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Prosjektretning

Changes in tryptophan and kynurenine concentrations in pig plasma after in vivo immune activation and controlled tissue trauma

Endringer i tryptofan og kynurenin konsentrasjoner i griseplasma etter in vivo immunaktivering og kontrollert vevstraume

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Veileder: Janicke Nordgreen

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Summary (English)

Title: *Changes in tryptophan and kynurenine concentrations in pig plasma after in vivo immune activation and controlled tissue trauma.*

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Abbreviations

5 - HT	5 – Hydroxytryptamine
AA	Amino acid
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
BBB	Blood – brain barrier
CRP	C – reactive protein
HPLC/MS	High performance liquid chromatography/mass spectrometry
IDO	Indoleamine 2,3 – dioxygenase
Kyn	Kynurenine
KYNA	Kynurenic acid
LAT – 1	L – type amino acid transporter 1
LNAA	Large neutral amino acids
LPS	Lipopolysaccharide
PD	Parkinson's disease
SAL	Saline
TDO	Tryptophan 2,3 – dioxygenase
TH	Tryptophan Hydroxylase
Trp	Tryptophan
QUIN	Quinolinic acid

Abstract

The main purpose of this thesis is to describe the changes in plasma concentrations of kynurenine and tryptophan in pig plasma after an LPS - injection and a surgical procedure. Pigs are frequently exposed to stressors, such as infections and tissue trauma in production settings. From other species we know that changes in amino acid metabolism is linked to changes in mood and behavior. However, the time-course of changes in amino-acid metabolism has rarely been described in pigs and doing so is an important step towards understanding how immunological stressors can change pig physiology, mood and behavior. The pigs were exposed to two different events that triggered a stress response and activated the immune system; 1) surgery to insert a permanent central venous catheter and 2) an injection of LPS. We measured the amount of kynurenine and tryptophan in their plasma. In addition, we had data on the CRP levels in the same plasma samples. The effect of surgery and LPS on these physiological variables were analyzed in JMP. As predicted, there was an increase in the amount of kynurenine and a decrease in the amount of tryptophan over time in relation to LPS. However, tissue trauma and LPS did not have the same effect on the kyn/trp ratio, which was not as predicted. Our results also showed that there was an association between CRP, tryptophan and kynurenine as predicted.

1. Introduction

Pigs are frequently exposed to stressful events, such as tissue trauma and inflammation, and their mood and behavior can be affected as a result. Changes in mood and behavior not only influence the individual, but also the group that the individual belongs to. Tailbiting is a common behavioral problem in pigs, which occurs under suboptimal conditions, where the pigs are exposed to increased stress loads. There is evidence of a correlation between changes in brain physiology and such behavior (Valros et al., 2015).

Stress is commonly defined as a bodily response to acute challenges (McEwen, 2017), such as an infection or a surgical procedure. After an acute challenge, it requires a certain amount of physiological effort to reestablish the normal in the individual, and this process is known as *allostasis*. The allostatic systems can be either overstimulated or malfunction. This condition is referred to as *allostatic load*, and a chronic allostatic load/stress response can lead to pathology over time (McEwen, 1998).

Changes in physiology due to stress and inflammation can influence our mood and behavior. Cytokines are mediators of inflammation and are thought to bring about these physiological changes. Behavioral changes that occur during immune activation are mediated by complex interactions between various cytokines (Avitsur and Yirmiya, 1999). When an animal is sick, it will show sickness behavior. Sickness behavior is a strategy to cope with infection and other immunological challenges, mediated by changes in physiology (Dantzer et al., 2008). In humans, cytokines can regulate social behavior in both pathological (infections) and non- pathological conditions (Hennessy et al., 2014). For instance, immune activation due to inflammation is reported to induce feelings of social disconnectedness in addition to depressed mood in humans

(Eisenberger et al., 2010). Cytokine immunotherapy in humans has also been shown to induce depressive symptoms in individuals with no prior signs of mood alterations (Hughes et al., 2012). The neurodegenerative and inflammatory hypothesis of depression also makes it clear that cytokines can cause changes in mood (Hughes et al., 2012, Dantzer et al., 2008, Eisenberger et al., 2010, Maes et al., 2009, Myint et al., 2007, Dantzer et al., 2011).

There are several mechanisms that could explain how the immune system, through cytokines, influences mood and behavior. Many of these mechanisms include tryptophan metabolism (Hoglund et al., 2019, Dantzer et al., 2011, Dantzer et al., 2008, Le Floc'h et al., 2011). Tryptophan is the precursor of both kynurenine and serotonin. Tryptophan depletion is associated with depression in humans (Hughes et al., 2012). Alterations in the levels of tryptophan, serotonin and kynurenine can cause mood changes and reduced well-being (Hughes et al., 2012, Myint et al., 2007, Eisenberger et al., 2010).

Tryptophan is an essential amino acid. Compared to the other 20 amino acids in the body, tryptophan is found in the lowest amount in both proteins and plasma. Only 10-20 percent of the body content of tryptophan is present as free form in plasma. Unlike other amino acids, tryptophan is found in blood and plasma mainly bound to albumin (Le Floc'h et al., 2011). The amount of tryptophan available for kynurenine and serotonin synthesis can change quite easily, due to the fact that the body content of tryptophan is low, and that tryptophan is in demand to fulfill a diversity of physiological functions (Le Floc'h et al., 2011). It is estimated that 95% of the tryptophan fraction that is not used for protein synthesis is metabolized through the kynurenine pathway, while less than 5% is transformed into serotonin (Botting, 1995, Ruddick et al., 2006).

Tryptophan is transported into the brain, through the blood brain barrier (BBB) by the transporter LAT -1, which also transports large neural amino acids (LNAA). Tryptophan entry into the brain is thus regulated by the tryptophan/LNAA ratio (Pardridge, 1998).

Tryptophan is converted into kynurenine by two enzymes; TDO and IDO, as illustrated in figure 1. IDO is ubiquitous in the body (brain tissue, intestinal tissue, placenta and immune cells), while TDO is mainly found in the liver (Botting, 1995). While TDO is quite specific for tryptophan, IDO also has a substrate affinity for serotonin and melatonin in addition to tryptophan (Shimizu et al., 1978). The activity of TDO is regulated by the availability of tryptophan, due to an allosteric binding site, and the presence of glucocorticoids. It has been shown that increased tryptophan levels reduce the degradation of TDO, and that the administration of hydrocortisone increases TDO synthesis (Schimke et al., 1965). IDO, unlike TDO, is not activated by glucocorticoids and does not have any activating sites for tryptophan analogues. However, IDO is activated by cytokines, such as IFN gamma (Botting, 1995). IDO has been activated by LPS in a porcine model (Wirthgen et al., 2014). Unregulated overexpression of the IDO enzyme can result in a systemic tryptophan depletion and an accumulation of neurotoxic metabolites of the kynurenine pathway, for instance quinolinic acid (Takikawa, 2005). IDO also has antimicrobial and antitumor properties, because of its ability to deplete cells of tryptophan, and tryptophan is necessary for normal protein synthesis and cell growth (Botting, 1995).

Tryptophan is converted into serotonin by the enzyme tryptophan hydroxylase, which exists in two forms; TPH2 (tryptophan hydroxylase 2), mainly found in neurons, and TPH1 (tryptophan hydroxylase 1), found in enterochromaffin cells in the intestine (Hoglund et al., 2019). The TPH enzymes are not saturated at physiological tryptophan levels, which means that the relationship

between tryptophan and serotonin is concentration dependent, and that brain serotonin can be increased following extra dietary tryptophan and tryptophan levels in plasma (Young and Gauthier, 1981).

The kynurenine pathway (KP) consists of the conversion of tryptophan to kynurenine and the conversion of kynurenine to other metabolites, as illustrated in Figure 1.

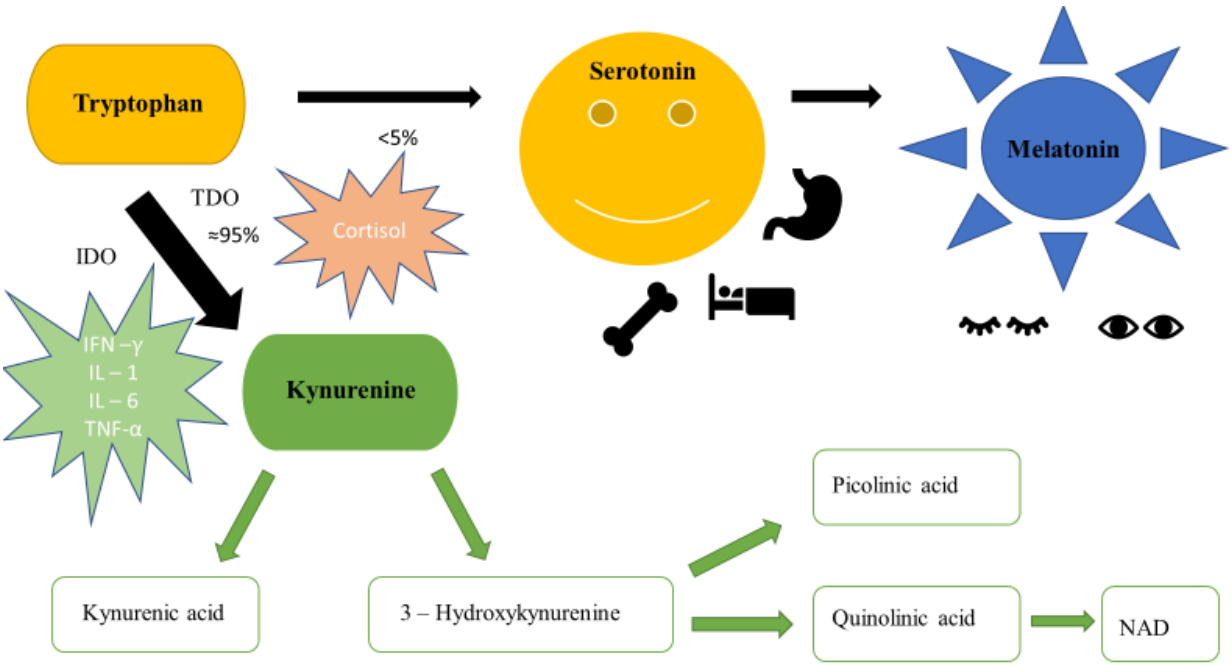

Figure 1. Major metabolic pathways of tryptophan. IDO (indoleamine 2, 3 - dioxygenase), TDO (tryptophan 2, 3 – dioxygenase). It is estimated that 95% of the tryptophan fraction that is not used for protein synthesis is metabolized through the kynurenine pathway, while less than 5% is transformed into serotonin. The activity of IDO is influenced by cytokines, while the activity of TDO is influenced by glucocorticoids.

Kynurenine is the precursor of both neurotoxic, immune regulatory, and antioxidative metabolites (Dantzer et al., 2008, Le Floc'h et al., 2011, Hoglund et al., 2019). One of the neurotoxic metabolites is quinolinic acid. Quinolinic acid (QA) is an NMDA receptor agonist. The NMDA receptor activation seems to play a role in LPS – induced depressive- like behavior in mice (Walker et al., 2013). Quinolinic acid and other metabolites of kynurenine also play a part in aging and different neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and Alzheimer's disease (AD) (Tan et al., 2012). As mentioned above, kynurenine is also the precursor of antioxidant metabolites that are not neurotoxic, such as kynurenic acid. Reduced kynurenic acid has been shown to play a part in human female mood alterations (Meier et al., 2018). An imbalance in the production of downstream KP metabolites is suggested to be the basis of the neurodegenerative hypothesis of depression and the inflammatory hypothesis of depression in humans (Hughes et al., 2012, Maes et al., 2009, Myint and Kim, 2003, Dantzer et al., 2011).

Serotonin, or 5 – hydroxytryptamine, is a monoamine neurotransmitter that is known to influence mood (Young and Leyton, 2002, Jenkins et al., 2016). Serotonin also has multiple other functions such as the regulation of appetite, sleep, GI motility and bone metabolism (Hoglund et al., 2019). Acute tryptophan depletion (ATD) has been used as a technique to investigate the link between tryptophan and serotonin levels, where lowered dietary tryptophan levels has led to a lowering of brain serotonin levels in several species, including mice (Biskup et al., 2012) and primates (Young et al., 1989).

Exposure to LPS simulates a real-life infection with a bacterial endotoxin, which results in the production of proinflammatory cytokines (TNF-α, IL-1 and IFN-γ) by macrophages and

endothelial cells. LPS is one of the strongest immune stimulants known, because it activates nearly every immune mechanism in the body (Zachary, James K., McGavin, M. Donald 2012, 2007, 2001, 1995, 1988, *Pathologic Basis of Veterinary Disease*, Fifth edition, Penny Rudolph, 3251 Riverport Lane, St. Louis, Missouri 63043, Section 1, Chapter 3, p. 92, BOX 3-2, p.135; Chapter 4 p.163, Fig. 4-17, cont`d).

Several articles describe changes in physiology and behavior after exposure to low doses of LPS (Eisenberger et al., 2010, Nordgreen et al., 2018, Walker et al., 2013). In mice, a single dose injection of LPS (LPS+SAL) has resulted in elevated serum kynurenine levels at 24 and 48 hours, and elevated serum kyn/trp ratio levels at 48 hours post injection (Larsson et al., 2016). The dose of LPS used, was similar to that which has shown to induce sickness behavior and depressive like symptoms in mice (O'Connor et al., 2009).

Tissue trauma during surgery also results in immune activation. Damaged tissue goes through four phases to repair the injury: hemostasis, acute inflammation, granulation and remodeling. During the acute inflammation phase, cytokines like IL – 1, TNF – α and IFN – γ, are produced (Zachary, James K., McGavin, M. Donald 2012, 2007, 2001, 1995, 1988, *Pathologic Basis of Veterinary Disease*, Fifth edition, Penny Rudolph, 3251 Riverport Lane, St. Louis, Missouri 63043, Section 1, Chapter 3, p. 92, BOX 3-2, p. 135; Chapter 4 p.163, Fig. 4-17, cont`d).

After its discovery in the 1930's (Tillett and Francis, 1930), C – reactive protein (CRP) has been used successfully as a marker of inflammation, whether caused by infection or tissue trauma (Abernethy and Avery, 1941, Mortensen, 2001). It is therefore interesting to compare the levels of CRP to kynurenine and tryptophan levels from before and after tissue trauma and in relation to the LPS injection, to see whether there is an association. Based on the previously stated

knowledge, one could hypothesize that an increase in CRP would correlate with the release of pro - inflammatory cytokines which activate IDO and leads to an increase in levels of kynurenine and possibly tryptophan depletion. Both CRP and other serum acute phase proteins are used as markers of inflammation in pigs, for instance when considering swine health status (Chen et al., 2003) or the inflammatory response from tail biting in pigs (Heinonen et al., 2010).

An increase in cytokines and glucocorticoids, during inflammation and stress, can lead to an increase in the activity of IDO and TDO, and thus, an increase in kynurenine levels and a reduction in tryptophan levels. However, it is important to emphasize that kynurenine is produced all the time, not only when the individual is exposed to stressors, given that 95% of the tryptophan fraction that is not used for protein synthesis, is converted down the kynurenine pathway as mentioned above. It is the long-term change of this pathway that might lead to pathological changes, for instance a systemic depletion of tryptophan and serotonin, which again could alter mood and behavior over time.

Aims and objectives

In this project, our main aim was to describe the effect of in vivo immune activation following an LPS injection and tissue trauma (surgery), on the concentrations of tryptophan and kynurenine in pig plasma. We also wanted to compare the effect of tissue trauma and LPS on the kynurenine/tryptophan ratio, and test whether there is an association between the levels of CRP, kynurenine and tryptophan. We predicted: 1) That there will be an increase in the amount of kynurenine and a decrease in the amount of tryptophan over time, in relation to the LPS injection and surgery, 2) That tissue trauma and LPS will have a similar effect on the kyn/trp ratio and 3) That there is an association between CRP, kynurenine and tryptophan.

2. Materials and Methods

The samples analyzed to test the hypotheses in this thesis was taken from the pigs described in (Nordgreen et al., 2018). Thus, parts of the materials and methods section is based on descriptions in that paper. The analyses of tryptophan and kynurenine presented here were carried out specifically for this thesis and have not been presented before.

2.1 Experimental design, ethical permit, animals and husbandry

The experiment was approved by the national animal research authority (FOTS ID 7002). Sixteen female pigs were included in the experiment. The pigs were kept in a facility for a period prior to the experiment, so that they could get used to the environment and the people around them. The pigs had ad lib access to feed and water. The feeders were weighed and refilled every afternoon. To document feed intake. the amount of concentrate pellet feed was weighed in the morning every 24h. All pigs went through surgery to insert a permanent venous catheter in their jugular vein, to be able to sample blood at different points in time. Blood samples in relation to surgery were taken just before surgery, and then 24 and 48 hours after surgery. After surgery, half of the pigs were assigned to the LPS group, and the other half to the saline control group. LPS or saline was injected 5 or 6 days after surgery. Blood was sampled 30 min before the LPS injection (time point, 0, baseline) and 1,2,3,4,6,8,12,24 and 72 hours after injection. The blood samples were analyzed for tryptophan, kynurenine and CRP.

In this thesis, I will focus on the results following blood sampling at different time points post-LPS injection and in relation to the surgical procedure. Sixteen female pigs were used in the study. The breed was a mixture between Landrace Yorkshire and Duroc. All pigs were transported to the facility unit from their farm on the day of weaning, approximately at five

weeks of age. According to the farmers, the pigs had no previous history of illness or received medication. The pigs of the two different replicates came from two different farms within one hour`s driving distance from the research facility. There were eight individuals in each replicate, consisting of four sister pairs. One pig per sister pair received the LPS injection, and the other sister received the SAL injection (control). To balance out the allocation of treatment, an equal amount of pigs were placed further away from and closer to the entrance of the room. The sister pairs were not entirely separated and could have limited tactile contact through the fence dividing the pens, as well as see and hear each other through the pen divide. Their diet consisted of a standard piglet diet (Ideal Junior, Norgesfor, Oslo, Norway). The pens measured 115cmx163cm and had solid concrete flooring and a rubber mat covering parts of the pen. Wood shavings and hay were added after the pens were cleaned every day. The room had a temperature of between 18 to 22 degrees Celsius. The venous catheters were flushed with heparinized saline four times per day, at 09:00, 12:00, 15:30 and 21:00, and also after sampling.

2.2 Surgical procedure and anesthetic protocol

A full description of the surgical procedure in the sixteen pigs, and an overview of all substances and doses used for premedication, anesthesia, infection prevention, liquids, and post- operative pain relief is to be found in the main article that this thesis is based on (Nordgreen et al., 2018). The pigs were monitored by a trained veterinary anesthetist until full recovery. The surgery itself (central venous catheterization) was conducted by an experienced surgeon. All sixteen pigs went through the surgery. Easy access to the catheter was a prerequisite for the entire study. To ensure this, protective layers (bandages and a custom - made backpack) was constructed in a way as to

ensure easy access. NSAIDs and antibiotics were administered postoperatively for two days following the procedure, to ensure maximal post-operative pain relief and avoid infection.

2.3 LPS injection

On the day before injection, the pigs were weighed, their average weight varying between 25,9 +/- 3,5 kg. The lyophilized LPS (from Escherichia coli 0111: B4 – Sigma-Aldrich, Darmstadt, Germany) was prepared prior to the injection day. A Hamilton glass syringe was used to inject the LPS solution into the catheter. In the experiment, the smallest dose of LPS proven to give an effect was used, 1.5 µl kg^{-1}. Right after injection, the catheter was flushed with 10 mL of sterile saline water to ensure maximum effect of the LPS injection, by making sure that the entire dose would reach the circulation.

2.4 C – reactive protein (CRP) analysis

The central laboratory at NMBU's Faculty of Veterinary Medicine (*www.Sentrallaboratoriet.no*) analyzed the CRP in serum, by using a PEG (polyethylene glycol) enhanced immunoturbidimetric assay and an Advia®1800 Chemistry System (Siemens, AG, Erlangen, Germany). The basic principle of this analysis focuses on the reaction between the plasma serum and a specific antiserum that forms a precipitate, which is measured turbidimetrically at 340 nm.

2.5 Analysis in the lab of plasma with HPLC/MS

The purpose of the HPLC/MS analysis is to separate different components with different chemical and physical properties from a mixture, and to identify and analyze the separated components. The high-performance liquid chromatography (HPLC) separates the compounds that consist of multiple components, while the mass spectrometry (MS) is used to identify the individual components, based on structural properties. The combined techniques are ideal for

chemical analysis of complex biological samples, such as the quantification of tryptophan and kynurenine in blood plasma. The HPLC separation consists of a stationary phase and a mobile phase. The mixture of analytes moves through a column from the mobile phase to the stationary phase. The stationary phase consists of inhibitory compounds that slows down and separates the components in the mixture. There are different columns; the longer the columns, the more time it takes to analyze, but it makes it clearer which chemicals are eluting at different times. Elution is the process of separating one substance from another by means of a solvent. After separation, the analytes move through a PDA (photo dye array), where light hits the different components and scans the different wavelengths of the components (spectrophotometry). The absorbance of the different components (where in the electromagnetic field that the components will absorb light) is measured. Each component has its own unique absorbance (A). The time point of when the components are eluting out of the tube is registered. The relation between A and time can be illustrated in a figure with time as the X – axis and absorbance as the Y – axis. After the PDA step, the analytes move through an ESI (electrospray ionizer) with nitrogen gas as the nebulizing gas, which serves the purpose of ionizing the components with either a plus or minus charge. Then the charged analytes move through the mass spectrometer, consisting of four charged rods (quadrupole), which make up a sealed area. The quadrupole changes charge a thousand times a second, alternating between plus and minus charge. The analytes move through the MS, where 99% hit the quadrupole. To elucidate the mass of the analyte (mass analysis), there is a detector within the mass spectrometer, that detects which charge the quadrupole was at, when the analyte hit the detector. A small molecule uses less energy to deflect, in terms of charge, and is quickly deflected. A heavy molecule uses more energy to deflect and is slowly deflected. Thus, a low

charge equals a light/small molecule with minimal charge and minimal shift in charge, while a high charge equals a heavy/big molecule with more charge and a more evident shift in charge. After the analytes have moved through the MS and hit the detector, a computer shows the different ions that have come through the machine. It will show the strength of the signals at the Y – axis and the molecular weights at the X – axis, and this information can be compared to an already existing register, to identify the analytes.

Below follows a description of the specific method used in this thesis, developed and performed by the Head Engineer of the Pharmacology group at the Faculty of Veterinary Medicine, Department of Paraclinical sciences (PARAFAG), Daniela Dulgheriu.

A sensitive and selective high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) method for quantification of TRP and KYN was developed. The pure compounds and their respective corresponding stable isotope labelled standards were provided from CDN Isotopes (Quebec, Canada), Sigma-Aldrich (Darmstadt, Germany) and TRC (Toronto, Canada). All chemicals were of at least HPLC grade and supplied by VWR International (Fontenay sous Bois, France). Purified water (18.2 MΩ) was obtained from a Milli-Q water purifying system Merck Millipore (Bedford, MA, USA). All samples were thawed on ice and the respective corresponding isotope labelled internal standards added according to the weight/volume of each sample. The plasma samples were precipitated with acetonitrile 1:5 (v/v) after addition of the internal standard mixture. After being vortexed for 30 s and centrifuged at 12.000 x g for 15 min, 50 µL of plasma samples were subsequently transferred into a new 15 mL polypropylene centrifuge tube and evaporated to dryness at 40 ºC

under a nitrogen stream in a water bath (Zymark Turbo Vap LV, Oregon, USA). The dry residue was reconstituted in 100 µL dilution solution of 10 % methanol/water (v/v) with 0.1 % formic acid and 0.05 % ascorbic acid, filtered with Spin-X centrifuge tube filter, 0.22 µm (Costar, UT, USA) and transferred to a HPLC vial with insert (Agilent, Santa Clara, CA, USA). The HPLC-ESI-MS/MS system was performed using an Agilent 1100 setup consisting of a binary pump, degasser and autosampler with thermostat (Agilent Technologies, Santa Clara, CA, USA) coupled to an API 4000 triple- quadrupole mass spectrometer (AB Sciex, Ontario, Canada) equipped with turbo ion spray. The temperature of the autosampler was set at 5 ºC. Chromatographic separation was carried out on a reversed phase Synergy-Fusion column, 100 x 2.1 mm, 2.5 µm particles (Phenomenex, CA, USA) with a Fusion-RP guard column. The column temperature was 25 ºC. The mobile phase consisted of 0.1 % acetic acid in water (A) and acetonitrile/methanol (50 % v/v) (B). The separated compounds were detected in positive and negative electrospray ionization-multiple reaction monitoring (MRM) mode using the respective $[M+H]^+$ (protonated) and $[M-H]^-$ (deprotonated) ions in two separate run analyses, selecting one precursor ion to two products ion transitions for each compound. Chromatographic separation of the analytes detected in positive mode was achieved using a gradient program as follows: 1 % B to 25 % B for 3.5 min; 80 % B from 8.75 to 15 min; post run for 5 min. The flow rate was 0.2 mL/min and the injection volume were 10 µL. A separate run analysis was performed in negative mode with the following gradient program: 3 % B to 30 % B for 1.5 min; 50 % B from 3.5 to 5 min; 80 % B from 5.1 to 20 min; 10 min post run analysis. The flow rate was 0.22 mL/min and the injection volume were 15 µL. The ESI-MS/MS parameters were optimized as following: The ion source was operated using turbo ion spray voltage at 5000 V or -4500 V; curtain gas (N2) at

20 or 30 psi; ion source temperature at 300 ºC, nebulizer gas (N2) at 50 psi and turbo gas (N2) at 50 psi. TRY (m/z 205 > 188), 13C-TRY (m/z 206 > 189), d5-TRY (210 > 192) (used for plasma samples only), KYN (m/z 209 > 192) and KYN-d4 (m/z 213 > 196). Mass to charge ratio (m/z) represents the quantity actually measured by the mass spectrometer. The software used for controlling this equipment, acquiring and processing the data was Analyst Version 1.7 (AB Sciex, Ontario, Canada). Since the analytes are endogenous components in biological matrices, the validation parameters (detection limit, linearity, precision, accuracy, recovery and matrix effects) were determined by spiking the plasma as matrix with the corresponding stable isotope labelled standards analogues of each compound as standard. We verified that each internal standard produces a MS signal standard identical to that of the corresponding standard. Possible matrix effects were determined by comparing data from calibration curves in diluent to matrix-matched ones in different ranges for each compound. Standard stock solutions were prepared in methanol at 1 mg/mL, except kynurenine, which was dissolved in dimethyl sulfoxide. Working solutions were prepared in dilution solution (10 % methanol/water (v/v) with 0.1 % formic acid and 0.05 % ascorbic acid). All the solutions were stored at -20 ºC. The calibration standards were prepared in dilution solution as surrogate matrix based on the correction factors calculated for each compound related to their respectively matrix effects and recovery values. The linear ranges were: 0-500 ng/mL for KYN and 0-15000 ng/mL for TRY corresponding to 50µl plasma. The calibration curves were constructed based on the peak area ratio of the analytes to internal standards versus the nominal concentration ratio (analyte to internal standard). The concentration in each sample was calculated using the peak area ratio and linear regression analysis. The response for each compound was linear and gave correlation coefficients (R2) of 0.99 or better.

Limit of detection (LOD) was based on 3 x signal to noise (S/N) ratio and a lower limit of quantification (LLOQ) was determined as the lowest concentration validated. LLOQs ranged between 1 and 10 ng/mL for all the compounds. The intra-assay precision and accuracy for this method were determined by analysis of six replicates of the quality control (QC) samples spiked at the same level. Two QC samples were used for each run of analysis and prepared in a real brain homogenate by spiking with a known concentration of each analyte in order to evaluate the inter-assay precision and accuracy of the method. The extraction recoveries were between 50 % and 95 % for both analytes. The use of the stable isotope-labelled internal standard is one of the approaches to correct for matrix effects and improve the accuracy and precision of the analytical method.

2.6 Data processing and statistical analysis

To analyze our dataset, we used a mixed model in JMP. Mixed models include both random and fixed effects and can be used when there are repeated measurements on an individual in space or time. In this case, we had blood values at different points in time, with repeated measurements on the same individual. The individual pig was therefore included as a random factor in all models. We have analyzed two datasets; one dataset with several datapoints in time looking at difference in treatment (LPS or SAL), and one dataset that focuses on pre-surgery and post-surgery levels of analytes. The explanatory factors of Y (X) are treatment and time. Treatment is a categorical variable with two levels: LPS- injected pigs (LPS) and saline – injected pigs (SAL). Time is a categorical variable with three levels in the surgery dataset (A, B, C), and eight levels in the LPS dataset (0,2,4,6,8,12,24,48).

Treatment, time and the interaction between treatment and time was included as fixed effects. In the LPS and SAL dataset, we were mostly interested in the interaction between time and treatment, as it tests whether the two groups have different levels over time. In the other dataset, all pigs underwent surgery, and thus the model includes pig as a random factor, and time as a fixed effect, with time being the variable at interest. The outcome (Y – values) of our dataset were tryptophan (trp), kynurenine (kyn) and the ratio between them, the kynurenine tryptophan ratio (kyn/trp). We also tested the correlation between the kyn/trp ratio and CRP levels.

All variables fulfilled the assumptions needed to run a mixed model: independence, homogeneity of variance and normality of residuals, or were transformed to do so. The transformations that were necessary to fulfill the assumptions are listed and described for each variable below. Significant results are presented with a P-value, F ratio and degrees of freedom.

3. Results

3.1 The Effect of LPS on Tryptophan Levels

The treatment differences (11 in total) were numerically largest at 2, 3, 4, 6 and 8 hours after injection (Figure 2a). There was a significant interaction effect $F(treat*time)_{10, 110} = 4{,}64$; $p<0{,}0001$. We used a t – test for comparing the two treatments at the 11 different time points. To control for the effects of multiple testing, we calculated a new Bonferroni corrected critical p - value by dividing 0.05 with the number of comparisons (11). This gave us a new critical p – value of 0,0005. The tryptophan levels were significantly lower in the LPS than in the SAL pigs at 2,3,4,6 and 8 hours post-injection (p-value= 0, 0002;, <0,0001;, <0,0001;, <0,0003 and <0,0001).

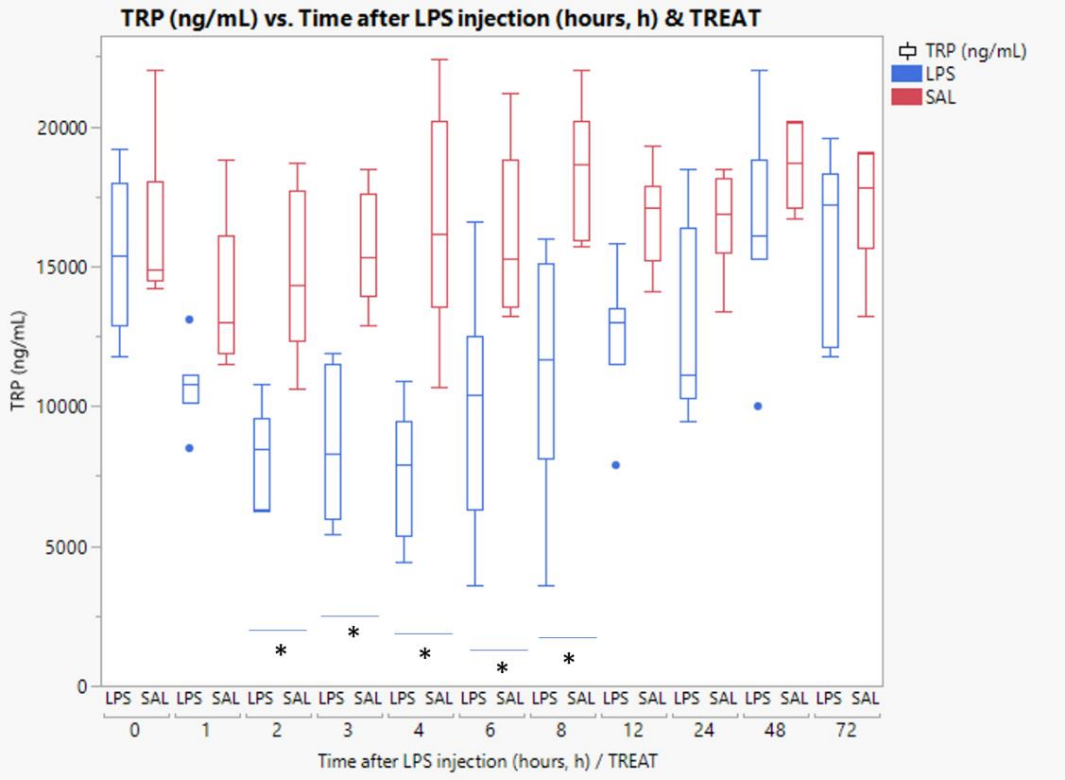

Figure 2a. Difference in levels of tryptophan in individuals treated with LPS (blue) and individuals treated with SAL (red). The asterisks indicate significant differences between the LPS injected pigs and the saline-injected controls.

3.2 The Effect of LPS on Kynurenine Levels

The datapoints were log transformed to fulfill the assumptions. The treatment differences were numerically largest at 4, 6, 8 and 12 hours after injection (Figure 2b). There was a significant interaction effect $F(treat*time)_{10, 110} = 14,48$; $p<0,0001$. The kynurenine levels were significantly higher in the LPS than in the SAL pigs at 4,6,8 and 12 hours post-injection (p = 0, 0005;, 0,0003;, 0,0007 and 0,0072). The treatments did not significantly differ at any other time points.

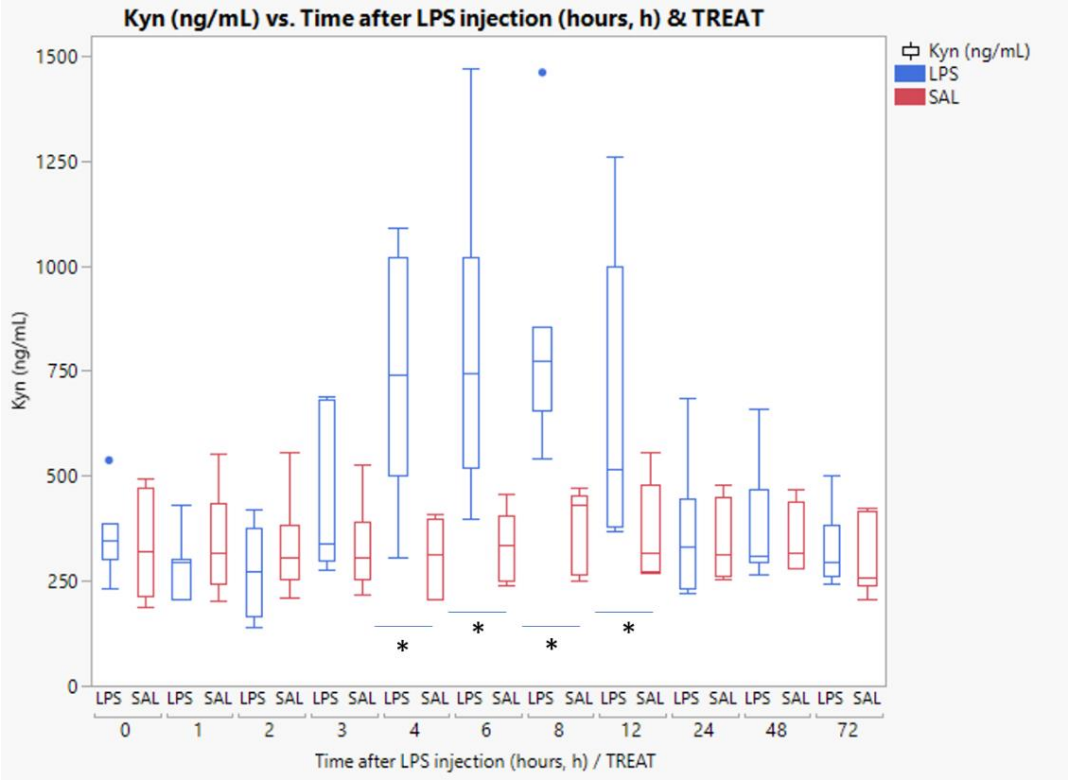

Figure 2b. Difference in levels of kynurenine in individuals treated with LPS (blue) and individuals treated with SAL (red). The asterisks indicate significant differences between the LPS injected pigs and the saline-injected controls.

3.4 The Effect of LPS on the Kyn/Trp ratio

The datapoints were log transformed to fulfill the assumptions. The treatment differences were numerically largest at 3, 4, 6, 8 and 12 hours after injection (Figure 2c). There was a significant interaction effect $F(treat*time)_{10, 110} = 7,87$; $p<0,0001$. The p-values for the treatment comparisons at timepoints 3,4, 6, 8- and 12-hours post-injection were 0,0007, <0,0001, <0,0001, <0,0001 and 0,0013. The treatments did not significantly differ at any other time points.

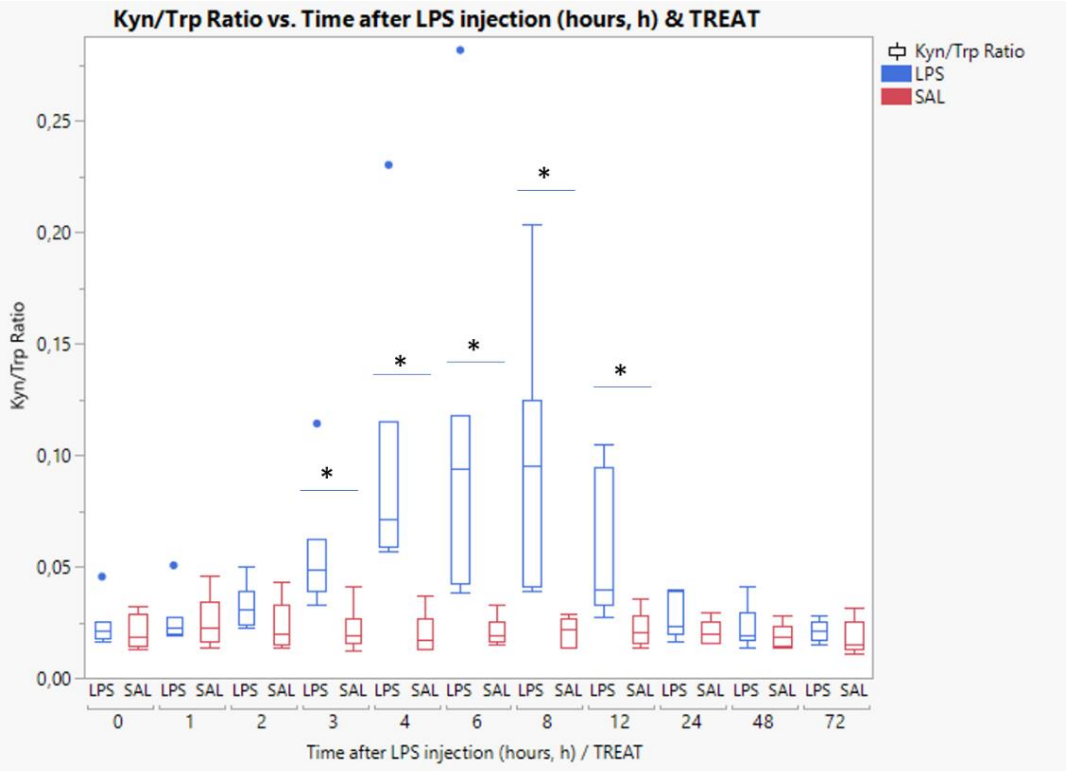

Figure 2c. Difference in the kyn/trp ratio in individuals treated with LPS (blue) and individuals treated with SAL (red). The asterisks indicate significant differences between the LPS injected pigs and the saline-injected controls.

3.5 The Effect of Surgery on Tryptophan Levels

Levels of tryptophan increased from pre to post-surgery (Figure 3a). There was a significant effect of time $F(time)_{2,27.85}=13,47$; $p<0,0001$. We used a t – test for comparing the different levels of tryptophan at the three different time points. To control for the effects of multiple testing, we calculated a new Bonferroni corrected critical p – value by dividing 0,05 with the number of comparisons (3). This gave us a new critical p – value of 0,0167. The p – values for the time points were <0,0001 (Before surgery/After fasting to 48h after surgery); 0,00143 (24h after surgery to 48h after surgery) and 0,0139 (Before surgery/After fasting to 24 hours after surgery).

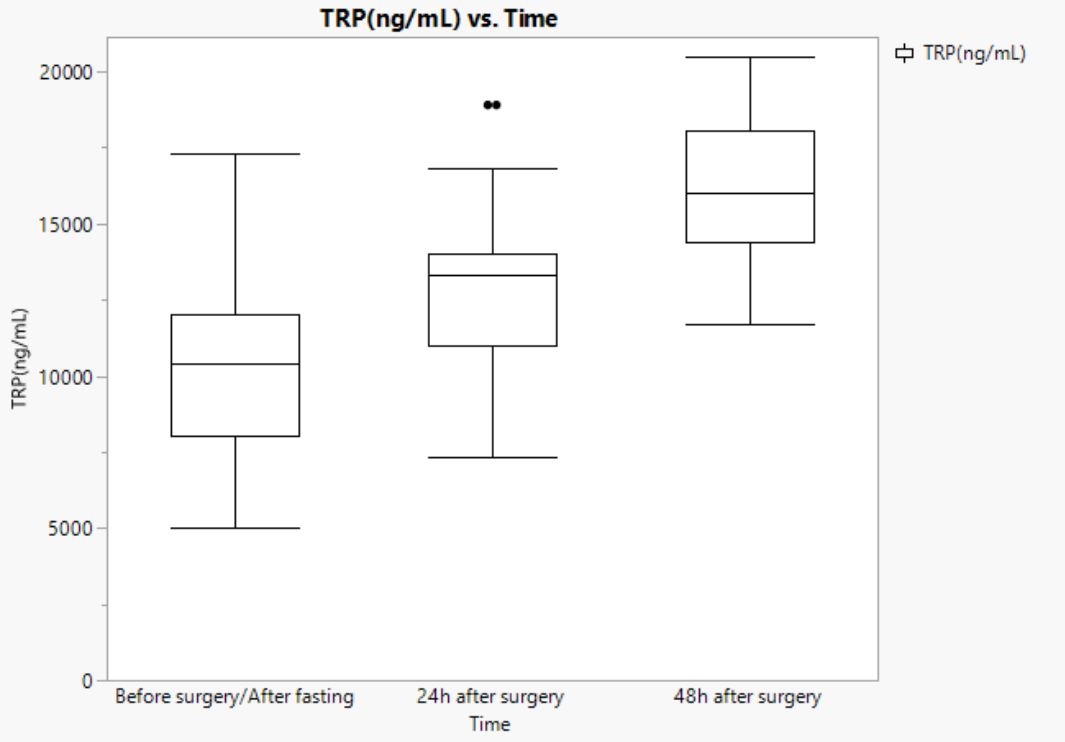

Figure 3a. Levels of tryptophan from Before surgery/After fasting to 24h and 48h after surgery. All differences were significant.

3.6 The Effect of Surgery on Kynurenine levels

Levels of kynurenine increased from pre to post surgery (Figure 3b). There was a significant effect of time $F(time)_{2,26.84}=9,2506$; p =0,0009. The p – values for the time points were 0,0005 (Before surgery/After fasting to 48h after surgery); 0,0023 (Before surgery/After fasting to 24h after surgery) and 0,4949 (24h after surgery to 48h after surgery). The results for 24h after surgery to 48h after surgery was not significantly different.

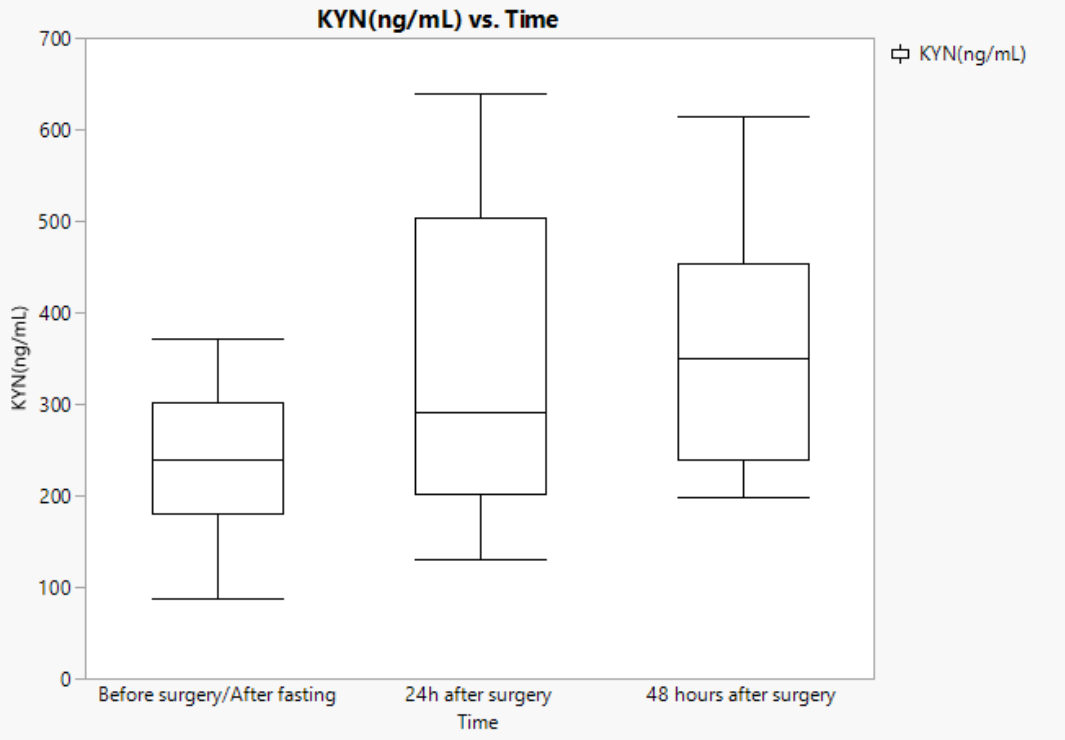

Figure 3b. Levels of kynurenine from Before surgery/After fasting to 24h and 48h after surgery.

3.7 The Effect of Surgery on the Kyn/Trp ratio

None of the results were significantly different (Figure 3c). There was a non-significant effect of time $F(time)_{2,26.86}=1,2420$; p=0,3049. The p -values for the time points were 0,5280 (Before surgery/After fasting to 48h after surgery); 0,3524 (24h after surgery to Before surgery/After fasting) and 0,1301(24h after surgery to 48h after surgery).

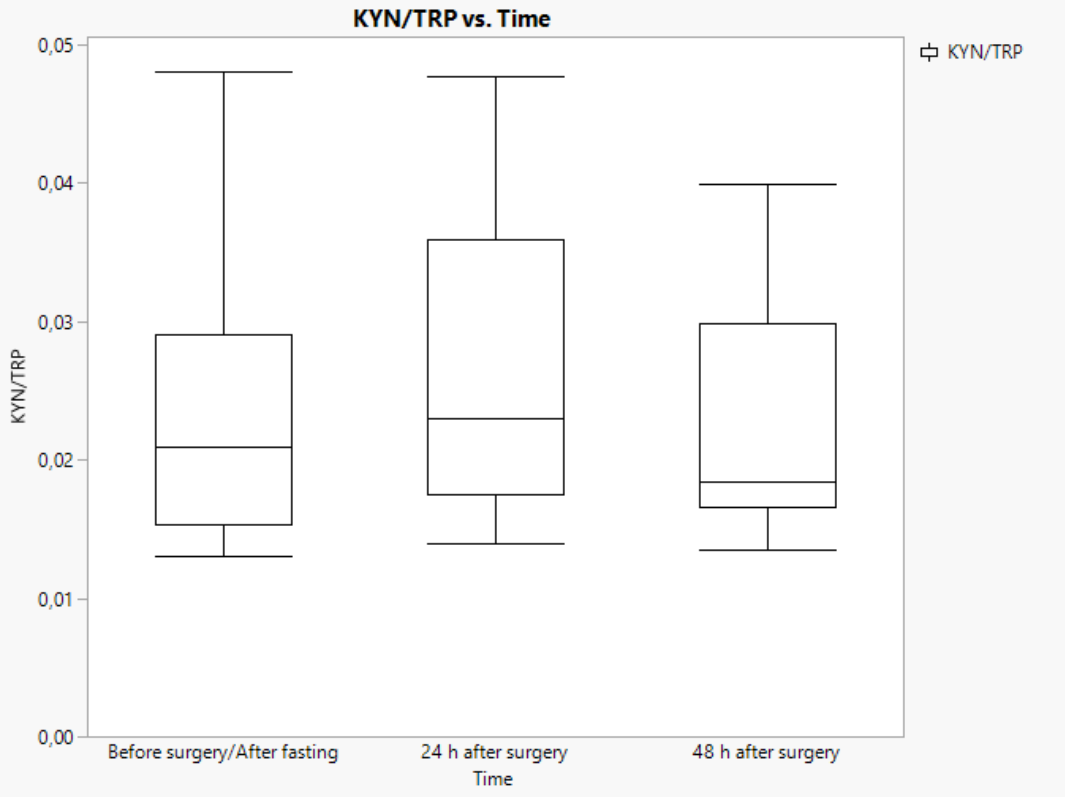

Figure 3c. The Kyn/Trp ratio from Before surgery/After fasting to 24h and 48h after surgery.

3.8 The Correlation between the Kyn/Trp ratio and CRP after LPS - injection

There was a positive and significant correlation between CRP and the Kyn/Trp ratio at 12h after LPS - injection (r: 0,4683, p – value: 0,0390) (Figure 4a). There were no other significant correlations.

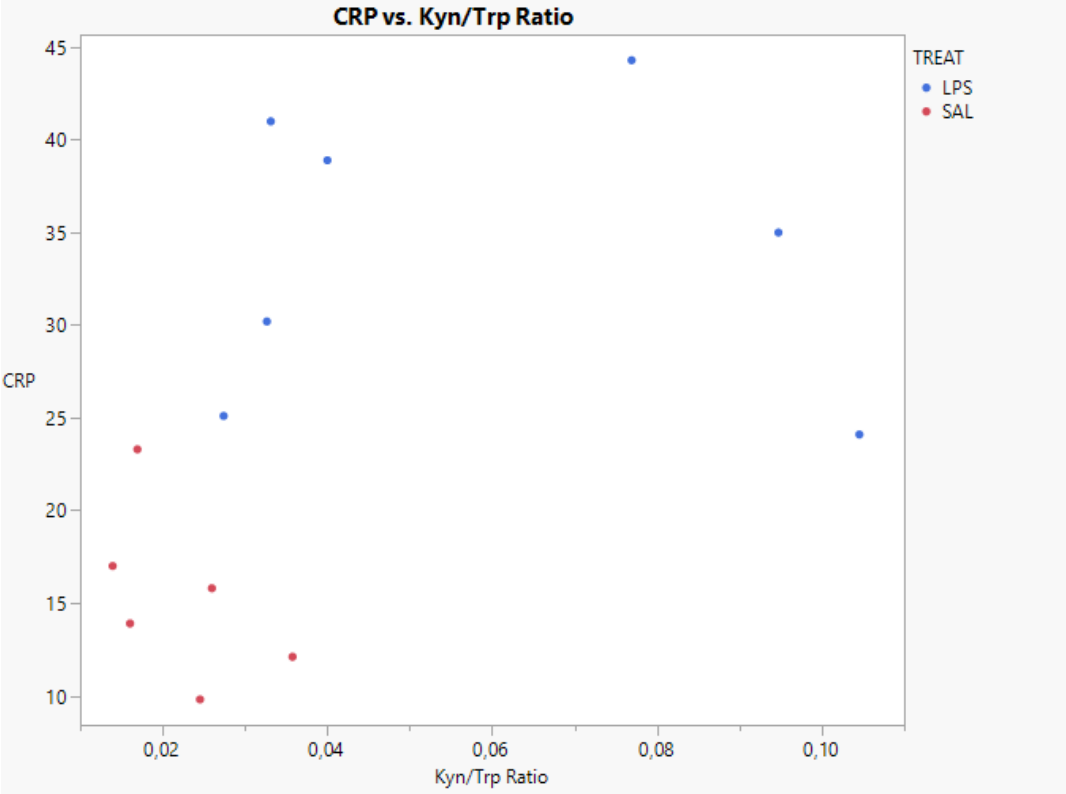

Figure 4a. The positive and significant correlation between the Kyn/Trp ratio and CRP after LPS injection. The brightness of the colors indicates the difference in CRP concentrations in the LPS and SAL group.

3.9 The Correlation between the Kyn/Trp ratio and CRP after Surgery

There was a negative and significant correlation between CRP and the Kyn/Trp ratio at 24h after surgery (r: -02476, p:0,0562) (Figure 4b). There were no other significant correlations.

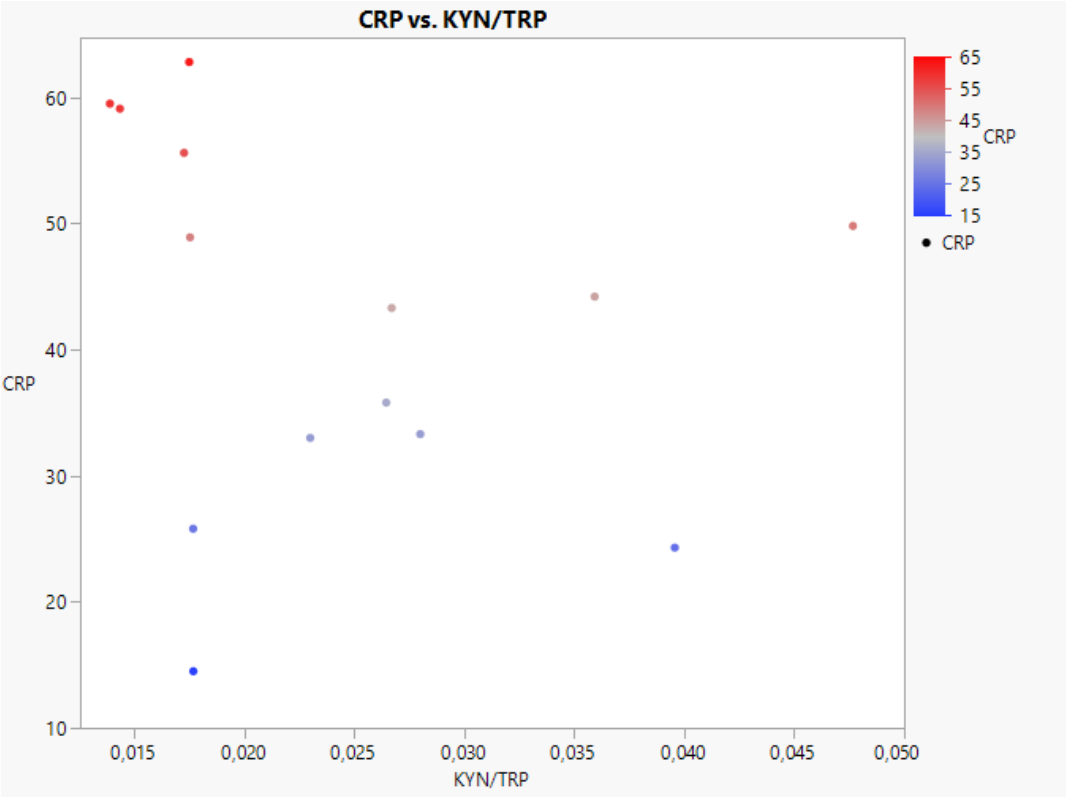

Figure 4b. The negative and significant correlation between the Kyn/Trp ratio and CRP after surgery. The brightness of the colors indicates the difference in CRP concentrations.

4. Discussion

The kyn/trp ratio was increased at 3, 4, 6 and 8 hours after LPS injection, and did not differ from baseline at 12 hours and onwards. The increase in the kyn/trp ratio was driven both by a kynurenine increase and a tryptophan decrease. Surgery did not influence the kyn/trp ratio at 24 and 48 hours after surgery. There was a positive correlation between the kyn/trp ratio and CRP at 12 hours after LPS - injection, and a negative correlation between the kyn/trp ratio and CRP at 24 hours after surgery.

As predicted, there was an increase in the amount of kynurenine and a decrease in the amount of tryptophan over time in relation to LPS. However, tissue trauma and LPS did not have the same effect on the kyn/trp ratio, which was not as predicted. There was a correlation between the kyn/trp ratio and CRP in relation to surgery and the LPS injection, but the correlations were in opposite directions. The positive correlation between the kyn/trp ratio and CRP after LPS – injection indicates that there is an association between the kyn/trp ratio and CRP in relation to an in vivo immune activation. The negative correlation between the kyn/trp ratio and CRP indicates that the effect of time is probably not related to immune activation, but rather to the fasting that happened before surgery and the increased food intake after surgery. It could also be that surgery actually did increase the kyn/trp ratio, but that the effect was over at 24 hours, and was not detected because we did not sample in the time span between the first sample and 24 hours. However, we can assume that there is an association between CRP, tryptophan and kynurenine as predicted, based on our results.

A possible explanation for the lack of effect on the kyn/trp ratio after surgery, could be that the pigs were fed after surgery, naturally increasing their tryptophan levels over time. To have

avoided this, the blood samples could have been taken the day before. Another possible explanation could be the use of NSAIDs (non – steroidal anti – inflammatory drug) postoperatively, due to its anti-inflammatory properties; inhibition of prostaglandin e2 and NFκB transcription factor involved in the transcription of cytokines, which could have interacted with the results.

In our experiment, the kyn/trp ratio increased at 3, 4, 6 and 8 hours after LPS injection. In another experiment with pigs (Wirthgen et al., 2014), the kyn/trp ratio increased at 3 hours after LPS injection and reached a maximum after 6 hours. Compared to our results, the increase started at the same time after injection but reached a maximum at slightly different time points (8 hours in our results compared to 6 hours). In a later study (Wirthgen et al., 2018), where the kyn/trp ratio was used as a marker for IDO activity, the LPS injection resulted in an increase of the kyn/trp ratio at 6 hours after administration. There seems to be a close to similar time-course to the changes in the kyn/trp ratio after LPS injection in pigs.

In mice who received a single dose of LPS, there was an increase in the serum kyn/trp ratio at 48 hours post injection (Larsson et al., 2016), which is a much later increase than in pigs. LPS induced depressive-like behavior mediated by the IDO activity in mice has also been investigated, and LPS increased the kyn/trp ratio here as well (O'Connor et al., 2009).

The above-mentioned experiments indicate that an increased IDO activity leads to an increase in the kyn/trp ratio, suggesting a tryptophan decrease and kynurenine increase after an acute immune challenge with LPS. However, the response is short-lived and there is a missing link between the short-lived response and the behavioral changes that have been observed a long time after the physiological signs of an immune challenge have disappeared. One would expect that a

short-lived physiological response would give short lived behavioral changes. However, this does not seem to be the case. The delayed behavioral changes observed after an acute immune challenge included submissive behavior in the LPS injected pigs (Munsterhjelm et al., 2019). Reduced tryptophan levels leading to reduced serotonin levels have been associated with increased aggression and reduced dominance behavior in humans (Young and Leyton, 2002). Since establishing dominance relationships among pigs in a group is an important part of exercising natural behavior in pigs, it is easy to suspect that submissive behavior, due to inflammation, could alter the social hierarchy and lead to more conflict and confusion, increasing the probability of damaging behaviors. The article (Munsterhjelm et al., 2019) also discusses how the increased attention from non-sick animals towards sick animals could make them victims of tailbiting, and how recovered pigs could have a higher risk of becoming biters, due to the changes in their physiology.

The changes in tryptophan and kynurenine concentrations in relation to each other, seem to be the starting point of a series of changes, that might lead to long-term emotional and behavioral alterations. After the increase in the kyn/trp ratio is over following an acute immune challenge, what happens next is unknown. It might be possible that this increase is just the first step in a series of steps that we cannot detect in such a short amount of time.

A reduced amount of serotonin following increased activation of the kynurenine pathway and tryptophan depletion, is one possible explanation of lowered mood, but there is evidence that the explanation is much more complex, and that reduced serotonin is not the only causal factor leading to lowered mood (Hughes et al., 2012). For instance, when looking at our results in pigs, the increase in kynurenine and decrease in tryptophan levels happen quickly and then subside,

when the pigs are exposed to an acute stressor. Within less than 12 hours, the physiological effects of LPS is gone. However, if the pigs are exposed to long-term stressors, one could imagine that the physiological effect would be more persistent, and lead to long-term changes in tryptophan and kynurenine levels. Systemic tryptophan depletion and kynurenine increase over time could be a possible explanation for the long-term mood and behavioral changes in pigs. It is also important to acknowledge that there might be individual differences in terms of resilience towards these changes.

However, there is still no clear explanation for lingering behavioral changes after an acute immune challenge. The above-mentioned change in social motivation could either come directly from the LPS injected pigs or indirectly from how the non-injected pigs reacted to the LPS injected pigs, as discussed in (Munsterhjelm et al., 2019). Munsterhjelm also points to changes in neurotransmitters and cytokines in the brain as a possible mechanism for delayed effects of an LPS immune challenge. There is evidence that cytokines definitely have the capacity to change social interactions, and that there are species differences when it comes to how non-sick animals interact with sick animals (Hennessy et al., 2014). Neuroinflammation could also cause long term changes in behavior. Several kynurenine metabolites down the kynurenine pathway are neurotoxic and can wreak havoc in the brain, so this is absolutely a plausible explanation for a delayed effect. However, more research is needed to find the exact mechanisms behind these changes.

Another possible mechanism could be from a more cognitive perspective, that a stressful event could trigger an emotional response great enough to induce learning and memory, which would explain why the injected pigs would act differently long after an LPS injection.

Physiologically, there is a link between the kynurenine pathway and brain function. Quinolinic acid is an NMDA receptor agonist. The NMDA receptor plays an important part in memory and learning (Riedel et al., 2003). Therefore, one could assume that an imbalance in the kynurenine pathway could have some influence on memory and learning through changes in synaptic plasticity, which could contribute to altering behaviors. However, this would likely happen after a longer period of time.

There is still a lot to discover about the exact mechanisms and causal relationships between immune stimulation and long-term behavioral changes. We can examine the neurotransmitter and cytokine levels in the brain, as already done in (Nordgreen et al., 2018), and metabolites of the kynurenine pathway could be measured and linked to behavioral changes. Also, there are few studies that have looked at pigs over a longer period of time, for instance throughout their entire life, to see the physiological and behavioral changes over a life span. This could also be useful to investigate. However, the methods involved would have to be as gentle as possible, so as not to disturb the natural course of the pig's life. Also, there are studies that investigate how the IDO activity can be influenced, either indirectly or directly by anti-inflammatory or antagonistic drugs (O'Connor et al., 2009). However, there is still a lot to discover when it comes to charting the exact effects of the kynurenine pathway and metabolites on mood and behavior. If specific causal relationships are established, it would be easier to find out which part of this pathway to target, either medicinally or otherwise. A timeline of physiological changes and possible mechanisms for long-term changes is presented below (Figure 5).

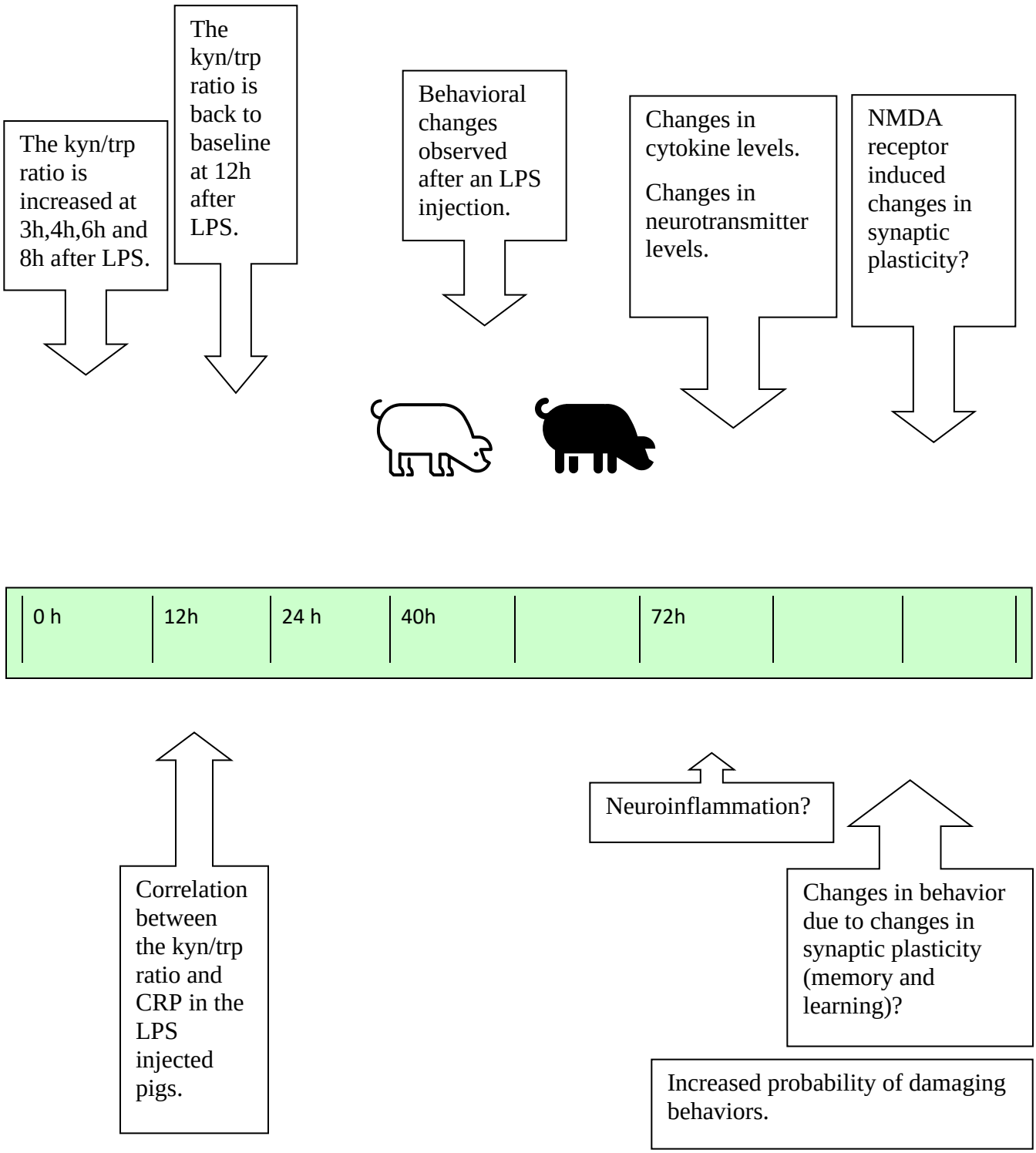

Figure 5. Timeline of physiological changes and possible mechanisms for long-term changes.

In this experiment, we measured physiological parameters that may influence emotional state and behavior in pigs. There are several ways of measuring emotional state in animals, and there are species differences. For instance, humans can verbally convey how they feel from a subjective point of view, while animals cannot convey this information in the human language. Therefore, linguistic self - report is often used in humans, while methods that measure behavioral and physiological changes, is often used to monitor the emotional state in non – human animals. There are those who claim that these methods can be difficult to interpret and that the nuances of emotions are sometimes lost, suggesting that there are limitations to what physiological measurements can convey. Therefore, some has suggested a more cognitive based approach (Paul et al., 2005). There is room for improvement when it comes to developing methods that accurately register and convey animals' emotions and affect, and that are easy to interpret as well. A scientific assessment of the emotional states of animals have been attempted through QBA (qualitive behavioral assessment), which supports the use of cognitive based approaches for the assessment of animal emotionality (Rutherford et al., 2012). Research on animal behavioral expression presents behavior as an expression process, supporting the validity of subjective (first person) observation as a complementary approach to the study of animal emotion (Wemelsfelder, 2001, Wemelsfelder, 1997). Measurements of mood in animals based on physiological changes alone, might not be a good enough indicator of emotional state and affect, and there are those who argue the importance of a multidimensional approach (Ramos and Mormède, 1997).

However, since behavioral changes is often an expression of mood and emotional state, and there is a connection between physiological changes and behavior, it seems reasonable to imply that physiological measurements are useful, if not necessarily sufficient on their own.

The levels of tryptophan and kynurenine could be used as an indicator of pig well- being, if the changes were less short-lived than we see in our results. However, if these short-lived changes are just the beginning of a series of changes that will be visible over time, for instance, due to changes in synaptic plasticity, they could be important in deciding what the behavior, mood and physiology of a healthy, happy pig should look like, and whether these "multifactorial characteristics" changes throughout being a commercialized pig, and thus be useful as an indicator of pig well – being after all. If we could standardize physiological measurements in healthy, happy pigs, it would be easier to locate the stressed, sick, or miserable pigs. It could also be interesting to compare the physiological and behavioral response of these pigs to wild boars or free-living pigs.

I believe that a physiological measurement of animal welfare would be useful in commercialized settings where good animal welfare is paramount and not always easy to quantify. However, if behavior and mood are directly linked to the concentrations of bioactive substances, one could develop a specific form where certain behaviors linked to for instance low tryptophan or high kynurenine, could be registered and acted upon. There are still a lot to discover when it comes to mood altering physiological changes; how to measure them and what they actually mean long term. I believe that it is important to obtain this basal knowledge to ensure sufficient animal welfare in commercialized settings. If absolute values (reference values) are found in pigs in

relation to specific behaviors and moods, this could be used as a physiology-based tool to measure welfare in pigs.

Our results contribute to a better understanding of the physiology of pigs that are exposed to stressful events such as inflammation or surgery. This is valuable information to better understand the physiological consequences of short- and long-term stress exposure in pigs.

5. Conclusion

The connection between physiology and mood is important in all species. In humans, increased knowledge of the interaction between physiology and mood, can improve our understanding of mental health, social interactions, irritability and all aspects of human beings that have to do with mood and the interplay between signaling substances in the brain and body. Examples of where this knowledge is valuable is for instance, in studies about premenstrual syndrome in women (Ryu and Kim, 2015), Alzheimer's disease and depression. In production animals, this knowledge can contribute to a better understanding of the physiological origin of damaging behaviors (eg. tail biting) in pigs, make it clear that mood is an important aspect of welfare, and help to better define what can be considered good animal welfare, both for the individual and the group, from a physiological point of view.

Conflict of interest

The authors declare no competing interests.

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Sammendrag (Norsk)

Tittel: Endringer i tryptofan og kynurenin konsentrasjoner i griseplasma etter in vivo immunaktivering og kontrollert vevstraume.

Forfatter: Cathinka Celine Jørgensen

Veileder: Janicke Nordgreen, Førsteamanuensis, Veterinærhøgskolen, Institutt for parakliniske fag, Faggruppe farmakologi.

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