragmented into product ions. Analysis of these product ions not only allows us to characterise the sequence of these precursor ions better but also facilitates better quantification of specific target peptides, hence the proteins from which they originate (e.g., [192, 193, 195]).

II.1.3 Quantification

Several quantification methods using peptide digests are possible with LC-MS/MS (Fig. 12). Quantification can be accomplished with a global approach to determine the abundance of all peptides in the samples, for example, with the use of isobaric tags. Alternatively, the abundance of only specific targeted peptides in samples can be assessed, using techniques like parallel reaction monitoring (PRM).

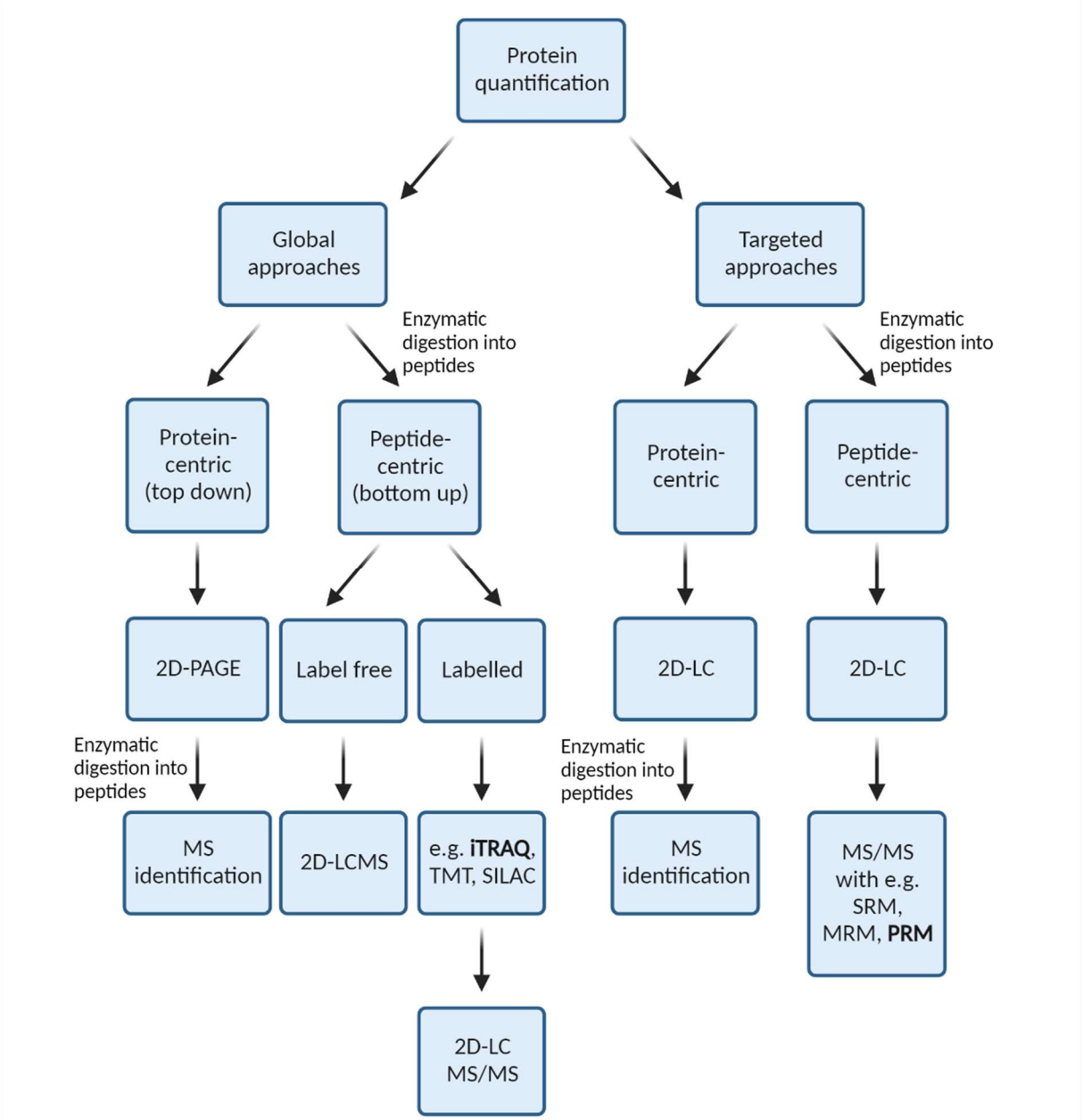


Figure 12. Overview of different methods for protein quantification using mass spectrometry. *The techniques in bold, more specifically iTRAQ (isobaric tags for relative and absolute quantification) and PRM (parallel reaction monitoring) were used in this project. Created with BioRender.com.*

II.1.3.1 *Isobaric labels for quantification*

Tandem MS is used for quantification of peptides with isobaric tags. Several isobaric tags are available, like iTRAQ or TMT (Tandem Mass Tag). These tags are chemically identical, but all differ in the ratio of isotopes between a reporter and a balancer part. For example, the iTRAQ 8-plex kit contains 8 such labels and thus allows for the parallel analysis of eight different samples at the same time (Fig. 13). When a sample is labelled, one chemical tag is added and

attaches to all peptides in an enzymatic digest of one sample via the amino moieties. Several labelled samples, all labelled with one of the eight different tags from the 8-plex kit, can be combined and analysed simultaneously. Since these tags are isobaric and chemically identical, labelled peptides do not show a shift in LC and a mass shift in MS. However, when these peptides are fragmented in MS/MS mode, each precursor peptide not only forms product ions (b- and y-type) but it also releases its reporter ion with a unique mass (either 113.1, 114.1, 115.1, 116.1, 117.1, 118.1, 119.1 or 121.1). As a result, the ratio of signal intensities from these reporter ions acts as an indication of the relative proportions of that peptide between the different labelled samples (e.g., [196]).

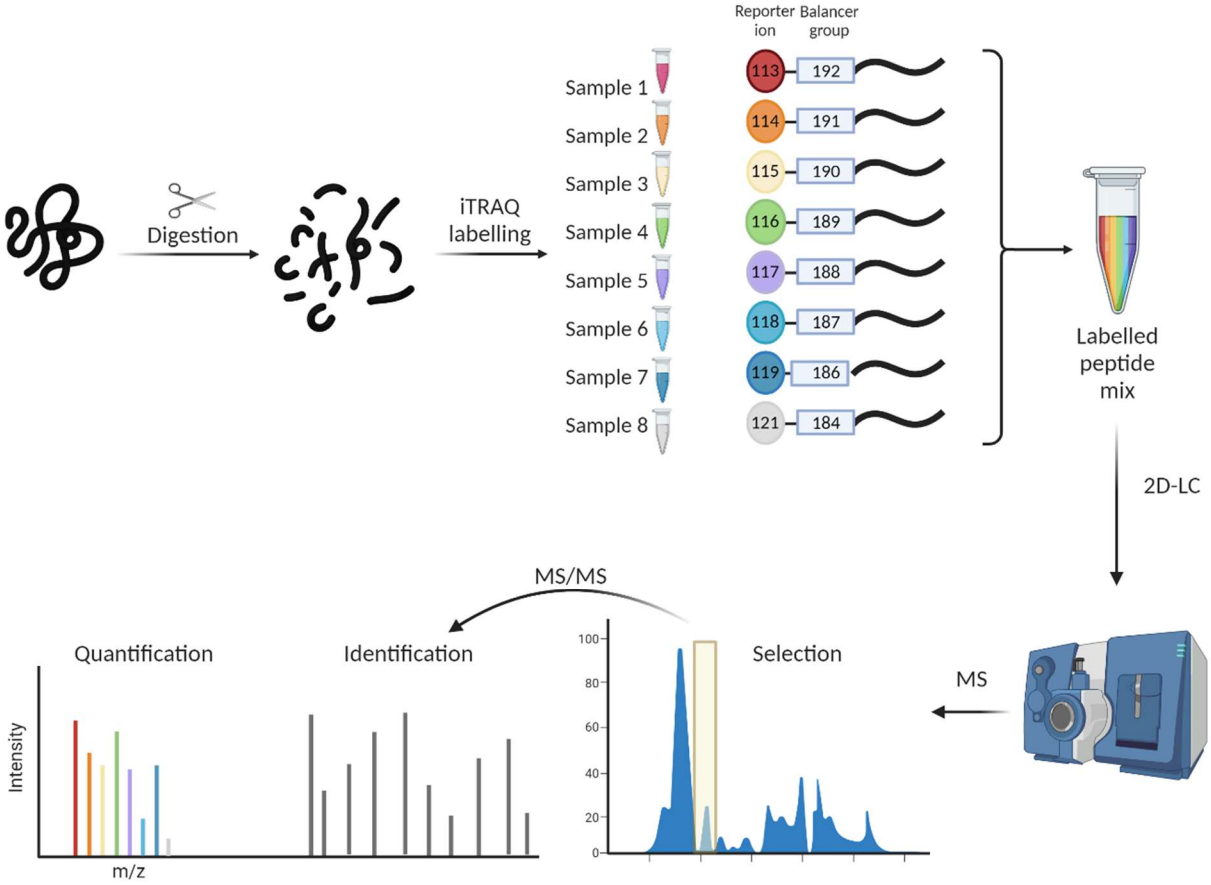


Figure 13. Quantification using iTRAQ-labelling. *Proteins are enzymatically digested into peptides. All peptides of the same sample are labelled with the same isobaric label. The eight labels all have a reporter ion with a different m/z, which is compensated in MW by the balancer part such that all labels are isobaric. After labelling, the labelled-peptide digests are combined and simultaneously fractioned using 2D-liquid chromatography (2D-LC). This digest is analysed with tandem-mass spectrometry (MS/MS). The generated spectra consist of a part used for peptide identification (peptide fragments, grey) and a part for peptide quantification (colours). The latter part originates from the released reporter ions during MS/MS. Each coloured peak*

reflects the abundance of a specific peptide in that specifically labelled sample. Created with BioRender.com.

II.1.3.2 *Parallel reaction monitoring (PRM)*

Alternatively, a targeted approach for peptide quantification can be performed. For PRM, an Orbitrap is often used (Fig. 14). This MS technique first separates the precursor ions using a high-resolution quadrupole mass analyser. This analyser consists of four cylindrical rods. Ions are separated based on the stability of their trajectories within the oscillating electric fields that are applied to the rods. The quadrupole is set to select a specific precursor ion after which the precursor ion is fragmented in the collision cell (Q2). Finally, all product ions are simultaneously scanned with high resolution and high accuracy in an orbitrap on the basis of their orbital motion frequency around an inner electrode (reviewed by [197]).

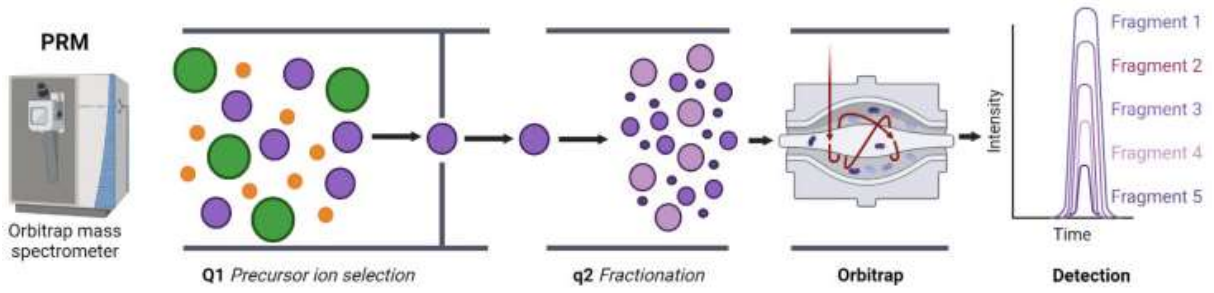


Figure 14. Parallel reaction monitoring (PRM). *A specific precursor ion is selected (Q1) and fragmented (Q2) after which the abundance and m/z of all fragments is recorded simultaneously in an orbitrap [198].*

II.1.4 Identification

Before proteins can be identified, their composing peptides should be identified first from MS and MS/MS spectra. The most commonly applied method is database searching, in which software matches observed spectra to theoretical or previously generated spectra from known peptides (Fig. 15). The accuracy of a possible peptide match is usually calculated and indicates how trustworthy this identification is. Only highly reliable peptide identifications are used for the following step, which is protein identification. Different kinds of software can be used to identify the peptide sequences and to match these to protein sequences in generated protein databases. Only the proteome of the desired species could be used to limit the number of possible matches. However, a correct match can only be made if the correct sequence is in

the search database. If an organism's genome sequence is incompletely present in the database, an underestimation of the total identifiable number of peptides could be the result (e.g., [195]). An additional global confidence assessment is identifying the false discovery rate (FDR). The generated spectra are not only searched against a target database but also against a "decoy" database. The latter is constructed by reversing or shuffling the protein sequences from the target database, leading to peptides with non-existing amino acid sequences. The number of false identifications estimates the reliability of the obtained identifications with the target database. For example, an FDR of 5%, means that 5% of the obtained peptide identifications are probably untrue.

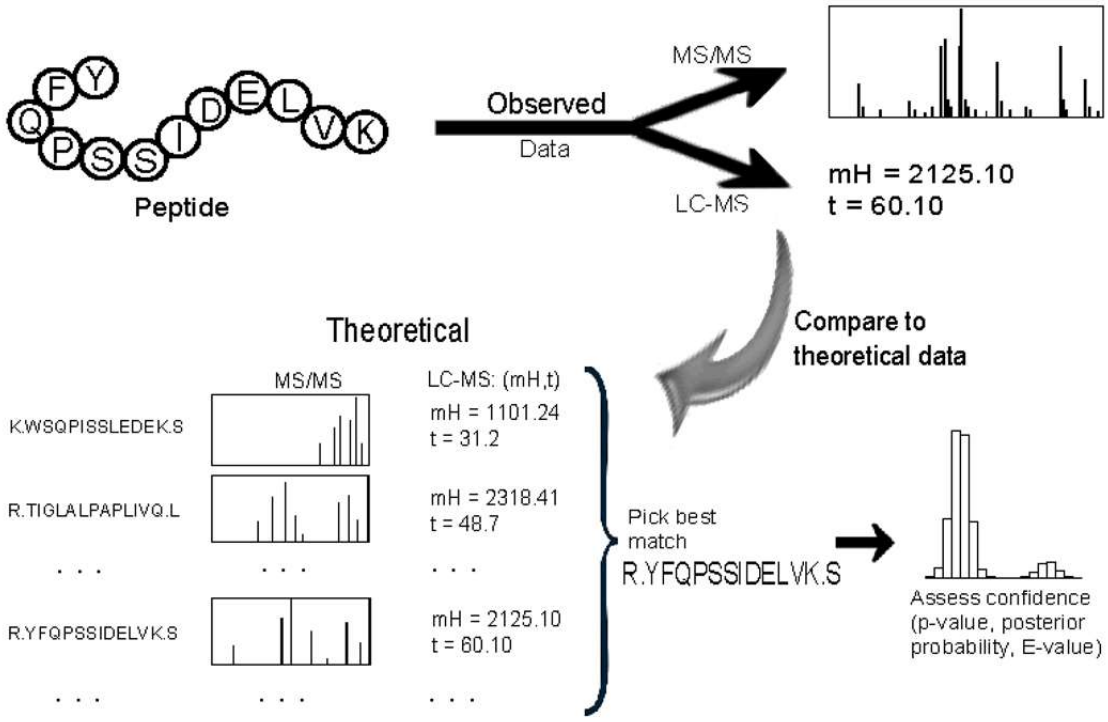


Figure 15. Protein identification. *Peptide and protein identification is usually performed by matching observed spectral/chromatographical measurements to theoretical or previously-observed information from a database. Mass (mH), in this case single charges peptides, and elution time (T) are used in high resolution LC-MS, while experimental fragmentation spectra are additionally matched from in silico predicted LC-MS/MS data. The quality of the identifications is assessed in the final step, including the use of a decoy database [195].*

Chapter III - Objectives

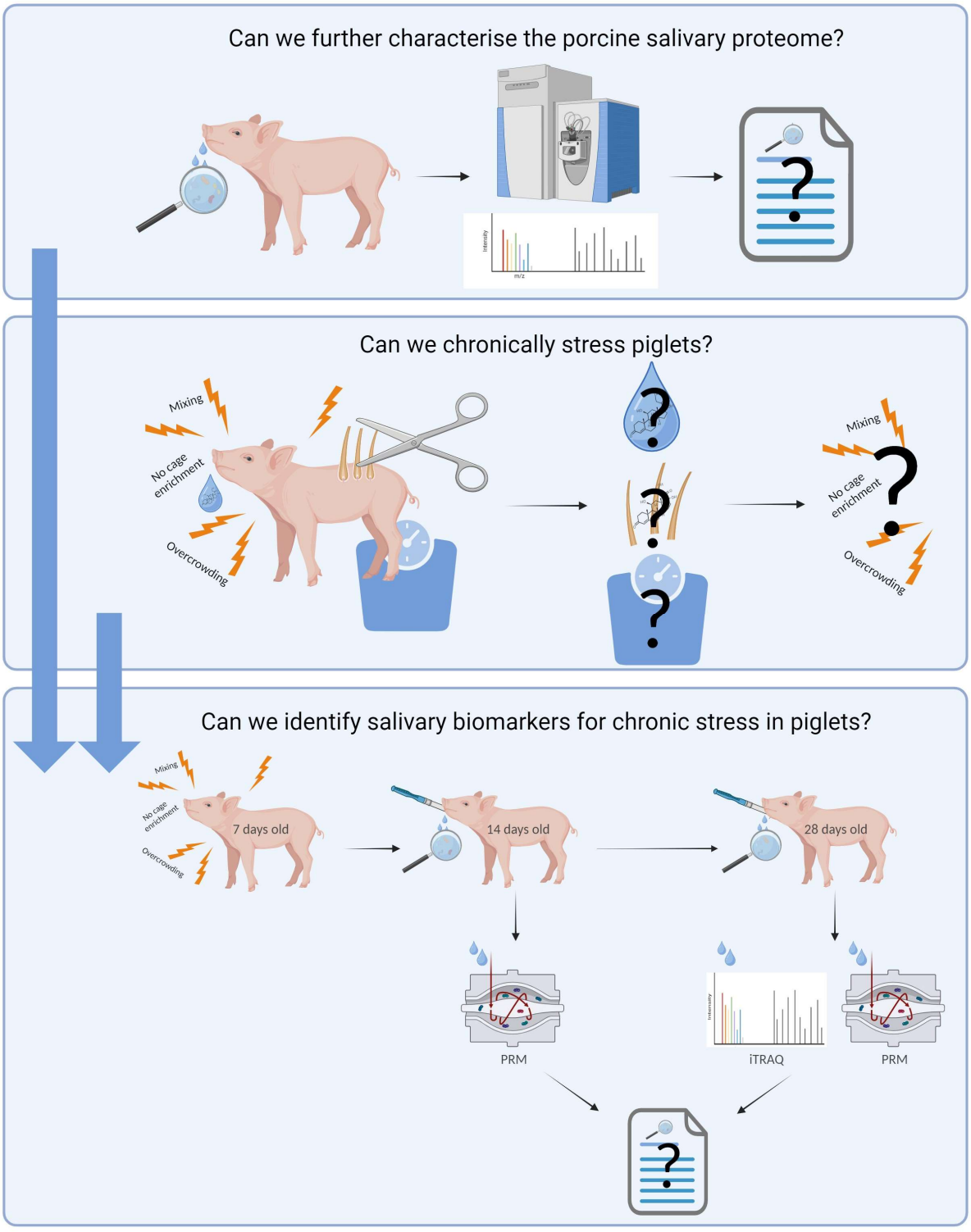
The goal of this doctoral thesis was to investigate whether saliva could be used to assess chronic stress in piglets.

Since knowledge of the protein composition of porcine saliva was scarce, the first goal of this thesis was to study and expand the knowledge about the porcine salivary proteome (**Chapter IV**). Since whole saliva, i.e., saliva secreted in the oral cavity is mixed with gingival crevicular fluid, buccal cells, microorganisms, and food remnants, gland-specific saliva was collected as ductal secretion. Shotgun proteomics was performed on mandibular/sublingual and parotid saliva to gain more insight into the proteome profile of porcine saliva.

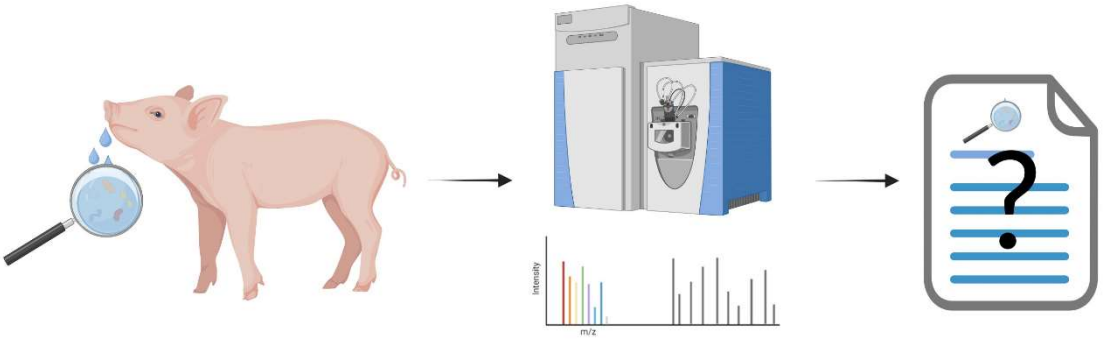
Once more insight into the porcine proteome was gathered, the use of saliva as a tool to monitor chronic stress started.

As mentioned before, some factors, like deprivation of cage enrichment, frequent mixing of animals and overcrowding could introduce chronic stress in pigs. However, to verify whether these stressors were successful, cortisol was investigated first, more specific, in hair and saliva (**Chapter V**).

Once the effectiveness of the stressors was confirmed, the salivary proteome of chronically stressed piglets was compared with that of control piglets (**Chapter VI**). To accomplish this, iTRAQ was applied in combination with a sensitive, high-resolution orbitrap MS/MS method. A subset of the proteins found in different relative concentrations was further validated using PRM.


Can we further characterise the porcine salivary proteome?
Can we chronically stress piglets?
Mixing
No cage enrichment
Overcrowding
Can we identify salivary biomarkers for chronic stress in piglets?
7 days old
14 days old
28 days old
PRM
iTRAQ
PRM

Chapter IV



Chapter IV - On the characterisation of the porcine gland-specific salivary proteome

Sara Prims, Geert Van Raemdonck, Charlotte Vanden Hole, Steven Van Cruchten, Chris Van Ginneken, Xaveer Van Ostade, Christophe Casteleyn

Adapted from Journal of Proteomics (2019), 10.1016/j.jprot.2019.01.016

Abstract

To expand the knowledge on the porcine salivary proteome, secretions from the three major salivary glands were collected from anaesthetised piglets. Pilocarpine and isoproterenol were simultaneously injected intraperitoneally to increase the volume and protein concentration of the saliva, respectively. The protein composition and relative protein-specific abundance of saliva secreted by the parotid gland and by the mandibular and monostomatic sublingual gland, were determined using iTRAQ. When combining two detection methods, MALDI-TOF/TOF MS and Q-Exactive orbitrap MS/MS, a total of 122 porcine salivary proteins and 6 mammalian salivary proteins with a predicted porcine homolog were identified. Only a quantitative and not a qualitative difference was observed between both ductal secretions. The 128 proteins were detected in both secretions, however, at different levels. Twenty-four proteins (20 porcine and 4 mammalian with a predicted porcine homolog) were predominantly secreted by the parotid gland, such as carbonic anhydrase VI and α-amylase. Twenty-nine proteins (all porcine) were predominantly secreted by the mandibular and sublingual glands, for example salivary lipocalin and submaxillary apomucin protein. Data are available via ProteomeXchange with identifier PXD008853.

IV.1 Significance

In humans, more than 3000 salivary proteins have been identified. To our knowledge, previous studies on porcine saliva only identified a total of 34 proteins. This research increased the total number of identified proteins in porcine saliva to nearly 150. This insight into the porcine salivary proteome will facilitate the search for potential biomarkers that may help in the early detection of pathologies and follow-up of animal welfare. Moreover, it can also endorse the value of a porcine animal model and contribute to a better understanding of the animal's physiology. Additionally, this was the first study to collect and analyse gland specific saliva of pigs. The obtained relative-quantitative knowledge of the identified proteins is valuable when comparing data of stimulated (chewing on a device) vs. unstimulated (passive) saliva collection in the future, since salivary stimulation changes the relative contribution of the major salivary glands to the whole saliva in the oral cavity. For example, carbonic anhydrase VI, which is present in higher concentrations in parotid saliva, has a higher concentration in stimulated whole saliva because of the larger contribution of the parotid gland after stimulation by chewing.

IV.2 Introduction

Saliva is an easily accessible body fluid that is widely accepted as a potential medium to assess an individual's health status (e.g., [94]). Using saliva has multiple advantages. It can be collected non-invasively, relatively stress-free and several samples can be taken over a short time period. As a result, saliva collection from challenging populations such as children, disabled or anxious persons and animals could be preferred over blood sampling. In addition, taking a saliva sample only requires limited training, so there is no need for highly trained staff. Saliva consists for 99% of water, complemented with a wide spectrum of proteins, peptides, hormones, nucleic acids and electrolytes [151-153]. In particular the proteins are investigated as potential salivary biomarkers. The two criteria to be met by a biomarker are (1) the possibility to measure the presence or the abundance of an individual protein, or a set of proteins, and (2) that its presence or abundance indicates normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [199]. In humans, more than 3000 salivary proteins have been identified of which approximately 27% derive from the blood stream through diffusion, filtration or active transport (e.g., [168-170]).

This means that not only local pathologies including Sjögren's syndrome [200], oral squamous cell carcinoma [201] or dental caries [202], but also systemic diseases, such as diabetes type 2 [203], lung cancer [204] and cardiovascular pathologies [205], could possibly be detected by a set of salivary biomarkers.

Also in veterinary medicine, interest in salivary biomarkers has tremendously risen during the last decade. In pig production, saliva is already being used to detect specific infections such as porcine circovirus type 2 (PCV2) and PRRSV (e.g., [206-208]). However, the search for more general biomarkers for infections and non-infectious adverse conditions, such as stress, intensifies. To facilitate this search, knowledge of the porcine salivary proteome is prerequisite. To our knowledge, previous studies on porcine saliva identified a total of 34 proteins (Supplementary file 1) [72, 173-177, 181, 183, 189, 207, 209-230]. Of these 34 proteins, 21 were identified for the first time using gel-based proteomics [173, 175, 177, 210], 12 using immune- or enzymatic assays or techniques relying on antibodies [72, 174, 176, 178, 183, 213, 219, 222, 226], while only one protein was identified using off-gel proteomics [211]. Since this number represents only a fraction of the number identified in human saliva, it is expected that more porcine salivary proteins are to be found. Insight into the porcine salivary proteome would not only facilitate the search for potential biomarkers, it can also endorse the value of a porcine animal model and contribute to a better understanding of the animal's physiology.

To further characterise the proteome of porcine saliva, uncontaminated saliva needs to be collected. However, whole saliva, i.e. saliva that is secreted in the oral cavity and mixed with gingival crevicular fluid, buccal cells and microorganisms is contaminated with a.o. food remnants. To avoid the latter, salivary sampling in humans is always preceded by a fasting period and a rinse of the oral cavity. Since this is difficult to achieve in pigs, uncontaminated saliva can only be collected from anesthetised pigs in the form of gland-specific saliva, more specifically as a ductal secretion, before contamination with a.o. food remnants can occur. In pigs, saliva is mainly produced by three major paired salivary glands, i.e. the parotid gland, the mandibular gland and the sublingual gland. The latter has a monostomatic and a polystomatic compartment. The minor labial, lingual and buccal glands secrete smaller amounts of saliva [143].

The goal of the present study was to expand the knowledge of the porcine salivary proteome by using shotgun proteomics applied to gland-specific saliva. In addition, the relative

abundance of the identified proteins was determined by means of an isobaric labelling method combined with complementary tandem mass spectrometry.

IV.3 Materials and methods

IV.3.1 Animals

Four piglets (Belgian Landrace × Piétrain), two animals of either sex, were transported from a local farm to the University of Antwerp at the age of 7 days. They were housed on commercial brooders (Rescue Decks®, S&R Resources LLC, Mason, USA) and artificially reared on milk formula (BIGGILAC PL+, AVEVE, Antwerp, Belgium), which was provided ad libitum, until the age of 21 days. Piglets had free access to water and were maintained under standard environmental conditions (12h/12h light/dark cycle, temperature adjusted to age). The animals were observed daily to document their general health status (body temperature, body weight, food and water consumption, general behaviour and signs of disease (e.g. diarrhoea) or distress (e.g. apathy)). All experiments were approved by the Ethical Committee for Animal Experiments of the University of Antwerp, Belgium (2014-01) and were in accordance to the European Directive (2010/63/EU).

IV.3.2 Sample and data collection

The 21-day-old animals were anesthetised by means of an intramuscular injection (0.22 mL/kg) of a mixed solution containing Zoletil 100® (tiletamine 50 mg/mL, zolazepam 50 mg/mL; Virbac, Louvain, Belgium) and Sedaxyl® (xylazine hydrochloride 20 mg/mL; VMD, Arendonk, Belgium). To collect saliva from the parotid gland, a modified Lashley cup [231] was three-dimensionally printed (Materialise HQ, Louvain, Belgium) in stainless steel in order to meet the required dimensions. The central part of the Lashley cup was positioned over the parotid papilla, which is a protrusion of the buccal mucosa located at the level of the third to fourth upper premolar [143]. The outer part was vacuumed to secure the cup against the buccal mucosa. The ipsilateral sublingual caruncle was cannulated using a 26 gauge I.V. catheter to collect mixed saliva originating from both the mandibular and monostomatic sublingual glands. Pilocarpine (2 mg/kg; Sigma Aldrich, Diegem, Belgium), which is a parasympathicomimetic drug (M_3-receptor agonist), and isoproterenol (2 mg/kg; Sigma Aldrich), which is a symphaticomimetic drug (β-receptor agonist), were simultaneously injected intraperitoneally. This dual stimulation is thought to mimic salivary reflex secretion

since both the parasympathetic and the sympathetic stimulation of acinar cells are necessary to induce salivary secretion [141]. The gland-specific secretions were collected in iced low-protein binding microcentrifuge tubes (Thermo Scientific, Brussels, Belgium) that were weighed before and after collection to estimate the collected volume, assuming that the specific gravity of saliva is 1.0 g/cm^3 (e.g., [232, 233]). Additionally, the collection time was recorded to estimate the salivary flow rate. Multiplying the concentration by the flow rate enabled us to determine the protein secretion rate, which could be a useful factor to normalise relative abundance data. All collected samples were immediately stored at -80°C until further analysis.

IV.3.3 Sample preparation for shotgun proteomics

To gain a more detailed insight into the salivary proteome, proteins from the eight salivary samples were labelled using an 8-plex kit of iTRAQ reagents and buffers according to the manufacturer's guidelines (Applied Biosystems Sciex Inc., MA, USA). In brief, appropriate volumes containing 100 µg of protein were taken from the four parotid and the four mandibular/sublingual samples, after determination of the total protein concentration using a bicinchoninic acid assay (BCA, Thermo Scientific). From each of these eight samples, proteins were extracted by means of acetone precipitation to discard any salts or lipids. The obtained protein pellets were resuspended in 500 mM triethylammonium bicarbonate (TEAB). Hydrogen bonds were disrupted and disulphide bonds reduced using 2% sodium dodecyl sulphate (SDS) and 50 mM tris-(2-carboxyethyl) phosphine (TCEP), respectively. To alkylate thiols reversibly, the samples were incubated with 200 mM methyl methanethiosulfonate (MMTS). Subsequently, trypsin (Promega, Leiden, The Netherlands) was added to digest proteins during the overnight incubation step at 37°C. Finally, the eight samples were labelled using the eight different iTRAQ reagents (label 113: piglet 1, mandibular/sublingual saliva; label 114: piglet 1, parotid saliva; label 115: piglet 2, mandibular/sublingual saliva; label 116: piglet 2, label 117: parotid saliva; piglet 3, mandibular/sublingual saliva; label 118: piglet 3, parotid saliva; label 119; piglet 4, mandibular/sublingual saliva; label 121: piglet 4, parotid saliva) and combined, resulting in one sample for further analysis by 2D-LC-MS/MS.

IV.3.4 First-dimensional separation

The combined sample was fractionated in a first dimension by strong cation exchange (SCX) chromatography using a Waters Alliance e2695 HPLC system with Photo Diode Array detector (Waters NV/SA, Zellik, Belgium). After acidification to a pH of 2.7, the sample was loaded onto a polysulfoethyl-aspartamide SCX-column (2.1 mm x 200 mm; 5 µm particles; PolyLC Inc., Columbia, MD, USA). Three different solvents (solvent A: 10 mM KH_2PO_4, 20% ACN (pH 2.7); solvent B: 10 mM KH_2PO_4, 650 mM KCl, 20% ACN (pH 2.7) and solvent D: 10 mM KH_2PO_4, 650 mM KCl, 20% ACN (pH 4.7)) were used in order to separate the combined peptide sample according to their charge. First, only solvent A was used for 10 min followed by a salt gradient (7.5 – 30%) of solvent B during 45 min and a pH gradient (30-100%) of solvent D for 15 min, with a final 5 min step of only solvent D to eluate highly charged peptides. During the entire gradient, a flow rate of 200 µL/min was kept constant. In total, ten fractions were collected of which the total peptide concentration was determined using the area under the curve (AUC). These fractions were lyophilised and frozen until further analysis.

IV.3.5 Second-dimensional separation and peptide analysis

IV.3.5.1 *Micro-capillary RP-HPLC and MALDI-TOF/TOF analysis*

The ten SCX fractions were resuspended in solvent A (95% water, 5% ACN, 0.1% formic acid (FA)) to allow for further fractionation using a micro-capillary HPLC system (Agilent 1100 series, Agilent Technologies, Waldbronn, Germany). A guard column (0.3 mm x 5 mm; particle size 5 µm; Zorbax 300 SB-C18, Agilent Technologies) was connected to a C18 analytical RP column (0.3 mm x 150 mm; particle size 3.5 µm; Zorbax 300 SB-C18, Agilent Technologies). A total of 15 µg, from each of the ten previously generated fractions, was loaded using solvent A followed by an elution with solvent B (10% water, 90% ACN, 0.1% FA) using the capillary pump at a flow rate of 6 µL/min. One technical replicate was performed. The gradient intensified from 5% to 55% in the first 56.7 min and quickly rose to 90% in the subsequent 3.3 min. This fractionation step separated each of the ten fractions into 350 spots (800 nL/spot) on an Opti-TOF® MALDI-plate (28 columns x 25 rows, 700 spots, 2 samples per plate; Applied Biosystems). Afterwards, a matrix consisting of 2.5 mg/mL α-cyanohydroxycinnamic acid in 70% ACN with an internal calibrant (63 pmol/mL human Glu-1-fibrinopeptide B, m/z 1570.6670) was used to cover the spots.

Spotted C18 fractions were analysed using a matrix assisted laser desorption/ionization (MALDI) AB4800 proteomics analyser (Applied Biosystems). Spots that generated precursors with a signal-to-noise (S/N) ratio above or equal to 100 after MALDI-TOF (MS) analysis (reflectron mode; 25 x 20 laser shots per spot; mass range: 800 – 3000 Da; laser intensity: 3300) were selected for MALDI-TOF/TOF (MS/MS) analysis (25 x 20 laser shots per spot; laser intensity: 4350). A maximum of 50 unique precursors per spot were selected for fragmentation in a collision cell (1 kV collisions (positive mode) with air), starting from the precursor with the lowest S/N-ratio.

IV.3.5.2 *Nano-capillary RP-HPLC and Q-Exactive orbitrap MS/MS analysis*

The ten SCX fractions that were used for the previous analysis using MALDI-TOF/TOF were desalted by using solid phase extraction (SPE). GracePure™ SPE C18 Columns (W. R. Grace & Co.-Conn., Maryland, USA) were placed onto a vacuum manifold and subsequently conditioned (three times with 100 µL methanol) and equilibrated (twice with 100 µL LC-MS H_2O) before the fractions were loaded (two times, reloading the eluate), washed (twice with 100 µl (20% methanol, 80% LC-MS H_2O)) and eluted (twice with 100 µL (40% methanol, 40% ACN, 20% 0.1% HCl in LC-MS H_2O)). The eluted peptides were subsequently lyophilised and frozen until further analysis. Each SCX fraction was separated in a second dimension by RP-C18 chromatography on an Easy nanoLC system using an Acclaim C18 PepMap®100 column (75 µm x 2 cm, 3 µm particle size) connected to an Acclaim PepMap™ RSLC C18 analytical column (50 µm x 15 cm, 2 µm particle size) (Thermo Scientific). Before loading, the vacuum-dried peptide pellets were dissolved in mobile phase A (2% ACN and 0.1% FA), and spiked with 20 fmol Glu-1-fibrinopeptide B (Glu-fib, Protea biosciences, Morgantown, WV, USA). Of each fraction, 1 µg of peptides was loaded onto the column. One technical replicate was performed. A linear gradient of mobile phase B (0.1% FA in 95% ACN) from 2% to 45% in 55 min followed by a steep increase to 100% mobile phase B in 5 min was used at a flow rate of 300 nl/min. Liquid chromatography was followed by MS and was performed on a Q-Exactive plus mass spectrometer equipped with a nanospray ion source (Thermo Fisher, Waltham, MA, USA). The high-resolution mass spectrometer was set up in an MS/MS mode in which a full scan spectrum (350 – 1850 m/z, resolution 70,000) was followed by a maximum of five high energy collision activated dissociation (HCD) tandem mass spectra (100 to 2000 m/z). The normalised

collision energy was set at 33%. A dynamic exclusion list of 15 s for data dependent acquisition was applied.

IV.3.6 Database searching

Proteome Discoverer 2.1 software (Thermo Scientific) was used to export the acquired MS/MS spectra to Mascot generic files. All MS/MS spectra were analysed by Mascot (version 2.5.1; Matrix Science, London, UK) (Supplementary file 4: raw output Mascot) using the Swiss-Prot database (Mammalia, 2017, 65789 entries) or the *Sus scrofa* database was generated based on characterised porcine proteins and the porcine genome (*Sus scrofa* database (reviewed + unreviewed), generated from Uniprot (2018/01/10), 50045 entries). To be complete, a search using the NCBI database (taxonomy *Sus scrofa* generated from NCBI (2018/01/10), 87942 entries) was additionally performed. Since this database did not lead to the identification of more proteins than the Uniprot *Sus scrofa* database and because the latter database is more curated, only the results of the Uniprot search are presented in this manuscript. Methylmethanethiosulfonate binding to cysteine and iTRAQ 8-plex labelling of lysine and the N-terminus were specified as fixed modifications. Oxidation of methionine and iTRAQ 8-plex labelling of tyrosine were set as variable modifications. Beside these common used variable modifications in salivary research, additional searches have been performed using deamidation (NQ), pyro-glutamic formation (E) and/or possible phosphorylations (STY). These variable modifications only had a minimal influence on the outcome of the protein identification search. Therefore, this additional data is not shown in this manuscript. Analysis was performed based on trypsin digestions, fragment ion mass tolerance of 0.8 Da, and a precursor tolerance of 200 ppm for MALDI-TOF/TOF-spectra and 8 ppm for Q-Exactive orbitrap-MS/MS-spectra. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [234] partner repository with the dataset identifier PXD008853. To avoid misidentifications due to sample contamination the common Repository of Adventitious Proteins was consulted (cRAP) (http://www.thegpm.org/crap/).

IV.3.7 Data analysis

Scaffold Q+ (version 4.7.5; Proteome Software Inc., Portland, USA) was used to validate MS/MS-based peptide and protein identifications [235]. Peptide identifications were only

accepted if they could be established at a probability greater than 95% by the stringent Peptide Prophet algorithm [236] with Scaffold delta-mass correction. This additional selection step reduced the number of peptides identified by Mascot, preserving only peptides with a high confidence. Protein identifications were accepted if they met the same probability criterion and contained at least one Scaffold-selected peptide. Protein probabilities were assigned by the Protein Prophet algorithm [237]. The FDR was less than 3% for all Mascot searches. All keratins were removed from the output list. A BLAST analysis was performed on all uncharacterised proteins (BLASTP 2.8.0+, All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects Program, *Sus scrofa* (taxid:9823)) [238]. Additionally, all peptides that led to a protein identification with the mammalian database, but not with the porcine database, were also subjected to a BLAST analysis. Only peptides with a 100% identity and query coverage were preserved. Relative quantification was reported by Scaffold Q+ based upon detected iTRAQ reporter ions during tandem mass spectrometric analyses. Since no absolute qualification is possible using this method, the first sample with iTRAQ label 113 was chosen as a reference label and generally received an abundance of 1 for each protein. All other samples/labels were compared to this reference. The output is a number that indicates a ratio that is relative to this reference sample.

IV.3.8 Statistical analysis

Mixed models were fitted to identify differences between both ductal secretions for the following parameters: concentration, flow rate, protein secretion rate and obtained secretion ratios of all identified proteins. As a consequence, salivary gland was used as a fixed factor. Since one pig produces two samples, data are not independent from each other. Therefore, this fixed factor was nested within an animal to operate as a random intercept in order to account for the variation between the animals. All data were analysed using JMP® Pro 12 (SAS Institute Inc., North Carolina, USA). A *P*-value smaller than or equal to 0.05 was considered statistically significant.

IV.4 Results

IV.4.1 Concentration and flow rate

Collected saliva from the parotid gland had an average protein concentration of 2.85 ± 0.72 mg/mL with an average flow rate of 12.70 ± 7.12 µL/s, meaning that 39 ± 27 µg of proteins were secreted per second (Table 1). The mandibular and sublingual glands together secreted saliva with a concentration of 1.67 ± 0.45 mg/mL at a flow rate of 8.16 ± 3.80 µL/s, resulting in a protein secretion rate of 14.62 ± 10.14 µg/s. The protein concentration of both gland-specific saliva samples differed significantly (*P*-value = 0.031), with parotid saliva displaying a higher concentration. Neither the salivary flow rate (*P*-value = 0.303) nor the protein secretion rate (*P*-value = 0.143) differed significantly between both saliva samples.

Table 1. *Salivary protein concentrations, flow rates and protein secretion rates of the collected gland-specific samples. The gender of each 21-day-old animal is specified. An asterisk (*) indicates statistically significant differences.*

Animal	Gender	Gland	Protein concentration (mg/mL)	Flow rate (mL/s)	Protein secretion rate (µg/s)
Piglet 1	Female	Mandibular/sublingual	1.10	0.0061	6.68
Piglet 2	Female	Mandibular/sublingual	1.56	0.0045	7.03
Piglet 3	Male	Mandibular/sublingual	2.14	0.0132	28.18
Piglet 4	Male	Mandibular/sublingual	1.88	0.0088	16.61
Piglet 1	Female	Parotid	2.54	0.0090	22.89
Piglet 2	Female	Parotid	3.74	0.0180	67.34
Piglet 3	Male	Parotid	3.06	0.0193	58.96
Piglet 4	Male	Parotid	2.07	0.0045	9.32
Average mandibular/sublingual gland ± SD			1.67 ± 0.45*	0.0082 ± 0.0038	14.62 ± 10.14
Average parotid gland ± SD			2.85 ± 0.72*	0.0127 ± 0.0071	39.63 ± 27.93

IV.4.2 Qualitative data

IV.4.2.1 *Identified proteins*

Analysing the samples with MALDI-TOF/TOF led to an identification of 21 proteins when using a mammalian Swiss-Prot database as identification source (Fig. 1, Table 2). Although the technique allowed for the identification of proteins that had not been characterised in porcine saliva before, this number was unexpectedly low. This might be caused by interspecies homology of some proteins since the search algorithm only assigns a unique sequence from

the database to a listed identification, hence an underestimation of identifications by Mascot may have occurred. As stated before by others, using larger protein databases requires a higher number of peptide spectra for unambiguous assignment of proteins [239]. To prevent this, a porcine protein database was assembled. Because only 1424 reviewed porcine proteins were available, also unreviewed porcine proteins were included in the porcine database. This database led to the identification of 41 porcine proteins, confirming the identification of 20 proteins that were detected using the mammalian database and adding 21 new porcine identifications. The identification of 1 mammalian protein, being disintegrin/metalloproteinase domain-containing protein 9, was not confirmed. However, a BLAST analysis using a porcine database with the identified peptides indicated that this mammalian protein has a predicted porcine homolog so it was added to the list of identified proteins as mammalian protein with a predicted porcine homolog. To test whether a more sensitive detection method might be advisable, the samples were analysed again, now using a Q-Exactive orbitrap MS instrument. An additional 86 proteins were identified when the search results of both the mammalian and the porcine databases were combined. In total, 122 highly confident porcine proteins and 6 mammalian proteins with a predicted porcine homolog could be identified combining both techniques and databases. However, 25 proteins were reported as uncharacterised proteins, meaning that these proteins lacked annotation. A BLAST analysis with a larger porcine database was conducted to gain insight into the potential function of these proteins. The amino acid sequence of all uncharacterised proteins aligned with a (predicted) protein in the *Sus scrofa* target database. All proteins had an identity percentage that equalled or exceeded 95. Only three proteins, including Ig kappa light chain V-C region (PLC18), MHC class II antigen and envelope glycoprotein, had a slightly lower identity of 87%, 94% and 78%, respectively. Usually, an identification is only considered to be reliable if at least two significant peptides are identified using Mascot. However, when using the stringent Peptide and Protein Prophet algorithm by Scaffold only reliable peptide identifications remain and we therefore allowed protein identifications based on one ‘Scaffold’ peptide. This is in line with the view of others who promote the use of protein inference engines instead of implementing rigid protein inference rules [240]. To give an idea, of all 157 proteins identified based on one significant peptide identification by Mascot, 41 proteins passed the stringent Peptide and Protein Prophet algorithm by Scaffold (Supplementary file 2). Additionally, 21 proteins had more than one significant peptide

identifications by Mascot but only 1 peptide was assigned to be reliable by Scaffold (Supplementary file 2 and 4). In total, 62 proteins are identified based on one 'Scaffold' peptide. For 45 of these proteins, this peptide was detected in all samples (Supplementary file 2). For 12 proteins this single peptide was only detected in a part of the samples. Finally, for 5 proteins, no iTRAQ-labelled peptides were identified.

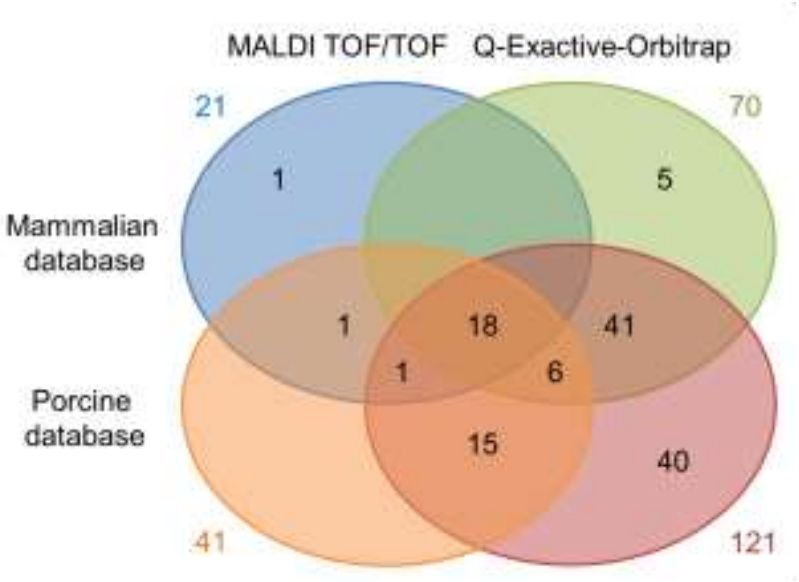


Figure 1. *Venn diagram of identified proteins combining two analytical methods (MALDI TOF/TOF and Q-Exactive orbitrap MS/MS) and two databases (mammalian and porcine database).*

Table 2. *List of identified proteins in gland-specific saliva of 21-day-old piglets with their molecular weight (MW). A BLAST analysis was performed on all uncharacterised proteins, which are identifiable by the word BLAST in front of their names. The unique peptides identified using MALDI TOF/TOF and/or Q-Exactive orbitrap MS/MS were analysed using the Peptide and Protein Prophet algorithm with Scaffold delta-mass correction. This additional selection step only preserves the identified unique peptides with a high confidence. Proteins indicated with a '†' were only identified using a mammalian database. All the mammalian peptides that led to a protein identification were also subjected to a BLAST analysis using a more complete porcine database. Only peptides with a 100% identity and query coverage were preserved. The predicted porcine protein names are given behind the mammalian homologue.*

#	Protein name	MW (kDa)	Number of unique peptides identified using MALDI-TOF/TOF	Number of unique peptides identified using Q-Exactive orbitrap	Gland by which the protein is predominantly secreted (not normalised to protein secretion rate)	*P*-value	Gland by which the protein is predominantly secreted (normalised to protein secretion rate)	*P*-value
1	Carbonic anhydrase VI (*Sus scrofa*)	36	9	12	Parotid gland	< 0.0001	Parotid gland	0.0445
2	Salivary lipocalin (*Sus scrofa*)	22	8	13	Mandibular/ sublingual gland	0.0026		0.2749
3	Lactoperoxidase (*Sus scrofa*)	80	17	29	Parotid gland	0.0037		0.0874
4	Submaxillary apomucin (*Sus scrofa*)	1184	24	33	Mandibular/ sublingual gland	< 0.0001		0.1444
5	Serum albumin (*Sus scrofa*)	70	11	29		0.1085		0.5493
6	Chromosome 6 open reading frame 58 (*Sus scrofa*)	39	6	7	Parotid gland	0.0034		0.0829
7	Cystatin (*Sus scrofa*)	16	3	9		0.1241		0.1633
8	Coagulation factor V (*Sus scrofa*)	256	8	23		0.2357		0.1099
9	Prolactin induced protein (*Sus scrofa*)	12	3	4		0.1975		0.1853
10	BLAST: BPI fold-containing family A member 2 isoform X2 (*Sus scrofa*)	26	7	6	Parotid gland	0.0027	Parotid gland	0.0348

11	α-amylase (*Sus scrofa*)	56	8	11	Parotid gland	0.0435		0.1138
12	BPI fold-containing family A member 1 (*Sus scrofa*)	26	4	6	Mandibular/ sublingual gland	0.0150		0.2869
13	Long palate lung and nasal epithelium protein 1 (*Sus scrofa*)	52	3	16		0.5029		0.0784
14	Angiotensinogen (*Sus scrofa*)	51	5	14	Parotid gland	0.0423		0.1277
15	BLAST: thrombospondin-1 precursor (*Sus scrofa*)	130	5	15	Parotid gland	0.0490		0.0503
16	Basic proline-rich protein (*Sus scrofa*)	62		2	Parotid gland	0.0016		0.0713
17	Carboxylic ester hydrolase (Fragment) (*Sus scrofa*)	39	3	6	Parotid gland	0.0207		0.0971
18	BLAST: zymogen granule protein 16 homolog B (*Sus scrofa*)	19	5	3		0.0992		0.9980
19	Serotransferrin (*Sus scrofa*)	77	3	9		0.1010		0.7975
20	BLAST: secretoglobin family 1D member 1 precursor (*Sus scrofa*)	12	2	2	Mandibular/ sublingual gland	0.0041		0.1327
21	Cholinesterase (*Sus scrofa*)	52	3	3	Parotid gland	0.0027		0.0524
22	BLAST: vitelline membrane outer layer protein 1 homolog precursor (*Sus scrofa*)	22	4	5	Parotid gland	0.0003		0.0645
23	Lysozyme C-3 (*Sus scrofa*)	17	5	3		0.2129		0.1863
24	BLAST: LOW QUALITY PROTEIN: IgGFc-binding protein (*Sus scrofa*)	265	2	8	Mandibular/ sublingual gland	0.0001		0.9814
25	Calcium-activated chloride channel regulator 1 (*Sus scrofa*)	88	2	9	Mandibular/ sublingual gland	< 0.0001		0.6041
26	Statherin (*Sus scrofa*)	8		1		0.317		0.3088
27	BLAST: double-headed protease inhibitor, submandibular gland-like (*Sus scrofa*)	13	2	2	Mandibular/ sublingual gland	< 0.0001		0.4171
28	BLAST: LOW QUALITY PROTEIN: nucleobindin-2 (*Sus scrofa*)	56	2	9		0.0885		0.1699

29	BLAST: multidrug resistance protein 1 isoform X1 (*Sus scrofa*)	139		1		0.3023		0.9001
30	Fstl1 (*Sus scrofa*)	35	1	4		0.0546		0.0684
31	Beta-2-microglobulin (*Sus scrofa*)	13	2	2		0.2423		0.1470
32	Sulfhydryl oxidase (*Sus scrofa*)	81	2	8		0.2057		0.1495
33	Cysteine rich secretory protein 3 (*Sus scrofa*)	27	2	3	Mandibular/ sublingual gland	0.0125		0.1793
34	Chromosome 16 open reading frame 89 (*Sus scrofa*)	41	1	3	Parotid gland	0.0167		0.0976
35	Mucin 7, secreted (*Sus scrofa*)	35	1	2	Mandibular/ sublingual gland	0.0019		0.2205
36	Alpha-2-glycoprotein 1, zinc-binding (*Sus scrofa*)	36		3		0.2497		0.2385
37	Tachykinin 4 (*Sus scrofa*)	12		3		0.0049		0.1027
38	Stromal cell derived factor 4 (*Sus scrofa*)	41		4		0.0633		0.0962
39	Alpha-1-acid glycoprotein (*Sus scrofa*)	23	3	3		0.2751		0.7296
40	Lectin, galactoside-binding, soluble, 3 binding protein (*Sus scrofa*)	61	1	6		0.2109		0.1278
41	Alpha-1-antitrypsin (*Sus scrofa*)	47		3		0.9583		0.0934
42	BLAST: acidic mammalian chitinase precursor (*Sus scrofa*)	52	1	3	Mandibular/ sublingual gland	0.0010		0.5021
43	Serpin family I member 1 (*Sus scrofa*)	46		3		0.3437		0.1328
44	Tissue inhibitor of metalloproteases-2 (*Sus scrofa*)	25	1	1	Parotid gland	< 0.0001		0.0736
45	Alpha-2-HS-glycoprotein (*Sus scrofa*)	39		3	Mandibular/ sublingual gland	0.0262		0.3736
46	Clusterin (*Sus scrofa*)	52		6		0.0768		0.8086
47	BLAST: cadherin-1 precursor (*Sus scrofa*)	98		2	Parotid gland	0.0035	Parotid gland	0.0371

48	BLAST: deleted in malignant brain tumors 1 protein precursor (*Sus scrofa*)	147	1	1		0.1337		0.4588
49	Inhibitor of carbonic anhydrase (*Sus scrofa*)	78		3		0.7833		0.1392
50	Actin, gamma 1 (*Sus scrofa*)	42		2		0.2965		0.1295
51	RNA exonuclease 1 homolog (*Sus scrofa*)	130		1		0.2943		0.1709
52	Peptidyl-prolyl cis-trans isomerase (*Sus scrofa*)	24		5		0.8271		0.1434
53	BLAST: nucleobindin-1 precursor (*Sus scrofa*)	49		4		0.2963		0.1740
54	Myosin binding protein C, slow type (*Sus scrofa*)	136		1		0.6791		0.1455
55	Serum amyloid A protein (*Sus scrofa*)	15	3					
56	Saposin-B-Val (*Sus scrofa*)	58		3	Mandibular/ sublingual gland	0.0005		0.3885
57	Long palate lung and nasal epithelium protein 2 (*Sus scrofa*)	50		2		0.0723		0.6492
58	Alpha-2-macroglobulin (*Sus scrofa*)	167		1		0.0574		0.6115
59	Peptidylglycine alpha-amidating monooxygenase (*Sus scrofa*)	106	1	2	Parotid gland	0.0019		0.0930
60	Apolipoprotein A-I (*Sus scrofa*)	30		2	Mandibular/ sublingual gland	0.0001		0.8329
61	Lipocalin-1 (*Sus scrofa*)	19		1		0.1064		0.2681
62	Polyubiquitin-C (*Sus scrofa*)	60	1	2		0.5676		0.1261
63	Proteasome (Prosome, macropain) 26S subunit, non-ATPase, 1 (*Sus scrofa*)	106	1	1	Mandibular/ sublingual gland	0.0445		0.6302
64	Odorant-binding protein (*Sus scrofa*)	18		3	Mandibular/ sublingual gland	0.0410		0.4435
65	Pheromaxein C subunit (*Sus scrofa*)	10		2	Mandibular/ sublingual gland	0.0419	Mandibular/ sublingual gland	0.0474

66	Desmocollin 3 (*Sus scrofa*)	100		1		0.4719		0.1098
67	Chitinase 3 like 2 (*Sus scrofa*)	48		1		0.5950		0.1242
68	Potassium voltage-gated channel subfamily J member 6 (*Sus scrofa*)	48		1				
69	IgG heavy chain (*Sus scrofa*)	50		1		0.0899		0.8832
70	Multiple coagulation factor deficiency 2 (*Sus scrofa*)	24		2		0.0734		0.0996
71	Transthyretin (*Sus scrofa*)	16		2		0.0785		0.7136
72	BLAST: Ig kappa chain V-C region (PLC18) (fragment) (*Sus scrofa*)	24		2		0.0811		0.7478
73	Protein S100-A12 (*Sus scrofa*)	11		2		0.3953	Parotid gland	0.0223
74	Peroxiredoxin 5 (*Sus scrofa*)	17		2	Mandibular/ sublingual gland	0.0117		0.4362
75	α-amylase (*Sus scrofa*)	57		2		0.4547		0.1522
76	SIL1 nucleotide exchange factor (*Sus scrofa*)	51		1		0.5231		0.1533
77	Ankyrin repeat domain 24 (*Sus scrofa*)	117		1	Mandibular/ sublingual gland	0.0139		0.3668
78	Tetraspanin (*Sus scrofa*)	26		1		0.1770		0.3003
79	BLAST: pheromaxein C subunit precursor (*Sus scrofa*)	10		1		0.1491		0.5125
80	Carboxylic ester hydrolase (Fragment) (*Sus scrofa*)	37		1	Parotid gland	0.0327		0.0946
81	Haemoglobin subunit beta (*Sus scrofa*)	16		2		0.3100	Parotid gland	0.0071
82	Ribonuclease 4 (*Sus scrofa*)	17		2		0.1350		0.1661
83	BLAST: LOW QUALITY PROTEIN: serpin A3-8 (*Sus scrofa*)	46		1		0.0579		0.7671
84	Cystatin-B (*Sus scrofa*)	11		1		0.6146	Parotid gland	0.0328
85	Cathepsin B (*Sus scrofa*)	37		1	Mandibular/ sublingual gland	0.0021		0.9881

86	Peptidoglycan-recognition protein (*Sus scrofa*)	24		1	Mandibular/ sublingual gland	0.0445		0.2895
87	Insulin-like growth factor-binding protein 4 (*Sus scrofa*)	28		1		0.5712		0.0942
88	O-acyltransferase (*Sus scrofa*)	61		1				
89	BLAST: collagen alpha-1(V) chain precursor (*Sus scrofa*)	184		1		0.6123		0.7351
90	BLAST: chitinase domain-containing protein 1 precursor (*Sus scrofa*)	45		1		0.2409		0.1268
91	Allograft inflammatory factor 1 like (*Sus scrofa*)	16		1				
92	Testis specific serine kinase substrate (*Sus scrofa*)	64		1				
93	BLAST: LOW QUALITY PROTEIN: myosin-10 (*Sus scrofa*)	26		1	Mandibular/ sublingual gland	< 0.0001		0.2346
94	alpha-1,2-Mannosidase (*Sus scrofa*)	73		1	Parotid gland	0.0009	Parotid gland	0.0452
95	Angiomotin (*Sus scrofa*)	118		1	Mandibular/ sublingual gland	0.0163		0.5369
96	BLAST: interleukin-15 receptor subunit alpha isoform X2 (*Sus scrofa*)	18		1		0.2861		0.1176
97	Lysozyme C-1 (*Sus scrofa*)	15		1		0.1854		0.5069
98	Elongation factor 1-alpha (*Sus scrofa*)	50		1	Mandibular/ sublingual gland	0.0054		0.4159
99	B-cell CLL/lymphoma 9 protein (*Sus scrofa*)	149		1		0.9861		0.2702
100	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (*Sus scrofa*)	80		1	Parotid gland	0.0172		0.0577
101	Dystroglycan (*Sus scrofa*)	95		1	Parotid gland	0.0120		0.0865
102	Ariadne RBR E3 ubiquitin protein ligase 1 (*Sus scrofa*)	64		1	Mandibular/ sublingual gland	0.1160		0.3289

103	Syncoilin, intermediate filament protein (*Sus scrofa*)	55		1	Mandibular/ sublingual gland	0.0038		0.1321
104	Son of sevenless-like 1 (*Sus scrofa*)	152		1		0.1792		0.1137
105	Sorting nexin 13 (*Sus scrofa*)	98		1		0.2861		0.1782
106	CutA divalent cation tolerance homolog (*Sus scrofa*)	22		1		0.2277		0.2313
107	Cell growth regulator with EF-hand domain 1 (*Sus scrofa*)	26		1	Mandibular/ sublingual gland	0.0115		0.5943
108	BRICHOS domain containing 5 (*Sus scrofa*)	26		1		0.2418		0.1378
109	Heat shock protein HSP 90-alpha (*Sus scrofa*)	85		1	Mandibular/ sublingual gland	0.0092		0.6126
110	BLAST: envelope glycoprotein, partial (*Sus scrofa*)	32		1		0.1327		0.3865
111	Uncharacterized protein (*Sus scrofa*)	9		1		0.4493	Parotid gland	0.0118
112	Ribonuclease T2 (*Sus scrofa*)	23		1		0.3783		0.1606
113	Solute carrier family 38 member 10 (*Sus scrofa*)	111		1		0.4536		0.1858
114	Peptidyl-prolyl cis-trans isomerase A (*Sus scrofa*)	18		1		0.1367		0.1856
115	Sphingomyelin phosphodiesterase acid like 3B (*Sus scrofa*)	45		1	Mandibular/ sublingual gland	0.0363		0.4969
116	Alpha-1-antichymotrypsin 3 (fragment) (*Sus scrofa*)	23		1	Mandibular/ sublingual gland	0.0419		0.3825
117	BLAST: MHC class II antigen, partial (*Sus scrofa*)	12		1	Parotid gland	0.0002	Parotid gland	0.0276
118	60S ribosomal protein L3 (*Sus scrofa*)	46		1		0.4286		0.1857
119	BLAST: heat shock cognate 71 kDa protein (*Sus scrofa*)	71		1		0.3749		0.0920

120	Proteasome 26S subunit, non-ATPase 7 (*Sus scrofa*)	28		1		0.0839		0.2144
121	BLAST: heat shock protein HSP 90-beta (*Sus scrofa*)	83		1		0.1906		0.1520
122	Protease, serine 22 (*Sus scrofa*)	36		1		0.3704		0.0745
123	Formin-2 (*Mus musculus*)[†] - PREDICTED: Formin-2 (*Sus scrofa*)	167		2	Parotid gland	0.0057		0.0868
124	Methylcytosine dioxygenase TET2 (*Homo sapiens*)[†] - PREDICTED: methylcytosine dioxygenase TET2-like (*Sus scrofa*)	223		1	Parotid gland	0.0017	Parotid gland	0.0419
125	Alanine--tRNA ligase, cytoplasmic (*Rattus norvegicus*)[†] - PREDICTED: alanine--tRNA ligase, cytoplasmic (*Sus scrofa*)	106		1	Parotid gland	0.0319		0.0851
126	Sterol O-acyltransferase 2 (*Homo sapiens*)[†] - PREDICTED: LOW QUALITY PROTEIN: sterol O-acyltransferase 2 (*Sus scrofa*)	59		1				
127	Disintegrin and metalloproteinase domain-containing protein 9 (*Homo sapiens*)[†] - PREDICTED: disintegrin and metalloproteinase domain-containing protein 9 (*Sus scrofa*)	90	1			0.4119		0.7649
128	Complement C1q tumor necrosis factor-related protein 3 (Homo sapiens)[†] - PREDICED: complement C1q tumor necrosis factor-related protein 3 precursor (*Sus scrofa*)	27		1	Parotid gland	0.0021	Parotid gland	0.0611

IV.4.2.2 *Classification of identified proteins*

The obtained porcine salivary proteome was plotted against a gene ontology database to generate an overview of the proteins' functions (Fig. 2). From a total of 101 recognisable genes, 61 could be classified in 7 different molecular functions. The majority of the proteins for which these genes encode had catalytic or binding functions (Fig. 2A). These 101 genes were involved in 12 different biological processes resulting in 126 functional hits of which nearly half were involved in cellular or metabolic processes, while only 0.8% could be linked to immunity (Fig. 2B). Only 72 functional hits were assigned to 7 different cellular compartments. A large number of the salivary proteins could be assigned to the extracellular region. However, proteins that were components of the cell, membranes and organelles, were also identified (Fig. 2C).

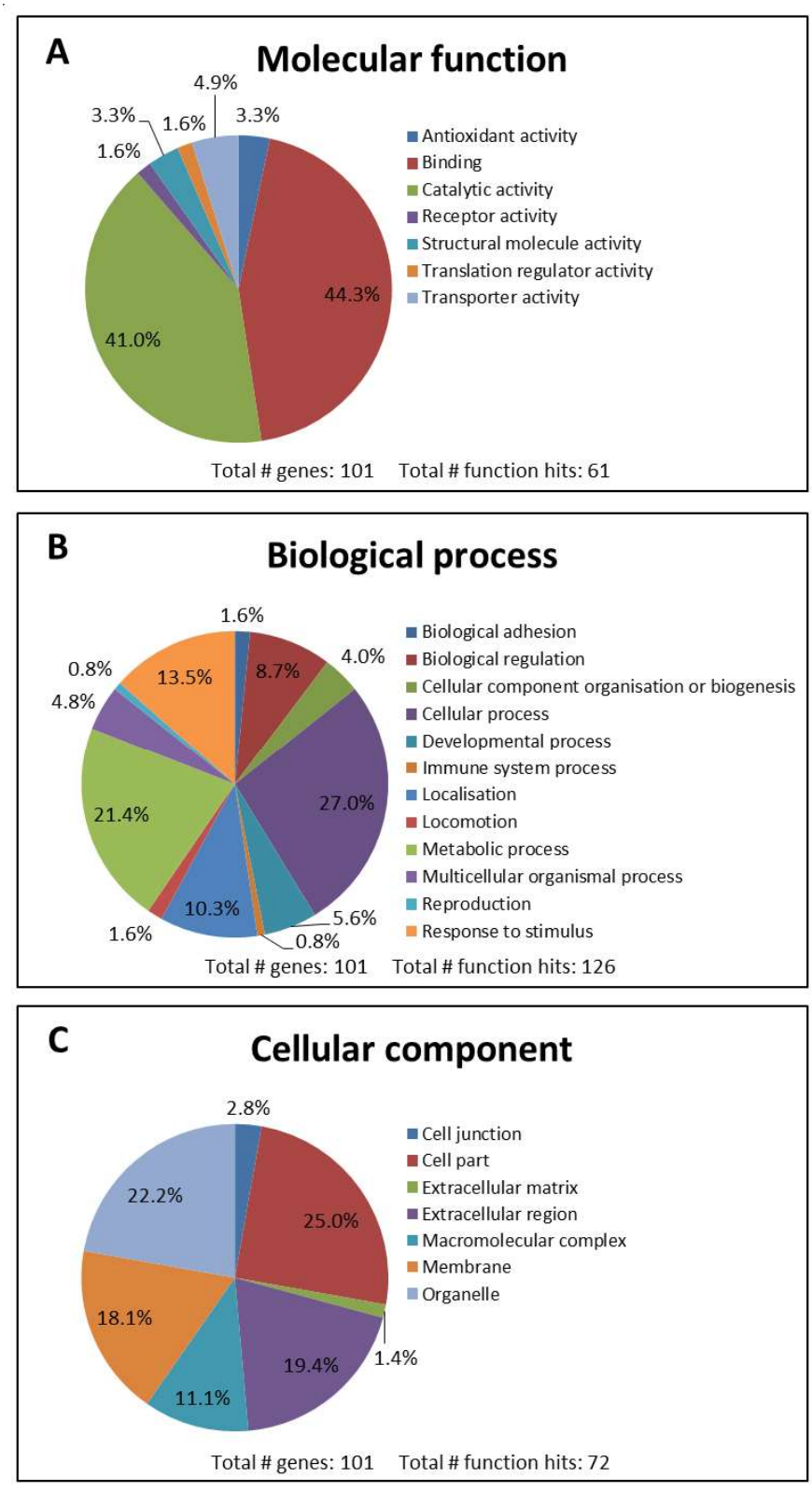


Figure 2. *Functional categorisation of the identified porcine salivary proteins, according to their molecular function (A), the biological process in which they are involved (B) or their cellular localisation (C).*

IV.4.3 Quantitative data

iTRAQ-analysis revealed that all identified salivary proteins were secreted through both glandular ducts, albeit in different concentrations. Of the 128 identified proteins, 24 (18.8%) proteins (20 porcine and 4 mammalian with a predicted porcine homolog) were predominantly (*P*-value < 0.05) secreted by the parotid gland, while 29 (22.7%) proteins (all porcine) were predominantly (*P*-value < 0.05) secreted by the mandibular and sublingual glands (Fig. 3, Table 2, Supplementary file 2). However, these obtained values are absolute, meaning that one assumes that these glands produce saliva at the same flow rate and with the same concentration of proteins and therefore equally contribute to the composition of whole saliva present in the oral cavity. Since this is not the case, we opted to normalise the obtained values for each protein with the initial protein secretion rate (µg/sec) of each gland. This normalisation reduced the number of proteins predominantly (*P*-value < 0.05) secreted by the parotid gland to 10 (7.%) (9 porcine proteins and 1 mammalian proteins with a predicted porcine homolog), while only 1 (0.8%) of the identified porcine proteins appeared to be secreted in higher concentrations by the mandibular and sublingual glands (Table 2, Supplementary file 2).

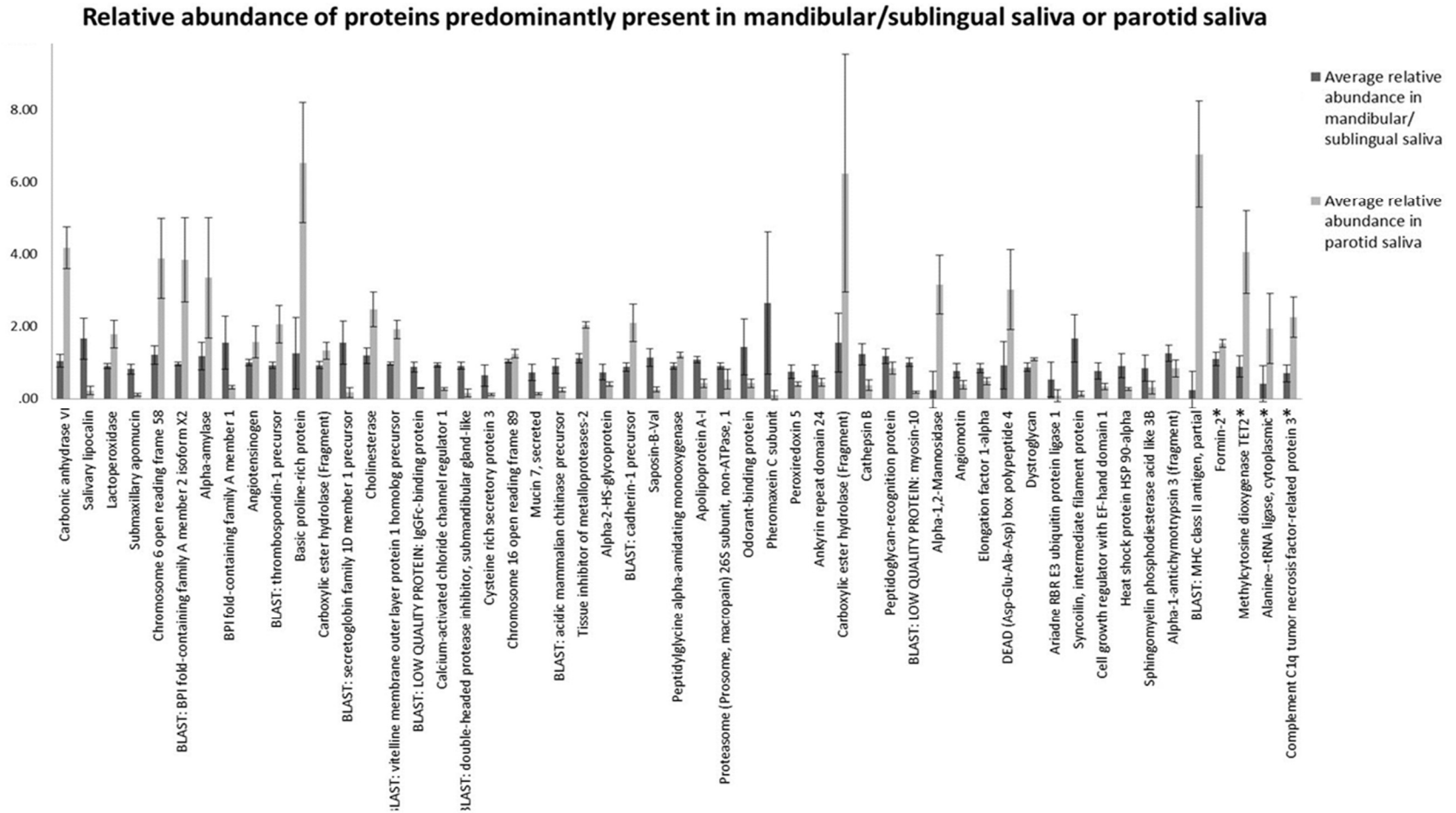


Figure 3. *Overview of proteins that are present in significant higher concentrations in saliva secreted by either the parotid gland or the mandibular and sublingual gland of 21-day-old piglets. Results are the averages of the relative abundance of each protein ± SD. Proteins indicated with an asterisk are mammalian proteins with a porcine homolog.*

IV.5 Discussion

One of the aims of this study was to extend the list of identified porcine salivary proteins. To be able to analyse saliva that is not contaminated with a.o. food remnants, gland-specific saliva was collected. Since it is very difficult to collect ductal secretions in conscious animals, the use of anesthetised animals is recommended (e.g., [241-244]). Unfortunately, salivary flow is low in resting subjects and can even be absent during anaesthesia [141, 233, 245, 246]. Therefore, dual stimulation using pilocarpine and isoproterenol was applied. Pilocarpine stimulates the salivary flow similar to acetylcholine by binding to the M_3-muscarinic receptors on the acinar cells (e.g., [27]). Simultaneously, noradrenaline, mimicked by isoproterenol, binds β_1-adrenoreceptors on acinar cells, which eventually leads to the release of stored proteins into the secreted saliva (e.g., [27]).

IV.5.1 Concentration and flow rate

In this study, the protein concentration was the highest in the parotid saliva, which is in line with previous findings in rats after dual stimulation with isoproterenol and pilocarpine [243, 247]. Moreover, in humans, the parotid gland consistently secretes the highest concentration of proteins, irrespective of stimulation [248, 249]. In contrast, no difference in flow rates of both ductal secretions was observed in the present study. Previous research in humans and rats, however, showed that the combined flow rate of the mandibular and sublingual glands is consistently higher compared to that of the parotid gland, irrespective of stimulation [141, 247, 249]. Whether this discrepancy is due to the used anaesthetics or the applied stimuli, or whether pigs display no differences in flow rate between the different ductal secretions needs to be further investigated. Pentobarbital is a frequently used, non-volatile anaesthetic for animals that could serve as an alternative to the used anaesthetics (e.g., [243, 250]). On the other hand, direct nerve stimulation could be tested as an alternative for chemical stimulation in the anaesthetised animal (e.g., [251, 252]).

IV.5.2 Qualitative data

IV.5.2.1 *Identified proteins*

Previous studies on porcine saliva could only identify about 32 proteins, while 122 porcine proteins and 6 mammalian proteins with a predicted porcine homolog were detected in the present study. Of these 128 proteins, 40 porcine proteins were identified by both the MALDI-TOF/TOF MS and the Q-Exactive orbitrap MS-technique. It is not surprising that the more sensitive Q-Exactive orbitrap MS/MS technique was able to identify the vast majority of proteins. Two proteins, of which one porcine and one mammalian protein with a predicted porcine homolog, were only detected with the LC-MALDI-TOF/TOF technique, emphasising the need for different ionisation methods to obtain a maximum number of proteins, as has been reported before [239, 253]. This study was also the first to use a gel-free technique to fractionate porcine salivary proteins instead of 2-DE to perform shotgun proteomics [173, 175, 210], which could explain the increased number of protein identifications. Both techniques are well-known to be complementary. However, a disadvantage of the gel-free technique is that information about possible post-translational modifications is harder to obtain [254, 255].

An additional explanation for the identification of a larger number of proteins, compared to previous studies, is the use of gland-specific saliva instead of whole saliva. Proteins that are secreted into the saliva by specific glands become diluted once they mix with each other and other fluids in the oral cavity. Moreover, buccal cells and bacteria are usually removed from whole saliva by means of centrifugation or filtration. This step, which was not performed in the present study, could remove macromolecular aggregates or proteins that are bound to bacteria or mucus [256]. For instance lactoperoxidase, two lysozymes and statherin, which are salivary proteins known to be involved in complex formation, were found in the present but not in previous studies that used whole saliva [257, 258].

The importance of using complementary methods not only applies to the techniques, but also to the protein identification database search. Unfortunately, only a mammalian Swiss-Prot database was initially available for protein identification. When using this multispecies database, the presence of proteins with a highly conserved amino acid sequence could

cause an underestimation of the number of identified proteins. The reason for this is that the search algorithm will only assign a unique sequence from the database to a detected peptide. This hypothesis appeared to be confirmed since the newly generated porcine database enabled the identification of 55 additional proteins. However, it should be mentioned that the mammalian database only contained reviewed proteins while the porcine database was composed of both reviewed and non-reviewed proteins. Nevertheless, this database appeared to be incomplete since 6 proteins that were found using the mammalian database were not identified using the porcine database. Expanding and further annotating the porcine database will most probably lead to a higher number of identified proteins in the future. This theory confirms why, despite the large number of good-quality mass spectra (5144 spectra for the experiment using MALDI-TOF/TOF MS and 89045 spectra for the experiment using Q-Exactive Orbitrap) only a limited number of proteins is identified.

To our knowledge, from the 34 proteins that were previously detected, 13 were not present in the list of the 128 proteins generated in the present study. Multiple reasons for this discrepancy are possible. Firstly, this exploratory proteomic study analysed gland-specific saliva that was derived from the three major salivary glands, while most preceding experiments investigated whole saliva that was collected from the oral cavity. Even though the minor glands only produce 1 to 4% of the total salivary volume, their secretions contain some unique proteins [259]. Additionally, whole saliva includes proteins from gingival crevicular fluid, which piles up in the gingival sulci. Though the formation process of this fluid is still under debate, it is considered to be a serum transudate that originates from the gingival plexus of blood vessels in the gingival corium (e.g., [260, 261]). It is known that a.o. enolase proteins and protein S100-A8 and S100-A9 are present in gingival crevicular fluid of humans, but not in saliva from the major salivary glands [262]. These findings suggest that the previously reported proteins in whole saliva that were not detected in the present study, could originate from either the minor salivary glands or the gingival crevicular fluid. Secondly, the present study analysed the saliva of 21-day-old piglets, whereas older pigs were the subjects of previous studies. It could be hypothesised that the salivary proteome

of 21-day-old pigs is still immature and therefore only contains a limited number or a different profile of proteins. This hypothesis has been confirmed in other species (e.g., [243, 263-265]). On the other hand, some proteins that in humans only appear in saliva at a later stage in life, were already present in our young pigs. Developmental dissimilarities could be the cause of these early life differences between human and porcine salivary proteomes. Indeed, pigs already have teeth when they are born, while babies only start teething at the age of 6 to 7 months [143]. Thirdly most previously identified proteins were identified using gel-separation followed by MS-identification, but targeted approaches, such as enzyme-linked immunosorbent assay (ELISA), Western blot, time-resolved immunofluorometric assay (TR-IFMA) or enzymatic assays, were also used. It has already been proven that a shotgun proteomics approach sometimes fails to identify proteins that are detectable with a targeted approach and vice versa [266].

IV.5.2.2 *Classification of identified proteins*

The porcine salivary proteins that were identified in this study cover a wide range of molecular weights from which the distribution (46.1% ≤ 40 kDa, 43.0% between 40 and 120 kDa, 10.9% ≥ 120 kDa) largely corresponds to the salivary proteome of human saliva [168]. The majority of these proteins is involved in binding or catalytic activities, which is in line with previous interpretations of the salivary proteome of e.g. humans, rats, mice, dogs, horses, cattle, goats and sheep [168, 266-269]. Additionally, nearly half of all proteins in these investigated proteomes are involved in either metabolic or cellular processes, as was also observed in this study on piglets. More variation was observed when grouping the salivary proteins according to the cellular localisation, but given the limited size of some salivary proteome datasets, conclusions should be drawn with caution [168, 266-269]. To our knowledge, 81.3% of all identified porcine salivary proteins can also be found in saliva of other species (Supplementary file 2) [168, 213, 266-276], indicating that 24 proteins are newly identified salivary proteins.

IV.5.3 Quantitative data

In contrast to humans, no proteins are exclusively secreted by either the parotid gland or the mandibular and sublingual gland in the piglet, although expression levels can vary (Supplementary file 2) [168, 256]. Therefore, in our study, only a quantitative and not a qualitative difference was observed between both ductal secretions. Information about variation in secretion rate or concentration differences of specific proteins in gland-specific saliva is scarce. *Veerman* and his group [248] found that a.o. amylase and proline-rich proteins are secreted at a higher concentration by the parotid gland in humans, which is also the case in pigs. The importance of this quantitative information lies in the fact that the contribution of each gland changes when the salivary flow is stimulated and therefore changes the composition of saliva present in the oral cavity [141]. In humans, the minor glands contribute only 4% of the total salivary volume that is secreted in rest, while the parotid glands contribute 28% and the mandibular/sublingual glands 68%. When the salivary flow is stimulated by tasting, smelling or chewing food, this ratio shifts, increasing the share of the parotid gland to 53%, while reducing the portions of the mandibular/sublingual glands and the minor glands to 46% and 1%, respectively [141]. This means that e.g. amylase and basic proline-rich protein will have a higher concentration in whole saliva after stimulation because of the larger contribution of the parotid gland. Therefore, comparing proteomics data from stimulated (chewing on a device) vs. unstimulated collection (passive collection) should be performed with caution. However, it should be mentioned that not only the volumetric contribution of the major salivary glands to whole saliva changes during stimulated secretion. The composition of unstimulated and stimulated gland-specific saliva may also diverge. In humans, stimulation with 2% citric acid influences the protein profile of human mandibular and sublingual saliva, but not that of the parotid saliva [249]. It would be valuable to collect both stimulated and unstimulated gland-specific saliva in order to confirm this trend for pigs. Additionally, it is known that the concentration of some salivary proteins is subjected to a circadian rhythm [221, 277]. However, in humans, salivary flow rate is also subjected to this circadian rhythm. Both whole saliva and parotid saliva show a similar rhythm, but with a different amplitude and

acrophase. These differences result in an altered percentage contribution of parotid saliva to whole saliva throughout the day with the largest contribution of 32% at 11 a.m. and the lowest contribution at midnight. Therefore, data collected at different time points should be compared with caution. Surprisingly, flow rate does not show a circadian rhythm when salivary flow is stimulated [278].

One should be careful when interpreting the above-mentioned data that are not normalised. These values are absolute, meaning that one assumes that these glands produce saliva at the same flow rate and with the same protein concentration. Since this is not the case, we opted to normalise the obtained values for each protein with the initial protein secretion rate of each gland. Even though this parameter displays some variation, these normalised values represent a better indication of the protein proportion that each gland contributes to the total protein mixture in whole saliva. Unfortunately, correcting for protein secretion rate is a relatively new concept and is not frequently used [150], so information for comparison is scare. Normalisation of values obtained for o.a. amylase and basic proline-rich proteins levelled out any differences in secretion levels between both ductal secretions. In contrast, for some proteins, such as carbonic anhydrase VI and cadherin-1, normalisation did not change the fact that the parotid gland contributed the largest quantity of this protein to whole saliva. While some proteins displayed no difference in relative abundance before normalisation, such as protein S100-A12 and cystatin-B, they appear to have a higher contribution to whole saliva through the parotid gland after normalisation for flow rate. As mentioned before, these data were collected under non-physiological conditions. It would be valuable in future studies to collect gland-specific saliva under physiological conditions in order to see whether the anaesthesia and chemical stimulation influences the composition and/or protein secretion rate.

An additional advantage of the used method is that knowledge of inter-individual variation is obtained. Proteins such as serpin family I member 1 and peptidylglycine alpha-amidating monooxygenase display very low variation between animals, bearing in mind that only four animals were used. In contrast, proteins statherin and collagen alpha-1(V) chain precursor, for example, are present in very variable concentrations in our porcine samples.

To further explore the salivary proteome of pigs in the future, alternative detection methods or protocol adaptations could be valuable. An example is treating the samples with peptide ligand libraries to reduce the risk of highly abundant proteins masking the presence of low abundance proteins during LC-MS analysis [279, 280]. Another intervention that would facilitate protein identification is pre-treating the salivary samples with PGNase [281]. It is known that salivary proteins are heavily glycosylated, and to eliminate interferences of this post-translational modification during sample preparation, salivary proteins could be deglycosylated [282].

IV.6 Conclusions

During the present study 122 porcine proteins and 6 mammalian proteins with a predicted porcine homolog were identified of which 111 had never been detected in porcine saliva before. The functional profile of this salivary proteome is similar to that of other species. iTRAQ analysis detected only a quantitative and not a qualitative difference between both ductal secretions. Consequently, the 128 proteins were detected in both secretions, however at different levels. This relative-quantitative knowledge of the gland-specific salivary proteome is valuable when comparing data between stimulated (chewing on a device) and unstimulated (passive collection) secretions. It needs to be mentioned that normalisation to the initial protein secretion rate of each gland may alter which gland contributed the largest quantity. Even though this parameter displays some variation, these normalised values represent a better indication of the protein proportion that each gland contributes to the total protein mixture in whole saliva.

IV.7 Supplementary files

Supplementary file 1. List of previously found porcine salivary proteins. *The condition for which this protein might have a potential value as a biomarker and the method of detection are presented.*

#	Protein	Potential biomarker for	Detection method
1	Adenosine deaminase	Infection, stress (restraint)	2DE + MALDI-TOF/TOF [173, 175, 177]; de novo sequencing [173]; enzymatic assay [228, 230]
2	Alpha-1-antichymotrypsin*	Rectal prolapse	2DE + MALDI-TOF/TOF [210]
3	Alpha-enolase-like, partial		2DE + MALDI-TOF/TOF [175]
4	Basic proline-rich protein*		RP-HPLC-ESI-IT-MS [211, 212]
5	Carbonic anhydrase VI*	Rectal prolapse, stress (restraint)	2DE + MALDI-TOF/TOF [173, 175, 177, 210]; de novo sequencing [173]
6	Cathelin		2DE + MALDI-TOF/TOF [175]
7	Cholinesterase*	Stress (restraint, transport)	Alternative Ellman's method [213]
8	Cholesterol esterase		WB [178]
9	Chromogranin A	Stress (restraint, isolation, regrouping)	TR-IFMA [59, 72, 213, 214, 217, 277]; ELISA [177, 209]
10	C-reactive protein	Infection with PRRSV, endotoxemia	TR-IFMA [173, 175, 214, 217-223, 229, 230]
11	Cystatin-B*		2DE + MALDI-TOF/TOF [173, 175]
12	Double-headed protease inhibitor, submandibular gland*	Infection, stress (restraint)	2DE + MALDI-TOF/TOF [173, 175, 177]; de novo sequencing [173]
13	Haptoglobin	Infection with PRRSV, rectal prolapse, endotoxemia	TR-IFMA [59, 173, 175, 210, 214, 217, 218, 221-224, 229, 230]; 2DE + MALDI-TOF/TOF [175, 210]
14	Haemoglobin subunit alpha*	Stress (restraint)	2DE + MALDI-TOF/TOF [177]
15	Haemoglobin subunit beta*		2DE + MALDI-TOF/TOF [175]
16	IgA	Stress (restraint, isolation), endotoxemia, infection	2DE + WB [173]; ELISA [183, 207, 214, 217, 225, 227]
17	IgG*	Infection	2DE + WB [173]; ELISA [207, 225, 227]

#	Protein	Potential biomarker for	Detection method
18	IgM	Infection, stress (restraint)	2DE + WB [173]; ELISA [207, 225, 227]; 2DE + MALDI-TOF/TOF [177]
19	Interleukin-18	Stress (restraint)	ELISA [174]
20	Leptin	Stress (restraint), feed ingestion, inflammation	TR-IFMA [176]
21	Light chain of immunoglobulins*	Stress (restraint)	2DE + MALDI-TOF/TOF [177]
22	Lipase	Stress (restraint), lameness	Enzymatic assay [178]
23	Lipocalin-1*	Infection, stress (restraint)	2DE + MALDI-TOF/TOF [173, 175, 177, 230]
24	Odorant-binding protein*	Stress (restraint, transport)	2DE + MALDI-TOF/TOF [175, 230]; 2DE + LTQ Linear Ion Trap MS [189]
25	Pancreatic alpha-amylase*	Stress (restraint); infection	2DE + MALDI-TOF/TOF [173, 175]; kinetic spectrophotometric assay [181]; ELISA [209]
26	Prolactin-inducible protein homologue*	Stress (restraint)	2DE + MALDI-TOF/TOF [173, 175, 177]; de novo sequencing [173]
27	Prolyl 4-hydroxylase β-polypeptide		2DE + MALDI-TOF/TOF [175]
28	Protein S100-A8	Infection, stress (restraint)	2DE + MALDI-TOF/TOF [175, 177]
29	Proteins S100-A9	Infection, stress (restraint)	2DE + MALDI-TOF/TOF [175, 177]
30	Protein S100-A12, calgranulin C*	Infection, stress (restraint)	2DE + MALDI-TOF/TOF [173, 175, 177]
31	Salivary lipocalin*	Infection, stress (restraint)	2DE + MALDI-TOF/TOF [173, 175, 177, 230]; de novo sequencing [173]
32	Serotransferrin*		2DE + MALDI-TOF/TOF [175]
33	Serum albumin (fragment)*	Infection; stress (restraint, transport)	2DE + MALDI-TOF/TOF [173, 175]; 2DE + LTQ Linear Ion Trap MS [189]
34	Serum amyloid A*	Infection with PRRSV, stress (road transport, isolation)	ELISA [209]; TR-IFMA [224, 226, 229][36, 43,46]

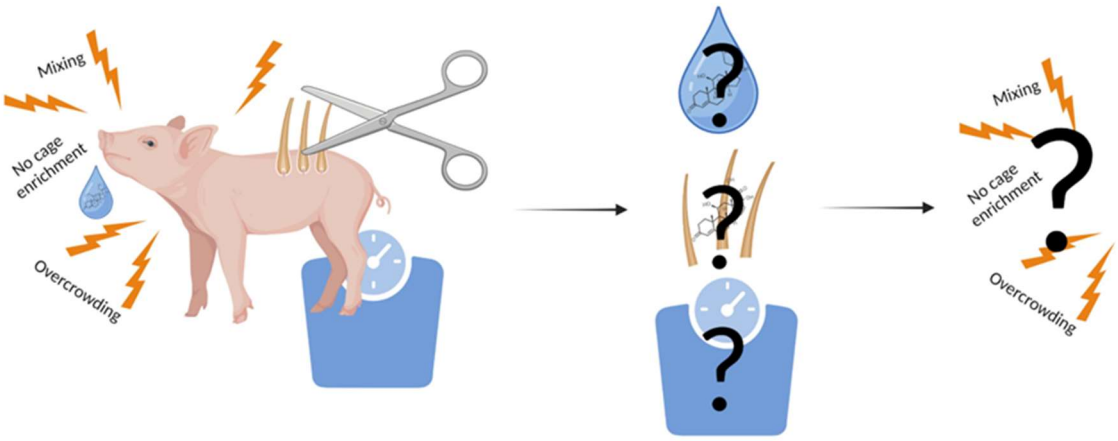
*Salivary proteins that were also identified in the present study.

Supplementary file 2. Identified proteins in porcine gland-specific saliva. See link below.

Supplementary file 3. Raw output Scaffold selections. See link below.

Supplementary file 4. Mascot output and Scaffold selection. See link:
https://eur01.safelinks.protection.outlook.com/?url=http%3A%2F%2Frepository.uantwerpen.be%2Fdocstore%2Fd%3Airua%3A22252&data=05%7C02%7CSara.Prims%40uantwerpen.be%7C7d8384a21f9b4cbb13a108dc3dc92733%7C792e08fb2d544a8eaf72202548136ef6%7C0%7C0%7C638453181356345738%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C0%7C%7C%7C&sdata=Hu8qMfWTJBz%2F%2FuduEZqqkyZACa1PFXabY%2BFVbU2Lqcw%3D&reserved=0

Chapter V


Mixing
No cage enrichment
Overcrowding
Mixing
No cage enrichment
Overcrowding

Chapter V -Hair or salivary cortisol analysis to identify chronic stress in piglets?

Sara Prims, Charlotte Vanden Hole, Steven Van Cruchten, Chris Van Ginneken, Xaveer Van Ostade, Christophe Casteleyn

Adapted from The Veterinary Journal (2019), 10.1016/j.tvjl.2019.105357

Abstract

Hair cortisol might better represent chronic stress than salivary cortisol in piglets. To test this hypothesis, 24 female, 7-day old piglets were allocated to two groups and artificially reared. The piglets in the stressed group were exposed to overcrowding (0.10 m^2/piglet) and frequent mixing with unfamiliar piglets until the age of 28 days. The control group remained in an unchanging group at a density of 0.29 m^2/piglet. After 3 weeks, stressed animals had gained significantly less weight (median, here and throughout, 6.43 kg) than the control animals (7.58 kg; *P*-value = 0.021). Additionally, hair from the stressed group contained significantly higher cortisol concentrations (87.29 vs. 75.60 pg/mg hair; *P*-value = 0.005), whereas salivary cortisol concentrations did not significantly differ between groups (0.30 vs. 0.25 µg/dL saliva; *P*-value = 0.447). Weight gain and hair cortisol concentrations were significantly correlated (*P*-value = 0.036, ρ = -0.430), but neither of these parameters were correlated with salivary cortisol concentrations (*P*-value = 0.929, ρ = 0.019 and *P*-value = 0.904, ρ = 0.026, respectively).

V.1 Introduction

Monitoring chronic stress is of value in assessing animal welfare and in searching for factors that could limit animal performance and/or increase susceptibility to infectious diseases [48]. Cortisol is predominantly used as a biomarker to assess chronic stress, as it is released upon activation of the HPA axis [79]. However, interpreting cortisol concentrations in biological fluids, such as saliva, has certain constraints, since they are influenced by various factors including a circadian rhythm [32]. Furthermore, cortisol concentrations might rise in response to an acute stressor and could therefore merely present a snapshot of an animal's physiological state [33]. Since cortisol concentrations in hair accumulate over time [80, 81], we hypothesised that this parameter might be a better indicator of chronic stress than salivary cortisol concentrations.

V.2 Materials and methods

To test this hypothesis, 24 female piglets (Belgian Landrace × Piétrain), born from eight litters, were transported from a local farm to the University of Antwerp at the age of 4 days (August/September 2016). Only female piglets were selected. The stressful event of castration for male piglets could interfere with our study design because it may not merely cause an acute activation of the HPA axis [283] but could also sensitise the pigs for later stressors [284, 285]. On the other hand, oestrogen enhances HPA function, possibly making female piglets more susceptible to stress [286]. They were housed in commercial brooders (Rescue Decks, S and R Resources LLC) and reared on milk formula (BIGGILAC PL+, AVEVE), which was provided ad libitum. These piglets were litter-matched, and randomly assigned to either the control (n = 8) or the stressed group (n = 16) by handpicking ear tag numbers from a bag. The latter group was exposed to three stressors: overcrowding, mixing with unfamiliar piglets and deprivation of environmental enrichment [48]. These animals were housed at a density of 0.10 m^2/animal, which is below the legal minimum of 0.15 m^2/piglet

(< 10 kg; 2001/88/EC)[3]. On 32 occasions, piglets from the stressed group were randomly allocated to be mixed between brooders by selecting ear tag numbers from a bag. The time of mixing was also randomly allocated by selecting ear tag numbers from a bag. Environmental enrichment was not provided to piglets in the stressed group. The control piglets were housed in stable groups at a density of 0.29 m^2/animal with balls and ropes as environmental enrichment. Animals were observed twice a day to document behaviour, body condition, lesions, and faecal composition. In case of any deviation from normal behaviour, body condition, the presence of lesions or the presence of diarrhoea a veterinarian would be consulted. Since all animals remained in good health this was not necessary. Humane endpoints were determined a priori (parameters that would lead to euthanasia: not standing up when startled, body condition score of one, pale oral mucosa and capillary refill time of gingiva longer than 3 s, or body temperature below 36°C) but were not reached. Prior to commencement, all experiments were approved by the Ethical Committee for Animal Experiments of the University of Antwerp, Belgium (Approval date 4 May 2016; Approval number: 2016-41) and were in accordance with the European Directive (2010/63/EU)[4].

Piglets were weighed at the start (7 days old) and at the end of the experiment (28 days old). Saliva was collected at day 28 between 8:30 am and 9:30 am by allowing piglets to chew on a synthetic cylindrical collection pad (Micro·SAL, Oasis Diagnostics; [287]). All specimens were stored at -80 °C until further analysis. Cortisol concentrations were determined in duplicate in a single assay using a commercially available cortisol saliva ELISA (IBL-International) validated for pig saliva [288]. To determine cortisol accumulation in hair during the experiment, the dorsum (approximately 35 cm x 10 cm) of each piglet was shaved with clippers at day 7 to set the baseline. Mechanical forces resulting in scratching

[3] See: EUR-Lex Access to European Union law https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32001L0088 (Accessed 13 August 2019).

[4] See: EUR-Lex Access to European Union law https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32010L0063 (Accessed 13 August 2019).

and rubbing that could elevate cortisol concentrations occur relatively infrequently in this region [289]. At day 28, the dorsal region was shaved again. The collected hairs were washed twice for 3 min with 10 mL isopropanol to remove dust and sebum [290]. After 5 days of drying, specimens were ground using a mortar and pestle. Because hair cortisol concentrations are known to vary between different regions of the back, the entire specimen was homogenised [81]. From this specimen, 50 mg was taken and added to 1.8 mL methanol. After incubation for 24 h, the specimens were centrifuged for 15 min at 1500 g [290]. From the supernatant, 1.3 mL was lyophilised and resuspended in 300 µL of phosphate buffered saline. Cortisol concentrations were determined using the same ELISA described above. Intra-assay coefficients of variation for both cortisol assays were <5%.

For weight gain and salivary and hair cortisol concentration analysis, a mixed model was applied to identify potential differences between both groups. Sow was included as a random factor. In order to meet normality and/or homoscedasticity assumptions, data from hair and salivary cortisol concentrations were log transformed. A nonparametric Spearman's assay was conducted to identify possible correlations between the three parameters. All data were analysed using JMP Pro 12 (SAS Institute) and reported as medians and $25^{th}/75^{th}$ percentiles; *P*-value ≤ 0.05 was considered statistically significant. Power analysis confirmed that, for the parameters studied, the chosen sample size resulted in power >80%.

V.3 Results and discussion

Median weight gain for the control group over the 21-day study period (7.58 kg; 6.79 kg/8.22 kg, $25^{th}/75^{th}$ percentiles respectively, here and throughout) was significantly higher than that of the stressed group (6.43 kg; 5.94/7.20 kg; *P*-value = 0.021; Fig. 1). Salivary cortisol concentrations at day 28 in the control group (0.25 µg/dL saliva; 0.21/0.39 µg/dL saliva) did not differ from the stressed group (0.30 µg/dL saliva; 0.27/0.51 µg/dL saliva; *P*-value = 0.447). In two animals, salivary cortisol concentrations were atypically high. Most likely this was due to an acute response to a stressful stimulus, rather than chronic stress.

Of note, the correct timing for salivary sampling could be a point of discussion. It is possible that differences between groups might be greater if sampling occurs when baseline cortisol concentrations are low, i.e. in the evening. However, the circadian rhythm of cortisol does not mature until 20 weeks [32], making the identification of daily baseline cortisol concentration in young animals unpredictable. Additionally, a previous study has demonstrated that decreased welfare leads to a blunted circadian rhythm [54], making the determination of the optimal timepoint for sampling problematic. As a result, the identification of chronic stress in pigs requires a cortisol measurement technique that is less sensitive to short-term fluctuations. In our study, in contrast to salivary cortisol concentrations, hair from stressed pigs contained higher concentrations of cortisol (87.29 pg/mg hair; 78.55/99.61 pg/mg hair) than hair from control animals (75.60 pg/mg hair; 69.95/78.42 pg/mg hair; *P*-value = 0.005), although there was a wide range of hair cortisol concentrations in the stressed group (range, 64.26 - 107.45 pg/mg hair). This variation might be explained by differences in susceptibility to stress because of different coping styles [24].

The concentrations found in this study are higher than the previously reported values of older boars [81]. Although concentrations of free cortisol are higher in boars and barrows than in gilts, these concentrations decrease with age [32]. This possibly explains why the cortisol concentrations detected in our 4-week-old gilts were higher. Nevertheless, the effects of gender, age and breed on hair cortisol concentrations should be further investigated. Additionally, it is important to emphasise that cortisol accumulation during the last days of the experiment was still present in the hair roots located in the skin. Since the mean depth of the hair follicles was 1.32 ± 0.04 mm and the estimated hair growth rate/month is 10.01 ± 0.24 mm, it would have taken 4 days before this cortisol accumulation was measurable in hair. However, the effect of the stressors was large enough to be detected, since cortisol concentrations in hair correlated significantly and negatively with weight gain (*P*-value = 0.036, ρ = - 0.430). Neither of these parameters correlated significantly with cortisol concentrations in saliva (*P*-value = 0.904, ρ = 0.026 and *P*-value = 0.929, ρ = 0.019, respectively). Nevertheless, salivary specimens taken over a

longer time period might have correlated with weight gain and hair cortisol concentration in stressed piglets [81].

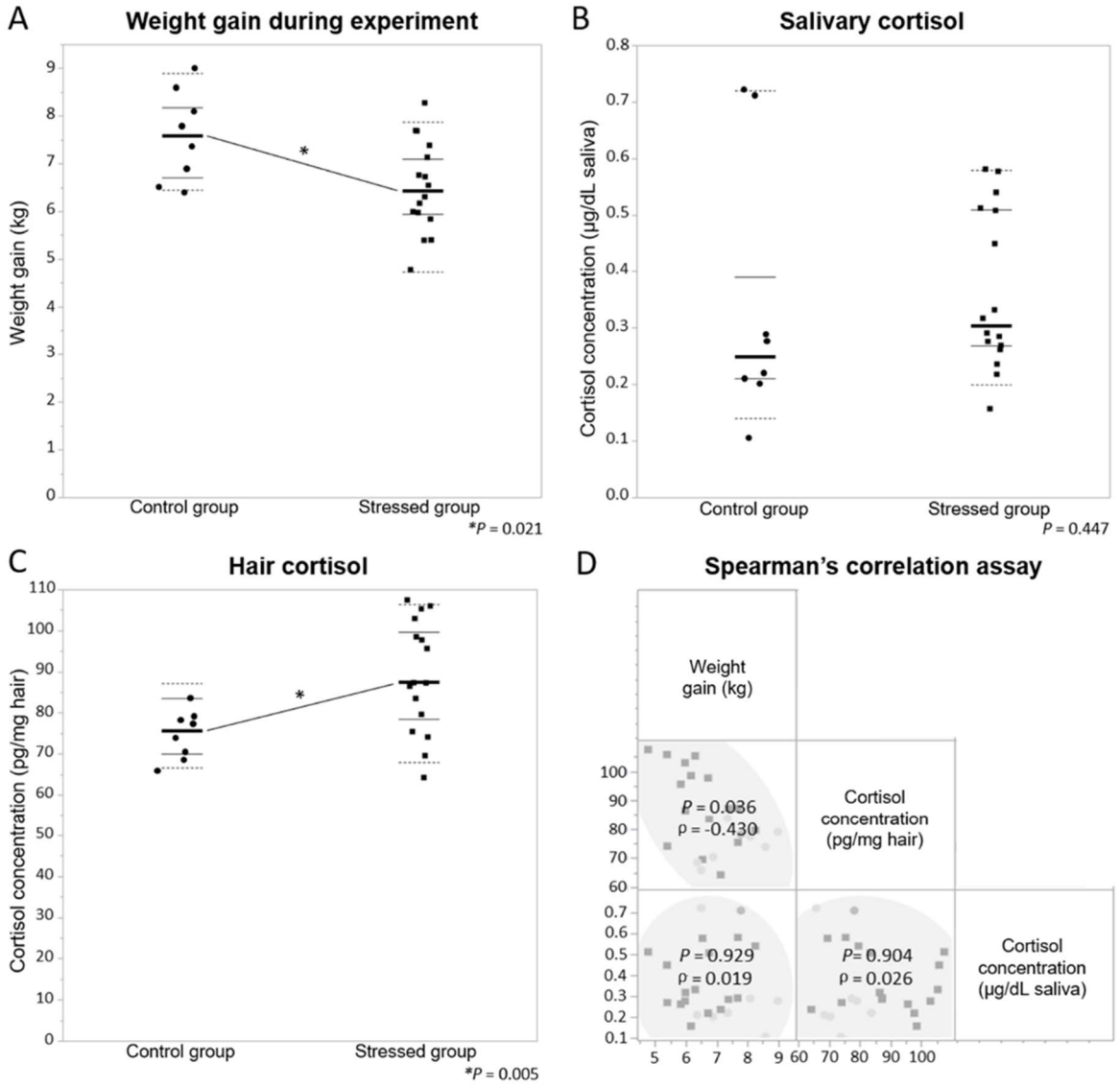


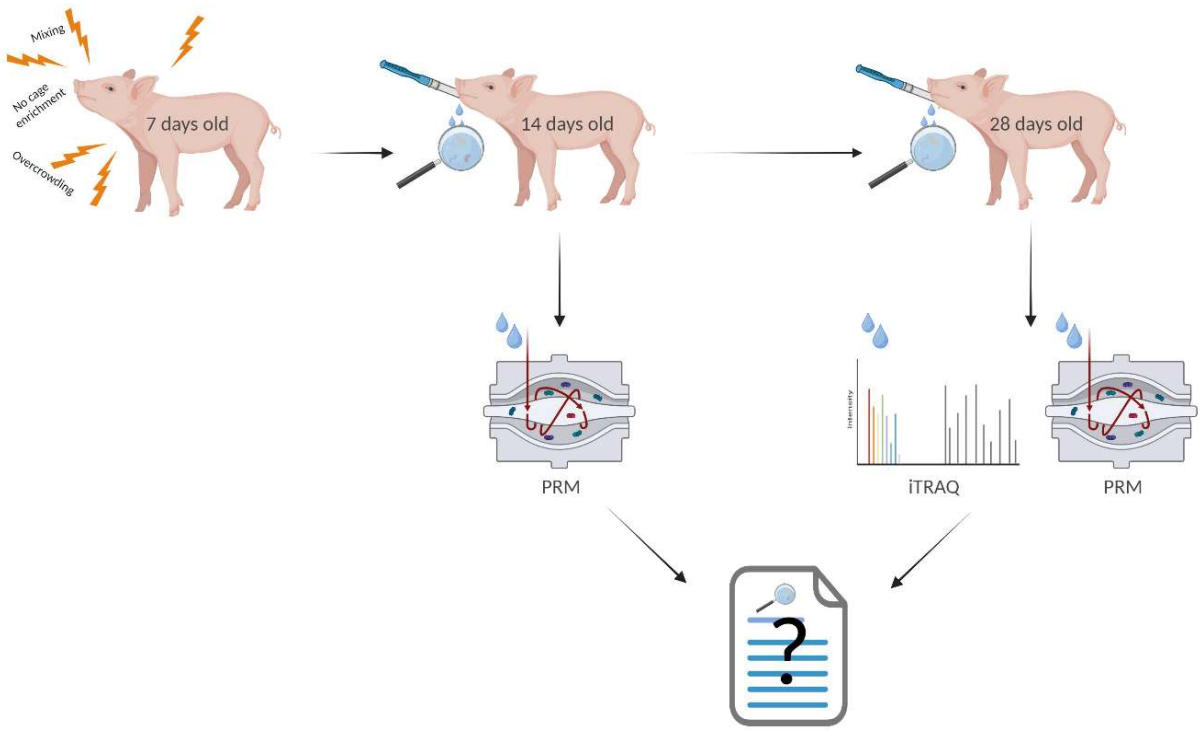
Figure 1. A. *Four-week-old piglets exposed to three stressors (overcrowding, mixing with unfamiliar piglets and privation of environmental enrichment) gained significantly less weight after 3 weeks compared to control piglets.* ***B.*** *At day 28, cortisol concentrations in saliva were not significantly different between the two groups.* ***C.*** *The stressed group had significantly higher values of hair cortisol compared to their control littermates.* ***D.*** *A nonparametric Spearman's assay indicated that weight gain and hair cortisol concentrations correlated significantly, while neither of these parameters correlated significantly with salivary cortisol. Control group (n = 8); stressed group (n = 16). Significant*

differences (linear mixed models, P≤0.05) are indicated by an asterisk and a line. For each group the median (thick line), the 25th and 75th percentiles (thin lines) and the 5th and 95th percentiles (dotted lines) are shown.

V.4 Conclusion

Despite inter-individual variations, there was a significant negative correlation between hair cortisol concentrations and weight gain in stressed piglets. This was not the case for salivary cortisol concentrations and salivary and hair cortisol concentrations were not correlated at the end of the 28-day study period. Hair cortisol concentrations could be used to identify chronically stressed piglets at a group level.

Chapter VI


Mixing
No cage enrichment
Overcrowding
7 days old
14 days old
28 days old
PRM
iTRAQ
PRM

Chapter VI - Chronic exposure to multiple stressors alters the salivary proteome of piglets

Sara Prims, Xaveer Van Ostade, Miriam Ayuso, Martin Dom, Geert Van Raemdonck, Steven Van Cruchten, Christophe Casteleyn, Chris Van Ginneken

Adapted from PLoS One (2023), doi.org/10.1371/journal.pone.0286455

Monitoring chronic stress in pigs is not only essential in view of animal welfare but is also important for the farmer, given that stress influences the zootechnical performance of the pigs and increases their susceptibility to infectious diseases. To investigate the use of saliva as a non-invasive, objective chronic stress monitoring tool, twenty-four 4-day-old piglets were transferred to artificial brooders. At the age of 7 days, they were assigned to either the control or the stressed group and reared for three weeks. Piglets in the stressed group were exposed to overcrowding, absence of cage enrichment, and frequent mixing of animals between pens. Shotgun analysis using an iTRAQ for tandem mass spectrometry performed on saliva samples taken after three weeks of chronic stress identified 392 proteins, of which 20 proteins displayed significantly altered concentrations. From these 20 proteins, eight were selected for further validation using PRM. For this validation, saliva samples that were taken one week after the start of the experiment and samples that were taken at the end of the experiment were analysed to verify the profile over time. We wanted to investigate whether the candidate biomarkers responded fast or rather slowly to the onset of chronic exposure to multiple stressors. Furthermore, this validation could indicate whether age influenced the baseline concentrations of these salivary proteins, both in healthy and stressed animals. This targeted PRM analysis confirmed that alpha-2-HS-glycoprotein was upregulated in the stressed group after one and three weeks, while odorant-binding protein, chitinase, long palate lung and nasal epithelium protein 5, lipocalin-1, and vomeromodulin-like protein were present in lower concentrations in the

saliva of the stressed pigs, albeit only after three weeks. These results indicate that the porcine salivary proteome is altered by chronic exposure to multiple stressors. The affected proteins could be used as salivary biomarkers to identify welfare problems at the farm and facilitate research to optimise rearing conditions.

VI.1 Introduction

Pigs are exposed to several stressors in their lives, such as regrouping (e.g., [1, 2]), castration (e.g., [4]), and road transport (e.g., [5]). Other stressors like restricted floor space [53, 100], inappropriate light and temperature [43], or lack of sufficient and/or qualitative enrichment [55, 291] can also occur. When the stressor exceeds a certain threshold in duration and magnitude, the body's homeostasis is disturbed. The equilibrium can be re-established by behavioural and physiological adaptive responses or by removing the stressor. However, failure to generate sufficient adaptive responses could lead to chronic stress, implying compromised animal welfare and suboptimal pig production due to a suppressed immune system (e.g., [6, 7]), reduced zootechnical and breeding performance (e.g., [3, 8-10, 113]). Thus, identifying and eliminating stress is essential for both the pig and the farmer.

To evaluate chronic exposure to stressors in pigs, behavioural assessments are often implemented despite being labour-intensive and difficult to interpret (e.g., [95]). Alternative methods, such as evaluating hyperkeratosis and ulcer formation in the stomach, are only feasible *post mortem* [292]. Cortisol concentrations in hair can be a good indicator of chronic stress in pigs [81, 293]. However, the sample preparation is labour-intensive and time-consuming. As a result, an objective, fast method that preferably relies on quantifiable biomarkers is sought after. Such biomarkers are routinely examined in blood, the most studied biological fluid in the past (e.g., [294, 295]). Unfortunately, blood sampling requires trained staff and, more importantly, induces stress on the pig [296]. In contrast, saliva collection does not require qualified personnel, is non-invasive, and is stress-free. Moreover, the proteome of pig saliva contains a wealth of proteins, as we demonstrated before [297], from which some could serve as biomarkers for chronic

exposure to stressors. Therefore, saliva was investigated as a potential biological sample to detect stress in pigs and is nowadays preferred over blood analysis (e.g., [32, 298, 299]). Most studies, however, focused on acute short-term stress, whereas only limited experiments investigated salivary profile differences in chronically stressed pigs. Cortisol (e.g., [298]), chromogranin A [55, 216], and serum amyloid A [224] have already been targeted in porcine saliva using antibody-based techniques concerning chronic stressor exposure. At the same time, only one study investigated the salivary proteome in an untargeted way using high-resolution MS [179]. The latter looked for candidate biomarkers in relation to compromised animal welfare due to lameness [179]. To our knowledge, no data on the salivary proteome of pigs in which chronic stress was experimentally induced are available. Therefore, the present study aimed to compare the salivary proteome of piglets chronically exposed to different stressors, including overcrowding, deprivation of cage enrichment, and frequent mixing of non-familiar individuals, with that of control piglets that were left undisturbed. We used iTRAQ in combination with a sensitive, high-resolution orbitrap MS/MS method. Because antibody-based assays on porcine proteins are scarce, a subset of the identified salivary proteins found in different relative concentrations was further validated using PRM.

VI.2 Materials and methods

VI.2.1 Animals and housing

All experiments were approved by the Ethical Committee for Animal Experiments of the University of Antwerp, Belgium (2016-41) and according to the European Directive (2010/63/EU). Twenty-four healthy female piglets (Belgian Landrace × Piétrain), with an average body weight within one standard deviation from the mean, born from eight sows, were transported from a local farm to the University of Antwerp at the age of 4 days (Fig. 1). All animals received an intramuscular iron injection (Iron(III) Dextran, 200 mg/piglet, Uniferon, Pharmacosmos, Holbaek, Denmark) on day 3. No antibiotics or vaccines were administered prior or during the studied period. They were allowed to adjust to the new environment until the start of the experiment at the age of 7 days. Only female piglets were

selected. The stressful event of castration for male piglets could interfere with our study design because it may not merely cause an acute activation of the HPA axis [283] but could also sensitise the pigs for later stressors [284, 285]. On the other hand, oestrogen enhances HPA function, possibly making female piglets more susceptible to stress [286]. The piglets were housed on commercial brooders (Rescue Decks®, S&R Resources LLC, Mason, USA) and artificially reared on milk formula (BIGGILAC PL+, AVEVE, Antwerp, Belgium), which was provided ad libitum until the age of 28 days (end of the experiment). They had free access to water and were maintained under standard environmental conditions (12h/12h light/dark cycle, temperature adjusted to age).

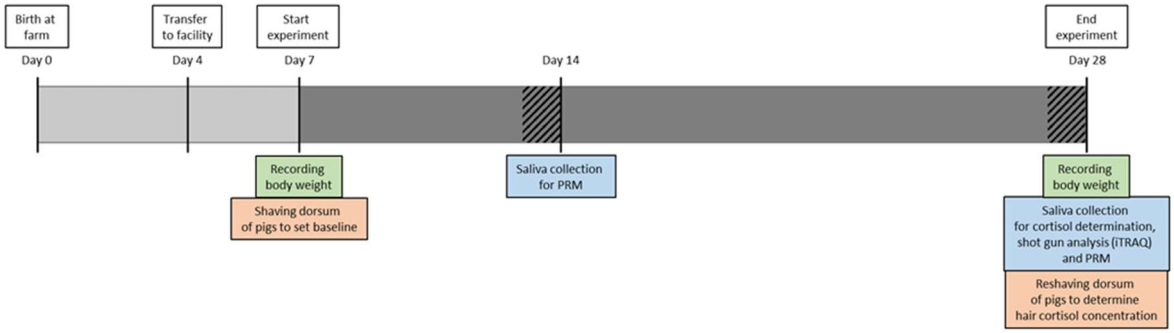


Figure 1. Timeline of the experiment. *From the age of 7 days until the end of the experiment at the age of 28 days, animals of the stressed group were exposed to three stressors, including overcrowding, deprivation of cage enrichment, and mixing with unfamiliar animals (dark grey). The latter was paused for 24h before saliva collection (hatched area). The time points at which the body weight was recorded for weight gain (green), the hair was shaven for its cortisol determination (orange), and saliva was sampled for iTRAQ analysis, cortisol determination, and PRM validation (blue) are indicated.*

Litter-matched piglets were randomly assigned to either the control group (n = 8) or the stress group (n = 16). The latter group was simultaneously exposed to three known stressors: overcrowding (e.g., [52, 100]), mixing with unfamiliar piglets (e.g., [48, 113]), and absence of cage enrichment (e.g.[55]) for 21 days, from the age of 7 days until the age of 28 days. The animals of the stressed group were housed in two subgroups of eight animals, each subgroup in a smaller brooder reducing the stocking density to 0.10 m^2/animal, bringing it under the European guideline of a minimum of 0.15 m^2/piglet (< 10 kg;

2001/88/EC)[5]. Additionally, throughout the experiment on 32 random time points during the daytime, piglets of the stressed group were exchanged between the two brooders to disturb the social hierarchy and induce social stress. Finally, environmental enrichment was not provided to piglets in the stressed group. The control piglets were housed in groups of four at a density of 0.29 m^2/animal with balls and ropes as environmental enrichment. Animals were observed twice a day, paying attention to behaviour, body condition, lesions, and faecal composition.

VI.2.2 Physiological parameters

To determine the effectiveness of the applied stressors, some previously established chronic stress indicators were used, i.e., bodyweight gain and cortisol concentrations in saliva and hair.

Bodyweight was distributed evenly between both experimental groups when transported to our facility on day 4 (control group: 1.89 ± 0.40 kg; stressed group: 2.05 ± 0.41 kg). Bodyweight was recorded at the start of the experiment at the age of 7 days and at the end when the animals were 28 days old (Fig. 1). Saliva was collected at 14 days of age (early time point in the experiment) and 28 days of age (end of the experiment) between 8:30 am and 9:30 am. The piglets were not exposed to mixing stress 24 h before saliva collection to avoid acute stress. Piglets were allowed to chew on a synthetic cylindrical collection pad mounted on a handle (Micro·SAL, Oasis Diagnostics) validated for cortisol analysis [287]. Saliva was recovered from this pad by placing it in a syringe-like compression chamber, pushing the plunger firmly downwards, and transferring the saliva into a clean Eppendorf tube. All samples were aliquoted and stored at -80°C until further analysis.

[5] See: EUR-Lex Access to European Union law https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32001L0088 (Accessed 1 September 2022).

Cortisol concentrations were determined in duplicate in a single assay using a commercially available cortisol saliva ELISA (IBL-International, Hamburg, Germany) validated for pig saliva, following the manufacturer's guidelines [288].

To determine cortisol accumulation in the hair during the three-week experiment, the dorsum (about 35 cm x 10 cm) of each piglet was shaved at the start of the experiment (7 days old) with clippers, and the hair was discarded to set the baseline. The dorsum was chosen since mechanical forces resulting in scratching and rubbing that could elevate cortisol concentrations in hair locally infrequently occur in this region [289]. After three weeks (28 days old), at the end of the experiment, the dorsal area was shaved again. The collected hairs, in which cortisol had accumulated during the experiment, were washed twice for 3 min with 10 mL isopropanol on an orbital shaker to remove dust and sebum [290]. After 5 days of drying, the hair samples were ground using a pestle and mortar. Because cortisol levels are lower in hair from the craniodorsal area than in hair from the dorsolumbar region, the whole sample was homogenised before further analysis [81]. From this sample, 50 mg was taken and added to 1.8 mL methanol. After incubation for 24 h, the samples were centrifuged for 15 min at 1500 g [290]. From the supernatant, 1.3 mL was lyophilised and resuspended in 300 µL of phosphate-buffered saline. Cortisol concentrations were determined using the same ELISA as used for the determination of cortisol levels in saliva. Intra-assay coefficients of variation for both cortisol assays were < 5%.

VI.2.3 Shotgun proteomics on saliva

VI.2.3.1 *Sample preparation*

Proteins in the individual saliva samples were labelled using iTRAQ-labels, reagents, and buffers. Working with the 8-plex kit allowed us to pool eight samples and simultaneously analyse them in one run since the mass spectrometer is able to distinguish proteins from different samples after tandem MS analysis [300]. Consequently, three parallel analyses were performed so the relative abundance of all 24 individually labelled samples could be

determined. Each parallel analysis contained samples of both the control and the stressed group. The protocol was according to the manufacturer's guidelines (Applied Biosystems Sciex Inc., MA, USA) and similar to what we described previously [297]. In brief, the total protein concentration of all saliva samples was determined using a bicinchoninic acid assay (BCA, Thermo Scientific, San Jose, CA, USA). Volumes containing 100 µg of protein were purified by means of acetone precipitation to discard any salts and lipids. The resulting protein pellets were resuspended in 500 mM TEAB. Hydrogen bonds were disrupted, and disulphide bonds were reduced using 2% SDS and 50 mM TCEP, respectively. The samples were incubated with 200 mM MMTS to alkylate thiols reversibly. Subsequently, trypsin (Promega, Leiden, The Netherlands) was added in a one-to-ten ratio (g/g) to digest proteins during the overnight incubation step at 37°C. Afterward, the eight samples were labelled using the eight different iTRAQ reagents. All eight individually labelled samples were combined, resulting in one batch for further analysis by 2D-LC-MS/MS.

VI.2.3.2 *First-dimensional separation*

The combined sample was fractionated in a first dimension by strong cation exchange (SCX) chromatography using a Waters Alliance e2695 HPLC system with Photo Diode Array Detector (Waters Corporation, Zellik, Belgium). After acidification to a pH of 2.7, the sample was loaded onto a polysulfoethyl-aspartamide SCX-column (2.1 mm x 200 mm; 5 µm particles; PolyLC Inc., Columbia, MD, USA). Three different solvents (solvent A: 10 mM KH_2PO_4, 20% CAN (pH 2.7); solvent B: 10 mM KH_2PO_4, 650 mM KCl, 20% ACN (pH 2.7) and solvent D: 10 mM KH_2PO_4, 650 mM KCl, 20% ACN (pH 4.7)) were used to separate the combined peptide sample according to their charge. First, only solvent A was used for 10 min followed by a salt gradient (7.5 - 30%) of solvent B for 45 min and a pH gradient (30 - 100%) of solvent D for 15 min, with a final 5 min step of only solvent D to eluate highly charged peptides. During the entire gradient, a flow rate of 200 µL/min was kept constant. In total, ten fractions were collected, of which the total peptide concentration was determined using the AUC. These were lyophilised and frozen until further analysis.

VI.2.3.3 *Second-dimensional separation and Q-Exactive Orbitrap MS/MS analysis*

The ten SCX fractions were resuspended in LC-MS H_2O to desalt the peptides using solid phase extraction (SPE). GracePure™ SPE C18 Columns (W. R. Grace & Co.-Conn., Maryland, USA) were placed onto a vacuum manifold and subsequently conditioned (three times with 100 µL methanol) and equilibrated (twice with 100 µL LC-MS H_2O) before the fractions were loaded (two times, reloading the eluate), washed (twice with 100 µL (20% methanol, 80% LC-MS H_2O)) and eluted (twice with 100 µL (40% methanol, 40% ACN, 20% 0.1% HCl in LC-MS H_2O)). The eluted peptides were subsequently lyophilised and frozen until further analysis. Each SCX fraction was separated in a second dimension by RP-C18 chromatography on an Easy nanoLC system using an Acclaim C18 PepMap®100 column (75 µm x 2 cm, 3 µm particle size) connected to an Acclaim PepMap® RSLC C18 analytical column (50 µm x 15 cm, 2 µm particle size) (Thermo Scientific). Before loading, the vacuum-dried peptide pellets were dissolved in mobile phase A (2% ACN and 0.1% FA). Of each SCX fraction, 1 µg of peptides were loaded onto the column. One technical replicate was performed for each sample. A linear gradient of mobile phase B (0.1% FA in 95% ACN) from 2% to 45% in 55 min, followed by a steep increase to 100% mobile phase B in 5 min, was used at a flow rate of 300 nL/min. Liquid chromatography was followed by MS, performed on a Q-Exactive Plus mass spectrometer equipped with a nanospray ion source (Thermo Scientific). The high-resolution mass spectrometer was set up in an MS/MS mode in which a full scan spectrum (350 to 1850 m/z, resolution 70,000) was followed by a maximum of five HCD tandem mass spectra (100 to 2000 m/z). The normalised collision energy was set at 33%. A dynamic exclusion list of 15 s for data-dependent acquisition was applied. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [234] partner repository with the dataset identifier PXD037193 (https://www.ebi.ac.uk/pride/archive).

VI.2.3.4 *Database searching*

All generated MS/MS spectra were analysed by means of MaxQuant software version 1.6.1 [301] using the *Sus scrofa* database that was generated based on both the characterised

porcine proteins and the porcine genome (*Sus scrof*a database (reviewed + unreviewed), generated from UniProt (2020/03/30), 120,806 entries). Analysis was performed based on trypsin digestions. Methyl methanethiosulfonate binding to cysteine and iTRAQ 8-plex labelling of lysine and the N-terminus were specified as fixed modifications. Oxidation of methionine and iTRAQ 8-plex labelling of tyrosine were set as variable modifications. Only proteins or protein groups with at least two unique peptides were further investigated. MaxQuant sometimes reports protein groups instead of a single protein. These groups are clusters of proteins that could not be distinguished from each other based on the identified peptides [301]. The leading protein of this group is the protein with the best match and the protein that is referred to in this manuscript. The FDR was set at 1% and guarded using a reversed decoy database. All human keratins and other possible contaminants were removed from the output list. A BLAST analysis was performed on all uncharacterised proteins (BLASTP 2.8.0+, All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects Program, *Sus scrofa* (taxid:9823)) [238]. These proteins are further identifiable by the word BLAST in front of their names. The fold-change difference of each protein was calculated, proteins whose relative abundance had a fold-change of at least 1.5 where further investigated.

VI.2.3.5 *Statistical analysis*

Mixed models were fitted to identify differences between experimental groups for the following parameters: body weight gain, cortisol concentrations in saliva and hair, and relative concentrations of specifically identified proteins. The sow was implemented as a random factor to account for the dependence of littermates. To determine differences between concentrations of specific salivary proteins, the different iTRAQ runs were added as a random factor for normalisation since three parallel runs were necessary to analyse all 24 samples. To meet normality and homoscedasticity assumptions, data from hair and salivary cortisol levels and some specific salivary protein concentrations were log-transformed. A Spearman rank correlation test was performed to investigate the

relationship between all assessed parameters. All data were analysed using JMP® Pro 13 (SAS Institute Inc., North Carolina, USA) and reported as medians and 25^{th}/75^{th} percentiles. A *P*-value ≤ 0.05 was considered statistically significant.

VI.2.4 Candidate biomarker validation using PRM

To confirm the salivary protein profile differences between stressed and control piglets, eight of the proteins from the discovery iTRAQ-experiment were selected for further validation. Since no commercially available ELISAs or antibody-based quantification techniques were available for the selected porcine proteins, we opted to validate these eight selected proteins using PRM. This is a targeted mass spectrometry approach, often used to validate candidate biomarkers. During this analysis specific peptides are selected and fragmented. The abundance of each fragment is detected with very high sensitivity and specificity (e.g., [197]).

VI.2.4.1 *Selection of proteins*

The selection of proteins was based on different parameters. Preferably the proteins had a high fold-change difference and/or a small *P*-value and/or had a known function or involvement in processes that are affected by stress, such as immunity, feeding behaviour, or reproduction capacity (e.g., [3, 6-10]). Moreover, some proteins were chosen because they had previously been reported to show altered concentrations in saliva after acute stress or compromised welfare conditions in pigs or other species [177, 179, 189, 302]. The final criterium was the possibility of detecting an adequate amount of proteotypic peptides for the target protein group during PRM optimisation, i.e., shotgun analysis and unscheduled PRM. For each of the final eight target proteins, three to five of these proteotypic peptides were listed to be monitored using an inclusion list (Supplementary file 1). These are the peptides that will be selected during the scheduled PRM analysis based on their known retention time and m/z for relative quantification.

Peptides from serotransferrin were also relatively quantified to detect potential blood contamination (e.g., [303]). Chewing and oral problems can cause small wounds in the

piglet's oral cavity through which blood can leak during saliva collection. Some candidate biomarkers, like alpha-2-HS-glycoprotein, but also cortisol, are present in low concentrations in saliva but in much higher concentrations in blood (e.g., [33, 304]). Therefore, even small amounts of blood contamination can result in artificially high levels of these components in saliva [305]. Serotransferrin is also a protein that is present in higher concentrations in the blood than in saliva. Consequently, we used it as a marker for blood contamination. Although it is known that several factors, such as age, gonadal hormones, salivary flow rate and chewing also affect serotransferrin levels in saliva [306], it is suggested as the best indicator for blood contamination [303]. Noteworthy is that all saliva samples were visually inspected, and no discoloured (pink or red) samples were included in the analysis.

Finally, specific peptides for two additional control proteins were added to the inclusion list. These proteins are apomucin and sulfhydryl oxidase, which are chosen to identify large differences in the background proteome due to sampling, individual sample preparation, or parallel analysis. Candidates for salivary control proteins are amylase, mucins, albumin, or IgA. However, the abundance of all of these proteins except for mucins is altered by acute stress [181, 183, 189]. Therefore, we opted for apomucin, which was previously detected in porcine saliva by our research group [297]. The variation in the abundance of this protein between animals was small. However, since it was more abundant in mandibular and sublingual secretions than in parotid saliva, chewing could introduce more variation of the concentration in whole saliva present in the oral cavity. The reason for this is that chewing increases the contribution of the parotid gland to the whole saliva therefore diluting and decreasing the concentration of apomucin in whole saliva [141]. Thus, we included sulfhydryl oxidase as a second protein with low variation between animals and with equal concentrations in mandibular/sublingual and parotid saliva [297].

To see whether there was a difference in the abundance profile over time in these eight candidate biomarkers, we determined their relative abundance in salivary samples taken from the piglets at the age of 14 days (one week after the start of the experiment) and 28 days (three weeks after the start).

VI.2.4.2 *Sample preparation*

All saliva samples of days 14 and 28 were enzymatically digested according to the following protocol. All samples where thawed and volumes containing 50 µg of protein were denatured at 90°C for 5 minutes. The samples were allowed to cool down and 2.5 µL of 50 mM of TCEP was added followed by a 1-hour incubation step at 55°C. To alkylate thiols irreversibly, 5 µL of 375 mM iodoacetamide (Biolsolve BV, Valkenswaard, The Netherlands) were added to each sample and incubated for 30 minutes. All protein mixtures were purified by acetone precipitation. The obtained protein pellets were resuspended in 500 mM TEAB. Subsequently, trypsin was added to digest proteins during an overnight incubation at 37°C. All peptide samples were lyophilised and frozen until further purification using C18 spin columns (Thermo Scientific), according to the manufacturer's instructions, except using FA (Merck KGaA, Darmstadt, Germany) instead of trifluoroacetic acid. The purified digest was lyophilised again and frozen.

VI.2.4.3 *Nano reversed phase liquid chromatography and mass spectrometry*

The digested peptides were reconstituted in 0.1% FA and analysed on a Q-Exactive Plus mass spectrometer (Thermo Scientific) connected to a nanoAcquity UPLC system (Waters Corporation). For each sample, a tryptic digest of peptides equivalent to 0.5 µg total protein was loaded on a 200 cm micro Pillar Array Column (µPAC™, PharmaFluidics, Ghent, Belgium) retrofitted to a NanoSpray Flex source. Peptides were eluted at a flow rate of 750 nL/min using the following gradient: 1% to 40% ACN in 0.1% FA/H_2O for 30 min, 40% to 99% ACN for 5 min, 99% to 1% ACN for 5 min and 35 min at 1% ACN in 0.1% FA/H_2O. Analytes were transferred to the gaseous phase with positive ion electrospray ionisation at 1.9 kV. Precursors were targeted with a 0.8 m/z isolation window around the m/z of interest. Precursors were fragmented in HCD mode with normalised collision energy of 28. A single MS1 scan was performed at a mass resolution of 17,500, an automatic gain control (AGC) target of 10^6 ions and a maximum C-trap fill time of 200 ms. Subsequently, 10 PRM scans were performed at a resolution of 70,000, an AGC target of 10^5 ions and a maximum injection time of 250 ms. Retention-time scheduling of PRM (sPRM) was adopted, which allowed for the analysis of all peptides in a single LC-MS analysis.

VI.2.4.4 *Data analysis*

Skyline 20.1 [307] was used to analyse all PRM raw data. Only peptides with a idotp score ≥ 0.8 were included, meaning that they were proven to be of good quality after comparison of the experimental transitions to the theoretical spectral library that was generated by Prosit [308]. All transitions for one peptide were added up. The sums of the proteotypic peptides of one protein were averaged to indicate its abundance within each sample. Mixed models were fitted using JMP® Pro 13 to identify differences between the stressed and control group on the one hand and the two sampling time points on the other hand. The interaction term between groups and time points was added as a fixed factor. The sow was included as a random factor to account for the dependence of littermates. The same piglets were sampled on days 14 and 28, so the piglet was nested in the sow and added as a random factor. This initial model was simplified by removing all non-significant effects using stepwise backward modelling. To meet normality and/or homoscedasticity assumptions, all data were log-transformed. Correlations were investigated using the non-parametric Spearman's assay. Correlations with a Spearman's rank correlation coefficient (ρ) higher than 0.4 (0.6 or 0.8) or smaller than -0.4 (-0.6 or -0.8) were moderate (strong or very strong) correlations. A *P*-value ≤ 0.05 was considered statistically significant.

VI.3 Results

VI.3.1 Physiological parameters

The piglets that were exposed to the stressors gained significantly less weight (*P*-value = 0.021) during the three-week experiment compared to the control piglets (Table 1). Cortisol concentrations in saliva at day 28 were not significantly different (*P*-value = 0.447) between both groups. The stressed group had significantly higher concentrations of cortisol (*P*-value = 0.005) in their hair compared to their control littermates. The total concentration of proteins in saliva did not differ between both groups and/or between ages (*P*-value = 0.531). Weight gain and cortisol concentrations in hair showed a significant negative correlation (*P*-value = 0.036, ρ = - 0.430). At the same time, neither of these parameters

correlated significantly with cortisol levels in saliva at day 28 (*P*-value = 0.904, ρ = 0.026 and *P*-value = 0.929, ρ = 0.019, respectively).

Table 1. Physiological parameters. *Weight gain over 21 days, cortisol concentrations in saliva at day 28, cortisol accumulation in the hair over 21 days (measured on day 28), and total protein concentration in saliva at day 14 and day 28. Values are displayed as the median of the group, with the 25th and 75th percentiles shown between brackets. Values that differed significantly between the control and the stressed group are indicated with an asterisk (P-value < 0.05).*

	Weight gain (kg)	**Concentration cortisol (µg/dL saliva)**	**Concentration cortisol (pg/mg hair)**	**Protein concentration in saliva (day 14) (µg/mL)**	**Protein concentration in saliva (day 28) (µg/mL)**
Control group	7.58 (6.79 - 8.22)*	0.25 (0.21 - 0.39)	75.60 (69.95 - 78.42)*	10352 (8337 - 11485)	6915 (5432 - 8305)
Stressed group	6.43 (5.94 - 7.20)*	0.30 (0.27 - 0.51)	87.29 (78.55 - 99.61)*	9040 (8142 - 10658)	7812 (5783 - 16690)

VI.3.2 Identified proteins using iTRAQ

In total, 421 protein groups were identified based on at least two unique peptide identifications. After the removal of all human keratins and possible contaminants, 392 proteins remained, of which 13 were uncharacterised proteins (Supplementary file 2). Of this protein list, 255 proteins were detectable in all three parallel iTRAQ runs, including samples of both the control and stressed groups. In total, the abundance of 26 proteins was up-or downregulated with a fold change of ≥ 1.5 between the saliva of control and stressed animals. Further statistical analysis of these 26 proteins using mixed models confirmed that 20 proteins showed a significant difference in salivary concentration between both treatment groups. Six of these 20 proteins were found in lower concentrations in the saliva of stressed animals, while 14 proteins were upregulated (Table 2).

Table 2. Identified salivary proteins with a significant fold difference. *List of proteins of which a significant fold change difference was seen during the exploration phase using iTRAQ labels. If the fold change difference is described as up, this indicates that the values were higher in the saliva of stressed animals compared to those of the control group. A BLAST analysis was performed on all uncharacterised proteins, which are identifiable by the word BLAST in front of their names. Asterisks indicate the proteins that were further validated using PRM.*

	Protein name	UniProt ID of lead protein	Number of unique peptides detected	Mol. weight [kDa]	Fold change difference	Fold change (stressed/ control)	Mixed model (*P*-value)
1	Odorant-binding protein*	P81245	2	17.71	2.22	Down	0.002
2	Long palate lung and nasal epithelium protein 5*	A7J153	10	54.11	2.18	Down	0.001
3	Chitinase*	I3LL32	9	51.97	1.6	Down	0.003
4	BLAST: Vomeromodulin-like protein*	F1S501	10	49.60	1.57	Down	0.001
5	Lipocalin-1*	P53715	9	19.37	1.56	Down	0.010
6	Vitelline membrane outer layer protein 1 homolog	F1RFV3	3	21.53	1.53	Down	0.010
7	Haemoglobin subunit beta	F1RII7	6	16.17	2.07	Up	0.008
8	Haemoglobin subunit alpha	P01965	9	15.04	1.95	Up	0.026
9	BLAST: Basic salivary proline-rich protein 1	A0A4X1U5H6	4	25.88	1.86	Up	0.038
10	CD5 molecule like	F1RN76	2	59.28	1.84	Up	0.001
11	Biliverdin reductase B	I3LQH7	4	22.21	1.72	Up	0.001
12	Basic proline-rich protein	Q95JC9	2	46.02	1.7	Up	0.009
13	Heat shock protein family A (Hsp70) member 9	F1RGJ3	2	70.12	1.68	Up	0.014
14	Parotid secretory protein*	Q6XZB6	9	25.98	1.66	Up	0.012
15	Apolipoprotein A-II	Q7YRR7	3	11.11	1.61	Up	0.001
16	Albumin	F1RUN2	25	67.14	1.6	Up	< 0.001
17	BLAST: Basic proline-rich protein 1	A0A5G2R9V5	3	17.54	1.59	Up	0.007
18	Carbonic anhydrase*	B7X727	9	36.31	1.57	Up	0.020
19	Alpha-2-HS-glycoprotein*	F1SFI7	5	38.79	1.55	Up	< 0.001
20	Ig lambda chain C region	P01846	3	11.00	1.53	Up	< 0.001

A functional analysis for the 392 filtered proteins was performed with the gene ontology database Panther (http://www.pantherdb.org/; Version 17.0). First, the genes that encoded the listed proteins were sought. From the 392 protein entries, 280 could be matched with their encoding gene. These genes could be assigned to 10 different molecular functions. Most of the genes encoded for proteins that had binding (40.1%) or catalytic functions (35.6%). These 280 recognisable genes were involved in 16 different biological processes. Of these, more than 80% were involved in cellular (50.1%) or metabolic (33.1%) processes, while only a small fraction of these genes encoded proteins that were involved in immunity (5.9%), growth (2.2%) or reproductive processes (0.6%).

VI.3.3 Biomarker validation using PRM

Eight proteins from the list of 20 were selected for further analysis using PRM. Alpha-2-HS-glycoprotein is a protein that was found in higher concentrations in the saliva of 4-week-old stressed animals after shotgun investigation. This observation was confirmed by the targeted validation (*P*-value = 0.003) (Fig. 2). This holds true for samples taken one week after the start of the experiment as well as at the end. It is noteworthy that the concentration of this protein rose with age (*P*-value = 0.008) in both the stressed and the control groups.

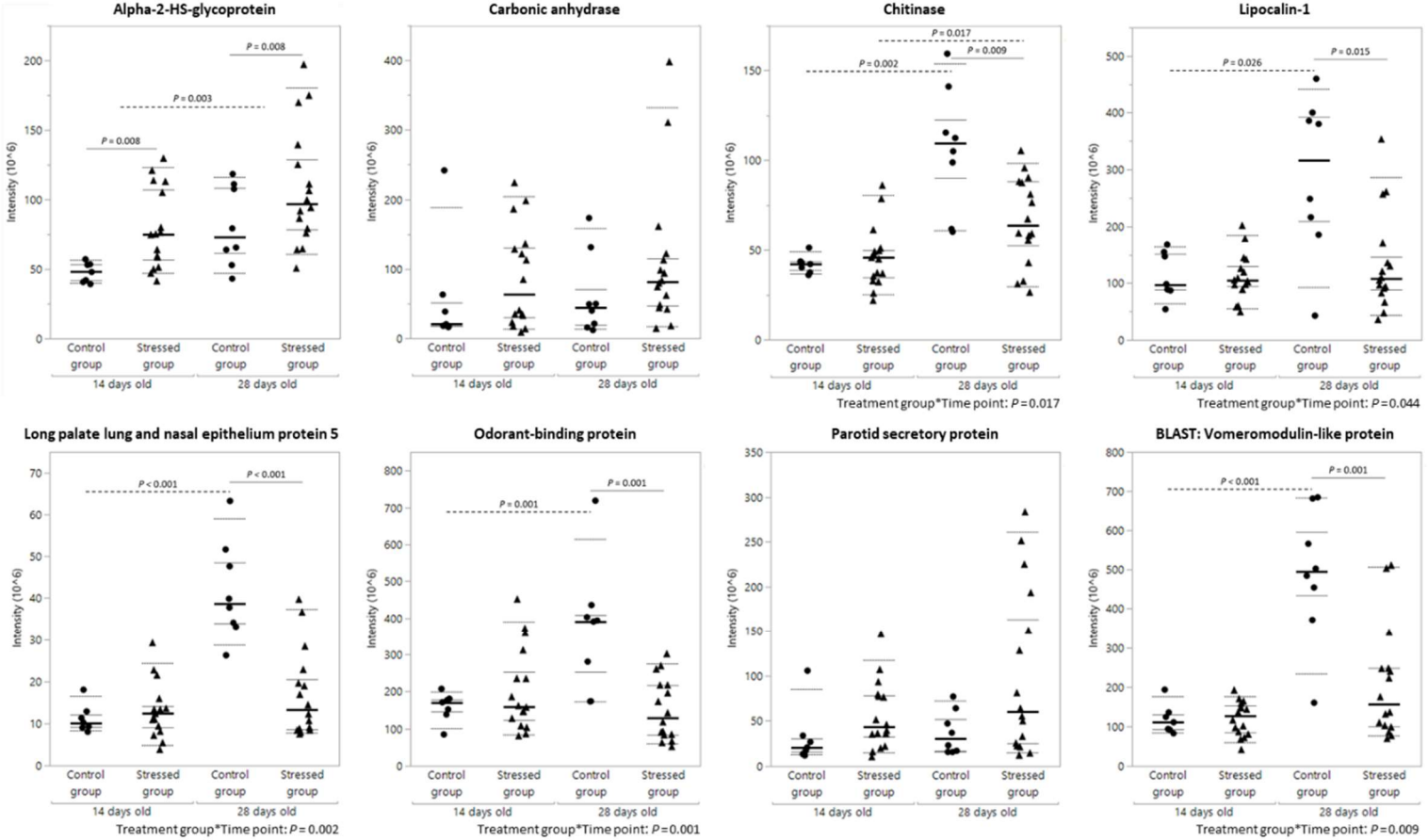


Figure 2. The abundance of proteins validated by PRM. *Statistically significant differences between samples taken at the age of 14 days and samples from day 28 are highlighted by dashed lines. Significant differences between the control group (circles) and the stressed group (triangles) are indicated with a full line. Significant interaction terms are placed underneath each graph. For each group (control group (n = 8); stressed group (n = 16)), the median (thick line), the 25th and 75th percentiles (thin lines), and the 5th and 95th percentiles (dotted lines) are shown.*

In contrast, the differences in concentration of the two other selected proteins with an upregulation in the saliva of stressed animals were not confirmed by PRM. Carbonic anhydrase and the parotid secretory protein did not show any difference between both experimental groups (*P*-value = 0.265 and *P*-value = 0.129, respectively) or between the different sampling points (*P*-value = 0.363 and *P*-value = 0.246, respectively).

The five other proteins that were validated using PRM all had a significant interaction of experimental groups and time points, meaning that the effect of stress was not the same at both time points. Post hoc analysis of the concentrations of chitinase showed a rise in concentration from the age of 14 days to 28 days (*P*-value = 0.002) in the saliva of the control group. This rise was less pronounced in the stressed group (*P*-value = 0.017), resulting in a significant difference in chitinase concentration between the two experimental groups on day 28 (*P*-value = 0.009) but not on day 14. Lipocalin-1, long palate lung and nasal epithelium protein 5, odorant-binding protein, and BLAST: vomeromodulin-like protein all had a similar profile. The latter was an uncharacterised protein that, after a BLAST analysis, appeared to be a homolog of the vomeromodulin-like protein (*Bison bison*). All four proteins showed a different effect of time on both experimental groups. Only a rise in concentration over time was observed in the control group (lipocalin-1: *P*-value = 0.026; long palate lung and nasal epithelium protein 5: *P*-value < 0.001; odorant-binding protein: *P*-value = 0.001 and BLAST: vomeromodulin-like protein: *P*-value < 0.001). In the stressed group, the concentration of these proteins remained the same. Therefore, the values of these four proteins were significantly lower in the saliva of the stressed piglets compared to those of the control group, albeit only on day 28 (lipocalin-1: *P*-value = 0.015; long palate lung and nasal epithelium protein 5: *P*-value < 0.001; odorant-binding protein: *P*-value = 0.001 and BLAST: vomeromodulin-like protein: *P*-value = 0.001).

Odorant-binding protein was the only salivary candidate biomarker with concentrations that correlated to the other determined physiological parameters (Supplementary file 3). The concentrations of this protein on day 28 determined by PRM correlated significantly with weight gain during the experiment (*P*-value = 0.048, ρ = 0.408). Nevertheless, the five

proteins that had a downregulation on day 28 all correlated significantly with each other. The strongest correlation was found between long palate lung and nasal protein 5 and BLAST: vomeromodulin-like protein with a Spearman's ρ of 0.944 (*P*-value < 0.001). Odorant-binding protein was the only protein with a significant downregulation that correlated with proteins that showed an upregulation after iTRAQ-analysis. Although PRM analysis could not confirm the difference between both treatment groups for carbonic anhydrase and parotid secretory protein, their values did correlate negatively with those of odorant-binding protein. Alpha-2-HS-glycoprotein was the only protein that did not correlate to any of the other candidate biomarkers. However, this protein did correlate positively with the abundance of serotransferrin on day 28 (*P*-value < 0.001, ρ = 0.747), while this was not the case for all other proteins. It is noteworthy that the values of serotransferrin also correlated with the concentration of cortisol that was detected in these salivary samples. No significant differences were found in the abundances of the selected control proteins apomucin (age, *P*-value = 0.375; condition, *P*-value = 0.058) and sulfhydryl oxidase (age, *P*-value = 0.286; condition, *P*-value = 0.107). However, the values of serotransferrin did rise with age (*P*-value = 0.001). No significant differences were observed between the total protein concentrations in the saliva samples, neither between treatment groups (*P*-value = 0.116) nor time points (*P*-value = 0.531). One animal stood out since the value of sulfhydryl oxidase in its saliva was 5 times higher than the median value of the 14-day old stressed group. The same animal also had a much higher value of serotransferrin on day 14. Since none of the eight validated biomarkers displayed values that deviated this much, this observation was not considered a problem for further data analysis and interpretation. For apomucin, one animal had exceeded the range for outliers of the mean ± 2.5 times the SD. This animal of the stressed group had 3 times higher values at day 28 compared to the average detected in this group. This animal had no extreme deviating values for the other examined proteins. Only two other values could be considered outliers. These were the highest value in the stressed group on day 28 for carbonic anhydrase and lipocalin-1. However, these were not from the same animal. Follow-up studies with larger sample sizes need to verify the working ranges for the biomarkers.

VI.4 Discussion

During this experiment, the control group gained, on average, significantly more weight during the 21-day study period than the piglets in the stressed group. This is not a surprise since it is known that the average daily weight gain of pigs is reduced by stress (e.g., [3]). A reduced feed intake can cause this reduction in weight gain since different stressors are known to result in lethargy and, therefore, lower feed intake [3]. In addition, the stress system can also interact with the appetite-satiety centres of the central nervous system [114, 115]. Weight gain correlated negatively with hair cortisol concentrations. Hair from stressed pigs contained significantly higher concentrations of cortisol. In contrast, no correlation between the physiological parameters, weight gain and hair cortisol concentrations, and cortisol concentrations in saliva was found. Salivary cortisol concentrations were not significantly higher after 21 days of exposure to multiple stressors. This lack of a difference was probably due to two higher saliva cortisol values in the control group. Most likely these reflected an acute response to a stressful stimulus, rather than chronic stress, although alternative biomarkers for acute stress, such as chromogranin A or IgA (e.g., [299]) were not analysed to confirm this hypothesis. Although, chromogranin A was previously also described as a marker for chronic stress in pigs making this marker less ideal for this purpose. Nevertheless, it could have been interesting to correlate the determined parameters and the determined profile of salivary proteins to the abundance of chromogranin A. Of note, the correct timing for salivary sampling could be a point of discussion. It is possible that differences between groups might be larger if sampling occurs when baseline cortisol concentrations are low, i.e., in the evening. However, the circadian rhythm of cortisol does not mature until 20 weeks of age [32], rendering the identification of daily baseline cortisol concentration in young animals unpredictable. Additionally, a previous study demonstrated that decreased welfare leads to a blunted circadian rhythm [54], making the determination of the optimal time point for sampling challenging. However, finding salivary biomarkers that are less sensitive to acute stressors and that are not subjected to a circadian rhythm would be ideal.

In this experiment, iTRAQ-analysis identified 392 proteins in porcine saliva of which many were original identifications. Together with the proteins that our group has identified before in gland-specific saliva and those identified by other researchers, the list of identified proteins of the porcine salivary proteome is approaching 500 [72, 171-180, 297]. Even though our knowledge of pig saliva is growing, this number is merely a fraction of the more than 3000 identifications of the human salivary proteome (e.g., [168-170]). Of the 392 protein identifications, the abundance of 20 proteins was different after a three-week exposure to different stressors, including overcrowding, deprivation of cage enrichment and frequent mixing of individuals between pens.

VI.4.1 Upregulated proteins

Fourteen proteins were found to be present in higher concentrations in the saliva of stressed animals. These include alpha-2-HS-glycoprotein, apolipoprotein A-II, basic proline-rich protein, biliverdin reductase B, carbonic anhydrase, CD5 molecule-like protein, haemoglobin subunit alpha and beta, heat shock protein family A (Hsp70) member 9, Ig lambda chain C region, parotid secretory protein, albumin and two uncharacterised proteins that had homology with basic salivary proline-rich protein 1 (*Homo sapiens*) and basic proline-rich protein 1 (*Homo sapiens*).

VI.4.1.1 *PRM-validated upregulated proteins*

Alpha-2-HS-glycoprotein, also called fetuin-A, is mainly synthesized by the liver and secreted into the bloodstream. It is involved in many different pathways. It is an inhibitor of insulin receptor tyrosine kinase, has adipogenic properties, and regulates bone remodelling and calcium metabolism in bones and teeth (previously reviewed [309]). Additionally, this glycoprotein plays an APP role exhibiting an anti-inflammatory function by inhibiting the production of proinflammatory mediators in macrophages [310, 311]. Because of its versatile function, alpha-2-HS-glycoprotein has been suggested as a biomarker for several human conditions, as previously reviewed [309]. High circulating levels of alpha-2-HS-glycoprotein were also found in the serum of humans with depressive

episodes and anxiety within the context of insulin resistance [312-314]. In contrast, lower concentrations were found in the bronchoalveolar lavage fluid of calves exposed to road transport and weaning [302]. Because this protein is so versatile, its concentration can be influenced by many different processes and therefore seems less specific. Another disadvantage is that the concentration of this glycoprotein could be influenced by blood contamination. Consequently, the results should be interpreted with caution. On the other hand, the values of alpha-2-HS-glycoprotein in saliva were already significantly higher after one week of exposure to the stressors. This is the only protein in this experiment that responded that fast. For this reason, this protein could be valuable as a candidate biomarker that responds relatively quickly to chronic stressor exposure. However, it should not be used as a single biomarker but rather as part of a set due to its low specificity.

The parotid secretory protein, like the basic proline-rich proteins, is a protein that is also stored in acinar granules [315] and predominantly secreted by the parotid gland [297]. The function of the parotid secretory protein is still unknown. It most probably belongs to the palate lung and nasal epithelium clone (PLUNC) family of mucosal secretory proteins that are predicted to be structurally similar to lipid-binding and host defence proteins. However, different members of this family may have different biological functions [316]. While higher saliva concentrations of these proteins have only been linked to autism spectrum disorder [317], increased secretion rates under stressful conditions could possibly be explained by beta-adrenergic stimulation [318, 319]. Although six stressed animals had much higher values of parotid secretory protein after three weeks compared to the control group, this effect was not consistent in all pigs.

The last protein for which the iTRAQ analysis indicated a positive fold-change difference is carbonic anhydrase. Carbonic anhydrase isoenzyme VI is the only secretory isoenzyme of its family that is expressed in the serous acinar cells of the parotid and mandibular glands [320]. Higher concentrations of this protein have been found in pooled saliva samples of pigs after snaring [177] and in pigs with non-infectious growth-rate retardation [171]. In contrast, others did not detect a significant effect on the carbonic anhydrase VI

concentration in saliva after snare restraint. Still, they did observe an increase due to lameness [178]. This discrepancy could be attributed to the presence of two different forms of carbonic anhydrase VI in porcine saliva. In the saliva of pigs with retarded growth two forms were identified of which only the larger form of this glycoprotein (36 kDa) was present in higher concentrations. In comparison, the smaller (33 kDa), assumed partially deglycosylated form, was nearly absent [171]. In our study, PRM validation failed to confirm any increase in concentration in stressed animals since only two animals of the stressed group presented elevated concentrations of this protein. Further studies should be conducted to clarify the role of carbonic anhydrase VI as a biomarker for animal welfare.

VI.4.1.2 *Non-validated upregulated proteins*

The basic proline-rich proteins upregulated in the iTRAQ analysis are predominantly secreted by the parotid gland [297]. These secretory proteins are stored in acinar granules [212]. Basic proline-rich proteins are often further cleaved into smaller fragments after secretion. This group of proteins and peptides has a role in the protection and repair of dental enamel, has antimicrobial capacities, and can bind feed components such as tannins (e.g., [321-323]) but have not been associated with stress before in pigs.

The amount of CD5 molecule-like protein in the saliva of the stressed animals was nearly twice as high as that of the control animals. This observation is in line with a previous study in which higher concentrations of this CD5 molecule-like protein were found in the saliva of lame pigs [179]. Unfortunately, this protein could not be further validated in our study since not enough specific peptides were identifiable during PRM analysis. Like the upregulation of CD5 molecule-like protein, also higher levels of haemoglobin subunit alpha and beta were detected in the saliva of lame animals [179], which is consistent with our results. These haemoglobin subunits were also found in higher concentrations in the saliva of pigs after exposure to an acute stressor, i.e. snaring restraint [177]. Snaring also led to higher concentrations of albumin in saliva, as did short road transport and 24 h isolation in a metabolic cage [189]. These findings are like the 1.6-fold upregulation of albumin observed in our study. The higher levels of albumin in stressed pigs can be the result of

higher cortisol levels since it has been reported that increased concentrations of cortisol could elevate albumin production [324]. Salivary albumin concentrations were also found in higher concentrations in pigs suffering from infection and/or inflammation [172, 175]. The four proteins described above, namely CD5 molecule-like protein, haemoglobin subunit alpha and beta, and albumin, have previously been suggested as salivary biomarkers for pig welfare and our findings reinforce this.

VI.4.2 Downregulated proteins

Salivary proteins that were found in lower concentrations after chronic exposure to stress are chitinase, lipocalin-1, long palate lung and nasal epithelium protein 5, odorant-binding protein, vitelline membrane outer layer protein 1 homolog, and an uncharacterised protein that was a homolog of the vomeromodulin-like protein (*Ursus maritimus*). The latter was never identified in porcine saliva before.

VI.4.2.1 *PRM-validated downregulated proteins*

In our study, chitinase was significantly decreased in the saliva of piglets exposed to stressors for three weeks. The family of chitinases are involved in inflammation, tissue remodelling and injury, and higher serum concentrations are associated to human diseases such as asthma (previously reviewed [325]). Importantly, the interpretation of these results must be performed with care. An effect of age on the concentration of this protein was observed in both treatment groups. However, this effect of age was lower in the stressed group leading to significantly lower levels of chitinase on day 28. Further investigation is needed to enable comparisons between different age groups.

Two members of the lipocalin family displayed a similar profile, i.e., lipocalin-1 and odorant-binding protein. The concentrations of these proteins rose with age under normal circumstances but not in a stressful situation. In consequence, significantly lower levels were observed in the saliva of 28-day-old stressed piglets when compared to control animals. Lipocalin-1 is mainly secreted by the porcine lachrymal glands and the lingual von Ebner's glands [326]. In addition, it was also detected in gland-specific saliva and had similar

concentrations in both mandibular/sublingual saliva and parotid saliva [297]. It protects the epithelia due to its role in the nonimmunological defence against micro-organisms and viruses and by controlling inflammatory processes [327]. Stress-related immunosuppression could explain these lower values [6, 7]. Of note is that the piglets in our study did not reach sexual maturity yet. However, very recently, it has been discovered that the concentration of lipocalin-1 in saliva fluctuates throughout the oestrus cycle, making interpretation of this biomarker difficult in sows [180]. Odorant-binding proteins are expressed by glands of the nasal cavity in vertebrates [328], but have also been found in gland-specific saliva of pigs, both in mandibular/sublingual saliva and in lower concentrations in parotid saliva [297]. These proteins are involved in mediating olfactory transduction, in chemical communication, and pre-mating recognition processes through pheromones [328, 329]. The suppression of this protein by a stressor could be linked to the negative effect of stress on reproduction capacity [9, 10]. Additionally, an increase in oxidative products associated with stress could contribute to its decrease [330]. The concentrations of both lipocalin-1 and odorant-binding protein were described to decrease after acute stress [177, 189], but also during disease [175]. The values of odorant-binding protein on day 28 correlated with all other PRM-validated proteins, except with alpha-2-HS-glycoprotein, and correlated with weight gain during the experiment (Supplementary file 3). This protein is locally expressed and secreted in the oral cavity and does not originate from the bloodstream. Its concentration can, therefore, not be altered by potential blood contamination of the sample. The only disadvantage of odorant-binding protein is that its concentration increases with age under non-stressed conditions, as those of lipocalin-1 and chitinase, making interpretation of results challenging. It is thus important to investigate the effect of age on these candidate biomarkers.

The vomeromodulin-like protein has similarities to odorant-binding protein, both in function and location [331, 332]. It is therefore, not surprising that the concentrations of this protein responded similarly to the exposure to stressors as the proteins of the lipocalin family. To our knowledge, no association between the vomeromodulin-like protein and stress has been described in studies using vertebrates.

Long palate lung and nasal epithelium protein 5 is the last protein with significantly lower values in the saliva of pigs reared under stressful conditions. Like the previously described lipocalin proteins, concentrations of this salivary protein rose with age under normal conditions, as in the control piglets, but failed to do so in response to the exposure to stressors. Knowledge about the function of this protein is scarce. However, like the parotid secretory protein, it is a member of the PLUNC family [316, 333]. Further insight into the function of this protein is advisable before this protein can be used as a biomarker for stressor exposure.

VI.4.2.2 *Non-validated downregulated proteins*

Vitelline membrane outer layer protein 1 homolog was previously found in porcine saliva [297], but never concerning stress.

VI.5 Conclusion

Even though much insight into the salivary proteome of pigs and how these proteins respond to stressful conditions has been gained, our study has some drawbacks. In this study, we only investigated female animals. To verify whether our findings can be generalised to both sexes, our results should be validated in males, both chemically castrated and uncastrated. Additionally, as mentioned before, the effect of aging under normal conditions should be studied before different age groups can be compared correctly. It is important to know until which age the concentrations rise when they plateau, and whether the start of this plateau is similar for each candidate biomarker. These questions still need to be answered before the candidate biomarkers can be used in practice to identify welfare problems at the farm or facilitate research to optimise rearing conditions.

Nonetheless, not a single biomarker but rather a set of different proteins needs to be analysed to identify a complex problem such as chronic stress. On the one hand, it is known that different stressors elicit different responses in biomarkers (e.g., [216]). To reduce the chance of false negatives or false positive identifications, proteins linked to different

pathways and processes should be analysed. However, also different individual coping mechanisms of the individual to the same stressor are reported (e.g., [24]). On the other hand, variation can arise from different sample processing methods, salivary flow rates or sample contamination (e.g., with blood, food or dirt).

Nevertheless, a possible set of biomarkers could include alpha-2-HS-glycoprotein, i.e., the confirmed upregulated protein whose abundance was affected even at the early timepoint, and odorant-binding protein, i.e., a downregulated protein that correlated to other physiological parameters. Chitinase and lipocalin-1, both linked to immunity, could be added to the set. Since chitinase has the least variation, it might be the preferred one out of the two. Lastly, long palate lung and nasal epithelium protein 5 is promising since this biomarker showed the highest difference between normal and stressful conditions and had the least variation. Additionally, to improve their use in stress evaluation, it is advisable that these proteins can be easily detected using antibody-based techniques.

To conclude, chronic exposure to different stressors altered the salivary proteome of piglets. Shotgun analysis using tandem mass spectrometry performed on saliva samples taken after three weeks of stress exposure identified 392 proteins, of which 20 proteins displayed significantly altered concentrations. Targeted PRM analysis confirmed that alpha-2-HS-glycoprotein was upregulated in the stressed group after one and three weeks, while odorant-binding protein, chitinase, long palate lung and nasal epithelium protein 5, lipocalin-1, and BLAST: vomeromodulin-like protein were present in lower concentrations in the saliva of the stressed pigs, however only after three weeks. The affected proteins could be used as salivary biomarkers after further validation to identify welfare problems at the farm and facilitate research to optimise rearing conditions.

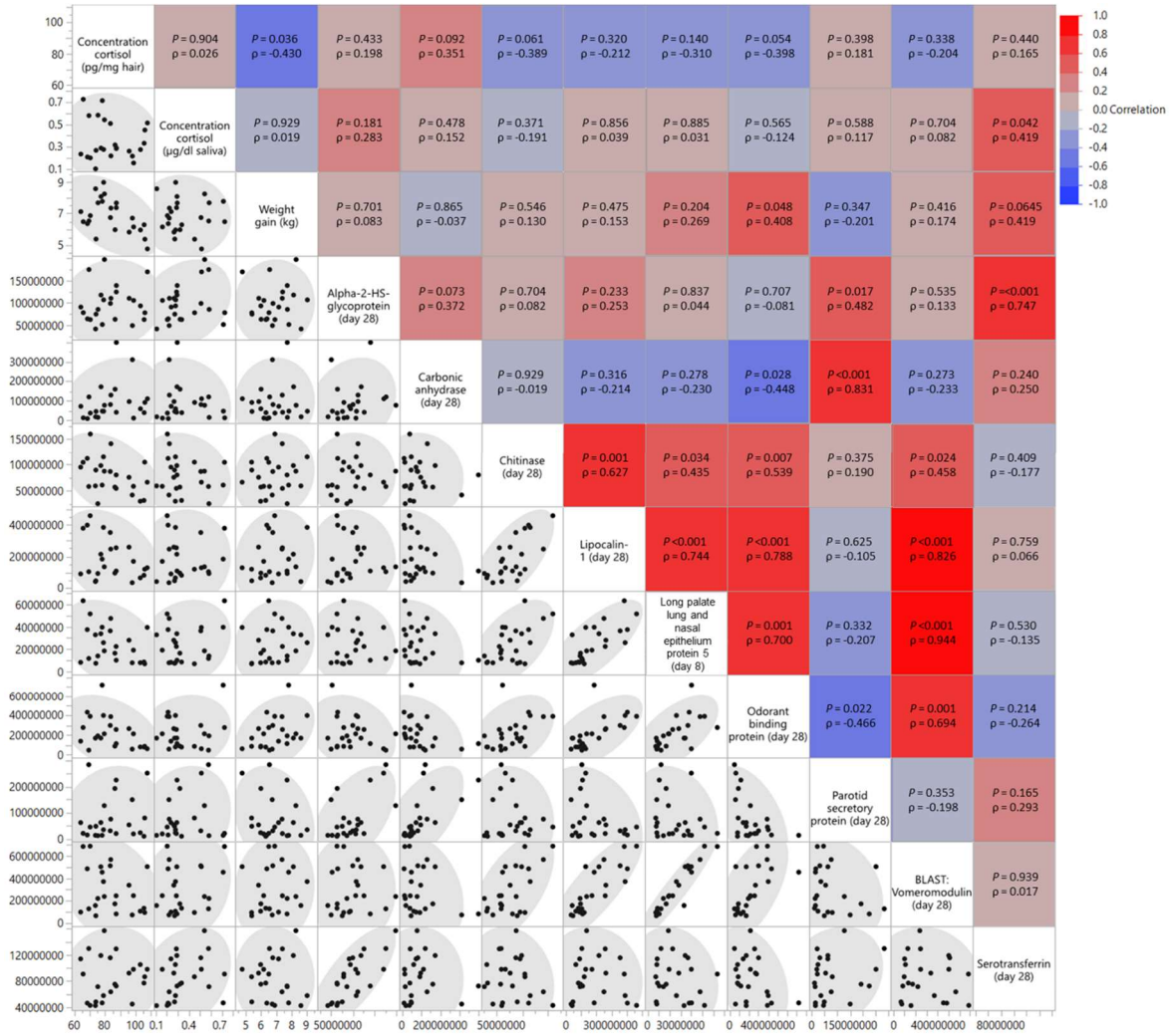
VI.6 Supplementary files

Supplementary file 1. *Peptides used for PRM analysis: characteristics and performance.*

Protein	UniProt ID of protein	Peptide	Mass [m/z]	CS [z]	Start [min]	End [min]
Alpha-2-HS-glycoprotein	F1SFI7	EPACDDVETEQAALAAVDYINK	1211.56	2	35.77	37.77
		HSFSGVASVESASGEAFHVGK	697.34	3	30.46	32.46
		QDGQFSVLFAK	620.32	2	35.04	37.04
		QLTEHAVEGDCDFHVLK	666.65	3	29.41	31.41
Carbonic anhydrase	B7X727	DYAENTYYSDFISHLK	983.45	2	36.98	38.98
		LTAPDGTQYIAK	639.34	2	28.3	30.3
		SVQYNPALR	524.28	2	26.15	28.15
Chitinase	I3LL32	GNEWVGYDNVK	640.80	2	28.72	30.72
		HLFTVLVQEMR	686.87	2	35.77	37.77
		QTFITSVIK	518.81	2	33.14	35.14
		QYGFDGLDFDWEYPGSR	1026.44	2	39.71	41.71
Lipocalin-1	P53715	AMTSDPEIPGK	573.28	2	26.2	28.2
		GLNPDIVRPQQSETCSPGGN	1063.50	2	28.78	30.78
		KPESVTPLILK	408.92	3	31.14	33.14
		TNQPFTFTAYDGK	745.35	2	32.19	34.19
		VVYILPSK	459.79	2	31.04	33.04
Long palate lung and nasal epithelium protein 5	A7J153	LEASVLELLR	571.84	2	39.03	41.03
		LLQAGGLVIEDAK	663.89	2	33.09	35.09
		NQLETDISDMFLK	777.38	2	39.35	41.35
Odorant-binding protein	P81245	GTDIEDQDLEK	631.79	2	25.36	27.36
		IGENAPFQVFMR	704.86	2	37.3	39.3
		QEGNTYDVNYAGNNK	843.87	2	24.83	26.83
		QEPQPEQDPFELSGK	864.91	2	32.72	34.72
Parotid secretory protein	Q6XZB6	AELESLQESESWQEAK	932.43	2	30.41	32.41
		GLETVEPVLQK	606.85	2	30.51	32.51
		LVENLGVSLFK	609.86	2	36.88	38.88
		VQEAENLLDK	579.80	2	27.93	29.93
BLAST: Vomeromodulin-like protein	F1S501	EAVDSTGLLDSNK	674.83	2	28.3	30.3
		GTSSLGILGGGGLVGGLGGTLSK	979.55	2	38.98	40.98
		SCDIELSDVNECK	784.83	2	28.09	30.09
		SLLGNVNVENLLVGLK	841.50	2	42.71	44.71
Serotransferrin	P09571	FDQFFGEGCAPGSQR	568.25	3	31.46	33.46
		TTYESYLGADYITAVANLR	1061.03	2	39.14	41.14
		WCTISNQEANK	675.81	2	25.52	27.52
Apomucin	P12021	DIVLDCPDGSTLPYR	860.91	2	32.94	34.94
		NSCLCCQEEDYEFR	955.36	2	28.72	30.72
		TVTYDYDIFQLK	753.38	2	37.14	39.14
Sulfhydryl oxidase	F1S682	SALYSSSDPLTLLQADTVR	1019.03	2	38.29	40.29
		TGSGATLPVAGADVQTLR	857.46	2	31.72	33.72

Supplementary file 2. List of identified proteins by iTRAQ-analysis in porcine saliva. See link:
https://eur01.safelinks.protection.outlook.com/?url=http%3A%2F%2Frepository.uantwerpen.be%2Fdocstore%2Fd%3Airua%3A22252&data=05%7C02%7CSara.Prims%40uantwerpen.be%7C7d8384a21f9b4cbb13a108dc3dc92733%7C792e08fb2d544a8eaf72202548136ef6%7C0%7C0%7C638453181356345738%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C0%7C%7C%7C&sdata=Hu8qMfWTJBz%2F%2FuduEZqqkyZACa1PFXabY%2BFVbU2Lqcw%3D&reserved=0

Supplementary file 3. Correlation matrix. *Correlation between the determined physiological parameters being the concentration of cortisol in hair and saliva and the weight gain during the three weeks of the experiment on the one hand, and the eight proteins validated by PRM, plus serotransferrin as an indicator for possible blood contamination, at the other hand side. A P-value smaller than 0.05 indicates a significant correlation between two parameters. The Spearman's rho (ρ) specifies the strength of the relation which can be either positive or negative.*



Chapter VII - General discussion

VII.1 Overview of the results

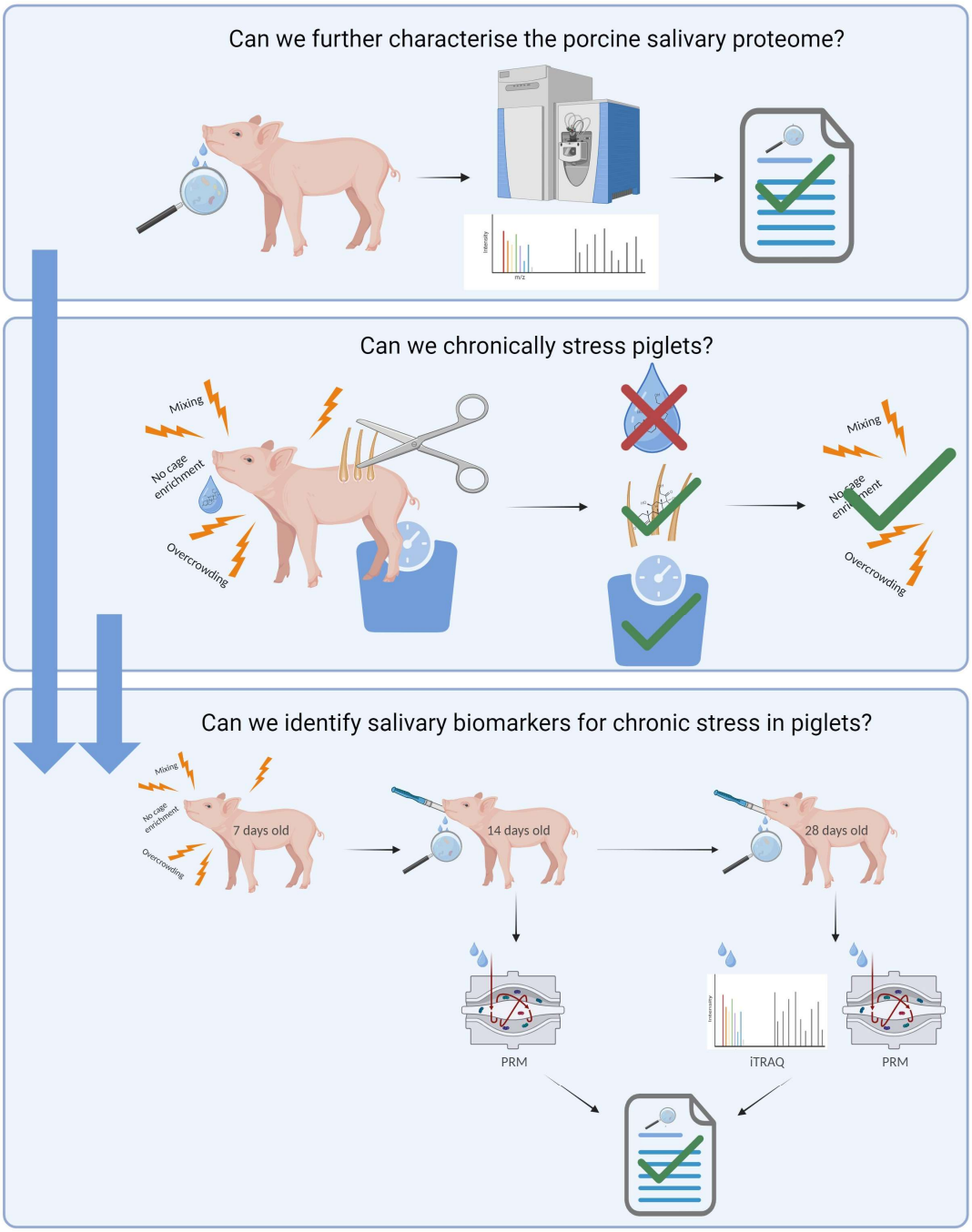


Figure 1. Visual overview of the different chapters.

Chapter IV - On the characterisation of the porcine gland-specific salivary proteome

A total of 122 porcine salivary proteins and six mammalian salivary proteins with a predicted porcine homolog were identified in gland-specific saliva collected from anaesthetised piglets (Fig. 1). All 128 proteins were detected in both ductal secretions albeit with different concentration: 24 proteins characterised the secretion of the parotid gland, while the mandibular and sublingual glands predominantly secreted 29 proteins.

Chapter V - Hair or salivary cortisol analysis to identify chronic stress in piglets?

Three weeks of exposure to multiple stressors, i.c. overcrowding, deprivation of cage enrichment, and mixing of animals led to a reduced weight gain. Additionally, hair from the stressed group contained higher cortisol concentrations, whereas salivary cortisol concentrations did not differ between groups. Weight gain and hair cortisol concentrations were correlated, but neither of these parameters was correlated with salivary cortisol concentrations.

Chapter VI - Chronic exposure to multiple stressors alters the salivary proteome of piglets

Shotgun analysis identified 392 proteins in the saliva of 28-day-old piglets. The relative abundance of 20 proteins was affected by three weeks of exposure to multiple stressors. From these 20 proteins, eight were selected for further validation. For this validation, saliva samples that were taken both one week and three weeks after the start of the experiment were analysed to verify the profile over time. This targeted PRM analysis confirmed that alpha-2-HS-glycoprotein was upregulated in the stressed group after one and three weeks, while odorant-binding protein, chitinase, long palate lung and nasal epithelium protein 5, lipocalin-1, and vomeromodulin-like protein were present in lower concentrations in the saliva of the stressed pigs, albeit only after three weeks.

VII.2 Study remarks

In chapter IV, gland-specific saliva was collected from anesthetised animals. Total protein concentration and the protein composition of gland-specific saliva were mapped. Although this study expanded the knowledge on the porcine gland-specific saliva and highlighted that differences in protein concentrations of several proteins between both ductal secretions exist, samples were collected under non-physiological conditions. Verifying the effect of other anaesthetics (e.g., [243, 250]) and direct nerve stimulation instead of chemical stimulation could be an option (e.g., [251, 252]). Ideally, gland-specific saliva would be collected under physiological conditions in awake animals. Recently, parotid saliva was collected after surgical cannulation through the cheek of adult pigs [334]. In contrast, cannulation of the ipsilateral sublingual caruncle below the tongue can be performed easily. However, keeping this cannula in place in awake animals may be challenging.

In the experiments described in chapters V and VI, piglets where chronically stressed for three weeks by continuous and repeated exposure to multiple stressors. As touched upon in the individual discussions of these chapters, the limitations of these experiments are the following. First of all, only one sex, i.c. the female was analysed. The importance of sex will be addressed later in this general discussion. Another concern is that animals were artificially reared in brooders to control the experimental environment. The stress of early deprivation of their mother, transport to our facility, and changing their diet into milk replacer would, as previously mentioned, be perceived as stressful (e.g., [51]). Thus, the control group is not a stress-free group. Therefore, baseline values of the candidate biomarkers will probably be dissimilar in stress-free piglets. In addition, it could be that the stress of artificial rearing masked the potential differences between the control and stressed groups. On the other hand, a number of significant differences were observed regardless of this artificial rearing, indicating that the effect of this additional stressor was not masking all effects.

VII.3 Is saliva a suitable biological matrix to assess piglet's welfare?

The answer to whether chronic stress altered the salivary proteome is affirmative. A fold change difference of 20 proteins was observed between chronically stressed and control animals. Further analysis of eight proteins confirmed that six are potential biomarkers for chronic stress. However, before we can claim this, further validation is needed. Since numerous factors can introduce variation of specific protein concentrations, the reliability and comparability of these potential biomarkers can be affected. A non-exclusive list of possible influencing factors is discussed in this section (Table 1). Assessing how these factors will affect the salivary concentrations of discovered candidate biomarkers to identify physical and psychosocial stress described in Chapter VI and in literature is critical.

Table 1. Overview of salivary biomarkers for physical and psychosocial stress and their influencing factors. *If the box remains blank, to our knowledge, no information is available.*

Biomarker	Up- or down-regula-tion	Acute or chronic stress	Other conditions	Circadian rhythm	Storage information	Influence of collection device	Effect of gland distribution	Effect of Age	Effect of sex	Effect of breed	Effect of season	Effect of oestrus cycle
Cortisol	Up[24]	Acute and chronic[24]	Physical activity[33]	Yes[340, 342, 32]	3 months (5°C)[346]	Yes[337]		Yes[341]	Yes[341]	Yes[357]		
Alpha-2-HS-glycoprotein	Up[353]	Chronic[353]					No (when normalised for protein secretion rate)[297]	Yes[353]				
Chitinase	Down[353]	Chronic[353]	Asthma[325]				No (when normalised for protein secretion rate)[297]	Yes[353]				
Lipocalin-1	Down[353, 177]	Acute and chronic[353, 177]	Disease[175]				No[297]	Yes[353]	Yes[230]			Yes[180, 355]
Long palate lung and nasal epithelium protein 5	Down[353]	Chronic[353]						Yes[353]				

Biomarker	Up- or down-regula-tion	Acute or chronic stress	Other conditions	Circadian rhythm	Storage information	Influence of collection device	Effect of gland distribution	Effect of Age	Effect of sex	Effect of breed	Effect of season	Effect of oestrus cycle
Odorant-binding protein	Down[353]	Acute and chronic[177, 189,353]	Disease[175]				No (when normalised for protein secretion rate)[297]	Yes[353]	Yes[230]			
BLAST: Vomeromodulin -like protein	Down[353]	Chronic[353]						Yes[353]				
IgA	Up[183]	Acute[183]	Infection			Yes[335]				No[356]		
IgM	Down[177]	Acute[177]	Infection									
α-amylase	Up[181]	Acute[181]		No[341]	<4 days (4°C), <3 months (-20°C)[344]	Yes[336]	No[297]	Yes[341]	No[341]			
IL-18	Up[174]	Acute[174]				Yes[337]						
Chromogranin A	Up[299]	Acute and chronic[299]		No[277]	2 days (4°C), 1 month (-20°C), up to 7 freeze-thaw cycles[278,277]			Not found (17 vs. 21 weeks)[277]	No[277]		Yes[277]	
Serum amyloid A	Up[189]	Acute and chronic[189, 34]					No[297]					
Testosterone	Up[25]	Acute[25]		No[343]		Yes[335]						

Biomarker	Up- or down-regula-tion	Acute or chronic stress	Other conditions	Circadian rhythm	Storage information	Influence of collection device	Effect of gland distribution	Effect of Age	Effect of sex	Effect of breed	Effect of season	Effect of oestrus cycle
Albumin	Up[189]	Acute[189]	Infection and in-flammation [172,175]				No[297]					
Salivary lipocalin	Down[177]	Acute[177]					No (when normalised for protein secretion rate)[297]		Yes[230]			
Prolactin inducible protein	Down[177]	Acute[177]					No[297]					
Adenosine deaminase	Down[177]	Acute and chronic[177]	Lameness, rectal prolapse, fatigue, inflammat ion (Up) [175]	Yes[341]	4 days (4°C), 1 month (-20°C) [345]			Yes Yes[341]	Yes[230, 353]	Yes[354]		
Carbonic anhydrase IV	Up[177]	Acute and chronic? [177,178]	Snaring (inconsisten t); non-infectious growth rate retardation [171]				Yes (higher concentratio ns in parotid saliva)[297]	Not between day 14 and day 28 [353]				Yes[180, 355]

Biomarker	Up- or down-regulation	Acute or chronic stress	Other conditions	Circadian rhythm	Storage information	Influence of collection device	Effect of gland distribution	Effect of Age	Effect of sex	Effect of breed	Effect of season	Effect of oestrus cycle
Protein S100-A8, calgranulin A, calprotectin (heterodimer with S100-A9)	Up[177]	Acute[177]	Inflammation, immune-mediated diseases, and sepsis (up)[175]									
Protein S100-A9, calprotectin, calgranulin B	Down[177]	Acute[177]	Inflammation, immune-mediated diseases, and sepsis (up)[175]									
Protein S100-A12, calgranulin C	Down[177]	Acute[177]	Inflammation, immune-mediated diseases, and sepsis (up)[175]	Yes[341]			Yes (higher concentrations in parotid saliva, when normalised for protein secretion rate)[297]	Yes[341]				
Double headed protease inhibitor SMG	Up[177]	Acute[177]										

Biomarker	Up- or down-regulation	Acute or chronic stress	Other conditions	Circadian rhythm	Storage information	Influence of collection device	Effect of gland distribution	Effect of Age	Effect of sex	Effect of breed	Effect of season	Effect of oestrus cycle
Haemoglobin	Up[177]	Acute and chronic[177]	Lame animals[179]									
Total esterase activity	Up[178]	Acute and chronic[178]	Pain discomfort [30,31]		<1 day (4°C), <1 month (-20°C) [344]							
Butyryl-cholinesterase	Up[213]	Acute and chronic[213]	Pain discomfort (TEA)[30,31]		<1 day (4°C), <1 month (-20°C)[344]							
Lipase	Up[178]	Acute and chronic[178]	Pain discomfort (TEA)[30,31]		<1 day (4°C) [344,345]							
Oxytocin	Down[363]	Acute and chronic[363]										
Total protein concentration	Up[150]	Acute [150]		Yes[341]			Yes[297]	Yes[341]	No[230]			

VII.3.1 Factors that could introduce variation in salivary biomarker concentration

VII.3.1.1 *Sample collection*

VII.3.1.1.1 Collection device

Individual collection of whole saliva from pigs is usually performed with a collection device that absorbs saliva present in the oral cavity. Literature shows that the most frequently used device is the collection sponge named Salivette® (Sarstedt) (Fig. 2). This device, initially developed for humans, exists in a soft cotton version and a harder synthetic version. For pigs, the collection sponge is usually attached to a metal wire, clipped onto forceps or tied to the middle of a rope that fixes the sponge into the pig's mouth. After sufficient absorption of saliva, the collection pad is placed in its container and centrifuged to collect the absorbed saliva and to remove debris and buccal cells. The main downside of the cotton sponge is the material. Cotton tends to interfere with the analysis of hormone levels such as that of testosterone, progesterone, and oestradiol resulting in artificially high values. In contrast, concentrations of other compounds such as secretory IgA (sIgA), amylase, melatonin, and IL-8 will be artificially low in samples collected with cotton devices [335-337].

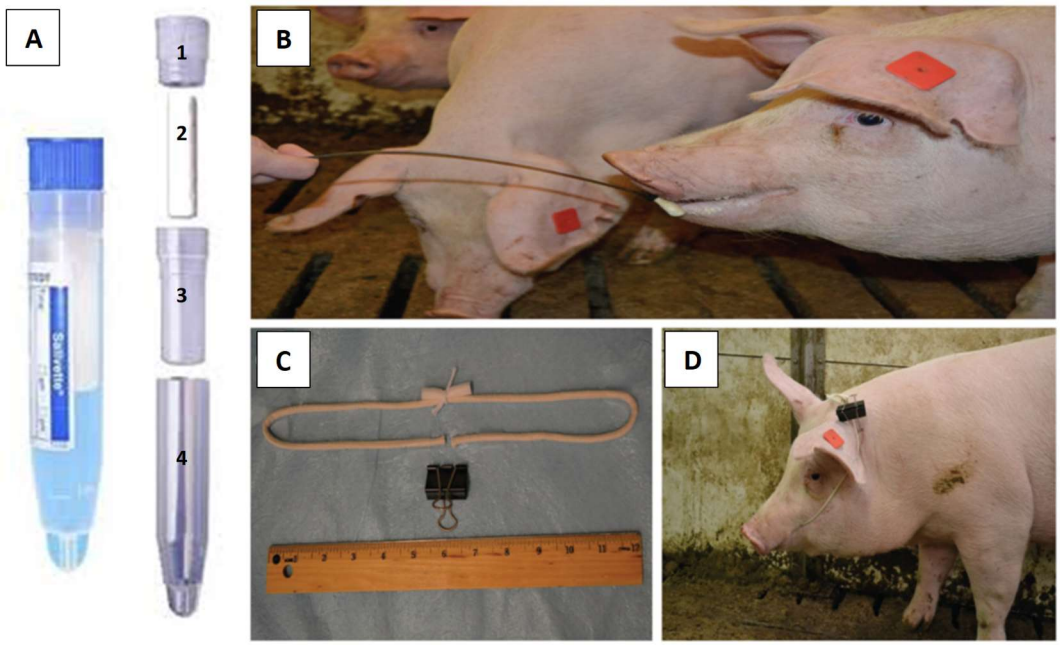


Figure 2. Saliva collection with a Salivette®. ***A.*** *Salivette collection device with 1. Stopper, 2. Absorbant pad, 3. Container with hole, 4. Centrifugation tube.* ***B.*** *Collection with the sponge clipped on a metal wire.* ***C*** *and* ***D.*** *Sponge attached to a rope that is fixated in the oral cavity. Modified from [155]*

The synthetic Salivette® is less prone to these side effects [337]. However, one of the major drawbacks is that the sponge is relatively hard. In our young animals, this caused bleeding of the gums, contaminating the saliva samples with blood compounds. This forced us to look for alternatives. The Super•SAL™ and RNAPro•SAL™ are also synthetic collection pads, but softer. These pads are mounted on a handle and are squeezed out after saliva collection (Fig. 3). The problem with these devices is that they have a low recovery percentage (Fig. 4). Since piglets naturally have low saliva volumes, further analysis is limited if the device retains a large part of this saliva. The Micro•SAL™ is a smaller device and ideal for saliva collection from subjects with low saliva volumes like, young children or small animals. Although, the device's material is thoroughly tested and shows no interference with a.o. cortisol [287], it should be further investigated whether the analysis of candidate biomarkers will be affected by these different collection devices.

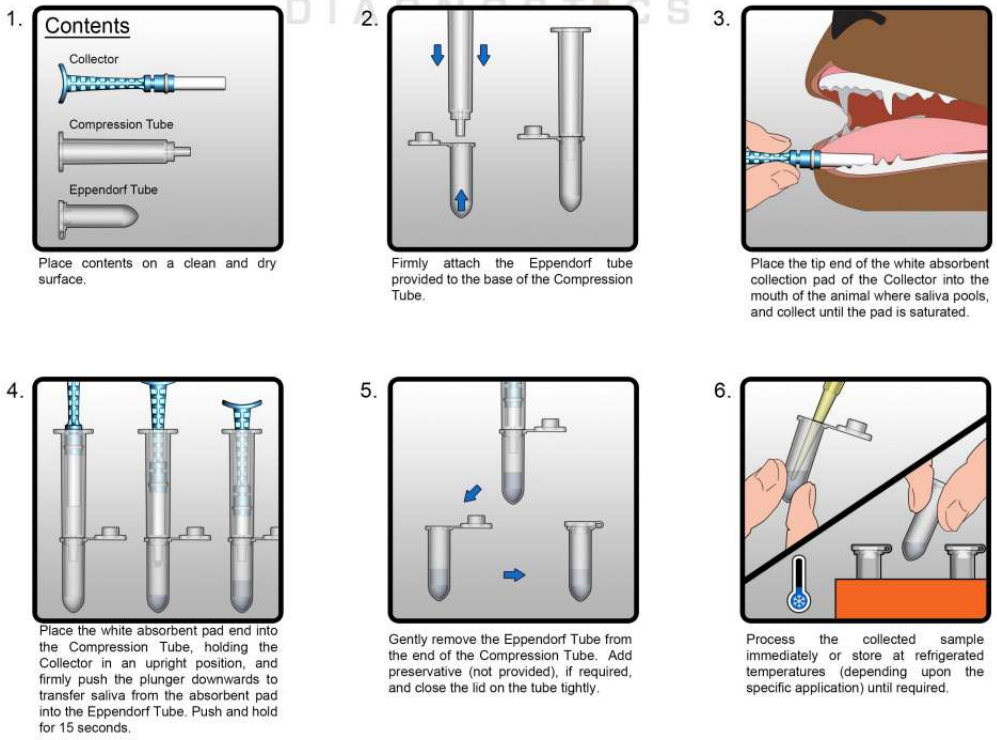


Figure 3. Saliva collection with the Micro•SAL™ for animals. *4saliva.com*

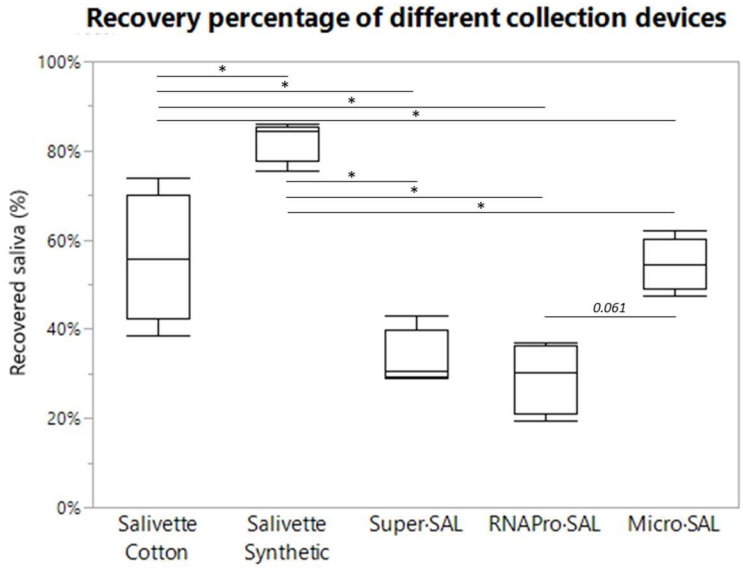


Figure 4. The saliva recovery percentage of five different collection sponges*. The recovery percentage of the synthetic Salivette® showed the highest recovery percentage (82.49 ± 4.80%), which differed significantly from all other values. However, the hard texture of this device made the piglet's gingiva bleed. The RNAPro●SAL™ had the lowest recovery percentage (29.18 ± 8.25%), which differed significantly from all other values except from those of the Super●SAL™ (Mean ± SD; n = 4; *p ≤ 0.05; Kruskal Wallis; non published personal data).*

VII.3.1.1.2 Chewing vs. non-chewing

In humans, saliva collection is most frequently performed without chewing, thus through passive drooling or by placing an absorbent sponge in the oral cavity without further manipulation. The above-mentioned sponges are initially not designed to be chewed on. For pigs, this is challenging. Chewing on objects that are in the oral cavity is a natural reflex, especially in young animals. This brings us back to Chapter IV, in which the importance of chewing was emphasised. To be precise, comparing data of stimulated (chewing on a device) vs. unstimulated (passive) saliva collection must be done cautiously since salivary stimulation changes the relative contribution of the major salivary glands to whole saliva in the oral cavity. This factor should be considered since we confirmed, as described in Chapter IV, that the concentration of several proteins differs between parotid saliva and mandibular/sublingual saliva. Therefore, proteins that are present in equal concentrations in both ductal secretions, such as lipocalin-1, α-amylase, serum amyloid A, albumin, and prolactin inducible protein could be preferred as biomarkers when whole saliva is sampled by means of sponges. In addition, alpha-2-HS-glycoprotein, chitinase, odorant-binding protein, and salivary lipocalin were also found in even concentrations in both ductal secretions when data were normalised to protein secretion rate. Carbonic anhydrase VI and proteins S100-A12 were found to be secreted with higher concentrations from the parotid gland and, therefore, their concentrations could be influenced by chewing. However, with this interpretation, we must remember that this data is the result of saliva that was collected under non-physical conditions.

The advantage of stimulated saliva is that it contains less mucins and is, therefore, less viscous, resulting in easier processing and management of the samples. As a downside, this stimulated saliva has a higher water percentage. As a consequence, the transudate proteins that derive from the bloodstream like, for example, protein S100-A8 and S100-A9 are diluted [262]. However, chewing may interfere with the salivary composition and therefore with the interpretation of the biomarkers that are used to monitor the direct activation of the sympathetic pathway, like α-amylase. Reflexive secretion due to chewing is a

mechanism that is independent of the central regulation of stress and, therefore, overrules the central effects of stress [150].

Lastly, sham chewing, a form of stereotypical behaviour that is characterised by chewing motions in the absence of ingesta, may interfere with the composition of saliva by making it watery. The identification of such sham chewing animals could therefore be valuable. Unfortunately, the correct identification is hampered by the low association between sham chewing and the presence of saliva foam around the mouth [338].

The impact of the factor chewing is significant and not receiving enough attention. It is of the utmost importance that saliva is collected in a standardised way to allow for correct comparison and interpretation. Preferably, biomarkers that are not or only minimally affected by this chewing-factor are chosen.

VII.3.1.1.3 Habituation

Another point of attention is that, although collecting saliva may appear relatively straightforward, bias due to sampling errors can be introduced. Healthy, thriving pigs are naturally curious, and they usually approach the investigator on their initiative. Consequently, saliva sampling can be performed without introducing stress. However, stressed animals can be lethargic or anxious and, therefore, reluctant to approach [3]. As a result, a sampling bias could be introduced if only spontaneously approaching animals are sampled at the farm. Approaching or chasing animals and grasping them for sampling purposes could affect the outcome. For example, a rise in salivary cortisol is already detectable soon after the exposure to the stressor is started, and remains high for at least 30 min (e.g., [339]). Additionally, multiple sampling in the same stable or herd or prolonged chasing of the animals could affect the concentrations of some salivary biomarkers. As a result, focussing on biomarkers which concentrations are not influenced by arousal and physical activity, or if they do, then only after a lag phase, is advised. Alternatively, animals could be trained and habituated to sampling, which is feasible in research settings. This habituation step was applied in the sampling strategy described in Chapters V and VI.

Piglets were trained once a day from arriving at our facility until the first sampling moment when they were 14 days old. Although several researchers were involved in the saliva sampling, the same piglets were consistently sampled by the same familiar researcher. However, even then, remaining calm and not forcing the animal during saliva sampling is key.

VII.3.1.1.4 Timepoint of collection during the day

Since a circadian rhythm influences some salivary components, the sampling moment should be planned with caution. It is well known that cortisol displays a circadian rhythm with a peak around noon [340]. Therefore, the correct reference values must be used. Salivary concentrations of some biomarkers for physical and psychosocial stress, like adenosine deaminase, protein S100-A12, and the total salivary protein concentration, all peak in the afternoon around 3 p.m. The best sampling moment was suggested to be between 10 a.m. and 12 a.m. [341]. However, some side information should be considered when working with biomarkers under the influence of a circadian rhythm. For example, it is known that the circadian rhythm for salivary cortisol is observable in female and male piglets as early as from the age of 6 and 10 days, respectively [342]. However, this rhythm only matures into a stable adult pattern around 20 weeks of age [32]. Therefore, the influence of the circadian rhythm may differ. Additionally, for cortisol, it is already known that decreased welfare leads to a blunted circadian rhythm [56]. Determining the optimal timepoint for sampling is sometimes problematic. Whether age and decreased welfare have the same impact on the circadian rhythm of other biomarkers needs to be evaluated.

At least in humans, the salivary flow rate is also subjected to this circadian rhythm. Both whole and parotid saliva have a similar rhythm but a different amplitude and acrophase. These differences result in an altered proportional contribution of parotid saliva to whole saliva throughout the day, with the most significant contribution of 32% at 11 a.m. and the lowest at midnight. Surprisingly, the human salivary flow rate does not show any circadian rhythm when the salivary flow is stimulated [278]. Whether this also holds for flow rates in pigs should be investigated.

To solve the problem of circadian rhythms on salivary biomarker concentrations, the ideal sampling moment could be determined, as suggested above. The impact of this circadian variation will be limited when the increase or decrease of the salivary concentrations in response to the affected welfare exceeds the maximum or minimum of the circadian variation throughout the day. The easiest solution is opting for biomarkers that do not present fluctuating salivary concentrations throughout the day, such as, α-amylase [341], chromogranin A [277] or testosterone [343]. An alternative is to take multiple samples over a longer period to adjust for this source of variation [81].

It might be clear that a potential circadian rhythm could affect the interpretation of the results. Therefore, it should be first determined whether the detected biomarkers described in Chapter VI display circadian fluctuations throughout the day. When this is the case, it should be assessed whether factors like age, sex, and welfare status affect these circadian salivary profiles.

VII.3.1.2 *Sample treatment and storage*

Once the saliva sample is collected, the handling and storage procedures should not affect the content of the sample. In general, it is recommended that salivary samples are processed as soon as possible. Protein degradation is a relatively fast process that starts in the mouth and continues through sampling and further processing. It should be prevented as much as possible. If immediate analysis is not possible, rapid storage on ice and subsequent freezing is advised (e.g., [299]). However, various salivary components may react differently to the same storage conditions.

For example, chromogranin A is stable at 4°C until 2 days in centrifuged porcine saliva [278]. At the same temperature, butyrylcholinesterase, lipase, and total esterase activity are stable less than 1 day, α-amylase less than 4 days, and adenosine deaminase for up to 4 days [344]. It has even been suggested that lipase and adenosine deaminase are stable at room temperature for 24 hours without preservatives [345]. No degradation of salivary

cortisol was found in human centrifuged saliva after 3 months at 5°C, while a decrease of nearly 10% per month was seen in samples stored at room temperature [346].

Besides fast storage on ice, the addition of a protease inhibitor or a reducing agent can be used to stabilise the saliva samples. Protease inhibitors prevent, a.o. the activity of degrading enzymes, while reducing agents prevent the formation of disulphide bonds that may cause misfolding, aggregation, or precipitation. However, these compounds may not prevent other sources of degradation. Possibly problematic is that these additives can interfere with further analysis, especially with LC-MS/MS. Polyols, such as sucrose, sorbitol, and glycerol, are alternative additives that increase protein stability or serve as cryoprotectants during freeze-drying. However, a 1:1 dilution is required, potentially causing detection level issues during later analysis [347].

Alternatively, alcohols such as ethanol can be added in denaturing and non-denaturing conditions for more protein stability at non-freezing temperatures. Although denaturing agents have the advantage of altering the physicochemical properties of the samples, resulting in the disruption of protein complexes and decreased viscosity, these compounds may interfere with accurate protein detection [347]. Often, samples are centrifuged, to reduce viscosity, and to remove buccal cells and bacteria that may interfere with sample stability. Unfortunately, this step could remove macromolecular aggregates or proteins bound to bacteria or mucus [256]. Similarly, adding acidic buffers can reduce the viscosity since it causes precipitation of mucins. However, this step may cause limitations similar to centrifugation due to the precipitation of proteins attached to mucins [256]. On the other hand, these acidic buffers will also precipitate α-amylases and carbonic anhydrases, which are proteins that are usually highly abundant and could prohibit identification/quantification of low abundant proteins [347]. A technique with a similar purpose is treating the samples with peptide ligand libraries that will reduce the risk of highly abundant proteins masking the presence of low abundance proteins during LC-MS analysis [279, 280].

Snap-freezing samples in liquid nitrogen is preferred over slow-freezing since the latter will induce aggregation and precipitation [347]. Samples can usually be stored for a prolonged period at -20°C or -80°C. For chromogranin A and adenosine deaminase, for example, no reduction in concentration after 1 year at both temperatures has been observed [277, 344]. For research purposes, prolonged storage at -80°C may be valuable, but this is less relevant for clinical purposes. However, some biomarkers for physical/psychosocial stress are stable less at -20°C than chromogranin A. For example, butyrylcholinesterase and total esterase activity are less than 1 month stable, α-amylase and lipase less than 3 months [344]. For longer storage it is advised to add glycerol (20-80%) and snap-freeze shortly after collection. Unfortunately, the requirement of liquid nitrogen on the sampling site makes this method untranslatable to farm settings [347].

Another factor that could influence the salivary compound concentrations is the number freeze-thaw cycles. While chromogranin A appears robust for up to seven freeze-thaw cycles [277] not all proteins appear to tolerate these repeated freeze-thaw cycles as good [222]. Aliquoting samples could be a solution to overcome this potential problem.

It might be clear that further research needs to be conducted on how the proposed candidate biomarkers in this study are affected by storage at room temperature, 4°C, or at -20°C and how the suggested stabilizing protocols can prevent possible degradation.

VII.3.1.3 *Saliva analysis*

There are different ways to analyse salivary biomarker concentrations. The most frequently used techniques probably include antibody-based techniques like ELISA's, TR-IFMA, and alphaLISA®'s (e.g., [72, 183, 348]), enzymatic assays for enzymes like α-amylase and adenosine deaminase (e.g., [349, 350]) and proteomic approaches, like HPLC, gel-based or liquid-based separation followed by MS/MS (e.g., [175]). Although all these techniques have the same purpose, namely determining the amount of a molecule, some variation in outcome between the different techniques is possible. For example, when cortisol was measured in the same samples with a competitive chemiluminescence enzyme

immunoassay and an indirect competitive alphaLISA assay, validated for porcine saliva, cortisol concentrations were 1.5 times higher when determined with the alphaLISA assay. Additionally, this latter technique has proven to be more sensitive to contamination with faecal matter than the chemiluminescence assay, resulting in artificially high detected cortisol values [351]. More details regarding the effect of contaminants are described further on.

Similar observations are found for the analysis of α-amylase in human saliva. The concentrations of this protein were determined either enzymatically or directly with a non-competitive indirect sandwich assay. Concentrations were consistently lower when the enzymatic activity was measured and displayed higher inter-individual variability. The correlation between both assays varied depending on the applied acute stressor. It was assumed that different isoforms of the enzyme were detected with the direct-protein assay and not with the enzymatic one. Since the difference between the control and the stressed group was more significant when the concentrations were measured with the enzymatic assay, this assay was suggested to be more sensitive for detecting changes induced by acute stress [352].

Although the above-mentioned techniques determine the concentrations in g/mL or units/mL, most MS-techniques are reported in concentrations or proportions to the total amount of proteins. Corrections for the total protein concentration in the sample should be made to allow comparison. More data regarding this normalisation can be found in the next section.

For MS techniques, some hurdles were experienced during the experiments. These primarily found their origins in the lack of a well-characterised porcine protein database. When protein identification was performed for the analysis described in Chapter IV (2018/01/10), only 50,045 (reviewed + unreviewed) entries were found in the Sus scrofa database accessible through UniProt. When protein identification was performed for biomarker detection, as described in Chapter VI (2020/03/30), 120,806 (reviewed +

unreviewed) entries were already accessible. At the end of 2023, a total of 398,092 entries were available at UniProt. Although this number has increased tremendously, from these nearly 400,000 entries, only 3,590 proteins are reviewed. These reviewed proteins are manually annotated, combing experimental results, computed features, and scientific conclusions. The unreviewed proteins are based on genome projects and are computational records with automatic annotation. Information on the latter unreviewed proteins is frequently updated and changed. The incomplete database resulted in issues during the PRM analysis. This quantification technique relies on quantifying protein-specific peptides (e.g., [197]). The described databases can be used to search for unique peptides for the target protein. However, if this database is incomplete and not fully annotated, this search becomes more challenging. We therefore noticed that it is advisable to analyse at least three but preferably more protein-specific peptides and compare their profiles. In some cases, the abundancy profile of a protein-specific peptide deviated from that of the other protein-specific peptides, suggesting that this peptide probably was not unique to the target protein.

VII.3.1.4 *Result normalisation*

When varying factors could influence the sample, normalisation is often performed. The discussion on what factor to use for normalisation is still under debate. An often-used method is normalising with a housekeeping protein, but as discussed in Chapter VI, some authors suggest amylase, mucins, albumin, or IgA. However, the abundance of these proteins, except for mucins, is altered by acute stress [181, 183, 189]. Other potential proteins that can be used for normalisation are apomucin or sulfhydryl oxidase. Variation for these proteins was limited in our studied animals, suggesting that they could be useful proteins for normalisation or for identifying a large difference in the background proteome. Unfortunately, chewing affects apomucin concentrations since this protein is more abundant in submandibular and sublingual secretions than in parotid saliva. This is not the case for sulfhydryl oxidase, which was found in equal concentrations in the studied ductal

secretion. Nevertheless, how stable this concentration is between stimulated and unstimulated saliva should be further explored.

Another often-used method is normalising for protein secretion rate, as was done for the gland-specific saliva in Chapter IV (e.g., [352]). This implies that the protein output per unit of time must be measured. Unfortunately, this factor cannot be determined in pigs since an absorbent collection device, such as sponges, needs to be used, but such sponges have a ceiling/saturation limit [150].

In addition, the total protein concentration could be used for normalisation. However, as mentioned before, stress will increase this total salivary protein concentration (e.g., [150]), although chewing may interfere with this factor. Food, dirt and faecal material contamination may also affect this parameter [351].

An alternative method that could be used to reduce the variation arising from different sample processing, different salivary flow rates or sample contamination (e.g., with blood, food, or dirt) is, combining the data, instead of looking at the proteins separately. For example, all six proteins of which PRM confirmed the profile differences as described in Chapter VI. By dividing the abundance of the upregulated protein by the average of the abundance of all the proteins that had a significant downregulation per animal, one value or ratio for each animal can be obtained (Fig. 5). This step reduces inaccuracies since one value is now generated, deriving from proteins within the same sample. Looking at the results described in Chapter VI, at the age of 14 days, the stressed groups' ratios of salivary proteins are 1.6 times higher compared to the ratios of the control group. However, at the age of 28 days, these ratios are 3.2 times higher in the saliva of the stressed group (note that, at 28 days, individual biomarker values are only between 1.5 and 2.3 times higher). Statistical analysis revealed that the interaction between age and condition was significant. Post-hoc tests indicated that the ratios were significantly different between the control and chronically stressed group, both on day 14 (*P*-value = 0.042) and day 28 (*P*-value = 0.001). Age affected for these values of the control group (*P*-value = 0.021) but remained the same

for the stressed group (*P*-value = 0.210). Interestingly, measuring the ratio between up- and downregulated biomarkers cancels the influence of variations from sample preparation, contamination, or flow rate since all individual values come from proteins within the same sample. Noticeable, the overlap of the ratios of all validated proteins between both experimental groups becomes smaller on day 28 compared to the data of the individual proteins.

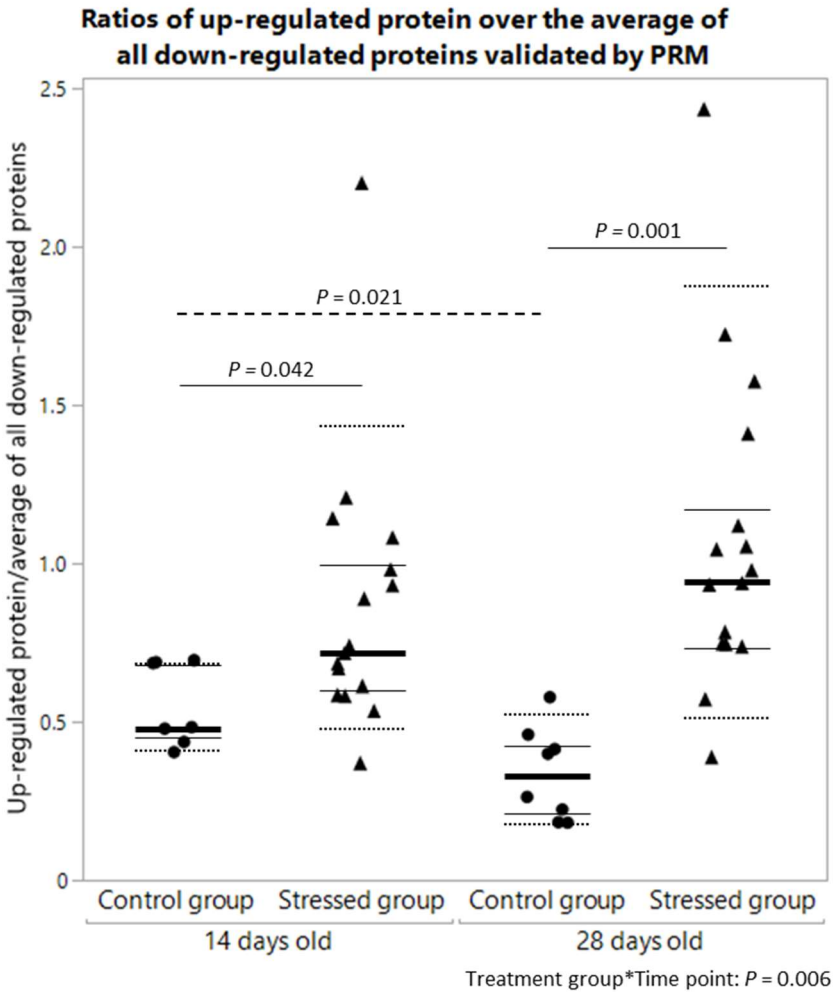


Figure 5. The ratios per animal between up- and downregulated proteins that were validated by PRM. *Statistically significant differences between samples taken at 14 days and samples from day 28 are highlighted by dashed lines. Significant differences between the control group (circles) (n = 8) and the stressed group (triangles) (n = 16) are indicated with a full line. The significant interaction term is placed beneath the graph. For each group,*

the median (thick line), the 25th and 75th percentiles (thin lines), and the 5th and 95th percentiles (dotted lines) are shown.

Note that the identification of up- and downregulated proteins in this study allows for the calculation of such ratios. Apart from being independent of protein concentration, the determination of ratios has two additional advantages, as mentioned above. Firstly, calculating ratios enlarges the differences between the control and stressed animals, making interpretation clearer (Table 2). Secondly, they are based on the values of at least two biomarkers and thus enhance the reliability of the outcome. Considering the development of a simple assay, these advantages could be substantial. The calculation presented above is performed using the average of all downregulated proteins, but ratios can also be calculated using one or two downregulated proteins. In such a case, the highest difference between protein ratios on day 28 of the stressed animals compared to the control animals is observed when the abundance of alpha-2-HS-glycoprotein is divided by the average of long palate lung and nasal epithelium protein 5 and BLAST: vomeromodulin-like protein (fold change difference: 3.85). This is followed by alpha-2-HS-glycoprotein divided by the downregulated proteins individually (fold change difference: 3.72 and 3.83, respectively). Odorant-binding protein is next, either individually divided by alpha-2-HS-glycoprotein (fold change difference: 3.61) or the combination with either long palate lung and nasal epithelium protein 5 or BLAST: vomeromodulin-like protein (fold change difference: 3.51 and 3.58 respectively) or alpha-2-HS-glycoprotein divided by the average of all three above mentioned downregulated proteins (fold change difference: 3.59).

Table 2. Example of a case in which the sample volume of the stressed group is 2x that of the control group.

Normal, undiluted, volumes

	Upregulated protein	**Downregulated protein**	**Downregulated protein/ upregulated protein**
Control Stressed	1 2.5	10 5	10 2
Stressed/control	= 2.5/1 = 2.5x difference	= 5/10 = 0.5x difference	= 10/2 = 5

Volume sample stressed group x2

	Upregulated protein	**Downregulated protein**	**Downregulated protein/ upregulated protein**
Control Stressed	1 5	10 10	10 2
Stressed/control	= 5/1 = 5x difference (2-fold difference to undiluted samples)	= 10/10 = 1 No difference (2-fold difference to undiluted samples)	= 10/2 = 5 (same result as undiluted samples)

VII.3.1.5 *Caution with interpretation and comparison of salivary biomarker concentrations*

Besides the above-mentioned interfering factors like circadian rhythms and type of collection, other factors can cause variations as well. However, unlike the previously mentioned factors that can be controlled during the sampling protocols, it is more challenging to restrict the influence of the factors that are described below. Each of these factors, a.o. age, sex, breed, season, oestrus cycle, contamination, or stress could affect the concentration of a specific salivary biomarker for physical/psychosocial stress and can influence the result interpretation substantially. Therefore, reference intervals should be made with these influencing factors in mind.

VII.3.1.5.1 Effect of age

The effect of different ages on the salivary composition of porcine saliva has also been studied to some extend and described in Chapter VI. The salivary concentrations of alpha-2-HS-glycoprotein, chitinase, lipocalin-1, long palate lung and nasal epithelium protein 5, odorant-binding protein, and vomeromodulin-like protein all increased from day 14 to day 28. The concentration of carbonic anhydrase and parotid secretory protein remained the same in both age groups [353]. In literature, differences in the concentrations of protein S100-A12, α-amylase, cortisol, and adenosine deaminase are described in salivary samples of post-weaning pigs, growing pigs, and finishing pigs. However, no pattern could be observed. For example, salivary adenosine deaminase concentrations continue to rise with age, while α-amylase was higher in post-weaning and growing pigs, and decreased toward the final age group [341]. For some biomarkers, like chromogranin A, no difference has been found between age groups (17 vs. 21 weeks old) [277].

The total protein concentration was the highest in the growing pigs [341], a parameter that is especially relevant when comparing concentrations (µg protein/mL saliva), usually the outcome of enzymatic activity assays or ELISA's, with the outcome of mass spectrometry that is usually expressed in µg protein/total µg salivary proteins. When the total protein concentration increases but the volume stays the same, this will not affect the concentration of a specific protein (µg protein/mL saliva). However, it could reduce the outcome of the mass spectrometry analysis. Therefore, monitoring and/or correcting for the total protein concentration in each sample is crucial.

VII.3.1.5.2 Effect of sex

Sex influences the salivary composition and, therefore, also the concentrations of salivary biomarkers for physical and psychosocial stress. This parameter often interacts with age and breed resulting in different subcategories (e.g., [230, 341, 349, 354]).

No effect of sex was detected for chromogranin A, protein S100-A12, α-amylase and the total salivary protein concentration [230, 277, 341]. This contrasts with adenosine

deaminase, lipocalin-1, odorant-binding protein, and salivary lipocalin, that presented the highest concentrations in female finishing pigs [230]. For some biomarkers, there was an interaction between age and sex, resulting in, for example, higher values of salivary cortisol in female growing pigs but not in post-weaning pigs, compared to age-matched intact male pigs [341]. For adenosine deaminase, the interaction of sex, age and breed results in even more subcategories [354]. A hormonal sexual influence is highly hypothesized. Therefore, the effect of castration or immunocastration on saliva profiles should be further investigated. On the other hand, the effect of the oestrus cycle should not be underestimated as well. Recent studies have proven that the composition of saliva differs between proestrus, oestrus, metestrus, and dioestrus. Proteins with varying expression profiles are lipocalin-1 and carbonic anhydrase [180, 355].

VII.3.1.5.3 Effect of breed

As mentioned before, breed often interacts with age and sex. For these parameters significantly different salivary concentrations of adenosine deaminase were detected for Large White x Duroc pigs and Iberian pigs. Nevertheless, the researchers pointed out that male Iberian pigs were castrated while the Large White x Duroc pigs remained intact, rendering a one-on-one comparison impossible. However, the total adenosine deaminase levels were found to be twice as high in the Iberian pigs [354]. Large White sows had higher salivary cortisol levels than Damin sows, while no difference was found in salivary IgA levels between both breeds [356]. The impact of many different breeds on a.o. plasma cortisol concentrations and other immune-related parameters has been studied before (e.g., [112, 357]) indicating that the effect of this variable on saliva profiles should be studied in more detail.

VII.3.1.5.4 Effect of season

Season may also affect the salivary composition, especially with the concept of heat and cold stress in mind. However, the milder seasons, spring and autumn, also affect the levels of, for example, chromogranin A, with higher levels observed in autumn [277]. The impact of this factor on the salivary composition is highly understudied.

VII.3.1.5.5 Effect of contamination

The effect of contamination on the measured biomarker concentrations was discussed briefly in Chapter VI. Chewing, biting and oral health problems can cause small wounds in the piglet's oral cavity through which blood can leak during saliva collection. Some candidate biomarkers, like alpha-2-HS-glycoprotein, cortisol, and α-amylase, are present in low concentrations in saliva but in much higher concentrations in blood (e.g., [33, 304]). Consequently, even small amounts of blood contamination can lead to artificially high levels of these components in saliva [305]. Additionally, haemoglobin, present in high concentrations in blood, can interfere with the determination of salivary testosterone and oxidative stress marker concentrations [358, 359]. Some of the pitfalls of blood contamination may be clear, but taking these into account requires that the concentration of blood is detectable in the salivary sample. Unfortunately, such detection method is currently not available. Visual inspection of saliva samples for discoloration due to blood is probably the most frequently used method since a volume contamination of 0.1-0.2% blood already leads to a tinted sample. Dipstick tests used to detect haemoglobin in urine have been suggested too. However, peroxidase present in saliva also catalyses the involved reaction, resulting in false positive results (e.g., [303, 360]). Haemoglobin and albumin have been suggested as blood contamination markers since the presence of these proteins is much higher in blood compared to saliva. Still, the determination of these proteins presents some sensitivity and reproducibility issues, and other factors also influence these concentrations [303]. Additionally, these proteins have been suggested as biomarkers of acute stress [177, 189], and were present in higher concentrations in the salivary samples of the chronically stressed animals described in Chapter VI. The upregulation that is due to stress hinders their possible use as biomarkers for blood contamination in the context of stress research. Serotransferrin is another protein present in higher concentrations in blood than in saliva. Although it is known that several factors, such as age, gonadal hormones, salivary flow rate and chewing affect serotransferrin levels in saliva [306], it is suggested as the best indicator for blood contamination [303], complemented with visual inspection.

Contamination by food, dirt or faecal material could occur, especially in farm settings. One drawback is that these contaminants may increase the total amount of proteins. This poses a problem when the amount of protein is determined, irrespective of the saliva volume but as a proportion of the sample's protein concentration, as with mass spectrometry. On the other hand, food particles may interfere with the composition of saliva. For example, α-amylase, a digestive enzyme responsible for starch cleavage, may be influenced by the ingestion of carbohydrate-rich food. Indeed, adding commercial food to porcine saliva samples reduced salivary α-amylase concentrations, with higher levels of contamination resulting in a stronger reduction. Higher food contamination levels increased cortisol, oxytocin, total protein concentration, and total esterase activities. Adenosine deaminase appeared to be unaffected. Other biomarkers associated with disease and oxidative stress were affected as well [351]. A study on horses' saliva determined that food contaminants such as oats, grass, and hay affect salivary components differently. Biomarkers suggested for physical and psychosocial stress in pigs, such as α-amylase, adenosine deaminase, and total esterase, appeared to be influenced by several types of food contamination [361]. Whether different feeds affect the salivary composition in pigs differently should be further explored. Special attention should be paid to the effect of milk since this has not been studied before.

The effect of faecal contamination on the determination of several salivary components has only been studied to some extent. Cortisol concentrations appeared to be increased in a dose-dependent matter. However, this was only the case when the analysis was performed with an alphaLISA and not when a chemiluminescence assay was used, once again stressing the variation that can be induced by several analytical methods. Oxytocin, total esterase activity, and the total protein concentration increased, α-amylase concentration was reduced, and adenosine deaminase remained constant [351].

Additionally, discoloration of the sample may also impact spectrophotometric methods [351, 361, 362]. Some researchers report that sampling was postponed if animals ate or drank at the scheduled sampling time in an attempt to obtain clean samples (e.g., [348]).

The idea of oral rinsing, also used in the sampling protocol described in Chapter VI, has been suggested and appears beneficial (e.g., [361]). An attempt to purify the sample via centrifugation, filtration or chemical clarification with chitosan can be performed. Of these three methods, centrifugation appears to be the best option [351]. The downside of this technique is that it could remove macromolecular aggregates or proteins that are bound to bacteria or mucus [256].

It may be clear that contamination can impact the outcome in a variety of ways and this source of variation should be further explored. Anyhow, attempting to collect clean samples and visual inspection with discarding of tinted samples is already a good start.

VII.3.2 What would be the ideal combination of salivary biomarkers to identify chronic physical/psychosocial stress?

During the last two decades, more insight has been gained into the composition of porcine saliva. In addition, the effect that different conditions, such as acute and chronic physical and psychosocial stress, different diseases, and inflammatory processes may have on it, has been elaborated. Moreover, many porcine salivary biomarkers for these conditions have been detected. However, as extensively pointed out in this discussion, many factors can influence the concentrations of these biomarkers. Therefore, more research is needed to identify the extent of these varying factors. Reference intervals should be set for every different combination of factors.

It should be noticed that more validation is needed to deduce a set of salivary biomarkers that can detect welfare problems or chronic stress. Since both are multifactorial, multiple aspects of the individual's response to these conditions should be monitored. The advantage of saliva as a biological matrix is that it can be used as an indicator of both the biological response to and the consequence of these circumstances. As regards the biological response, the most often used biomarkers for stress are the products of direct activation of the SAM and HPA axis, i.c. α-amylase and chromogranin A on the one hand, and cortisol on the other. Although their utility has drawbacks, these should be considered

for the salivary biomarker panel to identify welfare problems. These so-called activation biomarkers need to be complemented with consequence indicators. In this respect, odorant-binding protein (although also affected by disease), testosterone and vomeromodulin-like protein should be further explored since a decrease in their concentration reflects a reduction in reproductive capacity. Besides this effect of stress, it has been proven that it also has immunosuppressive consequences which can be detected in saliva by means of biomarkers. More specifically, reduced concentrations of immune-related markers could be a sign of chronic psychosocial and physical stress. Lower concentrations of chitinase, lipocalin-1 (although also affected by disease [175]), long palate lung and nasal epithelium protein 5, but also salivary lipocalin (although also affected by disease [175]), adenosine deaminase, protein S100-A9 and S100-A12 were found in lower concentrations in the saliva of stressed pigs [177]. However, lower immunity may result in higher infection rates and more diseases leading to an altered immune response. Additionally, while disease is often a consequence of stress it will also cause reduced welfare. Although not discussed in this thesis, biomarkers associated with infection and disease should be included in the assay. For example, most disease conditions correlate with elevated C-reactive protein (e.g., [220]). Although its saliva concentrations are influenced by a circadian rhythm, age, sex, and breed, this biomarker could be used as an indicator for a diseased state (e.g. [230, 354]). Noteworthy is that adenosine deaminase, protein S100-A9, and S100-A12 were described to be downregulated by acute stress but upregulated by disease and inflammation, suggesting that these proteins could have multiple purposes [175].

A final addition to the biomarker panel could be oxytocin. A reduction of this salivary biomarker could indicate a reduced welfare whereas increased values could suggest the opposite, i.e. positive welfare [363]. Initially, the idea of monitoring welfare, was to identify and prevent suffering. Recent advances in the field of ethology resulted in a broader view of this welfare concept and have emphasised the importance of positive experiences. This relatively new concept of positive welfare is still not well defined, but adding positive welfare biomarkers to the saliva biomarker panel will only be of added value.

VII.3.3 How to analyse salivary biomarkers?

The advantage of saliva as a biological matrix is that it usually does not need further processing. If a collection technique is used that does not require centrifugation, like the Micro·SAL salivary collection device that was used in the present doctoral research, saliva can either be analysed immediately or be stabilised for transport to clinical laboratories by means of preservatives. In the laboratory, analysis can be performed with ELISA's, TR-IFMA, or enzymatic assays, as mentioned before. However, finding and validating or developing assays that would enable the quantification of the discovered biomarkers described in Chapter VI is key. Recently, ELISA kits for porcine serum/plasma alpha-2-HS-glycoprotein, chitinase and lipocalin-1 have been marketed but are not yet validated for porcine saliva. Unfortunately, assays for long palate lung and nasal epithelium protein 5, odorant-binding protein and vomeromodulin-like protein are not commercially available.

As alternatives for lab-dependent analytical techniques, several options for on-site saliva analysis are possible. The most well-known portable sample analysis tool is the lateral flow test that is usually used to detect the presence or absence of a specific biomarker or pathogen, like in a urine pregnancy (hCG) or a COVID-19 (coronavirus) test, respectively. Although the described tests are qualitative in nature (positive or negative result), the lateral flow test has been modified now to generate a quantitative result. Usually, these quantitative tests require cartridges and specialised reading tools, or a smartphone with a calibrated camera (reviewed by [364]). One of the downsides of this technique is that only one biomarker, for example cortisol, can be quantified at a time.

Multiplexed immunoassays, or lab-on-a-chip, that detect several biomarkers at once could solve this problem. Such multiplexing technique is combined with a portable point-of-service device capable of rapid, sensitive, automated, and multiple biomarker detection that uses human saliva [365, 366].

The drawback of these one-site analytical techniques is the cost. Especially for welfare controls for welfare labels, this may pose an issue. Although 82% of the respondents of a

European survey declared that the welfare of farm animals should be better protected and that higher transparency regarding housing and living condition is needed, the willingness to pay for better housing conditions and welfare monitoring remains an obstacle. Surprisingly, willingness to pay for welfare-friendly animal-derived food products was the lowest for pigs followed by, fish, broilers, laying hens, dairy cows, and beef cows [367].

VII.3.4 What would be the ideal combination of parameters to assess welfare?

The added value of focussing on a panel of salivary biomarkers instead of just one should be clear by now. Yet, saliva should probably not be analysed exclusively, although the potential of saliva as a biological matrix to assess porcine welfare is high. Stress is a complex condition that requires a broad panel of parameters to determine it. The Welfare Quality protocol[6] was rolled out in 2009 to assess welfare at the farm. This protocol mainly focusses on causal and some consequence indicators such as mortality and weight gain, behaviour assessment, identifying stereotypies and (signs of) wounds/lesions or disease like coughing and sneezing. Some biological response indicators, for instance breathing patterns, are included in the protocol too. However, no other physiological parameters are assessed. The existing protocol could therefore greatly benefit from the addition of physiological assessment(s). From the biological matrixes suggested in the introduction, blood and saliva are the most versatile samples and could provide the most information. Although factors like sex, breed, season, etc., also have their implications in the reference values of blood biomarkers, the introduction of variation due to sampling and contamination is negligible for this matrix. It is the invasive nature of blood sampling that remains its most decisive disadvantage. And that is where saliva comes in the picture. If the issues with variation and contamination can be overcome or when biomarkers are selected that are less sensitive to variation, saliva is the go-to matrix for physiological assessments in welfare research. Since

[6] See: Welfare Quality® Assessment protocol for pigs http://www.welfarequalitynetwork.net/media/1018/pig_protocol.pdf (Accessed 15 December 2023).

urine, faeces, and hair accumulate biomarkers over a certain period of time, they are less sensitive to minor variations such as circadian rhythms. Of these three biological matrices, hair is the preferred matrix since it is less prone to microbial degradation and more straightforward to sample. However, the major drawback of this matrix is that the variety of analytes that can be measured in hair remains limited.

For research purposes, it is not always possible to determine a whole battery of parameters, especially behaviour assessment is a specialised discipline. Therefore, determining the presence of chronic stress could focus more on physiological assessments. The combination of salivary and hair analysis is, together with animal performance, a robust set of parameters to assess chronic stress in pigs.

VII.4 General conclusion

The research on welfare assessment tools for pigs knows a rich tradition, and yet no golden standard exists today. Many different indicators for chronic stress have their benefits but also their drawbacks. Although this also holds for saliva, this biological fluid remains a suitable matrix for assessing pigs' welfare. Six potential salivary biomarkers were detected in the standardised, experimental setting of this doctoral research in which piglets were chronically exposed to multiple stressors. These biomarkers can be regarded as consequence indicators. In the case of stress, they are related to a reduced HPG axis response (odorant-binding protein and vomeromodulin-like protein), an immunosuppressive status (chitinase, lipocalin-1, and long palate lung and nasal epithelium protein) and a heightened proinflammatory response (alpha-2-HS-glycoprotein) in the case of stress. Consequently, it is suggest that a panel of different biomarkers, which reflect different affected pathways, is valuable to detect stress. Moreover, if the ratios of the obtained values are considered, the strength of the salivary test increases. Unsurprisingly, further validation is needed, and more readily available analytical techniques should be developed.

At last, it should be emphasised that the target group of this PhD thesis was young piglets. The first weeks of a pig's life are intense and critical, and the piglets are highly vulnerable. It is concluded that saliva and hair are suitable matrixes to identify chronic stress in this understudied age group.

Summary

In pig farming, identifying potential stress in the animals at the farm has multiple advantages. It is known that stress, especially long-term chronic stress, will lead to a suppressed immune system, reduced zootechnical performances and disturbed breeding performances implying economical losses for the farmer. Therefore, a fast, easy, reliable, and objective tool to monitor stress can provide beneficial information for the farmer. Additionally, this tool can also help to evaluate the effect of different management strategies and interventions in agricultural research. Thirdly, easy tools to assess stress in pigs can be valuable to evaluate welfare on the farm, promote good practices and elicit transparency of the production process to the consumer. Already numerous methods to assess a pig's welfare exist, unfortunately, all with their own limitations. As a result, no definitive set of parameters or indicators of animal welfare exists. Therefore, the goal of this research project was to explore porcine saliva, more specific the salivary proteome, as a tool to identify chronic stress in piglets. Collection of saliva can be conducted fast and non-invasively. Since this analysis relies on physiological responses and adaptions to stress, it will be an objective tool to assess chronic stress in piglets.

Since knowledge of the protein composition, the proteome, of porcine saliva was scarce, the first goal of this thesis was to study and expand the knowledge about the porcine salivary proteome (**Chapter IV**). Considering that whole saliva, i.e., saliva that is secreted in the oral cavity is mixed with gingival crevicular fluid, buccal cells, microorganisms, and food remnants, salivary gland-specific saliva was collected as ductal secretion. Shotgun proteomics was performed on both mandibular/sublingual and parotid saliva to gain more insight into the proteome profile of porcine saliva. A total of 122 porcine salivary proteins and six mammalian salivary proteins with a predicted porcine homolog were identified in gland-specific saliva that was collected from anaesthetised piglets. A quantitative difference was observed between both ductal secretions. Twenty-four proteins were predominantly secreted by the parotid gland, while 29 proteins were predominantly

secreted by the mandibular and sublingual glands. Once more insight into the porcine salivary proteome was gathered, saliva could be exploited as a tool to monitor chronic stress. According to literature, some factors, like deprivation of cage enrichment, frequent mixing of animals and overcrowding could introduce chronic stress in pigs. However, to verify whether these factors could effectively be considered stressors, cortisol was investigated first, more specific, in hair and saliva, since increased secretion of this hormone is directly activated by stress (**Chapter V**). Three weeks of exposure to multiple stressors led to substantially less weight gain compared to control animals. Additionally, hair from the stressed group contained significantly higher cortisol concentrations, whereas salivary cortisol concentrations did not significantly differ between groups. Weight gain and hair cortisol concentrations were significantly correlated, but neither of these parameters were correlated with salivary cortisol concentrations.

Once the effectiveness of the stressors was confirmed, the salivary proteome of chronically stressed piglets was compared with that of control piglets (**Chapter VI**). Shotgun analysis identified 392 proteins in saliva of 28-day-old piglets. The relative abundance of 20 proteins was affected by three weeks of exposure to multiple stressors. From these 20 proteins, eight were selected for further validation with targeted Parallel Reaction Monitoring (PRM). For this validation, saliva samples that were taken both one week and three weeks after the start of the experiment were analysed to verify the profile over time. This PRM analysis confirmed that alpha-2-HS-glycoprotein was upregulated in the stressed group after one and three weeks, while odorant-binding protein, chitinase, long palate lung and nasal epithelium protein 5, lipocalin-1, and vomeromodulin-like protein were present in lower concentrations in the saliva of the stressed pigs, albeit only after three weeks.

It can be concluded that besides expanded knowledge on the porcine salivary proteome, differences in the salivary profile of chronically stressed young pigs could by detected. After further validation, the affected proteins could be used as salivary biomarkers to identify stress problems at the farm, elicit transparency regarding animal welfare to the consumer, and facilitate research to optimise rearing conditions.

Samenvatting

Voor de varkenshouderij heeft het verschillende voordelen om op de boerderij mogelijke stress bij de dieren te identificeren. Het is namelijk geweten dat stress, en in het bijzonder chronische stress, kan leiden tot de onderdrukking van het immuunsysteem, gereduceerde zoötechnische prestaties zoals verminderde dagelijkse groei, en verminderde fokprestaties met economische verliezen voor de varkenshouder als gevolg. Vandaar dat een snel, gemakkelijk, betrouwbaar en objectief hulpmiddel om stress te monitoren de landbouwer nuttige informatie kan opleveren. Een bijkomend voordeel is dat dit hulpmiddel agrarisch onderzoek zou kunnen vooruithelpen, meer specifiek door het evalueren van verschillende opfokstrategieën en de effecten van interventies na te gaan. Bijkomend zou een eenvoudige methode om stress bij varkens in kaart te brengen kunnen helpen bij het beoordelen van de welzijnsstatus op de landbouwbedrijven, bij het promoten van dierenwelzijn en bij het transparant maken van het productieproces naar de consument toe. Er bestaan reeds verschillende methodes om het welzijn van varkens te evalueren, maar helaas heeft elke techniek zijn limitaties. Hierdoor is er nog steeds geen definitieve set van parameters of indicatoren beschikbaar om het welzijn van varkens te beoordelen. Vandaar dat het doel van dit doctoraatsonderzoek was om de mogelijkheden van varkensspeeksel als medium om chronische stress bij biggen in kaart te brengen na te gaan. Speeksel kan relatief snel en op een niet invasieve manier gecollecteerd worden. Aangezien een speekselanalyse gebaseerd is op de fysiologische respons op en de adaptatie aan stress zal deze test op een objectieve manier in staat zijn om chronische stress bij biggen te identificeren.

Omdat de kennis over de eiwitsamenstelling, het proteoom, van varkensspeeksel beperkt is, was het eerste doel van dit doctoraatsonderzoek om meer inzicht te verkrijgen in het speekselproteoom van varkens (**Hoofdstuk IV**). Omdat geheel speeksel, dus speeksel dat gesecreteerd wordt in de mondholte, vermengd wordt met gingivale creviculaire vloeistof,

buccale cellen, micro-organismen en voedselresten, werd er voor geopteerd om speekselklierspecifiek speeksel te verzamelen onder de vorm van zuivere ductale secreten. Shotgun proteoomanalyse werd uitgevoerd op mandibulaire/sublinguale en parotisspeekselstalen om zo meer inzicht te verwerven in de eiwitsamenstelling van deze ductale secreten. In totaal werden 122 porciene speekseleiwitten en zes zoogdierspeekseleiwitten met een porcien homoloog geïdentificeerd in de klierspecifieke speekselstalen die werden verzameld bij biggen onder narcose. Een kwantitatief verschil in speekselsamenstelling kon worden waargenomen: 24 eiwitten werden voornamelijk gesecreteerd door de parotisspeekselklier, terwijl 29 eiwitten voornamelijk door de mandibulaire en sublinguale speekselklier gesecreteerd werden.

Nadat er meer inzicht in het speekselproteoom van het varken werd vergaard, werd de mogelijkheid om speeksel als hulpmiddel te gebruiken om chronische stress bij varkens te identificeren verder uitgediept. Volgens de literatuur zouden sommige factoren zoals het weerhouden van kooiverrijking, het frequent mengen van niet vertrouwde dieren en een te hoge hokdensiteit chronische stress veroorzaken bij biggen. Om na te gaan of deze stressoren effectief zijn, werd er eerst een cortisolanalyse uitgevoerd op zowel speeksel als haren van biggen, aangezien cortisol verhoogd wordt vrijgesteld ten gevolgen van stress (**Hoofdstuk V**). Drie weken blootstelling aan deze verschillende stressoren resulteerde in een verminderde gewichtstoename vergeleken met de controledieren. Er werd een verhoogde concentratie aan cortisol waargenomen in het haar van de gestresseerde dieren. Tussen beide groepen werd er echter geen verschil in cortisolconcentraties gedetecteerd in het speeksel. Gewichtstoename en cortisolconcentraties in haar correleerden significant, terwijl geen van beide parameters correleerde met de speeksel cortisolconcentraties.

Eens de effectiviteit van de stressoren was bevestigd werd het speekselproteoom van chronisch gestresseerde dieren vergeleken met dat van controle dieren (**Hoofdstuk VI**). Met behulp van shotgun proteoomanalyse werden 392 eiwitten geïdentificeerd in het speeksel van 28 dagen oude biggen, waarvan de relatieve abundantie van 20 speekseleiwitten werd beïnvloed door drie weken blootstelling aan verschillende

stressoren. Acht van deze 20 eiwitten werden geselecteerd voor verdere validatie. Hiervoor werden zowel speekselstalen die één week na de start van het experiment en stalen die op het einde werden verzameld geanalyseerd om het eiwitprofiel over een langere periode in kaart te brengen. Daartoe werd gerichte proteoomanalyse gebruikt, nl. parallel reaction monitoring (PRM). Deze PRM-analyse bevestigde dat alpha-2-HS-glycoproteïne werd opgereguleerd in het speeksel van de gestresseerde dieren, zowel na één als na drie weken, terwijl odorant-binding protein, chitinase, long palate lung and nasal epithelium protein 5, lipocalin-1, en vomeromodulin-like protein konden worden waargenomen in lagere concentraties in het speeksel van gestresseerde biggen, maar dit enkel na drie weken.

Dit proefschrift is er niet enkel in geslaagd meer inzicht te verschaffen in het speekselproteoom van varkens, er konden ook veranderingen in het speekselprofiel van jonge varkens worden aangetoond ten gevolge van chronische stress. De eiwitten die hierdoor worden beïnvloed kunnen, na verdere validatie, mogelijkerwijze gebruikt worden als biomerkers om chronische stress bij varkens te identificeren op het landbouwbedrijf. Zij kunnen ook het agrarisch onderzoek naar betere opfokmethodes faciliteren.

Dankwoord

It takes a village to raise a child. Dat heb ik ondertussen kunnen ondervinden maar er is ook een klein dorp nodig om een doctoraat te kunnen afronden. Deze thesis heb ik enkel kunnen afleveren dankzij de hulp van heel veel mensen. Ik zou alvast iedereen willen bedanken die voor me klaar stond met goede raad, wetenschappelijk advies, veel geduld, een portie relativeringsvermogen, een luisterend oor, een dosis humor of een lekker hapje en drankje om alles door te spoelen. Graag neem ik de tijd om enkele belangrijke personen extra in de bloemetjes te zetten.

I would like to start with thanking the external jury members, prof. dr. Ana-Maria Gutiérrez Montes and prof. dr. Friederike Ebner. I admire the expertise you gathered in your research domains and I am grateful that you were willing to invest time into reviewing this thesis. Bedankt interne juryleden voor jullie bijdrage aan dit werk. Hierbij wil ik prof. dr. Dirk Adriaensen bedanken voor de erg kritische blik op het geschreven werk. Bedankt prof. dr. Peter Delputte, om mij met een bredere blik naar mijn onderzoek te laten kijken. Prof. dr. Jean-Pierre Timmermans wil ik bedanken om, een voor mij bijzondere dag, nl. mijn publieke verdediging, in goede banen te leiden.

Ik zou graag mijn drie promotoren enorm willen bedanken. Hierbij wil ik graag starten met, Christophe, bedankt om me al die jaren geleden te overtuigen om toch aan een doctoraat te beginnen. Jij als startende Tenure track zocht je eerste doctoraatstudent en was initieel meer overtuigd dan ik dat ik een goede academische onderzoeker zou zijn. Aanvankelijk hebben we samen eerst het immuunsysteem van jonge biggen bestudeerd, maar ik ben je erg dankbaar dat je enkele maanden na de start van mijn doctoraatsperiode afkwam met de vraag “Wil je misschien iets doen met speeksel, eiwitten en varkens?”. Met deze brede vraag kon ik mijn eigen interesses achterna. Het was niet de gemakkelijkste weg en het heeft… euh… ook eventjes geduurd maar ik ben heel blij dat ik me dankzij jou heb mogen verdiepen in de wondere wereld van dierenwelzijn, speeksel en proteomics. Ook al ben je de laatste tijd nog maar sporadisch te vinden op onze universiteit, over de hele periode kon ik altijd bij jou terecht voor een grondige spellingscheck van al mijn geschreven werk.
Xaveer, bedankt dat ik dankzij jou zoveel heb bijgeleerd over proteomics. Ik mocht altijd langskomen bij problemen of vreemde resultaten en was altijd welkom bij PPES voor staalvoorbereidingen of analyses. Je eeuwige optimisme deed vaak wonderen, bedankt!
Chris, na het vertrek van Christophe bij onze groep heb jij me onder je vleugels genomen. Ondanks je overvolle agenda slaag je er toch telkens in om klaar te staan met een kritische blik op mijn draft-versies, een idee voor een andere invalshoek en een peptalk wanneer nodig. Ik heb al heel veel van je geleerd. Uiteraard ook bedankt dat je aan mij hebt gedacht toen je iemand zocht om het VLAIO-project te ondersteunen en toen Miriam besloot om een jaartje te gaan reizen. Nog groter is mijn dankbaarheid voor je blijk van vertrouwen

toen Miriam definitief vervangen moest worden. Ik hoop dat ik kan groeien tot de projectcoördinator die onze groep nodig heeft en verdient. Hopelijk hebben we dus samen nog vele jaren voor de boeg vol wetenschappelijke uitdagingen (en successen…) en gezellige babbels tussendoor. Steven, niet echt promotor maar altijd trouwe co-auteur, bedankt voor al je feedback over de jaren heen. Ook jou moet ik bedanken om me een kans te geven als nieuwe projectcoördinator van CoPeD. Ik ben me al helemaal aan het verdiepen in NAMS, toxicologie en minipigs, en ik kijk al uit naar wat de toekomst nog in petto heeft.

Katty en Gunther, waar moet ik beginnen. Jullie zijn de ruggengraat van CoPeD. De voorbije jaren kon ik telkens op jullie rekenen voor advies in het labo, helpende handen bij speekselende (en bijtende) biggen, creatieve oplossingen voor weer eens een probleem, … wat zou onze groep zijn zonder jullie. Gunther bedankt voor de hulp met de biggen, de tripjes naar 'den Brico' zodat we onze biggencontainer efficiënt konden inrichten, je kennis over kleuringen en originele oplossingen voor elke hindernis. Katty, bedankt voor je eeuwig optimisme, je bent echt het zonnetje van onze groep dat ons telkens energie geeft. En uiteraard bedankt dat ik telkens bij jullie terecht kon om kleine hoeraatjes te delen als iets lukte, maar ook mocht langskomen om even te ventileren als dat nodig was, of soms, letterlijk, bij jullie uit te huilen. Ik ben heel blij dat ik jullie als collega's heb.

De voorbije jaren heb ik wel wat bureaus afgeschuimd maar ik denk met een heel warm, haha, gevoel terug aan "de koude hoek", bureau U.017. Charlotte, ik denk dat wij daar het langste hebben samengezeten, uren gedebatteerd over statistiek maar ook over reizen en recepten. Onze tripjes naar de boerderij in Neerhespen en onze creatieve manier van tijd doden tijdens het wachten zullen me zeker bijblijven. Ik vind het nog steeds jammer dat je terug naar 'de Vlaanders' bent getrokken. Misschien ligt je nieuwe avontuur toch wat dichter bij Antwerpen en zien we je hopelijk vaker passeren. Alvast heel veel succes daarmee en met je zoektocht naar een nieuw stekje! Kevin, ik ben heel blij dat ik je mocht vergezellen in je VLAIO-avontuur. De tripjes naar boerderijen en meetings met de gebruikersgroep waren niet hetzelfde geweest zonder jou. Onze gedeelde interesses in lekkere koffie en een goed sterk biertje waren hierbij soms een bonus, al heeft je droge portie humor en relativeringsvermogen ook wonderen gedaan. Ik ben heel blij dat ik vooral voor dat laatste (en misschien ook voor je bakkunsten) nog steeds kan binnenwaaien in je bureau. Laura, jou gedeelde interesse in planten en 'dino-run' spelen als het internet uitviel zorgde ervoor dat je direct binnen "de koude hoek" paste. Je was een geweldige bureaugenoot en dankbare collega die altijd open stond voor advies, maar ook babbeltjes over o.a. verbouwen. Soms voelde ik me in de beginjaren een beetje je grote zus, maar ik ben heel blij dat we je na je doctoraat terug zien in de gang en dat jij er de laatste maanden was om die grote zussenrol even over te nemen en klaar te staan voor mij met advies en woorden van steun!

Het gaat al een tijdje terug maar de vorige "bewoners" van U.017, Hans, Véronique, Maartje moet ik ook bedanken. Jullie hebben me aan de start van mijn doctoraat wegwijs

gemaakt in het doctoraatsleven. Bedankt daarvoor. Bart, als begeleider van mijn masterproef maar ook erna heb ik heel veel van je geleerd. Bedankt! Els en Evy, ook met jullie heb ik een bureau gedeeld, ook jullie bedankt om me in het begin te helpen en de beginselen van wetenschappelijk onderzoek bij te brengen en uiteraard bedankt voor de gezellige babbels.

Miriam, I feel lucky that I was able to work with you. Although you were not involved in my project from the start, you were always there with constructive feedback that really improved the quality of my work. Thank you for that! I wish you all the best on your new adventure in Spain and hope our paths will cross again.

Ellen en Marjan (en Kevin uiteraard), jullie bureau is vaak een tussenstopje op weg naar de koelkast of als ik voel dat ik het even nodig heb. Bij jullie kan ik altijd terecht voor een “you can do it”, een luisterend oor of wat afleiding met babbels over reizen, verbouwen, vlinders opzetten, trouwen, over wat kunnen we eigenlijk niet kwetteren… Bedankt! Fien ook jij bedankt voor de steun en aanmoedigingen en voor de babbeltjes (voornamelijk over tuinieren en Gustjes) die vooral niets met mijn thesis te maken hadden. Marleen, Casper, Moayad, Denise, Chloe, Allan, Jente, Falk, Marina, Steve en, al eventjes geleden, Christel ook jullie bedankt voor de fijne samenwerkingen en de leuke gesprekjes *en passant*.

Lieselotte en Kris, we zijn nog niet heel lang bureaugenoten maar ik denk dat jullie me ineens van mijn meest intense kant hebben meegemaakt. In volle eindspurt naar dit doctoraat en onder een ferme dosis zwangerschapshormonen. Bedankt dat ik bij jullie mocht ventileren, uithuilen en dat jullie er waren met raad en steun. Ik beloof dat het er vanaf nu minder intens aan toe gaat gaan…

Geert, Ruben, Martin en Mops, ik denk niet dat ik jullie genoeg kan bedanken voor jullie hulp de voorbije jaren. Zonder jullie had ik bitter weinig proteomics werk kunnen afleveren. Geert, je hebt me de beginselen van proteomics aangeleerd. Ik heb onnoemlijk veel vragen op je afgevoerd en zelfs als je niet meer verbonden was aan onze universiteit nam je nog de tijd om die te beantwoorden. Bedankt dat ik mocht meeliften op je enorme kennis over eiwitanalyses. Ruben en Martin, ook jullie moet ik bedanken voor jullie hulp in het labo en met de data-analyses achteraf. Jullie hulp was van onschatbare waarde. Uiteraard ook bedankt voor de leuke praatjes tussendoor, ik voelde me altijd heel welkom bij PPES, dat deed deugd!

Lieve vrienden en vriendinnen, bedankt dat jullie na al die jaren nog steeds interesse toonden in “wat ik daar deed met varkentjes”. Bedankt voor alle gezellige uitstapjes en avondjes, ook al stonden die het laatste jaar wat op een laag pitje. Nu dat dit doctoraat eindelijk is afgerond prop ik jullie agenda terug vol met leuke plannen, het is maar dat jullie gewaarschuwd zijn :-).

Mama en papa, woorden schieten mij te kort als ik jullie wil bedanken. Bedankt dat jullie me alle kansen gegeven hebben om te groeien tot wie ik nu ben. Jullie staan altijd paraat, met een luisterend oor en een peptalk voor wat zelfvertrouwen, maar jullie staan ook altijd klaar met Tupperware vol lekker eten en opvang voor de kindjes in drukke tijden. Ik kan altijd op jullie rekenen en niets is te veel, ik weet hoe kostbaar dat is, en ben jullie daar ook heel dankbaar voor. Ik zie jullie graag.

Thomas, kleintje, buiten je wetenschappelijke bijdrage, nog altijd geweldig dat je mijn idee om zelf kleinere Lashley cups te maken niet zo gek vond en direct hebt uitgewerkt, ben ik ook blij dat ik je te pas en te onpas kan bellen om te ventileren of om te babbelen over alles behalve mijn doctoraat.

Opa, je was één van mijn meest trotse supporters, hopelijk heb je kunnen meevolgen van daarboven. Oma, bedankt om telkens interesse te tonen in de varkentjes en voor je tips om te ontspannen.

Nadine en Gabor, ik ben heel dankbaar dat ik jullie heb als schoonouders. Ook jullie stonden klaar met potten spaghettisaus en soep (die mogen nog blijven komen hoor…). Maar ik ben ook heel blij dat Nico en de kindjes zo vaak bij jullie terecht konden als ik weer eens een weekend schrijven had ingepland. Caro (en uiteraard ook Peter), bedankt voor je oprechte interesse tijdens de hele periode, en dat was niet enkel omdat je dierenarts bent. Ook bedankt omdat jullie heel goed zijn in mij vooral niet aan mijn doctoraat te laten denken! Bij uitbreiding ook bedankt aan de rest van mijn schoonfamilie!

Kleine baby, jij hebt de eindspurt helemaal op de eerste rij meegemaakt. Bedankt voor je gezelschap tijdens al die uren achter mijn laptop. Ik hoop dat ik je niet te veel heb blootgesteld aan een te hoge dosis cortisol?! Ik kan al niet wachten om je te ontmoeten kleine man! Julia en Gust, mama haar “grote toets” is nu eindelijk achter de rug. Bedankt voor jullie warme knuffels en woordjes van aanmoediging de voorbije maanden. Dat deed me telkens enorm deugd. Ook bedankt voor jullie kleurrijke geschilderde varkentjes die zo mooi op deze cover prijken. Ik kader de originele kunstwerkjes in als geweldig aandenken aan deze periode. Ik hou van jullie men engeltjes.

Nico, lieverd, jij bent mijn rots, bedankt daarvoor. Ik weet dat het afwerken van dit doctoraat altijd, maar vooral dit laatste jaar, best veel van je heeft gevraagd. Ik stuurde je heel veel weekends op pad met de kindjes zodat ik in rust kon schrijven en je hebt veel avonden alleen doorgebracht omdat ik dan nog meer kon schrijven. Daarbij kwam dan dat de momenten samen vaak een uiting van stress en emoties waren, en sorry, want er waren ook niet altijd propere sokken :-). Maar ik heb niets anders dan begrip, empathie, warmte en liefde van je teruggekregen met daarbovenop een goeie portie relativeringsvermogen. Ze zeggen dat samen een huis verbouwen een grote relatietest is, maar dat was toch peanuts vergeleken met het afwerken van een doctoraat. Ik had dit traject niet kunnen afleggen zonder jouw steun. Bedankt voor alles. Ik hou van je.

Curriculum vitae

Personalia

Last name	Prims
First name	Sara
Date of birth	01/04/1988
Nationality	Belgian
Email	sara.prims@uantwerpen.be

Career and Education

Project coordinator **2023 – current**
Laboratory of Comparative Perinatal Development
University of Antwerp

Ad interim Project coordinator **2022 – 2023**
Laboratory of Comparative Perinatal Development
University of Antwerp

VLAIO Project **2018 – 2021**
Laboratory of Comparative Perinatal Development
University of Antwerp
Project title: VLAIO HBC.2016.0786: Introduction and optimisation of innovative rearing concepts for resilient piglets

PhD candidate **2013 – 2024**
Laboratory of Comparative Perinatal Development
University of Antwerp
PhD thesis: Saliva as a biological matrix to assess piglet's welfare
Supervisors: Prof. dr. Chris Van Ginneken, Prof. dr. Xaveer Van Ostade, Prof. dr. Christophe Casteleyn

Master in Biomedical Science – Neuroscience **2011 – 2013**
University of Antwerp
Master thesis: Morphology of the brain and cognitive capacity of piglets in relation to their birth weight
Supervisors: Prof. dr. Chris Van Ginneken, dr. Bart Tambuyzer

Teaching Experience

Bachelor of Veterinary Science and Biomedical Sciences:

- General pathology

Master of Biomedical Sciences, Biochemistry and Biotechnology, and PhD students:

- Laboratory Animal Sciences

Student Supervision

Master thesis Ben Jurgens
Biomedical Sciences, University of Antwerp. Academic year: 2015 – 2016

Honours College project Tobias Debeuf
Veterinary Sciences, University of Antwerp. Academic year: 2015 – 2016

Master thesis Freya Molenberghs
Veterinary Sciences, Gent University. Academic year: 2014 – 2015

Master thesis Ivana Sunjic
Biomedical Sciences, University of Antwerp. Academic year: 2014 – 2015

Master thesis Niels Pintens
Biomedical Sciences, University of Antwerp. Academic year: 2013 – 2014

Additional Scientific Trainings & Certificates

- Training school in the Experimental Design & Statistical Analysis of Biomedical Experiments (FRAME)
- Method in research design (StatUA), University of Antwerp
- Writing academic papers in English (Linguapolis), University of Antwerp
- Basic Principles of Statistics (StatUA), University of Antwerp
- Analysis of grouped and longitudinal data using linear mixed (StatUA), University of Antwerp
- R-workshop (StatUA), University of Antwerp
- FELASA category C, University of Antwerp

Conferences

Oral presentations

- International Pig Veterinary Society - Belgian Branch, Merelbeke, 2019
- European Federation of Animal Science, Ghent, 2019
- 11th European Symposium on Saliva, Egmond aan Zee, 2017
- International Pig Veterinary Society - Belgian Branch, Merelbeke, 2016
- Research Day - Faculty Pharmaceutical, Biomedical and Veterinary Sciences, 2016

Poster presentations

- International Pig Veterinary Society - Belgian Branch, Merelbeke, 2018, awarded
- International Pig Veterinary Society - Belgian Branch, Merelbeke, 2016, awarded
- National Symposium on Applied Biological Sciences, Antwerp, 2016, awarded
- Digestive Physiology in Pig Symposium, Poland, 2015
- Conference - Belgium Immunology Society, Leuven, 2014

Publications in international peer-reviewed journals

First or second author

- **Prims, S.**, Van Ostade, X., Ayuso, M., Dom, M., Van Raemdonck, G., Van Cruchten, S., Casteleyn, C., Van Ginneken, C., 2023. Chronic exposure to multiple stressors alters the salivary proteome of piglets. PLoS One, under review
- **Prims, S.**, Vanden Hole, C., Van Cruchten, S., Van Ginneken, C., Van Ostade, X., Casteleyn, C., 2019. Hair or salivary cortisol analysis to identify chronic stress in piglets? The Veterinary Journal 252, 105357.
- **Prims, S.**, Van Raemdonck, G., Vanden Hole, C., Van Cruchten, S., Van Ginneken, C., Van Ostade, X., Casteleyn, C., 2019. On the characterisation of the porcine gland-specific salivary proteome. Journal of Proteomics 196, 92-105.
- **Prims, S.**, Jurgens, B., Vanden Hole, C., Van Cruchten, S., Van Ginneken, C., Casteleyn, C., 2018. The porcine tonsils and Peyer's patches: A stereological morphometric analysis in conventionally and artificially reared piglets. Veterinary Immunology and Immunopathology 206, 9-15.
- **Prims, S.**, Pintens, N., Vergauwen, H., Van Cruchten, S., Van Ginneken, C., Casteleyn, C., 2017. Effect of artificial rearing of piglets on the volume densities of M cells in the tonsils of the soft palate and ileal Peyer’s patches. Veterinary Immunology and Immunopathology 184, 1-7.
- **Prims, S.**, Tambuyzer, B., Vergauwen, H., Huygelen, V., Cruchten, S.V., Ginneken, C.V., Casteleyn, C., 2016. Intestinal immune cell quantification and gram type classification of the adherent microbiota in conventionally and artificially reared, normal and low birth weight piglets. Livestock Science 185, 1-7.
- Van Tichelen, K., **Prims, S**., Ayuso Hernando, M., Van Bockstal, L., Van Kerschaver, C., Vandaele, M., Degroote, J., Van Cruchten, S., Michiels, J., Van Ginneken, C., 2023. The effect of drenching (very) low birth weight piglets with a dense, concentrated milk replacer at farms with differing farrowing management, Animals 13(1).
- Van Tichelen, K., **Prims, S.**, Ayuso, M., Van Kerschaver, C., Vandaele, M., Degroote, J., Van Cruchten, S., Michiels, J., Van Ginneken, C., 2021. Drenching Bovine Colostrum, Quercetin or Fructo-Oligosaccharides Has No Effect on Health or Survival of Low Birth Weight Piglets. Animals 12 (1).

- Van Tichelen, K., **Prims, S.**, Ayuso, M., van Kerschaver, C., Vandaele, M., Degroote, J., van Cruchten, S., Michiels, J., van Ginneken, C., 2021, Handling Associated with Drenching Does Not Impact Survival and General Health of Low Birth Weight Piglets, Animals 11(2), 404
- Vergauwen, H., **Prims, S.**, Degroote, J., Wang, W., Casteleyn, C., van Cruchten, S., de Smet, S., Michiels, J., van Ginneken, C., 2016. In Vitro Investigation of Six Antioxidants for Pig Diets. Antioxidants 5.

Third or following author

- Buyssens, L., Valenzuela, A., **Prims, S.**, Ayuso, M., Thymann, T., Van Ginneken, C., Van Cruchten, S, 2023. Ontogeny of CYP3A and UGT activity in preterm piglets: a translational model for drug metabolism in preterm newborns, Frontiers in Pharmacology 14
- Vanden Hole, C., Van Ginneken, C., **Prims, S.**, Ayuso, M., Van Cruchten, S., Aerts, P., 2019. Does intrauterine crowding affect the force generating capacity and muscle composition of the piglet front limb? PLoS One 14.
- Vanden Hole, C., Ayuso, M., Aerts, P., **Prims, S.**, Van Cruchten, S., Van Ginneken, C., 2019. Glucose and glycogen levels in piglets that differ in birth weight and vitality. Heliyon 5.
- Vanden Hole, C., Cleuren, S., Van Ginneken, C., **Prims, S.**, Ayuso, M., Van Cruchten, S., Aerts, P., 2018. How does intrauterine crowding affect locomotor performance in newborn pigs? A study of force generating capacity and muscle composition of the hind limb. PLoS One 13.
- Vanden Hole, C., Goyens, J., **Prims, S.**, Fransen, E., Ayuso Hernando, M., Van Cruchten, S., Aerts, P., Van Ginneken, C., 2017. How innate is locomotion in precocial animals? A study on the early development of spatio-temporal gait variables and gait symmetry in piglets. Journal of Experimental Biology 220, 2706-2716.
- Vanden Hole, C., Aerts, P., **Prims, S.**, Ayuso, M., Van Cruchten, S., Van Ginneken, C., 2018. Does intrauterine crowding affect locomotor development? A comparative study of motor performance, neuromotor maturation and gait variability among piglets that differ in birth weight and vitality. PLoS One 13.
- Huygelen, V., De Vos, M., **Prims, S.**, Vergauwen, H., Fransen, E., Casteleyn, C., Van Cruchten, S., Van Ginneken, C., 2015. Birth weight has no influence on the morphology, digestive capacity and motility of the small intestine in suckling pigs. Livestock Science 182, 129-136.

Reference list

1. Knox, R., et al., *Effect of day of mixing gestating sows on measures of reproductive performance and animal welfare.* J Anim Sci, 2014. **92**(4): p. 1698-707.
2. Coutellier, L., et al., *Pig's responses to repeated social regrouping and relocation during the growing-finishing period.* Applied Animal Behaviour Science, 2007. **105**(1): p. 102-114.
3. Hyun, Y., et al., *Growth performance of pigs subjected to multiple concurrent environmental stressors.* J Anim Sci, 1998. **76**(3): p. 721-7.
4. Van Beirendonck, S., et al., *Behavior of piglets after castration with or without carbon dioxide anesthesia.* J Anim Sci, 2011. **89**(10): p. 3310-7.
5. Pineiro, M., et al., *Characterisation of the pig acute phase protein response to road transport.* Vet J, 2007. **173**(3): p. 669-74.
6. Morrow-Tesch, J.L., J.J. McGlone, and J.L. Salak-Johnson, *Heat and social stress effects on pig immune measures.* J Anim Sci, 1994. **72**(10): p. 2599-609.
7. Elenkov, I.J. and G.P. Chrousos, *Stress Hormones, Th1/Th2 patterns, Pro/Anti-inflammatory Cytokines and Susceptibility to Disease.* Trends Endocrinol Metab, 1999. **10**(9): p. 359-368.
8. Oostindjer, M., et al., *Effects of environmental enrichment and loose housing of lactating sows on piglet performance before and after weaning.* J Anim Sci, 2010. **88**(11): p. 3554-62.
9. Munsterhjelm, C., et al., *Housing during early pregnancy affects fertility and behaviour of sows.* Reprod Domest Anim, 2008. **43**(5): p. 584-91.
10. Hurtgen, J.P. and A.D. Leman, *Seasonal influence on the fertility of sows and gilts.* J Am Vet Med Assoc, 1980. **177**(7): p. 631-5.
11. Selye, H., *The alarm reaction.* Can Med Assoc J, 1936. **34**: p. 706.
12. Tan, S.Y. and A. Yip, *Hans Selye (1907-1982): Founder of the stress theory.* Singapore Med J, 2018. **59**(4): p. 170-171.
13. Cooper, C. and J.P. Dewe, *Stress: A Brief History*. 2004: Blackwell Publishing Ltd.
14. Chrousos, G.P. and P.W. Gold, *The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis.* Jama, 1992. **267**(9): p. 1244-52.
15. Goldstein, D.S., *Adrenal responses to stress.* Cell Mol Neurobiol, 2010. **30**(8): p. 1433-40.
16. Robinson, A., *Let's Talk About Stress: History of Stress Research.* Review of General Psychology, 2018. **22**.
17. Bernard, C., *Lectures on the Phenomena of Life Common to Animals and Plants*. 1974: Charles C. Thomas Pub Ltd.
18. Cannon, W.B., *The wisdom of the body.* 1939.
19. Cannon, W.B., *Bodily changes in pain, hunger, fear and rage: An account of recent researches into the function of emotional excitement*. 1925: D. Appleton.

20. Selye, H., *Stress and the general adaptation syndrome.* Br Med J, 1950. **1**(4667): p. 1383-92.
21. Selye, H., *A Syndrome produced by Diverse Nocuous Agents.* Nature, 1936. **138**(3479): p. 32-32.
22. Selye, H., *Thymus and Adrenals in the Response of the Organism to Injuries and Intoxications*. Br J Exp Pathol. 1936 Jun;17(3):234-48.
23. Goldstein, D.S., *The autonomic nervous system in health and disease*. 2000: Informa Health Care.
24. Koolhaas, J.M., et al., *Coping styles in animals: current status in behavior and stress-physiology.* Neuroscience & Biobehavioral Reviews, 1999. **23**(7): p. 925-935.
25. Kim, E.J. and J.J. Kim, *Neurocognitive effects of stress: a metaparadigm perspective.* Molecular Psychiatry, 2023.
26. de Kloet, E.R., M. Joëls, and F. Holsboer, *Stress and the brain: from adaptation to disease.* Nat Rev Neurosci, 2005. **6**(6): p. 463-75.
27. Boron, W.F. and E.L. Boulpaep, *Medical physiology a cellular and molecular approach*. 2 ed. ed. 2012, Philadelphia: Saunders. 1337.
28. Moberg, G.P. and J.A. Mench, *Biology of Animal Stress - Basic Principles and Implications for Animal Welfare*. 2000, New York, USA: Cabi Publishing.
29. McEwen, B.S. and E. Stellar, *Stress and the individual. Mechanisms leading to disease.* Arch Intern Med, 1993. **153**(18): p. 2093-101.
30. Chrousos, G.P., *The HPA axis and the stress response.* Endocr Res, 2000. **26**(4): p. 513-4.
31. Timmermans, S., J. Souffriau, and C. Libert, *A General Introduction to Glucocorticoid Biology.* Front Immunol, 2019. **10**: p. 1545.
32. Ruis, M.A.W., et al., *The Circadian Rhythm of Salivary Cortisol in Growing Pigs: Effects of Age, Gender, and Stress.* Physiology & Behavior, 1997. **62**(3): p. 623-630.
33. Cook, N.J., et al., *Salivary vs. serum cortisol for the assessment of adrenal activity in swine.* Canadian Journal of Animal Science, 1996. **76**(3): p. 329-335.
34. Keller-Wood, M.E. and M.F. Dallman, *Corticosteroid inhibition of ACTH secretion.* Endocr Rev, 1984. **5**(1): p. 1-24.
35. Wiepkema, P.R. and J.M. Koolhaas, *Stress and Animal Welfare.* Animal Welfare, 1993. **2**(3): p. 195-218.
36. Janssens, C.J., et al., *Chronic stress increases the opioid-mediated inhibition of the pituitary-adrenocortical response to acute stress in pigs.* Endocrinology, 1995. **136**(4): p. 1468-73.
37. Barnett, J.L. and P.H. Hemsworth, *The validity of physiological and behavioural measures of animal welfare.* Applied Animal Behaviour Science, 1990. **25**(1): p. 177-187.
38. Hewson, C.J., *What is animal welfare? Common definitions and their practical consequences.* Can Vet J, 2003. **44**(6): p. 496-9.
39. FAWC, *Farm Animal Welfare Council updates the five freedoms.* The Veterinary Record, 1992. **17:357**.

40. Guevara, R.D., et al., *Systematic review of animal-based indicators to measure thermal, social, and immune-related stress in pigs.* PLoS One, 2022. **17**(5): p. e0266524.
41. Sutherland, M.A., et al., *Heat and social rank impact behavior and physiology of PRRS-virus-infected pigs.* Physiol Behav, 2007. **90**(1): p. 73-81.
42. Hicks, T.A., et al., *Behavioral, endocrine, immune, and performance measures for pigs exposed to acute stress.* J Anim Sci, 1998. **76**(2): p. 474-83.
43. Canaday, D.C., et al., *Effect of variability in lighting and temperature environments for mature gilts housed in gestation crates on measures of reproduction and animal well-being.* J Anim Sci, 2013. **91**(3): p. 1225-36.
44. Herpin, P., M. Damon, and J. Le Dividich, *Development of thermoregulation and neonatal survival in pigs.* Livestock Production Science, 2002. **78**: p. 25-45.
45. O'Connor, E.A., et al., *The impact of chronic environmental stressors on growing pigs, Sus scrofa (Part 1): stress physiology, production and play behaviour.* Animal, 2010. **4**(11): p. 1899-1909.
46. Ni, J.Q., et al., *A critical review of advancement in scientific research on food animal welfare-related air pollution.* J Hazard Mater, 2021. **408**: p. 124468.
47. Bacou, E., et al., *Acute social stress-induced immunomodulation in pigs high and low responders to ACTH.* Physiol Behav, 2017. **169**: p. 1-8.
48. de Groot, J., et al., *Long-term effects of social stress on antiviral immunity in pigs.* Physiology & Behavior, 2001. **73**(1–2): p. 145-158.
49. Vandaele, M., et al., *Piglet performance and colostrum intake in litters either or not split-suckled during the first day or during the first three days of life.* Livestock Science, 2020. **241**: p. 104265.
50. De Vos, M., et al., *Artificial rearing of piglets: Effect on small intestinal morphology and digestion capacity.* Livestock Science, 2014. **159**(165-173).
51. Gimsa, U., M. Tuchscherer, and E. Kanitz, *Psychosocial Stress and Immunity-What Can We Learn From Pig Studies?* Front Behav Neurosci, 2018. **12**: p. 64.
52. Marco-Ramell, A., et al., *Identification of serum stress biomarkers in pigs housed at different stocking densities.* Vet J, 2011. **190**(2): p. e66-71.
53. Salak-Johnson, J.L., et al., *Wider stall space affects behavior, lesion scores, and productivity of gestating sows.* J Anim Sci, 2015. **93**(10): p. 5006-17.
54. de Jong, I.C., et al., *Effects of environmental enrichment on behavioral responses to novelty, learning, and memory, and the circadian rhythm in cortisol in growing pigs.* Physiology & Behavior, 2000. **68**(4): p. 571-8.
55. Casal, N., et al., *Effect of environmental enrichment and herbal compound supplementation on physiological stress indicators (chromogranin A, cortisol and tumour necrosis factor-alpha) in growing pigs.* Animal, 2016: p. 1-9.
56. LeDoux, J.E., *Emotion circuits in the brain.* Annu Rev Neurosci, 2000. **23**: p. 155-84.
57. Weinberg, J. and S. Levine, *Psychobiology of Coping in Animals: The Effects of Predictability*, in *Coping and Health*, S. Levine and H. Ursin, Editors. 1980, Springer US: Boston, MA. p. 39-59.
58. Bassett, L. and H.M. Buchanan-Smith, *Effects of predictability on the welfare of captive animals.* Applied Animal Behaviour Science, 2007. **102**(3): p. 223-245.

59. Ott, S., et al., *Different stressors elicit different responses in the salivary biomarkers cortisol, haptoglobin, and chromogranin A in pigs.* Research in Veterinary Science, 2014. **97**(1): p. 124-128.
60. Boumans, I.J.M.M., et al., *How social factors and behavioural strategies affect feeding and social interaction patterns in pigs.* Physiology & Behavior, 2018. **194**: p. 23-40.
61. Mostaço, G.M., et al., *Determination of piglets' rectal temperature and respiratory rate through skin surface temperature under climatic chamber conditions.* 2015. **35**: p. 979-989.
62. Schrader, L. and J. Ladewig, *Temporal differences in the responses of the pituitary adrenocortical axis, the sympathoadrenomedullar axis, heart rate, and behaviour to a daily repeated stressor in domestic pigs.* Physiol Behav, 1999. **66**(5): p. 775-83.
63. Shen, C., et al., *Identifying blood-based biomarkers associated with aggression in weaned pigs after mixing.* Applied Animal Behaviour Science, 2020. **224**: p. 104927.
64. Ferrari, P., *The role of 11β-hydroxysteroid dehydrogenase type 2 in human hypertension.* Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 2010. **1802**(12): p. 1178-1187.
65. Shimanoe, C., et al., *Perceived stress, depressive symptoms, and cortisol-to-cortisone ratio in spot urine in 6878 older adults.* Psychoneuroendocrinology, 2021. **125**: p. 105125.
66. Larson, C.T., W.B. Gross, and J.W. Davis, *Social stress and resistance of chicken and swine to Staphylococcus aureus challenge infections.* Can J Comp Med, 1985. **49**(2): p. 208-10.
67. Kanitz, E., et al., *A single exposure to social isolation in domestic piglets activates behavioural arousal, neuroendocrine stress hormones, and stress-related gene expression in the brain.* Physiology & Behavior, 2009. **98**(1): p. 176-185.
68. Tuchscherer, M., et al., *Effects of social status after mixing on immune, metabolic, and endocrine responses in pigs.* Physiology & Behavior, 1998. **64**(3): p. 353-360.
69. Schalk, C., et al., *Effects of repeated social mixing on behavior and blood immune cells of group-housed pregnant sows (Sus scrofa domestica).* Livestock Science, 2018. **217**: p. 148-156.
70. Pineiro, C., et al., *Pig acute-phase protein levels after stress induced by changes in the pattern of food administration.* Animal, 2007. **1**(1): p. 133-9.
71. Camerlink, I., et al., *Emotions after victory or defeat assessed through qualitative behavioural assessment, skin lesions and blood parameters in pigs.* Applied Animal Behaviour Science, 2016. **183**: p. 28-34.
72. Escribano, D., et al., *Measurement of chromogranin A in porcine saliva: validation of a time-resolved immunofluorometric assay and evaluation of its application as a marker of acute stress.* Animal, 2013. **7**(4): p. 640-7.
73. Colson, V., et al., *Grouping piglets by sex at weaning reduces aggressive behaviour.* Applied Animal Behaviour Science, 2006. **97**(2–4): p. 152-171.
74. Foury, A., et al., *Stress neuroendocrine profiles in five pig breeding lines and the relationship with carcass composition.* animal, 2007. **1**(7): p. 973-982.

75. Holliman, C.J., et al., *Technique for acute suprapubic catheterization of urinary bladder in the pig.* Am J Vet Res, 1982. **43**(6): p. 1056-7.
76. Palme, R., *Monitoring stress hormone metabolites as a useful, non-invasive tool for welfare assessment in farm animals.* Animal Welfare, 2012. **21**(3): p. 331-337.
77. Möstl, E., et al., *Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock.* Zentralbl Veterinarmed A, 1999. **46**(10): p. 621-31.
78. Khan, M.Z., et al., *A matter of time: evaluating the storage of fecal samples for steroid analysis.* General and Comparative Endocrinology, 2002. **128**(1): p. 57-64.
79. Chrousos, G.P., L.D. Loriaux, and P.W. Gold, *The concept of stress and its historical development.* , in *Mechanisms of physical and emotional stress*, G.P. Chrousos, L.D. Loriaux, and P.W. Gold, Editors. 1988, Plenum Press: New York. p. 3-7.
80. Koren, L., et al., *A novel method using hair for determining hormonal levels in wildlife.* Animal Behaviour, 2002. **63**(2): p. 403-406.
81. Casal, N., et al., *Analysis of cortisol in hair samples as an indicator of stress in pigs.* Journal of Veterinary Behavior, 2017. **19**: p. 1-6.
82. Baumgartner, A.M., et al., *Radioimmunoassay of hair for determining opiate-abuse histories.* J Nucl Med, 1979. **20**(7): p. 748-52.
83. Ryabukhin, Y.S., *Activation analysis of hair as an indicator of contamination of man by environmental trace element pollutants*. 1976: International Atomic Energy Agency (IAEA). p. 135.
84. Baumgartner, A.M., P.F. Jones, and C.T. Black, *Detection of phencyclidine in hair.* J Forensic Sci, 1981. **26**(3): p. 576-81.
85. Wheeler, M.J., et al., *The measurement of testosterone in hair.* J Endocrinol, 1998. **159**(1): p. R5-8.
86. Cirimele, V., et al., *Identification of ten corticosteroids in human hair by liquid chromatography–ionspray mass spectrometry.* Forensic Science International, 2000. **107**(1): p. 381-388.
87. Stalder, T. and C. Kirschbaum, *Analysis of cortisol in hair - state of the art and future directions.* Brain Behav Immun, 2012. **26**(7): p. 1019-29.
88. Pragst, F. and M.A. Balikova, *State of the art in hair analysis for detection of drug and alcohol abuse.* Clin Chim Acta, 2006. **370**(1-2): p. 17-49.
89. Meyer, J.S. and M.A. Novak, *Minireview: Hair cortisol: a novel biomarker of hypothalamic-pituitary-adrenocortical activity.* Endocrinology, 2012. **153**(9): p. 4120-7.
90. Henderson, G.L., *Mechanisms of drug incorporation into hair.* Forensic Science International, 1993. **63**(1): p. 19-29.
91. Ito, N., et al., *Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal axis and synthesize cortisol.* Faseb j, 2005. **19**(10): p. 1332-4.
92. Davidson, A., et al., *Do boars compensate for hunting with higher reproductive hormones?* Conserv Physiol, 2021. **9**(1): p. coab068.
93. Trevisan, C., et al., *Hair cortisol and dehydroepiandrosterone concentrations in naturally Taenia solium infected pigs in Tanzania.* General and Comparative Endocrinology, 2017. **246**: p. 23-28.

94. Kaufman, E. and I.B. Lamster, *The diagnostic applications of saliva: A review.* Crit Rev Oral Biol Med, 2002. **13**(2): p. 197-212.
95. Murphy, E., R.E. Nordquist, and F.J. van der Staay, *A review of behavioural methods to study emotion and mood in pigs, Sus scrofa.* Applied Animal Behaviour Science, 2014. **159**: p. 9-28.
96. McGlone, J.J., *Agonistic behavior in food animals: review of research and techniques.* J Anim Sci, 1986. **62**(4): p. 1130-9.
97. Camerlink, I. and S.P. Turner, *The pig's nose and its role in dominance relationships and harmful behaviour.* Applied Animal Behaviour Science, 2013. **145**(3–4): p. 84-91.
98. Cronin, G.M. and P.R. Wiepkema, *An analysis of stereotyped behaviour in tethered sows.* Ann Rech Vet, 1984. **15**(2): p. 263-70.
99. Telkanranta, H., J.N. Marchant-Forde, and A. Valros, *Tear staining in pigs: a potential tool for welfare assessment on commercial farms.* Animal, 2016. **10**(2): p. 318-25.
100. Salak-Johnson, J.L., et al., *Space allowance for gestating sows in pens: behavior and immunity.* J Anim Sci, 2012. **90**(9): p. 3232-42.
101. Forkman, B., et al., *A critical review of fear tests used on cattle, pigs, sheep, poultry and horses.* Physiology & Behavior, 2007. **92**(3): p. 340-374.
102. Ruis, M.A., et al., *Adaptation to social isolation. Acute and long-term stress responses of growing gilts with different coping characteristics.* Physiol Behav, 2001. **73**(4): p. 541-51.
103. Turner, S.P., et al., *The accumulation of skin lesions and their use as a predictor of individual aggressiveness in pigs.* Applied Animal Behaviour Science, 2006. **96**(3): p. 245-259.
104. Chaloupkova, H., et al., *Preweaning housing effects on behavior and physiological measures in pigs during the suckling and fattening periods.* J Anim Sci, 2007. **85**(7): p. 1741-9.
105. Held, S. and M. Spinka, *Animal Play and Animal Welfare.* Animal Behaviour, 2011. **81**: p. 891-899.
106. Boissy, A., et al., *Assessment of positive emotions in animals to improve their welfare.* Physiology & Behavior, 2007. **92**(3): p. 375-397.
107. Colson, V., et al., *Influence of housing and social changes on growth, behaviour and cortisol in piglets at weaning.* Physiology & Behavior, 2012. **107**(1): p. 59-64.
108. Düpjan, S., et al., *Differential vocal responses to physical and mental stressors in domestic pigs (Sus scrofa).* Applied Animal Behaviour Science, 2008. **114**(1): p. 105-115.
109. Cordeiro, A.F.d.S., et al., *Use of vocalisation to identify sex, age, and distress in pig production.* Biosystems Engineering, 2018. **173**: p. 57-63.
110. Alexandersen, S., et al., *Studies of Quantitative Parameters of Virus Excretion and Transmission in Pigs and Cattle Experimentally Infected with Foot-and-Mouth Disease Virus.* Journal of Comparative Pathology, 2003. **129**(4): p. 268-282.
111. Gieling, E.T., R.E. Nordquist, and F.J. van der Staay, *Assessing learning and memory in pigs.* Animal Cognition, 2011. **14**(2): p. 151-173.

112. Sutherland, M.A., et al., *Impacts of chronic stress and social status on various physiological and performance measures in pigs of different breeds.* J Anim Sci, 2006. **84**(3): p. 588-96.
113. Li, Y., et al., *Chronic social stress in pigs impairs intestinal barrier and nutrient transporter function, and alters neuro-immune mediator and receptor expression.* PLoS One, 2017. **12**(2): p. e0171617.
114. Dallman, M.F., et al., *The neural network that regulates energy balance is responsive to glucocorticoids and insulin and also regulates HPA axis responsivity at a site proximal to CRF neurons.* Ann N Y Acad Sci, 1995. **771**: p. 730-42.
115. Liu, J.P., et al., *Studies of the secretion of corticotropin-releasing factor and arginine vasopressin into the hypophysial-portal circulation of the conscious sheep. II. The central noradrenergic and neuropeptide Y pathways cause immediate and prolonged hypothalamic-pituitary-adrenal activation. Potential involvement in the pseudo-Cushing's syndrome of endogenous depression and anorexia nervosa.* J Clin Invest, 1994. **93**(4): p. 1439-50.
116. Adam, T.C. and E.S. Epel, *Stress, eating and the reward system.* Physiol Behav, 2007. **91**(4): p. 449-58.
117. Maniam, J. and M.J. Morris, *The link between stress and feeding behaviour.* Neuropharmacology, 2012. **63**(1): p. 97-110.
118. Reichmann, F. and P. Holzer, *Neuropeptide Y: A stressful review.* Neuropeptides, 2016. **55**: p. 99-109.
119. Hyun, Y., M. Ellis, and R.W. Johnson, *Effects of feeder type, space allowance, and mixing on the growth performance and feed intake pattern of growing pigs.* J Anim Sci, 1998. **76**(11): p. 2771-8.
120. Spurlock, M.E., *Regulation of metabolism and growth during immune challenge: an overview of cytokine function.* J Anim Sci, 1997. **75**(7): p. 1773-83.
121. Johnson, R.W., *Inhibition of growth by pro-inflammatory cytokines: an integrated view.* J Anim Sci, 1997. **75**(5): p. 1244-55.
122. Burguera, B., et al., *Dual and Selective Actions of Glucocorticoids upon Basal and Stimulated Growth Hormone Release in Man.* Neuroendocrinology, 1990. **51**(1): p. 51-58.
123. Raza, J., et al., *Direct effects of corticotrophin-releasing hormone on stimulated growth hormone secretion.* Clin Endocrinol (Oxf), 1998. **48**(2): p. 217-22.
124. Salak-Johnson, J.L., et al., *Space allowance for dry, pregnant sows in pens: body condition, skin lesions, and performance.* J Anim Sci, 2007. **85**(7): p. 1758-69.
125. Schnurrbusch, U., G. Grandjot, and W. Brede, *The Real Pig Handbook*. 4th Edition ed. 2018, Randburg, South Africa: Boehringer Ingelheim Animal Health GmbH.
126. Romero, L.M., M.J. Dickens, and N.E. Cyr, *The reactive scope model — A new model integrating homeostasis, allostasis, and stress.* Hormones and Behavior, 2009. **55**(3): p. 375-389.
127. Harkness, J.E. and M.D. Ridgway, *Chromodacryorrhea in laboratory rats (Rattus norvegicus): etiologic considerations.* Lab Anim Sci, 1980. **30**(5): p. 841-4.
128. Park, S.H., et al., *Localization of porphyrin in mouse Harderian glands.* Arch Histol Cytol, 1996. **59**(2): p. 189-95.

129. Chieffi, G., et al., *Cell biology of the harderian gland.* Int Rev Cytol, 1996. **168**: p. 1-80.
130. Marchant-Forde, A.E.M. and J.N. Marchant-Forde. *Social status and tear staining in nursery pigs*. in *48th Congress of the International Society for Applied Ethology*. 2014. Vitoria-Gasteiz, Spain.
131. DeBoer, S., et al., *An initial investigation into the effects of isolation and enrichment on the welfare of laboratory pigs housed in the PigTurn ® system, assessed using tear staining, behaviour, physiology and haematology.* Animal welfare, 2015. **24**: p. 15-27.
132. Whay, H.R., et al., *Preliminary assessment of finishing pig welfare using animal-based measurements.* Animal Welfare, 2007. **16**(2): p. 209-211.
133. Straw, B.E., et al., *Diseases of swine*. 1999. 1209.
134. Carroll, G.A., et al., *What can carcass-based assessments tell us about the lifetime welfare status of pigs?* Livestock Science, 2018. **214**: p. 98-105.
135. Hessing, M.J.C., et al., *Implications of individual behavioural characteristics on performance in pigs.* Livestock Production Science, 1994. **40**(2): p. 187-196.
136. Robertson, I.D., et al., *Risk factors for gastric ulcers in Australian pigs at slaughter.* Preventive Veterinary Medicine, 2002. **53**(4): p. 293-303.
137. Holinger, M., et al., *Grass silage for growing-finishing pigs in addition to straw bedding: Effects on behaviour and gastric health.* Livestock Science, 2018. **218**: p. 50-57.
138. Cappai, M.G., M. Picciau, and W. Pinna, *Ulcerogenic risk assessment of diets for pigs in relation to gastric lesion prevalence.* BMC Veterinary Research, 2013. **9**(1): p. 36.
139. Wondra, K.J., et al., *Effects of particle size and pelleting on growth performance, nutrient digestibility, and stomach morphology in finishing pigs2.* Journal of Animal Science, 1995. **73**(3): p. 757-763.
140. Casagrande Proietti, P., et al., *Detection of Helicobacter spp. in gastric, fecal and saliva samples from swine affected by gastric ulceration.* J Vet Sci, 2010. **11**(3): p. 221-5.
141. Proctor, G.B., *The physiology of salivary secretion.* Periodontol 2000, 2016. **70**(1): p. 11-25.
142. Dawes, C., et al., *The functions of human saliva: A review sponsored by the World Workshop on Oral Medicine VI.* Archives of Oral Biology, 2015. **60**(6): p. 863-874.
143. Nickel, R. and A. Schummer, *Mouth and pharynx of the pig* in *The viscera of the domestic mammals*, S. E., Editor. 1979, Verlag PaulParey: Berlin und Hamburg. p. 60-63.
144. Boshell, J.L. and W.H. Wilborn, *Histology and ultrastructure of the pig parotid gland.* American Journal of Anatomy, 1978. **152**(4): p. 447-465.
145. Zhou, J., et al., *Histological and Ultrastructural Characterization of Developing Miniature Pig Salivary Glands.* 2010. **293**(7): p. 1227-1239.
146. Scott, B.J., J. Bajaj, and R.W. Linden, *The contribution of mechanoreceptive neurones in the gingival tissues to the masticatory-parotid salivary reflex in man.* J Oral Rehabil, 1999. **26**(10): p. 791-7.

147. Lee, A., S. Guest, and G. Essick, *Thermally evoked parotid salivation.* Physiol Behav, 2006. **87**(4): p. 757-64.
148. Dunér-Engström, M., et al., *Autonomic mechanisms underlying capsaicin induced oral sensations and salivation in man.* J Physiol, 1986. **373**: p. 87-96.
149. Lee, V. and R.J.E.P. Linden, *An olfactory-submandibular salivary reflex in humans.* 1992. **77**.
150. Bosch, J.A., *The use of saliva markers in psychobiology: mechanisms and methods.* Monogr Oral Sci, 2014. **24**: p. 99-108.
151. Edgar, W.M., *Saliva: its secretion, composition and functions.* Br Dent J, 1992. **172**(8): p. 305-12.
152. Humphrey, S.P. and R.T. Williamson, *A review of saliva: normal composition, flow, and function.* J Prosthet Dent, 2001. **85**(2): p. 162-9.
153. de Almeida, P.d.V., et al., *Saliva composition and functions: a comprehensive review.* J Contemp Dent Pract, 2008. **9**(3): p. 72-80.
154. Tryon, A.F. and B.G. Bibby, *Preliminary studies on pig saliva.* Archives of Oral Biology, 1966. **11**(5): p. 527-531.
155. Akhtar, N., et al., *An efficient method for saliva collection from mature pigs to determine their enzymatic and electrolytic profiles.* J Vet Med Sci, 2018. **80**(1): p. 147-151.
156. Proctor, G.B. and G.H. Carpenter, *Salivary secretion: mechanism and neural regulation.* Monogr Oral Sci, 2014. **24**: p. 14-29.
157. Bardow, A., J. Madsen, and B. Nauntofte, *The bicarbonate concentration in human saliva does not exceed the plasma level under normal physiological conditions.* Clin Oral Investig, 2000. **4**(4): p. 245-53.
158. Singh, V., et al., *Comparison of relationship between salivary electrolyte levels and dental caries in children with Down syndrome.* J Nat Sci Biol Med, 2015. **6**(1): p. 144-8.
159. Song, M., et al., *Promising applications of human-derived saliva biomarker testing in clinical diagnostics.* Int J Oral Sci, 2023. **15**(1): p. 2.
160. Bahn, J.H., et al., *The landscape of microRNA, Piwi-interacting RNA, and circular RNA in human saliva.* Clin Chem, 2015. **61**(1): p. 221-30.
161. Dame, Z.T., et al., *The human saliva metabolome.* Metabolomics, 2015. **11**(6): p. 1864-1883.
162. Rzeznik, M., et al., *Identification of a discriminative metabolomic fingerprint of potential clinical relevance in saliva of patients with periodontitis using 1H nuclear magnetic resonance (NMR) spectroscopy.* PLoS One, 2017. **12**(8): p. e0182767.
163. Morgan, L., et al., *Saliva metabolome alterations after acute stress.* Scientific Reports, 2022. **12**(1): p. 18470.
164. Madsen, P.A., et al., *Non-targeted metabolomics of saliva to explore potential biomarkers for gastric ulceration in pigs fed hemp.* Animal, 2022. **16**(3): p. 100477.
165. Goudet, G., et al., *Salivary and urinary metabolome analysis for pre-puberty-related biomarkers identification in porcine.* Animal, 2018: p. 1-11.
166. Goudet, G., et al., *Steroidome and metabolome analysis in gilt saliva to identify potential biomarkers of boar effect receptivity.* Animal, 2020: p. 100095.

167. Fletcher, L., et al., *Identification of Candidate Salivary, Urinary and Serum Metabolic Biomarkers for High Litter Size Potential in Sows (Sus scrofa).* Metabolites, 2022. **12**(11).
168. Denny, P., et al., *The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions.* J Proteome Res, 2008. **7**(5): p. 1994-2006.
169. Bandhakavi, S., et al., *A dynamic range compression and three-dimensional peptide fractionation analysis platform expands proteome coverage and the diagnostic potential of whole saliva.* J Proteome Res, 2009. **8**(12): p. 5590-600.
170. Loo, J.A., et al., *Comparative human salivary and plasma proteomes.* J Dent Res, 2010. **89**(10): p. 1016-23.
171. Gutiérrez, A.M., et al., *Towards Understanding Non-Infectious Growth-Rate Retardation in Growing Pigs.* Proteomes, 2019. **7**(3): p. 31.
172. López-Martínez, M.J., et al., *A Proteomic Approach to Elucidate the Changes in Saliva and Serum Proteins of Pigs with Septic and Non-Septic Inflammation.* Int J Mol Sci, 2022. **23**(12).
173. Gutierrez, A.M., et al., *Proteomic analysis of porcine saliva.* Vet J, 2011. **187**(3): p. 356-62.
174. Muneta, Y., et al., *Interleukin-18 expression in pig salivary glands and salivary content changes during acute immobilization stress.* Stress, 2011. **14**(5): p. 549-56.
175. Gutierrez, A.M., et al., *Detection of potential markers for systemic disease in saliva of pigs by proteomics: a pilot study.* Vet Immunol Immunopathol, 2013. **151**(1-2): p. 73-82.
176. Schmidt, E.M., et al., *Development and validation of an assay for measurement of leptin in pig saliva.* BMC Vet Res, 2016. **12**(1): p. 242.
177. Gutierrez, A., et al., *Influence of different sample preparation strategies on the proteomic identification of stress biomarkers in porcine saliva.* BMC Veterinary Research, 2017. **13**(1): p. 375.
178. Tecles, F., et al., *Total esterase measurement in saliva of pigs: Validation of an automated assay, characterization and changes in stress and disease conditions.* Res Vet Sci, 2017. **114**: p. 170-176.
179. Escribano, D., et al., *Changes in saliva proteins in two conditions of compromised welfare in pigs: An experimental induced stress by nose snaring and lameness.* Res Vet Sci, 2019. **125**: p. 227-234.
180. Li, C., et al., *Identification of Estrus in Sows Based on Salivary Proteomics.* Animals, 2022. **12**(13).
181. Fuentes, M., et al., *Validation of an automated method for salivary alpha-amylase measurements in pigs (Sus scrofa domesticus) and its application as a stress biomarker.* J Vet Diagn Invest, 2011. **23**(2): p. 282-7.
182. Sellers, L. and A. Allen, *Studies on pig and sheep salivary mucins.* Biochemical Society Transactions, 1984. **12**(4): p. 650-650.
183. Muneta, Y., et al., *Salivary IgA as a useful non-invasive marker for restraint stress in pigs.* J Vet Med Sci, 2010. **72**(10): p. 1295-300.

184. Moriyoshi, M., et al., *Early Pregnancy Diagnosis in the Sow by Saliva Progesterone Measurement Using a Bovine Milk Progesterone Qualitative Test EIA Kit.* The Journal of veterinary medical science / the Japanese Society of Veterinary Science, 1996. **58**: p. 737-41.
185. Vining, R.F. and R.A. McGinley, *The measurement of hormones in saliva: possibilities and pitfalls.* J Steroid Biochem, 1987. **27**(1-3): p. 81-94.
186. Cappai, M.G., et al., *Different physical forms of one diet fed to growing pigs induce morphological changes in mandubular glands and local leptin (Ob) production and receptor (ObR) expression.* J Anim Physiol Anim Nutr (Berl), 2016. **100**(6): p. 1067-1072.
187. Sankarganesh, D., et al., *Pig pheromones and behaviors: A review.* Theriogenology, 2021. **175**: p. 1-6.
188. Hattab, J., et al., *Insights into the Oral Bacterial Microbiota of Sows.* Microorganisms, 2021. **9**(11).
189. Fuentes-Rubio, M., et al., *Porcine salivary analysis by 2-dimensional gel electrophoresis in 3 models of acute stress: a pilot study.* Can J Vet Res, 2014. **78**(2): p. 127-32.
190. O'Farrell, P.H., *High resolution two-dimensional electrophoresis of proteins.* J Biol Chem, 1975. **250**(10): p. 4007-21.
191. Lubman, D.M., et al., *Two-dimensional liquid separations-mass mapping of proteins from human cancer cell lysates.* J Chromatogr B Analyt Technol Biomed Life Sci, 2002. **782**(1-2): p. 183-96.
192. Issaq, H.J., et al., *Methods for fractionation, separation and profiling of proteins and peptides.* Electrophoresis, 2002. **23**(17): p. 3048-61.
193. Nägele, E., et al., *2D-LC/MS techniques for the identification of proteins in highly complex mixtures.* Expert Rev Proteomics, 2004. **1**(1): p. 37-46.
194. Betancourt, L.H., et al., *SCX charge state selective separation of tryptic peptides combined with 2D-RP-HPLC allows for detailed proteome mapping.* J Proteomics, 2013. **91**: p. 164-71.
195. Karpievitch, Y.V., et al., *Liquid Chromatography Mass Spectrometry-Based Proteomics: Biological and Technological Aspects.* Ann Appl Stat, 2010. **4**(4): p. 1797-1823.
196. Chen, X., et al., *Quantitative Proteomics Using Isobaric Labeling: A Practical Guide.* Genomics Proteomics Bioinformatics, 2021. **19**(5): p. 689-706.
197. Kulyyassov, A., M. Fresnais, and R. Longuespée, *Targeted liquid chromatography-tandem mass spectrometry analysis of proteins: Basic principles, applications, and perspectives.* Proteomics, 2021. **21**(23-24): p. e2100153.
198. Guerrero, L., A. Paradela, and F. Corrales, *Targeted Proteomics for Monitoring One-Carbon Metabolism in Liver Diseases.* Metabolites, 2022. **12**: p. 779.
199. Strimbu, K. and J.A. Tavel, *What are Biomarkers?* Curr Opin HIV AIDS, 2010. **5**(6): p. 463-466.
200. Tishler, M., et al., *Salivary and serum hyaluronic acid concentrations in patients with Sjogren's syndrome.* Ann Rheum Dis, 1998. **57**(8): p. 506-8.

201. Jou, Y.J., et al., *Proteomic identification of salivary transferrin as a biomarker for early detection of oral cancer.* Anal Chim Acta, 2010. **681**(1-2): p. 41-8.
202. Rudney, J.D., R.K. Staikov, and J.D. Johnson, *Potential biomarkers of human salivary function: a modified proteomic approach.* Arch Oral Biol, 2009. **54**(1): p. 91-100.
203. Rao, P.V., et al., *Proteomic identification of salivary biomarkers of type-2 diabetes.* J Proteome Res, 2009. **8**(1): p. 239-45.
204. Xiao, H., et al., *Proteomic analysis of human saliva from lung cancer patients using two-dimensional difference gel electrophoresis and mass spectrometry.* Mol Cell Proteomics, 2012. **11**(2): p. M111.012112.
205. Floriano, P.N., et al., *Use of saliva-based nano-biochip tests for acute myocardial infarction at the point of care: a feasibility study.* Clin Chem, 2009. **55**(8): p. 1530-8.
206. Ramirez, A., et al., *Efficient surveillance of pig populations using oral fluids.* Prev Vet Med, 2012. **104**(3-4): p. 292-300.
207. Kittawornrat, A., et al., *Kinetics of the porcine reproductive and respiratory syndrome virus (PRRSV) humoral immune response in swine serum and oral fluids collected from individual boars.* BMC Veterinary Research, 2013. **9**(1): p. 61.
208. Prickett, J., et al., *Prolonged Detection of PCV2 and Anti-PCV2 Antibody in Oral Fluids Following Experimental Inoculation.* Transbound Emerg Dis, 2011. **58**: p. 121 - 127.
209. Adcock, S.J.J., G.M. Martin, and C.J. Walsh, *The stress response and exploratory behaviour in Yucatan minipigs (Sus scrofa): Relations to sex and social rank.* Physiol Behav, 2015. **152, Part A**: p. 194-202.
210. Gutierrez, A.M., et al., *Detection and first characterization of an uncommon haptoglobin in porcine saliva of pigs with rectal prolapse by using boronic acid sample enrichment.* Animal, 2016: p. 1-9.
211. Patamia, M., et al., *Two proline-rich peptides from pig (Sus scrofa) salivary glands generated by pre-secretory pathway underlying the action of a proteinase cleaving ProAla bonds.* Peptides, 2005. **26**(9): p. 1550-9.
212. Fanali, C., et al., *Mass spectrometry strategies applied to the characterization of proline-rich peptides from secretory parotid granules of pig (Sus scrofa).* J Sep Sci, 2008. **31**(3): p. 516-22.
213. Tecles, F., et al., *Cholinesterase in porcine saliva: Analytical characterization and behavior after experimental stress.* Res Vet Sci, 2016. **106**: p. 23-8.
214. Escribano, D., et al., *Effect of repeated administration of lipopolysaccharide on inflammatory and stress markers in saliva of growing pigs.* Vet J, 2014. **200**(3): p. 393-7.
215. Escribano, D., et al., *Saliva chromogranin A in growing pigs: a study of circadian patterns during daytime and stability under different storage conditions.* Vet J, 2014. **199**(3): p. 355-9.
216. Ott, S., et al., *Different stressors elicit different responses in the salivary biomarkers cortisol, haptoglobin, and chromogranin A in pigs.* Res Vet Sci, 2014. **97**(1): p. 124-8.

217. Escribano, D., et al., *Changes in saliva biomarkers of stress and immunity in domestic pigs exposed to a psychosocial stressor.* Res Vet Sci, 2015. **102**: p. 38-44.
218. Gomez-Laguna, J., et al., *Haptoglobin and C-reactive protein as biomarkers in the serum, saliva and meat juice of pigs experimentally infected with porcine reproductive and respiratory syndrome virus.* Vet J, 2010. **185**(1): p. 83-7.
219. Gutierrez, A.M., S. Martinez-Subiela, and J.J. Ceron, *Evaluation of an immunoassay for determination of haptoglobin concentration in various biological specimens from swine.* Am J Vet Res, 2009. **70**(6): p. 691-6.
220. Gutierrez, A.M., et al., *C-reactive protein quantification in porcine saliva: a minimally invasive test for pig health monitoring.* Vet J, 2009. **181**(3): p. 261-5.
221. Gutierrez, A.M., et al., *Circadian pattern of acute phase proteins in the saliva of growing pigs.* Vet J, 2013. **196**(2): p. 167-70.
222. Gutierrez, A.M., S. Martinez-Subiela, and J.J. Ceron, *Evaluation of changes in haptoglobin and C-reactive protein concentrations caused by freezing of saliva and meat juice samples collected from healthy and diseased pigs.* Am J Vet Res, 2011. **72**(1): p. 11-7.
223. Gutierrez, A.M., et al., *Longitudinal analysis of acute-phase proteins in saliva in pig farms with different health status.* Animal, 2012. **6**(2): p. 321-6.
224. Soler, L., et al., *Response of salivary haptoglobin and serum amyloid A to social isolation and short road transport stress in pigs.* Res Vet Sci, 2013. **95**(1): p. 298-302.
225. Escribano, D., et al., *Validation of three commercially available immunoassays for quantification of IgA, IgG, and IgM in porcine saliva samples.* Res Vet Sci, 2012. **93**(2): p. 682-7.
226. Soler, L., A. Gutierrez, and J.J. Ceron, *Serum amyloid A measurements in saliva and serum in growing pigs affected by porcine respiratory and reproductive syndrome in field conditions.* Res Vet Sci, 2012. **93**(3): p. 1266-70.
227. Olsen, C., et al., *Effect of collection material and sample processing on pig oral fluid testing results.* Vet J, 2013. **198**(1): p. 158-63.
228. Gutierrez, A.M., et al., *Easy and non-invasive disease detection in pigs by adenosine deaminase activity determinations in saliva.* PLoS One, 2017. **12**(6): p. e0179299.
229. Soler, L., et al., *Towards a better understanding of salivary and meat juice acute phase proteins determination in pigs: an expression study.* Vet Immunol Immunopathol, 2013. **156**(1-2): p. 91-8.
230. Gutierrez, A.M., et al., *Gender influence on the salivary protein profile of finishing pigs.* J Proteomics, 2017. **178**: p. 107-113.
231. Lashley, K.S., *Reflex secretion of the human parotid gland.* J Exp Psychol, 1916. **1**: p. 461-493.
232. Vissink, A., et al., *An adaptation of the lashley cup for use in rat saliva collection.* Arch Oral Biol, 1989. **34**(7): p. 577-578.
233. Knudsen, J., et al., *Effects of isoflurane anesthesia and pilocarpine on rat parotid saliva flow.* Radiat Res, 2011. **176**(1): p. 84-8.

234. Vizcaino, J.A., et al., *2016 update of the PRIDE database and its related tools.* Nucleic Acids Res, 2016. **44**(D1): p. D447-56.
235. Searle, B.C., *Scaffold: a bioinformatic tool for validating MS/MS-based proteomic studies.* Proteomics, 2010. **10**(6): p. 1265-9.
236. Keller, A., et al., *Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search.* Anal Chem, 2002. **74**(20): p. 5383-92.
237. Nesvizhskii, A.I., et al., *A statistical model for identifying proteins by tandem mass spectrometry.* Anal Chem, 2003. **75**(17): p. 4646-58.
238. Altschul, S.F., et al., *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.* Nucleic Acids Res, 1997. **25**(17): p. 3389-402.
239. Bodnar, W.M., et al., *Exploiting the complementary nature of LC/MALDI/MS/MS and LC/ESI/MS/MS for increased proteome coverage.* J Am Soc Mass Spectrom, 2003. **14**(9): p. 971-9.
240. Claassen, M., et al., *Generic comparison of protein inference engines.* Mol Cell Proteomics, 2012. **11**(4): p. O110.007088.
241. Alterman, A., et al., *Functional and proteomic analysis of submandibular saliva in rats exposed to chronic stress by immobilization or constant light.* Arch Oral Biol, 2012. **57**(6): p. 663-669.
242. Fickel, J., et al., *Analysis of parotid and mixed saliva in Roe deer (Capreolus capreolus L.).* J Comp Physiol B, 1998. **168**(4): p. 257-64.
243. Koller, M.M., et al., *A biochemical analysis of parotid and submandibular salivary gland function with age after simultaneous stimulation with pilocarpine and isoproterenol in female NIA Fischer 344 rats.* Arch Oral Biol, 1992. **37**(3): p. 219-230.
244. Marshall, T., et al., *Electrophoretic analysis of stimulated cat parotid saliva.* Electrophoresis, 1993. **14**(12): p. 1328-32.
245. Ito, Y., et al., *Suppression of parasympathetic reflex vasodilatation in the lower lip of the cat by isoflurane, propofol, ketamine and pentobarbital: implications for mechanisms underlying the production of anaesthesia.* Br J Anaesth, 1998. **81**(4): p. 563-8.
246. Kang, J.G., et al., *A prospective, randomized comparison of the effects of inhaled sevoflurane anesthesia and propofol/remifentanil intravenous anesthesia on salivary excretion during laryngeal microsurgery.* Anesth Analg, 2008. **106**(6): p. 1723-7.
247. Martinez, J.R. and N. Cassity, *Secretory responses of the rat submandibular and parotid glands to sequential stimulation with pilocarpine and isoproterenol.* Arch Oral Biol, 1982. **27**(2): p. 159-166.
248. Veerman, E.C., et al., *Human glandular salivas: their separate collection and analysis.* Eur J Oral Sci, 1996. **104**(4 (Pt 1)): p. 346-52.
249. Jasim, H., et al., *The proteomic profile of whole and glandular saliva in healthy pain-free subjects.* Sci Rep, 2016. **6**: p. 39073.

250. Tobin, G., D. Giglio, and B. Gotrick, *Studies of muscarinic receptor subtypes in salivary gland function in anaesthetized rats.* Auton Neurosci, 2002. **100**(1-2): p. 1-9.
251. Lung, M.A., *Variations in blood flow on mandibular glandular secretion to autonomic nervous stimulations in anaesthetized dogs.* J Physiol, 1990. **431**: p. 479-93.
252. Edwards, C.M., P.P. Corkery, and A.V. Edwards, *Submandibular responses to stimulation of the parasympathetic innervation in anesthetized sheep.* J Appl Physiol, 2003. **95**(4): p. 1598-605.
253. Creaser, C.S., et al., *Capillary liquid chromatography/atmospheric-pressure matrix-assisted laser desorption/ionisation ion trap mass spectrometry: a comparison with liquid chromatography/matrix-assisted laser desorption/ionisation time-of-flight and liquid chromatography/electrospray ionisation quadrupole time-of-flight for the identification of tryptic peptides.* Rapid Commun Mass Spectrom, 2006. **20**(5): p. 829-36.
254. Amado, F.M., R.P. Ferreira, and R. Vitorino, *One decade of salivary proteomics: current approaches and outstanding challenges.* Clin Biochem, 2013. **46**(6): p. 506-17.
255. Hu, S., et al., *Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry.* Proteomics, 2005. **5**(6): p. 1714-28.
256. Walz, A., et al., *Proteome analysis of glandular parotid and submandibular-sublingual saliva in comparison to whole human saliva by two-dimensional gel electrophoresis.* Proteomics, 2006. **6**(5): p. 1631-9.
257. Soares, R.V., et al., *Salivary micelles: identification of complexes containing MG2, sIgA, lactoferrin, amylase, glycosylated proline-rich protein and lysozyme.* Arch Oral Biol, 2004. **49**(5): p. 337-43.
258. Gibbins, H.L., et al., *What interactions drive the salivary mucosal pellicle formation?* Colloids Surf B Biointerfaces, 2014. **120**: p. 184-92.
259. Siqueira, W.L., et al., *Proteome of human minor salivary gland secretion.* J Dent Res, 2008. **87**(5): p. 445-50.
260. Barros, S.P., et al., *Gingival crevicular fluid as a source of biomarkers for periodontitis.* Periodontol 2000, 2016. **70**(1): p. 53-64.
261. Huynh, A.H., et al., *Gingival crevicular fluid proteomes in health, gingivitis and chronic periodontitis.* J Periodontal Res, 2015. **50**(5): p. 637-49.
262. Ngo, L.H., et al., *Mass spectrometric analyses of peptides and proteins in human gingival crevicular fluid.* J Proteome Res, 2010. **9**(4): p. 1683-93.
263. Manconi, B., et al., *Modifications of the acidic soluble salivary proteome in human children from birth to the age of 48months investigated by a top-down HPLC-ESI-MS platform.* J Proteomics, 2013. **91**: p. 536-43.
264. Messana, I., et al., *Chrono-proteomics of human saliva: variations of the salivary proteome during human development.* J Proteome Res, 2015. **14**(4): p. 1666-77.
265. Cabras, T., et al., *Age-dependent modifications of the human salivary secretory protein complex.* J Proteome Res, 2009. **8**(8): p. 4126-34.

266. Ang, C.S., et al., *Global survey of the bovine salivary proteome: integrating multidimensional prefractionation, targeted, and glycocapture strategies.* J Proteome Res, 2011. **10**(11): p. 5059-69.
267. Lamy, E., et al., *Sheep and goat saliva proteome analysis: a useful tool for ingestive behavior research?* Physiol Behav, 2009. **98**(4): p. 393-401.
268. de Sousa-Pereira, P., et al., *Cross-species comparison of mammalian saliva using an LC-MALDI based proteomic approach.* Proteomics, 2015. **15**(9): p. 1598-607.
269. Karn, R.C., A.G. Chung, and C.M. Laukaitis, *Shared and unique proteins in human, mouse and rat saliva proteomes: Footprints of functional adaptation.* Proteomes, 2013. **1**(3): p. 275-289.
270. Delius, J., et al., *Label-free quantitative proteome analysis of the surface-bound salivary pellicle.* Colloids Surf B Biointerfaces, 2017. **152**: p. 68-76.
271. Booth, W.D. and C.A. White, *The isolation, purification and some properties of pheromaxein, the pheromonal steroid-binding protein, in porcine submaxillary glands and saliva.* J Endocrinol, 1988. **118**(1): p. 47-57.
272. Kanda, A., et al., *Isolation and characterization of novel tachykinins from the posterior salivary gland of the common octopus Octopus vulgaris.* Peptides, 2003. **24**(1): p. 35-43.
273. Goto, M., et al., *Immunohistochemical demonstration of acidic mammalian chitinase in the mouse salivary gland and gastric mucosa.* Arch Oral Biol, 2003. **48**(10): p. 701-7.
274. Hall, S.C., et al., *Alterations in the Salivary Proteome and N-Glycome of Sjogren's Syndrome Patients.* J Proteome Res, 2017. **16**(4): p. 1693-1705.
275. Abdollahi, M., et al., *Oxidative stress and cholinesterase inhibition in saliva and plasma of rats following subchronic exposure to malathion.* Comp Biochem Physiol C Toxicol Pharmacol, 2004. **137**(1): p. 29-34.
276. Sivadasan, P., et al., *Data from human salivary proteome – A resource of potential biomarkers for oral cancer.* Data in Brief, 2015. **4**: p. 374-378.
277. Escribano, D., et al., *Saliva chromogranin A in growing pigs: A study of circadian patterns during daytime and stability under different storage conditions.* The Veterinary Journal, 2014. **199**(3): p. 355-359.
278. Dawes, C. and B.Y. Ong, *Circadian rhythms in the flow rate and proportional contribution of parotid to whole saliva volume in man.* Arch Oral Biol, 1973. **18**(9): p. 1145-53.
279. Roux-Dalvai, F., et al., *Extensive analysis of the cytoplasmic proteome of human erythrocytes using the peptide ligand library technology and advanced mass spectrometry.* Mol Cell Proteomics, 2008. **7**(11): p. 2254-69.
280. Guerrier, L., P.G. Righetti, and E. Boschetti, *Reduction of dynamic protein concentration range of biological extracts for the discovery of low-abundance proteins by means of hexapeptide ligand library.* Nat Protoc, 2008. **3**(5): p. 883-90.
281. Thotakura, N.R. and O.P. Bahl, *Enzymatic deglycosylation of glycoproteins*, in *Methods in Enzymology*. 1987, Academic Press. p. 350-359.
282. Ramachandran, P., et al., *Comparison of N-linked Glycoproteins in Human Whole Saliva, Parotid, Submandibular, and Sublingual Glandular Secretions Identified*

using Hydrazide Chemistry and Mass Spectrometry. Clin Proteomics, 2008. **4**(3-4): p. 80-104.
283. Prunier, A., A.M. Mounier, and M. Hay, *Effects of castration, tooth resection, or tail docking on plasma metabolites and stress hormones in young pigs.* J Anim Sci, 2005. **83**(1): p. 216-22.
284. McGlone, J.J., et al., *The development of pain in young pigs associated with castration and attempts to prevent castration-induced behavioral changes.* J Anim Sci, 1993. **71**(6): p. 1441-6.
285. Raeside, J.I., R.M. Friendship, and O.E. Vrablic, *Effects of castration on early postnatal development of male accessory sex glands in the domestic pig.* Eur J Endocrinol, 1997. **137**(3): p. 287-92.
286. Handa, R.J., et al., *Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis.* Horm Behav, 1994. **28**(4): p. 464-76.
287. Shirtcliff, E.A., et al., *Salivary cortisol results obtainable within minutes of sample collection correspond with traditional immunoassays.* Clin Ther, 2015. **37**(3): p. 505-14.
288. Thomsson, O., et al., *Validation of an enzyme-linked immunosorbent assay developed for measuring cortisol concentration in human saliva and serum for its applicability to analyze cortisol in pig saliva.* Acta Veterinaria Scandinavica, 2014. **56**(1): p. 55.
289. Salaberger, T., et al., *Influence of external factors on hair cortisol concentrations.* General and Comparative Endocrinology, 2016. **233**: p. 73-78.
290. Davenport, M.D., et al., *Analysis of endogenous cortisol concentrations in the hair of rhesus macaques.* General and Comparative Endocrinology, 2006. **147**(3): p. 255-61.
291. Rodarte, L.F., et al., *The effect of environmental manipulation on behavior, salivary cortisol, and growth of piglets weaned at 14 days of age.* J Appl Anim Welf Sci, 2004. **7**(3): p. 171-9.
292. Holinger, M., et al., *Chronic intermittent stress exposure and access to grass silage interact differently in their effect on behaviour, gastric health and stress physiology of entire or castrated male growing-finishing pigs.* Physiol Behav, 2018. **195**: p. 58-68.
293. Prims, S., et al., *Hair or salivary cortisol analysis to identify chronic stress in piglets?* The Veterinary Journal, 2019. **252**: p. 105357.
294. Marple, D.N., et al., *Endocrine responses of stress susceptible and stress resistant swine to environmental stressors.* J Anim Sci, 1972. **35**(3): p. 576-9.
295. Becker, B., et al., *Serum cortisol concentrations in gilts and sows housed in tether stalls, gestation stalls and individual pens.* Ann Rech Vet, 1984. **15**(2): p. 237-42.
296. Merlot, E., A.M. Mounier, and A. Prunier, *Endocrine response of gilts to various common stressors: a comparison of indicators and methods of analysis.* Physiol Behav, 2011. **102**(3-4): p. 259-65.
297. Prims, S., et al., *On the characterisation of the porcine gland-specific salivary proteome.* Journal of Proteomics, 2019. **196**: p. 92-105.

298. Parrott, R.F. and B.H. Misson, *Changes in pig salivary cortisol in response to transport simulation, food and water deprivation, and mixing.* British Veterinary Journal, 1989. **145**(6): p. 501-505.
299. Cerón, J.J., et al., *Basics for the potential use of saliva to evaluate stress, inflammation, immune system, and redox homeostasis in pigs.* BMC Veterinary Research, 2022. **18**(1): p. 81.
300. Westbrook, J.A., et al., *Quantitation with chemical tagging reagents in biomarker studies.* Proteomics Clin Appl, 2015. **9**(3-4): p. 295-300.
301. Tyanova, S., T. Temu, and J. Cox, *The MaxQuant computational platform for mass spectrometry-based shotgun proteomics.* Nat Protoc, 2016. **11**(12): p. 2301-2319.
302. Mitchell, G.B., et al., *Stress alters the cellular and proteomic compartments of bovine bronchoalveolar lavage fluid.* Veterinary Immunology and Immunopathology, 2008. **125**(1): p. 111-125.
303. Kang, J.-H. and H.-S. Kho, *Blood contamination in salivary diagnostics: current methods and their limitations.* Clin Chem Lab Med, 2019. **57**(8): p. 1115-1124.
304. Demirhan, H., et al., *The Role of Fetuin-A and Electrolytes in the Etiology of Sialolithiasis.* Otolaryngol Head Neck Surg, 2017. **156**(5): p. 840-843.
305. Kivlighan, K.T., et al., *Quantifying blood leakage into the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva.* Horm Behav, 2004. **46**(1): p. 39-46.
306. Kang, J.-H., Y.-H. Lee, and H.-S. Kho, *Clinical factors affecting salivary transferrin level, a marker of blood contamination in salivary analysis.* BMC oral health, 2018. **18**(1): p. 49-49.
307. MacLean, B., et al., *Skyline: an open source document editor for creating and analyzing targeted proteomics experiments.* Bioinformatics, 2010. **26**(7): p. 966-968.
308. Gessulat, S., et al., *Prosit: proteome-wide prediction of peptide tandem mass spectra by deep learning.* Nature Methods, 2019. **16**(6): p. 509-518.
309. Dabrowska, A.M., et al., *Fetuin-A (AHSG) and its usefulness in clinical practice. Review of the literature.* Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 2015. **159**(3): p. 352-9.
310. Lebreton, J.P., et al., *Serum concentration of human alpha 2 HS glycoprotein during the inflammatory process: evidence that alpha 2 HS glycoprotein is a negative acute-phase reactant.* The Journal of Clinical Investigation, 1979. **64**(4): p. 1118-1129.
311. Dziegielewska, K.M., N.A. Andersen, and N.R. Saunders, *Modification of macrophage response to lipopolysaccharide by fetuin.* Immunol Lett, 1998. **60**(1): p. 31-5.
312. Ramsey, J.M., et al., *Sex Differences in Serum Markers of Major Depressive Disorder in the Netherlands Study of Depression and Anxiety (NESDA).* PLoS One, 2016. **11**(5): p. e0156624.
313. Bot, M., et al., *Serum proteomic profiling of major depressive disorder.* Transl Psychiatry, 2015. **5**(7): p. e599.

314. Mancuso, E., et al., *Effects of Alpha-2-HS-glycoprotein on cognitive and emotional assessment in prediabetic and diabetic subjects.* Journal of Affective Disorders, 2021. **282**: p. 700-706.
315. Gorr, S.U., S.G. Venkatesh, and D.S. Darling, *Parotid secretory granules: crossroads of secretory pathways and protein storage.* Journal of dental research, 2005. **84**(6): p. 500-509.
316. Abdolhosseini, M., et al., *Human parotid secretory protein is a lipopolysaccharide-binding protein: identification of an anti-inflammatory peptide domain.* Molecular and cellular biochemistry, 2012. **359**(1-2): p. 1-8.
317. Ngounou Wetie, A.G., et al., *Comparative two-dimensional polyacrylamide gel electrophoresis of the salivary proteome of children with autism spectrum disorder.* J Cell Mol Med, 2015. **19**(11): p. 2664-78.
318. Edwards, A.V. and D.A. Titchen, *Autonomic control of protein production by the parotid gland of the sheep.* Autonomic Neuroscience, 2003. **103**(1): p. 38-49.
319. Gorman, A.L. and A.J. Dunn, *β-adrenergic receptors are involved in stress-related behavioral changes.* Pharmacology Biochemistry and Behavior, 1993. **45**(1): p. 1-7.
320. Kivela, J., et al., *Salivary carbonic anhydrase isoenzyme VI.* J Physiol, 1999. **520 Pt 2**: p. 315-20.
321. Cabras, T., et al., *Structural and functional characterization of the porcine proline-rich antifungal peptide SP-B isolated from salivary gland granules.* J Pept Sci, 2008. **14**(3): p. 251-60.
322. Levine, M., *Susceptibility to Dental Caries and the Salivary Proline-Rich Proteins.* International Journal of Dentistry, 2011. **2011**: p. 953412.
323. Mehansho, H., et al., *Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels.* Proceedings of the National Academy of Sciences of the United States of America, 1983. **80**(13): p. 3948-3952.
324. Caldin, M., et al., *Serum acute phase protein concentrations in dogs with hyperadrenocorticism with and without concurrent inflammatory conditions.* Vet Clin Pathol, 2009. **38**(1): p. 63-8.
325. Lee, C.G., et al., *Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury.* Annu Rev Physiol, 2011. **73**: p. 479-501.
326. Garibotti, M., et al., *Porcine VEG Proteins and Tear Prealbumins.* Chemical Senses, 1995. **20**(1): p. 69-76.
327. Holzfeind, P., et al., *Structure and organization of the porcine LCN1 gene encoding Tear lipocalin/von Ebner's gland protein.* Gene, 1997. **202**(1): p. 61-67.
328. Pelosi, P., *Odorant-binding proteins.* Crit Rev Biochem Mol Biol, 1994. **29**(3): p. 199-228.
329. Marchese, S., et al., *Lipocalins of boar salivary glands binding odours and pheromones.* Eur J Biochem, 1998. **252**(3): p. 563-8.
330. Baldini, C., et al., *Proteomic analysis of the saliva: a clue for understanding primary from secondary Sjögren's syndrome?* Autoimmun Rev, 2008. **7**(3): p. 185-91.
331. Khew-Goodall, Y., et al., *Vomeromodulin, a putative pheromone transporter: cloning, characterization, and cellular localization of a novel glycoprotein of lateral nasal gland.* Faseb j, 1991. **5**(14): p. 2976-82.

332. Krishna, N.S., M.L. Getchell, and T.V. Getchell, *Expression of the putative pheromone and odorant transporter vomeromodulin mRNA and protein in nasal chemosensory mucosae.* J Neurosci Res, 1994. **39**(3): p. 243-59.
333. Bingle, C.D., R.L. Seal, and C.J. Craven, *Systematic nomenclature for the PLUNC/PSP/BSP30/SMGB proteins as a subfamily of the BPI fold-containing superfamily.* Biochem Soc Trans, 2011. **39**(4): p. 977-83.
334. Zeng, F., et al., *Genetically Engineered Pigs as Efficient Salivary Gland Bioreactors for Production of Therapeutically Valuable Human Nerve Growth Factor.* 2022. **11**(15): p. 2378.
335. Shirtcliff, E.A., et al., *Use of salivary biomarkers in biobehavioral research: cotton-based sample collection methods can interfere with salivary immunoassay results.* Psychoneuroendocrinology, 2001. **26**(2): p. 165-73.
336. DeCaro, J.A., *Methodological considerations in the use of salivary alpha-amylase as a stress marker in field research.* Am J Hum Biol, 2008. **20**(5): p. 617-9.
337. Gröschl, M., et al., *Evaluation of saliva collection devices for the analysis of steroids, peptides and therapeutic drugs.* J Pharm Biomed Anal, 2008. **47**(3): p. 478-86.
338. Conte, S., et al., *On-farm evaluation of methods to assess welfare of gestating sows.* Animal, 2014. **8**(7): p. 1153-61.
339. Giergiel, M., et al., *The Markers of Stress in Swine Oral Fluid.* J Vet Res, 2021. **65**(4): p. 487-495.
340. Ekkel, E.D., et al., *The circadian rhythm of cortisol in the saliva of young pigs.* Physiology & Behavior, 1996. **60**(3): p. 985-989.
341. Saco, Y., et al., *Influence of the circadian cycle, sex and production stage on the reference values of parameters related to stress and pathology in porcine saliva.* Porcine Health Manag, 2023. **9**(1): p. 42.
342. Gallagher, N.L., L.R. Giles, and P.C. Wynn, *The development of a circadian pattern of salivary cortisol secretion in the neonatal piglet.* Biol Neonate, 2002. **81**(2): p. 113-8.
343. Escribano, D., M. Fuentes-Rubio, and J.J. Cerón, *Salivary testosterone measurements in growing pigs: validation of an automated chemiluminescent immunoassay and its possible use as an acute stress marker.* Research in Veterinary Science, 2014. **97**(1): p. 20-25.
344. Escribano, D., et al., *Stability of selected enzymes in saliva of pigs under different storage conditions: a pilot study.* J Vet Med Sci, 2018.
345. Barranco, T., et al., *Changes of salivary biomarkers under different storage conditions: effects of temperature and length of storage.* Biochem Med (Zagreb), 2019. **29**(1): p. 010706.
346. Garde, A.H. and A.M. Hansen, *Long-term stability of salivary cortisol.* Scand J Clin Lab Invest, 2005. **65**(5): p. 433-6.
347. d'Amone, L., G. Matzeu, and F.G. Omenetto, *Stabilization of Salivary Biomarkers.* ACS Biomater Sci Eng, 2021. **7**(12): p. 5451-5473.

348. Ortín-Bustillo, A., et al., *Changes in S100A8/A9 and S100A12 and Their Comparison with Other Analytes in the Saliva of Pigs with Diarrhea Due to E. coli.* Animals (Basel), 2023. **13**(16).
349. Sánchez, J., et al., *Analytical characterization of trace elements (zinc, copper, cadmium, lead and selenium) in saliva of pigs under common pathological conditions in the field: a pilot study.* BMC Veterinary Research, 2020. **16**(1): p. 27.
350. Contreras-Aguilar, M.D., et al., *Detection and measurement of alpha-amylase in canine saliva and changes after an experimentally induced sympathetic activation.* BMC Vet Res, 2017. **13**(1): p. 266.
351. Franco-Martínez, L., et al., *Effects of pen faeces and feed contamination in biomarkers determination in oral fluid of pigs.* Res Vet Sci, 2022. **152**: p. 403-409.
352. Contreras-Aguilar, M.D., et al., *Influence of the way of reporting alpha-Amylase values in saliva in different naturalistic situations: A pilot study.* PLOS ONE, 2017. **12**(6): p. e0180100.
353. Prims, S., et al., *Chronic exposure to multiple stressors alters the salivary proteome of piglets.* PLoS One, 2023. **18**(5): p. e0286455.
354. Sánchez, J., et al., *Porcine Breed, Sex, and Production Stage Influence the Levels of Health Status Biomarkers in Saliva Samples.* 2019. **6**.
355. Xin, H., et al., *Quantitative proteomic analysis and verification identify global protein profiling dynamics in pig during the estrous cycle.* Front Vet Sci, 2023. **10**: p. 1247561.
356. Liu, H., et al., *Behavior and physiology of two different sow breeds in a farrowing environment during late 35-day lactation.* PLoS One, 2018. **13**(5): p. e0197152.
357. Sutherland, M.A., et al., *Breed and age affect baseline immune traits, cortisol, and performance in growing pigs.* J Anim Sci, 2005. **83**(9): p. 2087-95.
358. Kamodyova, N., et al., *Blood Contamination in Saliva: Impact on the Measurement of Salivary Oxidative Stress Markers.* Dis Markers, 2015. **2015**: p. 479251.
359. Durdiaková, J., et al., *The effects of saliva collection, handling and storage on salivary testosterone measurement.* Steroids, 2013. **78**(14): p. 1325-31.
360. Granger, D.A., et al., *Blood contamination in children's saliva: prevalence, stability, and impact on the measurement of salivary cortisol, testosterone, and dehydroepiandrosterone.* Psychoneuroendocrinology, 2007. **32**(6): p. 724-33.
361. Contreras-Aguilar, M.D., et al., *Effect of food contamination and collection material in the measurement of biomarkers in saliva of horses.* Research in Veterinary Science, 2020. **129**: p. 90-95.
362. Franco-Martínez, L., et al., *Effects of pen faeces and feed contamination in biomarkers determination in oral fluid of pigs.* Research in Veterinary Science, 2022. **152**: p. 403-409.
363. López-Arjona, M., et al., *Changes in oxytocin concentrations in saliva of pigs after a transport and during lairage at slaughterhouse.* Res Vet Sci, 2020. **133**: p. 26-30.
364. Miočević, O., et al., *Quantitative Lateral Flow Assays for Salivary Biomarker Assessment: A Review.* Front Public Health, 2017. **5**: p. 133.
365. Khanna, P. and D.R. Walt, *Salivary Diagnostics Using a Portable Point-of-Service Platform: A Review.* Clinical Therapeutics, 2015. **37**(3): p. 498-504.

366. Christodoulides, N., et al., *Lab-on-a-chip methods for point-of-care measurements of salivary biomarkers of periodontitis.* Ann N Y Acad Sci, 2007. **1098**: p. 411-28.
367. Alonso, M.E., J.R. González-Montaña, and J.M. Lomillos, *Consumers' Concerns and Perceptions of Farm Animal Welfare.* Animals (Basel), 2020. **10**(3).