Measuring cognitive-affective appraisal processes in pigs

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Table of contents

Chapter 1:

General Introduction

1.1 Animal welfare

"They eat without pleasure, cry without pain, grow without knowing it; they desire nothing, fear nothing, know nothing" (Malebranche, 1997). We have come a long way since these words of Nicolas Malebranche (1638 -1715) (see Harrison, 1992) for a philosophical discourse on Descartes and Malebranche).

With rising knowledge in scientific disciplines like neuroscience and ethology and an increased public concern of how animals are treated and housed, the first milestone in animal welfare science was set in the 20th century. In 1964, the British government set up a committee to report on housing conditions of farm animals. Results of these efforts were written down in the Brambell Report providing the concept of the "five freedoms" (Broom, 2011). These involve the freedom from hunger and thirst, the freedom from discomfort, the freedom from pain, injury and disease, the freedom from fear and distress and the freedom to express normal behaviour (Brambell, 1965; Farm Animal Welfare Council, FAWC, 2009). Influencing not only political regulations in the United Kingdom but throughout Europe, the "five freedoms" would form the basis in the effort to improve farming, animal welfare legislation and animal welfare assessment (Veissier et al., 2008; McCulloch, 2013). Since then, interest and effort in animal welfare has increased enormously - not only in society, but in politics and science as well which has brought up a diversity of approaches to describe animal welfare scientifically, for example, the "concept of needs" (Thorpe, 1965; Broom, 2011) or the "five domains model" (Mellor and Reid, 1994). However - as Appleby and Sandøe (2002) state - a brief definition of animal welfare seems to be impossible. Still, while screening the numerous references to animal welfare science there does seem to be a focus on three major alignments influencing the nature of animal welfare: biological function, natural living and affective state (Fraser et al., 1997; Appleby and Sandøe, 2002; Mellor, 2016).

The idea of affective states in non–human animals is not new with the first approaches to the topic dating back to Darwin (Webster, 2006). However, a significant shift in ideology only happened in the later 20th century. As Lawrence (2008) calls it, the "Wood-Gush era" had a drastic impact on the scientific field of affective states. Scientists developed first approaches to assess the subjective perspective of non-human animals like first studies on preference tests or frustration in farm animals (Duncan and Wood-Gush, 1971; Dawkins, 1977).

Nevertheless, many in the scientific community remained sceptical when considering the study of the affective state of non-human animals, a debate which is still - to some degree - ongoing. Therefore it is not surprising that the idea that mental state plays an important role in ensuring good welfare has only recently become the focus of attention in animal welfare studies. This is reflected by a rapid increase in publications in the last few years (Marchant-Forde, 2015), including aspects of affective states (Désiré et al., 2002; Boissy et al., 2007), motivation (Hughes and Duncan, 1988) or cognition (Dantzer, 2002; Marchant-Forde et al., 2009).

In the thesis presented, I as well consider the affective state to be an essential part when it comes to animal welfare, therefore the following section will give a brief introduction into this topic.

1.2 Affective states

The question of "What is emotion" bothered a whole range of scientists in the near and distant past (James, 1884; Simonov, 1986; Zemach, 2001; Cabanac, 2002; Scherer, 2005; Kagan, 2007, to name only a few studies) and despite being a relatively young field in science, manifold definitions describing emotion can be found throughout literature. There are two main scientific movements. First would be the "discrete emotions approach" which describes affective states as a set of "basic emotions" (such as fear, happiness, anger etc.) with each of them having their own unique features (Ekman, 1992). Critics of this theory highlight three major problems: (1) there is no consensus of how many basic emotions exist and what characterises them as being "basic" (Ortony and Turner, 1990); (2) the approach is not appropriate to find a satisfying explanation for a wide range of affective states (Mendl et al., 2010b) and (3) the construct of discrete emotions is rather based on a verbal classification than on empirical evidence (Barrett et al., 2007). In opposition to the discrete emotions approach, the second movement - initiated by Wilhelm Wundt - described emotional states by their value on each of three dimensional axes: valence, arousal and tension. In the field of dimensional approaches, various theories developed with differing models, based on four (Cabanac, 2002) three (Osgood, 1966), or two dimensions (Watson and Tellegen, 1985; Russell and Barrett, 1999).

In this thesis I will refer to an approach proposed by Mendl et al. (2010) who combined the dimensional and the distinct emotions approach into one integrative framework. The basis of the proposed framework is the "core affect", defined as "a neurophysiological state that is consciously accessible as a simple, non-reflective feeling" (Russell, 2009) which can be characterised in terms of two dimensions: the valence (negative vs. positive) and the arousal (high vs. low).

Even if it might be discussed (and in fact is one of the most controversially discussed topics in the modern field of animal welfare sciences) if affective states, or more specifically, the conscious experience of affective states are essential to assess animal welfare (see Dawkins, 2017 for a broad discussion) it remains undoubtedly that affective states at least play an important role (Dawkins, 1990; Duncan, 1993; Mendl and Paul, 2004). There is scientific consensus that affective states are shaped by adaptive processes which meet the different challenges evolution prepared (Nesse and Ellsworth, 2009) and hence from an ultimate view are essential for the biological fitness. Therefore, the main benefits of emotions (in relevance to animal welfare) are: (1) the modulation of autonomic and

endocrine responses to prepare the organism to react (e.g. fight/flight), (2) enabling communication (e.g. to form social bonds) and (3) evaluation of external/internal information (e.g. reward/punishment, past experiences, cognition) to prepare for the most appropriate reaction.

Concerning the proximate principles of affective states there is a variety of approaches which only scratch the surface of emotional regulation. In contrast to the theory of James and Lange (Coleman and Snarey, 2011), where an emotion is expected to be reflexively triggered by a specific stimulus which finally results in a physiological reaction, modern theories regard affective states as multifaceted comprising physiological, behavioural and subjective components (Paul et al., 2005). Scherer (2005) postulates five components as crucial: a neurophysiological component, a motivational component, a motor component, a subjective component and a cognitive component. Whereas the first players seem to be undisputedly accepted to be part of the affective regulation, the cognitive component is discussed controversially. Some regard cognitive and affective processes to be distinctly separated (Zajonc, 1980; Panksepp, 2003), based on discrete functionality as for example in the brain (Papez, 1937; Panksepp, 1998).

Rising knowledge of the neurophysiology indicates to blurring boundaries between affective and cognitive mechanisms. Two prominent scientists in the field of cognitive-emotion theories - Magda Arnold and Richard Lazarus - suggested the appraisal theory (Lazarus, 1982; Scherer, 2001). Here, an emotion is described to be causally determined by a cognitive evaluation. The appraisal is a process depending on a series of stimulus checks which evaluate the information presented by a specific situation (Scherer, 2001). This evaluation includes information of past experiences, memories or other attributes which consider the stimulus, for example its suddenness or familiarity (Mendl et al., 2009). Whereas these appraisal processes can rely on highly complex evaluation checks, which are most likely to be linked to conscious perception of an affective state (Leventhal and Scherer, 1987; Paul et al., 2005), they might as well be rapid, requiring a simpler level of evaluation (Lazarus, 1982; Leventhal and Scherer, 1987). Such rapid evaluations of an event are thought to produce an immediate, automatic reaction leading directly to the appropriate behavioural reaction or autonomic response. They are innate and adaptive and ultimately provide maximum fitness of the organism (LeDoux, 2009). What is most interesting is that they do not necessarily have to be experienced consciously (Lazarus, 1982; Paul et al., 2005), which makes them appropriate for non-human animals studies avoiding a discussion about if, or if not, animals are capable of conscious experience. The appraisal theory is basis of various studies analysing the affective state in non-

human animals (Désiré et al., 2002; Boissy et al., 2007). After presenting stimuli with specific characteristics which are associated to specific affective states in humans, the measured behavioural or physiological reaction is used as an indicator of the underlying affective state. However, this presumes that relationships between stimulus appraisals and emotions are the same in humans and non-human animals (Mendl et al., 2009), therefore the appraisal approach requires a species-specific validation.

In my thesis I focus on another aspect of the relation between emotion and cognition. There is strong evidence that these links between cognition and affective states function not only in one causal direction but in the other way as well (Mathews and MacLeod, 2002; Paul et al., 2005; Mendl et al, 2009). Therefore, affective states might influence cognitive processes; these shifts in cognitive performance can be measured. The cognitive performance can be defined clearly, and therefore be measured objectively. This is a clear advantage over the appraisal approach, as parameters like behaviour or physiology have their limitations in interpretation which I will discuss more in detail later on. The phenomenon of shifts in cognitive processes due to affective states is known as cognitive bias. As the cognitive bias is the basis of my thesis, here again I will come back to that more in detail presently.

For now, I will give a short definition of the terms used in this thesis. Throughout this document I will use the general term of "affective states", sometimes distinguishing between emotions and moods. Fundamental to these affective states is the "core affect" as has already been defined above (Russell and Barrett, 1999). In contrast to the core affect, emotions are defined as short-term affective states containing an additional component, namely they are directed to someone/something or they are triggered by something (Ekkekakis, 2013). Moods on the other hand, are defined as long lasting affective states, resulting from an accumulation of short term emotions. In contrast to the stimulus-focused emotion, moods are not directed to something specific but can be seen as a general state (Ekkekakis, 2013).

1.3 Regulating affective states

1.3.1 The brain

For a long time, it was an accepted assumption that the brain is a structured organ which reflects different emotion categories in distinct subcortical regions (see Lindquist et al., 2012 for a discussion of the "locationist approach"), equitable to the understanding of affective states in the "distinct emotion approach". With the emergence of theories describing "dimensional approaches" - as mentioned above - there developed different ideas of how these affective states might be regulated in the brain, too. In modern neuroscience it is an accepted view that the brain is characterised by highly connected structures, circuits and networks (Pessoa, 2017). The "theory of constructed emotion" (Lindquist et al., 2012; Barrett, 2017) even goes one step further, detaching itself from the categorization of emotions and considering the function of the brain per se as basis of affective regulation. The theory is grounded on the assumption that the main purpose of the brain is that of maintaining an allostatic state, meaning to ensure that all systems in an animal's body are in best balance to provide maximum fitness (Sterling, 2012; Barrett, 2017). To reach this aim the brain disposes of a network of neurons, communicating with each other by the help of different neurotransmitters. Each neuron is multipurpose. It can represent different characteristics according to the specific situation, it can reveal many-to-one connectivity (many neurons connect into one single neuron) or one-to-many connectivity (one neuron synapses into many other neurons). However, the whole organization of the neuronal network is characterised by high complexity and plasticity (McIntosh, 2004; Barrett, 2017; Pessoa, 2017). Additionally, subcortical regions of the brain are highly interconnected not only with other subcortical regions but with cortical structures as well (Pessoa, 2017) and therefore regulating autonomic, endocrine and immune systems (Barrett, 2017). The brain is creating a "mental model" of a specific (affective) situation in order to categorize the sensory event, as it is known for example in the perception of colors, objects or sounds (Lindquist et al., 2012; Barrett, 2017). Hereby it uses information of the environment and information about past experiences (as already known from appraisal theories), it considers the best action to deal with the situation and it encodes and consolidates unpredicted information as well. In humans the "mental model" of an affective state also includes cultural and language based information. As Barrett (2017) therefore concludes, emotional concepts relevant in humans (e.g. fear, disgust, happiness etc.) are constructed categories which cannot be transferred to non-human

animals in a one-to-one relationship. However, regardless of how an affective state is categorized in the brain (of non-human animals) and named in consequence: What matters for animal welfare should be the evaluation of a specific situation by each individual – putting it simple, the classification on an axis from positive to negative. So here again the dimension of the valence is of crucial relevance, therefore classifying an emotion on the valence/arousal dimension as proposed by Mendl et al. (2009) seems to be a promising approach. Altogether, this defines the brain as a dynamic network combining "functional integrated systems" (Pessoa, 2017) with flexibility and plasticity playing one major role, a feature which is well known in many biological systems (IDR-Team, 2010; Barrett, 2017).

Still, different brain regions were identified to play a superior role in affective/cognitive contexts. In fact, some of the prominent brain regions when it comes to affective control are found to be the most highly connected regions in the brain. Therefore, these might not be reduced to their specific function but may be seen as the control center to exchange information with subcortical and cortical structures and therefore to regulate the mechanisms of the organism to gain an allostatic state (Barrett, 2017; Pessoa, 2017).

The most prominent region in affect-regulation would be the amygdala. As part of the so-called limbic system, the amygdala is a structure located at the medial side of the anterior temporal lobe. It is highly interconnected with cortical structures gathering various information of all kind of sensory modalities (e.g. visual, olfactory, acoustical). The amygdala is found to be extensively connected to multiple structures of the brain stem and therefore has various effects on different systems like for example the autonomic system or different hormone circuits (Sah et al., 2003; Pessoa, 2017). In pigs, the amygdala is known for its role in controlling stressful situations and the behavioural reaction to aversive events. Authors could show that weaning stress resulted in a downregulation of glucocorticoid receptor binding (Kanitz et al., 1998), injections of acetylcholine into the amygdala evoked stress-like vocalizations (Manteuffel et al., 2007). On the other hand, the amygdala plays a superior role in the processing of positive emotions and rewards (for a review see Murray, 2007). In pigs, enrichment of the housing environment resulted in a downregulation of two specific receptors of the opioid-system, both in the amygdala and the hypothalamus (Kalbe and Puppe, 2010).

The hypothalamus is the ventral part of the diencephalon and its role is highly integrative. It activates autonomic, endocrine and behavioural responses and is

integrated in most diverse processes e.g. energy metabolism or hormone control (Saper and Lowell, 2014). Dysfunctions of the hypothalamus and therefore disturbances in the autonomic regulation are strongly related to mood disorders in humans (Drevets et al., 2008a).

This is also the case for the brain stem. The brain stem is the posterior part of the brain, mostly known for its role in mediating the reaction to a specific (affective) situation, especially by controlling behavioural, autonomic, and endocrine responses (LeDoux, 2000). There is a strong connection between the brain stem and amygdala, as well as between hypothalamus and amygdala (Nieuwenhuys et al., 2007).

The hippocampus, a structure located at the medial temporal lobe, is most prominent for its role in memory which makes it an important target for studies concerning Alzheimer's disease (Mattson, 2004). Not only memory is influenced by processes of the hippocampus – it also plays a crucial role in affective diseases and behaviour. Already the sheer volume of the hippocampus is positively correlated with depressive disorders in humans (Geerlings and Gerritsen, 2017). In pigs a study of Ursinus et al. (2013) could show that hippocampal serotonin activity is positively correlated to exploration behaviour in a novel object test.

The cingulate cortex, also part of the limbic system, is located at the medial side of the cerebral cortex, highly connected to different subdivisions to control cognitive, affective and motor functions and most prominent in processes of motivation (Bush et al., 2000). Interferences in functioning of the cingulate cortex are strongly related to mood disorders in humans (Drevets et al., 2008b).

The striatum and the prefrontal cortex both play a major role in decision making (Kable and Glimcher, 2009) and emotional processing. As increased activity in the striatum could be found to be associated with positive affective states like reward processing (Hare et al., 2005), while activity in the prefrontal cortex is more likely to be associated to negative emotional stimuli (Etkin et al., 2011).

Even if this indicates different brain structures to be of major importance in different parts of affective regulation (e.g. amygdala, hypothalamus and brain stem regulating the output information to prepare the organism to react; hippocampus and striatum on the other hand responsible for the further processing of the (cognitive) stimuli it remains important to keep in mind that modern neurosciences tends to concentrate more on the interactions between different structures then the specific task these structures fulfil. In the presented thesis the introduced brain tissues are monitored as representatives for the affective-

cognitive control mechanisms in the brain. Considering the above mentioned interactivity, focus was not laid on one or the other properties of the specific tissue but on manipulating serotonin (5-HT), as one major neurotransmitter relevant for the transfer of information in and between the different structures.

1.3.2 The serotoninergic system

Communication between neurons in the brain would be impossible without the help of chemical messenger substances called neurotransmitters. One such neurotransmitter would be serotonin (5-HT) which is involved in many of the different processes regulating affective behaviour. The 5-HT system is a very complex one, including at least 17 different types of receptors (Dayan and Huys, 2009) and interacting with other neurotransmitter circuits like the dopamine system (Boureau and Dayan, 2011) or brain structures like the extended amygdala (Marcinkiewcz et al., 2016). 5-HT finds it origin in the brainstem raphe nuclei and sends projections to nearly all brain structures. This already implies that 5-HT has not only one specific purpose but rather regulates a whole range of processes as for example sleep, feeding or pain perception (Curran and Chalasani, 2012). 5-HT is also an important player in regulating affective behaviour, in fact being the most prominent transmitter system in treating human affective disorders like anxiety, bipolarity and most importantly depression.

Generally, neurotransmitter transmission is divided in four main parts, the synthesis, the storage and release, the interaction at the post-synapse and the removal from the synaptic cleft (Siegelbaum and Hudspeth, 2000). Manipulations of the 5-HT system at these four stages were found to alter behaviour related to affective states, which makes it a preferred goal for pharmaceutical treatment as well as for studies concerning affective states in non-human animals.

Hasegawa et al. (2006) found the Flinders sensitive line rat model, the most popular rat model used in depression studies, to show impaired 5-HT synthesis. There is substantial evidence that a depletion of the 5-HT synthesis - and therefore a manipulation of 5-HT concentration - has an influence on affective behaviour. Mosienko et al. (2012) found decreased 5-HT concentrations in the brain to alter behaviour in mice. This was represented by decreased anxiety and an increased depression-like behaviour when tested in the forced swim test. Variation in the 5-HT synthesis is also known to have an impact on other species besides laboratory animals. Sheep with 5-HT depletion showed a decreased vocalization rate in an open field test and were found to show pessimistic behavioural responses in a cognitive bias test paradigm (Doyle et al., 2011). 5-HT depletion

studies in humans clearly indicate negative effects on the mood (Ruhé et al., 2007).

The release and storage of 5-HT may also be manipulated to alter affective behaviour. Petty et al. (1994) suggest a causal relationship between increased 5-HT release in the frontal neocortex to learned helplessness in the rat, an animal model used to study human depression. 5-HT release from dorsal raphe nuclei innervating the amygdala is discussed to increase anxiety behaviour whereas 5-HT release from nuclei innervating the dorsal periaqueductal grey inhibits unconditioned fear (Graeff et al., 1996). In humans, the administration of fenfluramine - a 5-HT agonist - results in an increase of anxiolytic responses in patients with a history of panic disorder (Targum and Marshall, 1989).

On the level of the post-synapsis, Heisler et al. (2007) could show that knockout mice for the 5-HT2C receptor show significant reductions in anxiety-like behaviour. An activation of the post-synaptic receptor 5-HT1A resulted in a reduction of depressive like symptoms in humans (Blier et al., 1997). The most prominent treatment of depressive patients would be the usage of selective 5-HT reuptake inhibitors (SSRI). They display their main function on the 5-HT transporter (5-HTT). The purpose of the 5-HTT is to transport 5-HT from the synaptic cleft back to the pre-synapsis and therefore to end the signal transduction. SSRI`s enhance the concentration of available 5-HT in the synaptic cleft and hence prolong signal transduction to the post-synapse. Ferris et al. (1997) could show that altered behaviour due to manipulation of 5-HT removal from the synaptic cleft is not restricted to humans. They found golden hamsters to show less aggressive behaviour when treated with a fluoxetine, which is also a selective 5-HT reuptake inhibitor. Only recently, human studies have shown 5-HTT to be a very interesting target in science concerning affective disorders, as there seems to be a genetic disposition for depressive behaviour, rooting in the 5-HTT gene (Canli and Lesch, 2007). Lesch et al. (1996) first found different versions of the 5-HTT gene, with a polymorphism in length, where the short version is directly linked to a decreased 5-HT synthesis. More interesting for affective studies is the fact that carriers of one or two short alleles show an increased tendency for anxiety related personality traits. In addition there are indications that a genotype with one or two copies of the short allele seem to be more susceptible to develop depression like symptoms due to stressful life events than the homozygous genotype for the long allele (Caspi et al., 2003). Such a gen-environment interaction is not restricted to the life-time of an individual but already plays a role in the prenatal phase, as Heiming and Sachser (2010) would show in a study with mice. Up to now, these polymorphisms of the 5-HTT gene

could be found for primates, rats and mice (Homberg and Lesch, 2011). To my knowledge, a comparable gene polymorphism has not yet been found in pigs.

Altogether it can be stated that the 5-HT system is quite complex - to make things even more intricate, modern theories state that neuromodulation itself (comparable to the structures in the brain) is rather flexible instead of a formation of "fixed" circuits. Bargmann (2012) states neurons seem to be connected to more synapses than would be relevant for functioning. He further states that these redundant connections are not random but rather provide alternative patterns of information processing, which means this offers the possibility to alternatively use different neurotransmitter pathways according to the specific situation.

However, manipulating the 5-HT system seems to be a promising approach which highlights the underlying mechanisms in affective processing. In my thesis, I concentrate on the first level of neurotransmission, the 5-HT synthesis. Manipulating the 5-HT availability in combination of measures of affective state should provide one of many steps in understanding the regulation of affective states in pigs.

1.4 Assessing affective states

There is a rising social and political interest in animal welfare making it even more important to find ways and indicators of which to judge and measure "good welfare". This is reflected by a rising number of political approaches to determine welfare indicators (e.g. AWIN, a European funded project finding animal welfare indicators; Tierschutzindikatoren, KTBL (Kuratorium für Technik und Bauwesen in der Landwirtschaft e.V.); "animal welfare indicators" - funding of the BMEL (Bundesministerium für Ernährung und Landwirtschaft, Germany); etc.). However, assessing animal welfare clearly depends on the point of view of the individual observer (Fraser et al., 1997).

Astrid Lindgren, an influential advocate for animal welfare in Sweden, once stated "Let [farm animals] see the sun just once (…). Let them get to breathe fresh air for once, (…)" (Anonymous, 1989; Fraser, 2008). She therefore would judge animal welfare from a rather naturalist view, a position which she shares with a presumably high number of humans and scientists in the animal welfare field (e.g. Rollin, 1995; defining the concept of 'telos', giving that the basic interests of an animal base on its nature (Verhoog et al., 2004). When asking a farmer or a veterinarian, they would probably emphasize animal welfare indicators with an aspect to the animal's functionality using indicators which define a health state or the performance (Fraser, 2008). Judging animal welfare therefore is in urgent need to be based on objective and valid indicators to ensure sustainable animal welfare assessment which is accepted by a majority in human society (Ohl and Van der Staay, 2012). Additionally, priority of the measured impact in animal welfare assessment should not lie on what one or the other human league thinks to be good welfare, but on the evaluation from the animal's perspective. Thus, strenuous efforts in finding the indicators which include affective states (or even the subjective experience of these affective states) should be taken.

As already described above, an emotional response, according to human studies, is manifested in three fundamental reaction components: the physiology, the behaviour and the subjective component. Characteristics of the first two components can be measured quite easily, even in non-human animals (Mendl et al., 2010b). Changes in affective state have an effect to the activity of the autonomous nervous system (Levenson, 2014; Krause et al., 2017), to the brain activity (Muehlemann et al., 2011; Gygax et al., 2013) or might change muscle activity which can be reflected in different facial expressions (Finlayson et al., 2016; Hintze et al., 2016) or a tendency to run away from a specific situation (Paul

et al., 2005). Behaviour, as well, is often consulted to make assumptions to the affective state, like play behaviour (Held and Špinka, 2011) or the behaviour in different behavioural tests (Franks et al., 2013). Although all of them are very important indicators, some of these have their limitations illustrating the arousal dimension in affective states rather than the valence (e.g. heart rate variability; behaviour).

In human studies, measurements of the subjective component are based on verbal "self-reports" (Scherer, 2005), a tool which is obviously not available in non-human animals. Additionally, as there is still no valid measurement tapping the conscious experience of an emotion there is still no consensus about the existence in non-human animals per se. Irregardless of which side of this debate one finds himself, it is a fact that affective states are highly subjective (which is the same in humans), as a direct measurement of these intrinsic processes is not possible thus far (with even verbal reports having their limitations in validity). Here, cognitive-emotional theories might be promising to fill this gap.

As already mentioned above there is a strong relationship between affective states and cognitive processes. Cognition can be described as the entirety of how animals receive and process the information from their environment, e.g. perception, learning, memory and decision making (Shettleworth, 2001). Contrary to the assumption that cognition and affective states are two incoherent things, modern theories refer to the interaction between both. Both of these share the same neuronal networks - sensory in and output - as well as the somatovisceral consequences (Sergerie and Armony, 2006; Okon-Singer et al., 2015). Duncan and Barrett (2007) even claim affect to be some form of cognition, as they state that a "non-affective thought" does not exist.

As already mentioned, this linkage between affect and cognitive processes is bidirectional, meaning that at each level appraisal mechanisms might be influenced by some form of cognitive bias (Paul et al., 2005). This phenomenon is known from human studies as the cognitive output is found to be influenced by the valence of the individual's affective state (Mathews and MacLeod, 1994; Mendl et al., 2009; Blanchette and Richards, 2010; Blanchette and Richards, 2013). These cognitive biases can be grouped in three categories - the attention bias, the memory bias and the judgement bias (see Paul et al., 2005 for a review of the distinct categories). To cut a long story short, it may be stated that humans being in a negative affective state (pessimism/affective disorders/ etc.) tend to pay more attention to threatening stimuli (Kindt and Van Den Hout, 2001), are more prone to negative memories (Gotlib and Krasnoperova, 1998) even if

results indicate differences between depressive and anxious disorders (with depression revealing an effect while anxiety does not) and they are more likely to judge an ambiguous situation negatively (Loewenstein et al., 2001). On the contrary, there is an association between optimism and the attention to positive stimuli (Segerstrom, 2001), positive affective states facilitates the recall of positive memories (Ashby and Isen, 1999) and leads to a rather positive judgement (Clore and Huntsinger, 2007). These cognitive biases are adaptive and biologically reasonable to maximize fitness (Nettle and Bateson, 2012) and there is evidence for them not to be restricted to humans but to occur in non-human vertebrates as well (Paul et al., 2005) and even could be detected in invertebrates (e.g. bees; Bateson et al., 2011). Measuring such cognitive biases therefore provides the possibility of getting access to the affective state of non-human animals indirectly while adding information of the valence and (theoretically) giving an impression of the subjective experience even in non-human animals (Mendl et al., 2009).

1.4.1 Cognitive Bias Test

Cognitive bias tests are promising tools when measuring affective states and therefore might be used to evaluate animal welfare (Würbel, 2009). In the last years, there has been a noticeable scientific boost in this field. There have been a few studies concentrating on the measure of attentional biases (Bethell et al., 2012; Brilot and Bateson, 2012; Lee et al., 2016). However, the majority of studies in non-human animals concentrate on the judgement bias.

The group around Mike Mendl and Liz Paul from Bristol University (UK) were the first to transfer the concept of judgement bias to non-human animal research. The principle is based on a training period (discrimination learning) where animals should learn to associate two stimuli either positively or negatively and to show a specific behavioural response to both of them. In a testing phase animals are exposed to ambiguous stimuli (i.e. probes) – the behavioural response should shift according the underlying affective state of the animal. Therefore, animals should present a rather optimistic behaviour when being in a positive affective state whereas they should react pessimistically when being in a negative affective state. In the pioneering study (Harding et al., 2004), they trained rats to show lever pressing when confronted with a tone signal which was positively associated to food and to refuse to press the lever when confronted with a different tone signal which was associated negatively (white noise). In testing, animals were housed in different systems (predictable vs. unpredictable) to induce opposing affective conditions. Presenting an ambiguous (neutral) tone signal to the animals resulted

in a pessimistic behaviour when housed in the unpredictable environment - e.g. the behavioural response resembled the reaction following the negative associated tone stimulus. Up to now, the cognitive bias approach has been used in many different species to assess the affective state, for example in rats (Burman et al., 2008), starlings (Bateson and Matheson, 2007) sheep (Destrez et al., 2012) and dogs (Starling et al., 2014), but also bees (Bateson et al., 2011). For a detailed review of the different species as well as the different methods used see Roelofs et al. (2016); for a review which discusses in depth the usage of the cognitive bias paradigm in farm animals in special, see Baciadonna and McElligott (2015).

Numbering 27.3 million in Germany alone (Statistisches Bundesamt, 2016), pigs are an important farm animal species so it would seem necessary to measure their affective state as an important parameter to ensure animal welfare. A first attempt to establish the cognitive bias approach in pigs was done by Douglas et al. (2012). This study is based on the original study of Harding et al. (2004). Animals were trained in a go/no-go task, auditory cues were used for discrimination learning and for testing of cognitive bias. The authors found the animals to show a more optimistic response when housed in an enriched environment when compared to barren housing conditions (not tested repeatedly) and a positive bias when switched from barren to enriched environment. However, authors in this study reported that pigs did not manage to learn pure tones of different frequencies as was originally proposed by Harding et al. (2004). They used complex auditory cues instead, which complicates interpretation of the results, as test cues might be considered rather as "novel" then being "ambiguous". A study of Beattie et al. (2000) could show, that environmental enrichment indeed can alter the behaviour of pigs, when confronted with "novelty", finding pigs from enriched environments to show shorter latencies when presented with a novel object. Testing cognitive bias, ambiguity is the key element though. A promising approach was developed by Burman et al. (2008). They presented a spatial test design in rats. As spatial perception has ecological relevance in many animal species (e.g. foraging behaviour), the spatial judgement test is most prominent in cognitive bias testing by now.

In pigs, however, Düpjan et al. (2013) - using a spatial go/no-go task - could not find a pessimistic behavioural response due to social isolation. The same negative results were found by Scollo et al. (2014) who failed to show a negative behavioural response due to increased stocking densities. In this study, authors report a learning process concerning the ambiguous cues (in this case: unrewarded), a result which could be found in a study of Carreras et al. (2015), as well. Here, authors used a spatial go/no-go task, testing animals twice researching

the consistency in time. They found animals to show a more optimistic behavioural response in the second trial, assuming that animals did learn the impact of the ambiguous cues, an effect which was previously observed in sheep (Doyle et al., 2010b).

These results already indicate that mechanisms behind the cognitive bias are not well understood yet. The influence of affective states on decision making in general is depending on various factors. One factor would be the quality of the decision itself. Blay et al. (2012) could show that there is an asymmetry between positive and negative affective state depending on the risk value of the decision which has to be made. They found negative affect to improve information search efficiency in humans when associated to a high risk, while positive affect would decrease search efficiency when risk is low. On the other hand, not only the quality of the decision but also the quality of the affective state might be decisive in order to receive a shift in decision making. Raghunathan and Pham (1999) found that even affective states of the same valence (sad vs. anxious) might result in different cognitive outcomes. Also, it is not clear if cognitive bias approaches represent emotional states or moods (i.e. long lasting emotional states) per se or rather measure shifts in affective states (i.e. from positive to negative and vice versa). It is most likely that species-specific cognitive abilities like their mental representation of time (e.g. time perception, episodic memory, ability of anticipatory planning) [Mendl and Paul, 2004] or their self-awareness might play a superior role influencing the mechanisms how affective states are regulated in a cognitive approach.

Apart from these general influences, the impact of affective states also depends on a range of factors including the environment as well as previous experiences that the subjects rely on, a fact that should be taken into account when designing cognitive bias tests. In pigs, the study of Douglas et al. (2012) mentioned above found an interaction of present and past environment, with pigs switching from an enriched environment to a barren environment to be more pessimistic than pigs which were housed in a barren environment from start on.

Additionally, designing cognitive bias test designs not only holds methodical pitfalls (e.g. finding the right cues, maintaining ambiguity, measuring the appropriate behavioural response etc.) as discussed by Roelofs et al. (2016) but statistical pitfalls among others as discussed in the review of Gygax (2014). However, Murphy et al. (2014) provides several promising studies, testing pigs in a cognitive bias approach using an active choice test. Here, tone cues were used to indicate either the positive or the negative event, and as behavioural response the

animals had to open one of two goal boxes. Although they did not manage to find an effect in the behavioural response due to a mild stressor (e.g. restraint), they could find more pessimistic answers of pigs with a low birth weight, compared to pigs with a high birth weight in a later study (Murphy et al., 2015). The active choice design works best using rewards of different values (opposing to a differentiation due to reward and punishment). Murphy et al. (2013a) managed to train the animals on quantities of rewards (e.g. 1 M&M chocolate vs. 4 M&M chocolates). In pre-studies conducted in our own group, we did not manage to reproduce these achievements across individuals. Furthermore, training the active choice design is quite time-consuming. The proposed design of Murphy et al. (2013a) found animals to have learned the task in 16 sessions on average (1 session/day; 13 trials/session).

The aim of the presented thesis was to establish a test design for pigs that is based on a go/no-go task. These are usually easier to apply to the training, which therefore shortens the training phase. The focus of the test design was to predominantly rule out problems associated with repeated testing (a problem which I will discuss in greater detail later on), while on the other hand dealing with the methodical difficulties found to occur in testing the affective states in pigs.

1.4.2 The open field / novel object test

The open field test (OF) is one of the most popular behavioural tests in animal science (Walsh and Cummins, 1976), providing information for the behavioural component of affective states. Basically, an animal is placed in a (novel) arena, and sometimes the test is combined introducing a novel object. The test was originally developed for laboratory animals such as rats and mice to measure the exploratory behaviour and general activity (Gould et al., 2009). Principally, the OF measures different states of fear, by using the fact, that rodents usually are uncomfortable in large open environments. For laboratory animals, there exist numerous studies validating the OF for its validity in detecting anxious like behaviour (for a review see Prut and Belzung, 2003). As the test is easy to apply, it seems appealing to transfer this test to other species. In fact it is used in many farm animal species to measure affective states, primarily in measuring fear behaviour. Here, the OF often is extended by introducing a novel object test (NO) measuring the reaction of the animals to an unfamiliar object. Forkman et al. (2007) provides a comprehensive review of fear tests (OFNO among others) used in farm animal species but also quite critically discusses the conclusion, which are drawn from the

results in the different studies. In cattle, for example, the same behavioural parameter might be interpreted different depending on different factors influencing the test situations. Activity measured in the OF is affected by the age, as older calves seem to be more active in the novel arena then younger ones (Boivin et al., 1992). While, according to Forkman et al. (2007), the OF is validated quite well in sheep, goats and poultry, studies on horses lack correlations with other tests and therefore must be interpreted with caution. In pigs the usage of the OF is discussed quite controversially. Indeed, different factors might complicate the interpretation of results. As Beattie et al. (1995) could show in a study with gilts, experiences during the rearing time might influence behaviour in the OF, as they found animals reared in barren environments to show significantly more locomotor behaviour compared to animals raised in enriched environments. An influence of enrichment in the rearing period to performance in the NO could be shown by Pearce and Paterson (1993) who found pigs to interact with the novel object less when they had access to toys in their rearing phase. Using pharmaceuticals to manipulate pig's internal state revealed contrasting results. As Andersen et al. (2000) found no effects of diazepam treated pigs in the OF, Donald et al. (2011) found animals treated with azaperone to show higher locomotion scores as well as higher rates of exploratory behaviour.

However, Puppe et al. (2007) showed that cognitive enrichment clearly influences behavioural reactivity in an OF, von Borell and Ladewig (1992) found that adrenocortical function is positively correlated with active behaviour in the OF and Donald et al. (2011) found the presence of a conspecific to positively influence exploratory behaviour in the OF. Considering these results, even if the interpretation has to be handled with caution, the OFNO seems to be a good tool to measure behavioural activity.

1.5 Rationale behind the presented studies

The aim of this thesis is to establish and validate a spatial judgement bias paradigm in pigs, in order to provide a valid tool measuring affective states in pigs objectively. The following chapter presents three studies which aim for a comprehensive picture, including neurophysiological, behavioural and cognitive components as well as practical solutions.

Study 1 provides an improved test design, dealing with the species-specific difficulties the cognitive bias provides in pigs (as described above in part 1.4.1). Focus of this study was to develop a spatial judgment bias design which enables repeated testing and which induces graded responses to graded probes.

As described above, mechanisms behind the cognitive bias are not well understood yet. However, 5-HT is an important key player in regulating affective states. Therefore, study 2 addresses a proposal by Mendl et al. (2009) who suggested validating the cognitive bias approach pharmacologically. In this thesis, a first attempt by depleting the 5-HT availability in the brain is presented. Using the improved test design (study 1) this manipulation was expected to result in a pessimistic behaviour.

Study 3 uses the cognitive bias approach with a rather practical orientation by measuring the effects of tryptophan (TRP) supplementation in the pig's diet on the affective behaviour. As TRP is the precursor in 5-HT synthesis a supplementation in the pig`s diet was expected to result in a higher availability of 5-HT in the brain and therefore to result in a positive behaviour in cognitive bias.

The following part will provide an introduction to the theoretical background and ideas behind the three studies presented in this thesis.

1.5.1 Study 1: Modification of a spatial judgement bias task for pigs

According to Murphy et al. (2014) behavioural tests measuring affective states should fulfil a range of requirements. Tests should be (a) ethological-relevant in the behavioural response which is expected, (b) they should be robust against side

effects and therefore specific to target the affective state, (c) they should detect subtle differences in affective state already, (d) be robust when tested repeatedly, (e) should be standardized and (f) able to be automatable. Testing cognitive bias in a go/no-go paradigm, Gygax (2014) proposes that animals are supposed to behave as follows: (1) They should show a no-go reaction to the negative associated cue and a go reaction to the positive associated cue; (2) they should show a monotonic graded response (from negative to positive) to ambiguous cues according to their resemblance of the reference cues and (3) the behavioural response to the reference cues should not change due to manipulation of the affective state while (4) the response to the ambiguous cues should be affected.

Even though the cognitive bias approach is a smart way to reveal information of the affective state indirectly - via the linkage to cognitive processes - it does have its practical pitfalls in fulfilling these requirements, particularly since there are species specific differences which have to be taken into account. The main difficulties include: animals having to learn the discrimination task which is the basis of the cognitive bias paradigm; finding the right cues for each species; finding the best parameter to measure; finding an adequate reward/punishment; avoiding a loss of ambiguity due to learning in the cognitive bias when tested repeatedly and maintaining a stable response in the test situation (Roelofs et al., 2016). To my knowledge, up to now there are eight studies (excluding those provided in this thesis) dealing with pigs in a cognitive bias approach. They reveal the problematic nature of the right cues and reinforcement (Douglas et al., 2012; Carreras et al., 2015), the long time required for training (Murphy, 2015; Murphy et al., 2014) or the problem to achieve consistent behavioural responses when testing repeatedly (Roelofs et al. 2017; Düpjan et al., 2013; Murphy et al., 2013b; Scollo et al., 2014; Carreras et al., 2015), the latter being one of the most frequent problems.

The first aspect was discussed by Düpjan et al. (2013). Within this study pigs were taught a spatial judgement bias task. The task consisted of opening a goal box in one position to either receive a reward (positive) vs. not opening the goal box on another position where they would receive nothing (negative). Testing the animal's judgement to ambiguous probes resulted in an undifferentiated behavioural response to three different probes, all of them resembling the answer in the rewarded training situation. When pigs are housed conventionally their environment is kept quite barren compared to their natural species-specific habitat. Additionally, pigs dispose of rather complex cognitive abilities (Marino and Colvin, 2015). Opening the goal box and the judgement bias task might therefore act as a cognitive enrichment by itself (Puppe et al., 2007; Zebunke et

al., 2011; Zebunke et al., 2013; Westlund, 2014) which could then mask the behaviour in the cognitive bias testing (ambiguous probes) by shifting it to a more positive outcome. Therefore, in the presented thesis I introduce a mild punishment - consisting of a plastic bag which was shaken in the pig´s face - in order to raise the cost for a potential wrong answer.

Achieving consistent behavioural responses when testing repeatedly is a common problem in cognitive bias testing. In most approaches the ambiguous cues used in the testing phase remain unreinforced. If 'no reward' is used as a punisher in the discrimination task, this might lead to difficulties of clearly distinguishing the ambiguous cue in the testing phase from this negative reference. An even bigger problem is the learning capacity of the animals. This effect was not only observed in pigs - as described above - but in other species such as starlings (Brilot et al., 2009), sheep (Sanger et al., 2011; Destrez et al., 2014) or dogs (Karagiannis et al., 2015). Doyle et al. (2010b) tested the behavioural reaction of sheep in a judgement bias task done without any experimental treatment. They found that with increasing time of the study animals tend to change their behaviour to a more pessimistic one. As it seems unlikely that animals changed their affective state (as environment etc. stayed stable), the authors discuss such a shift in behaviour to be the result of a learning process of the unrewarded outcome of the unreinforced cues. These results could be confirmed for pigs in a study by Carreras et al. (2015) who tested their animals repeatedly in order to evaluate the stability of behaviour over time. There are several approaches in order to find a solution for this problem. Murphy et al. (2013b) proposes to reinforce the ambiguous cues according to the animal´s expectation. But as Roelofs et al. (2016) state, this might lead to an overestimation of the positive cue and does result in associative learning rather than judging an ambiguous event. Another approach would be to minimize the amount of testing trials, as reported in studies on rats or sheep (Roelofs et al., 2016) – this might solve the problem of learning but on the other hand might also lead to an increased variation in behavioural responses. Furthermore, pre-studies in our group found pigs to be capable to learn the unrewarded outcome of test cues in the second presentation already. I decided to test another approach which already worked well in several other studies: the partial reinforcement (Matheson et al., 2008; Bethell et al., 2012; Neave et al., 2013; Bateson and Nettle, 2015). Here, while learning the discrimination task, reinforcement is intermitted thus leaving some of the training trials unreinforced. This increases the uncertainty of the potential outcome of ambiguous probes in the test phase and therefore might slow down the learning process meaning, that in consequence the time test cues are perceived ambiguous is extended. Using the

partial reinforcement requires a differentiation between reinforcement vs. the absence of such, therefore, here again, using "no reward" as a punisher seems to be unfavourable.

There are only a few parameters used, measuring the impact of the behavioural response in the cognitive bias. In some studies, authors use the latency to respond (Mendl et al., 2010a; Bateson and Nettle, 2015) whereas the accuracy or the amount of approaches is used in other cases (Doyle et al., 2010a; Keen et al., 2014). Some authors measure the behavioural response more specifically like in a study with dogs measuring the running speed (Karagiannis et al., 2015). Using a go/no-go paradigm, the choice of the right parameter is quite important. Parameters might be influenced by different effects. One would be the motivation to work for food or to avoid a negative stimulus (individual differences, differences due to time of day etc.). There might be age-related effects (at least for latency and running speed) or interactions with the experimental setup (e.g. testing pharmaceuticals which inhibit walking motivation). In my thesis, I calculate a relative latency, which is based on the relation to the daily means of the approaches to the reference cues, both, the rewarded and the punished cue. To clarify the relevance of this parameter I will give an example: The daily mean in approaching the rewarded cue would be one second for one animal, whereas showing a mean latency of 30 seconds reaching the punished cue; a second animal would show a daily mean of 20 seconds in approaching the rewarded cue, whereas it would completely avoid the punished cue (60 seconds). If both animals show a latency of 25 seconds to an ambiguous cue (using rewarded and punished cue as reference for its decision) the first animal would judge this test position to be more likely to have a negative impact (with its reaction resembling the reaction to the negative reference) whereas the second animal would judge this ambiguous situation rather positive (its reaction resembling the behaviour to the positive reference). Apart from using the relative latency, different behavioural parameters are measured (e.g. movement or exploration) in order to find behaviour traits correlated to the latency. This might be interesting in treatments interacting with food motivation or impaired locomotor ability.

To conclude, the main aspects of the first paper are (1) to find an adequate reward/punishment system to ensure that animals are (a) able to distinguish both, positively and negatively associated stimulus and (b) would judge the cost of the punishment higher than the profit of the experimental procedure itself; (2) testing animals repeatedly with animals showing a stable response over time and (3) finding the adequate parameter to measure the behavioural outcome. Altogether, this first study aims to provide a basic test design for a spatial judgement task

(SJT) in pigs which deals with the problems reviewed above (1.4.1). This test design was used further in order to validate the test design and to consider a practical relevant application (study 2 and 3).

1.5.2 Study 2: Pharmacological validation of the spatial judgement bias task for pigs

There is a variety of behavioural approaches to study affective states in animals. Most tests which are used in farm animal science are adapted from tests designed for laboratory animals or - as is the case in the cognitive bias approach - from human studies which target possible problems in interpreting the results when performed with animals. Therefore, the establishment of every behavioural approach requires a species specific validation. Taking the most prominent behavioural test as an example, behaviour in the OF test (originally developed as fear test for rodents) might be interpreted in various ways. While an increase in exploratory behaviour in mice might be interrelated with a decrease in anxiety and therefore a rather positive affective state, the same effect in pigs might be correlated with an increase in arousal. This does not hint directly to the valence of affective state (Donald et al., 2011). Using the cognitive bias paradigm, there are several examples where the results of studies do not coincide with the assumptions. A study of Doyle et al. (2010a) found sheep to show a positive bias after a restraint treatment which was found to be received as a significant stressor to these animals (as reflected by measurements of cortisol). Authors discuss these unexpected results to either reflect a positive affective state of the animals due to a "release response" displaying the end of a negative treatment, or to be due to an altered "risk taking threshold" resulting from the negative experience. Similar results were found in a study with goats when comparing groups which experienced poor welfare with groups receiving good care. Unexpectedly, female animals from the "poor welfare group" displayed a rather optimistic cognitive bias. Other studies do not find differences in cognitive bias at all, even if the conditions should induce opposing affective states (Wichman et al., 2012; Düpjan et al., 2013). In the study of Düpjan et al. (2013) pigs were exposed to repeated social isolation, which is known to be a severe stressor to this species. The treatment revealed no effect. In contrary, animals evaluated the test positions in the same manner as the positive reference. Authors reported food motivation to be high in this food rewarded task design. They therefore supposed the test

design itself might already have represented a cognitive enrichment (see Puppe et al., 2007; Zebunke et al., 2013 for a discussion) which in consequence could overlap effects of the negative treatment. Comparable results could be found by Svendsen (2012), where training and preparing mink for the cognitive bias task altered behaviour of animals already. There are many such examples. For a broad review see Gygax (2014), altogether these inconsistent findings confirm the urgent need for a species- and method specific validation of the test paradigm.

As Gygax (2014) states, these inconsistent findings might be due to a lack of an all-encompassing comprehension of the feedback between affect and cognition. Reliability of many established behavioural tests benefit from a validation (Bailoo et al., 2014) by pharmaceuticals like well-established anxiolytics or anxiogenics. Direct manipulation of the internal affective state via pharmacological agents - which results in the predicted outcome - should also help to strengthen the validity of the cognitive bias approach (Mendl et al., 2009). Indeed, there are attempts to show the cognitive bias being sensitive to pharmacological treatment.

In rats, pharmacological manipulation of serotonergic, noradrenalin and dopaminergic system resulted in shifts of the behavioural response (Rygula et al., 2014a). The same could be found for mazindol, a dopamine reuptake inhibitor, as well as for lithium, which is used in pharmacological treatment of affective disorders in humans (Rygula et al., 2014b; Rygula et al., 2015a). In sheep, Doyle et al. (2011) found animals to show more pessimistic judgement due to 5-HT depletion via administration of para-Chlorophenylalanine (pCPA), which inhibits the 5-HT synthesis. Kis et al. (2015) could show dogs displaying rather optimistic behaviour to ambivalent cues after oxytocin administration, whereas McGuire et al. (2015) could not confirm these results in rats. As studies manipulating the affective state pharmacologically are rare, the methods are differing and species specific effects might occur. Study 2 presents an experimental setup validating the proposed spatial judgement task (Study 1) by depleting the 5-HT availability in the brain, using a pharmacological treatment (pCPA). As described above (1.3.2) the 5-HT system plays a great role in regulating affective states, therefore depleting the concentrations is expected to induce a negative affective state, a result which could be confirmed in sheep already (Doyle et al., 2011). The study presented in my thesis additionally evaluated the efficiency of the treatment. Herefore, the 5-HT depletion was validated by measuring the 5-HT concentration in various brain areas relevant for affective-cognitive regulation (for a detailed description of the chosen brain areas and their main functions see 1.3.1), before and after pCPA administration. Furthermore, the behaviour of the animals was tested not only in the SJT, but in a standard behavioural test, the OFNO. The study therefore

compasses most different facets resulting from the 5-HT depletion via pCPA, taking various components (physiological, behavioural and subjective/cognitive) into account. It therefore not only validates the proposed test design, but contributes to our understanding of the basic mechanisms underlying affective-cognitive control in the pig.

1.5.3 Study 3: Application of the spatial judgement bias task in a practically relevant paradigm

Instead of a pharmacological manipulation of the 5-HT system (as proposed in study 2), study 3 of this thesis provides a rather practical approach to transfer the theoretical results to applied behavioural sciences. A dietary supplementation of tryptophan (TRP), an essential amino acid and the precursor of 5-HT, was used to raise 5-HT concentrations within the brain. Here again the efficiency of the treatment was validated measuring the 5-HT (and its substitutes) concentration in the brain (see 1.3.1 and 1.5.2). TRP administration via the feed is commonly accepted as feed additive to manipulate behaviour positively (DeNapoli et al., 2000; Höglund et al., 2005; Brunberg et al., 2016). In pigs TRP supplementation is known to influence weight gain (Le Floc´h et al., 2004), stress response (Koopmans et al., 2005) and behaviour (Liu et al., 2013; Li et al., 2006). To my knowledge there is no study directly focusing on measuring the affective state. Therefore, study 3 investigates the assumed effect of TRP-supplementation on affective states in pigs, which also contributes in the debate of the use of TRP-supplementation in the therapy of other non-human animals. Here again, the study is complemented by behavioural observations in an OFNO test, so as in study 2, it is supposed to present a comprehensive picture of the underlying affective state and the mechanisms underlying the affective-cognitive control in the SJT.

1.6 Hypotheses and goal of the thesis

Accepting affective states to play an essential part of animal welfare leaves it absolutely essential to find measurements of those intrinsic states. This is most important in order to judge the quality of different relevant situations (e.g. housing conditions), all in the light of providing maximum welfare conditions. Especially in farm animals, where animal welfare often has to compete with economical challenges, the quality of welfare is judged from different point of views (see 1.4. for a short digression on this topic). Therefore here particularly, the risk of observer biases should be minimized, which leaves methodologies measuring affective states in urgent need to be validated. The cognitive bias approach, as one promising method to gain insight in the affective state of non-human animals, is based on the linkage between affect and cognition, interacting by a whole range of networks in the brain. As already mentioned above, the underlying mechanisms of these complex interactions are not thoroughly understood yet. The goal of my thesis therefore was to provide a test design which is suitable for the usage in pigs, which is validated and which could be transferred to practically relevant issues and as overall subjective would reveal insights in the mechanisms underlying those cognitive – affective appraisal processes.

- It is hypothesized that training pigs in a spatial go/no-go task using a reward as positive and a mild punishment as negative reference and additionally introducing a partial reinforcement to the training process would enable repeated testing of the ambivalent probes and induces graded responses to graded probes (Study 1).
- Furthermore, hypothesis is, that a depletion of the 5-HT synthesis would decrease 5-HT concentration in brain areas relevant for cognitive-affective processes whereas the supplementation of TRP would increase 5-HT concentration respectively (Study 2 and 3)
- It is assumed that, using the SJT, a depletion of the 5-HT synthesis, would result in a pessimistic evaluation of the ambivalent probes (Study 2).
- Consequently, the hypothesis is that a surplus of 5-HT acquired by a TRP supplementation via the diet would result in an optimistic evaluation in the SJT (Study 3).
- Both manipulations of the 5-HT system (5-HT depletion, TRP supplementation) are expected to alter behaviour in the SJT and an open field/novel object (OFNO) test (Study 2 and 3).

In conclusion, the three studies presented in my thesis are expected to verify practicability, validity and applicability of a modified cognitive bias design in pigs. As 5-HT depletion is supposed to generate a negative affective state whereas TRP supplementation should induce a positive affective state the thesis also is expected to provide evidence of the sensitivity of the test design to both extremes of the valence dimension. Complementing the studies by measurements of the 5-HT concentration in selected brain areas, as well as including measurements of the behaviour in a standard behavioural test (OFNO) this thesis comprises the multifaceted characteristics of affective states and is expected to contribute to a better understanding of the complex mechanisms as one essential part in animal welfare science.

Chapter 2:

Experimental studies

2.1 Study 1: An improved design for testing cognitive bias in domestic pigs

Redrawn from Applied Animal Behaviour Science: An improved design for testing cognitive bias in domestic pigs

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Abstract

Public concern for farm animal welfare calls for reliable tools to measure it. Measuring cognitive bias, i.e., the influence of affective states on cognitive processing, has gained importance during recent years. However, current methods for the assessment of cognitive bias in domestic pigs, one of the most intensively housed farm animal species in Europe, need to be refined and validated. Hence, we developed a design to test pigs repeatedly and induce graded responses to graded probe cues. For this, we used a spatial judgement task in a go/no-go paradigm with partial reinforcement and a mildly aversive negative reinforcer. A total of 16 female juvenile German Landrace pigs underwent discrimination learning of the rewarded vs. punished goal box location; this was then repeatedly tested on three probe locations in between (eight times in four weeks). The subjects learned to discriminate between the two reference locations and showed graded responses (latency to open the goal box) to the probe locations. Analyses of the subjects' general behaviour implied that additional information on the animals' state can be derived. The subjects showed no signs of learning with respect to the outcome of probe cues but exhibited stable response levels during test weeks two to four. In conclusion, the design presented in this paper is suitable for testing affective valence based on judgement bias in domestic pigs and hence can contribute to our understanding of the welfare of this intensively farmed species.

Highlights

- spatial judgement tasks can be used to asses affective valence in animals
- current experimental designs for pigs need to be improved
- we developed a spatial go/no-go task with mild punishment and partial reinforcement
- pigs could be tested repeatedly and showed graded responses to graded probe cues
- this experimental design is suitable for future studies on affective valence in pigs

Introduction

Farm animal welfare has been an issue of increasing public concern in recent decades (e.g., European Commission, 2012), and reliable tools for measuring animal welfare are needed. Building on concepts such as the five freedoms (FAWC, 1992) the importance of allowing an animal to experience positive emotions while avoiding negative ones has emerged (Mendl & Paul, 2004). However, measuring emotions, and specifically differentiating positive and negative emotions, has been highly elusive. In 2004, Harding et al. first introduced an experimental design to measure cognitive bias, i.e., the effect of affective states on cognitive processes, such as judgement, on ambivalent stimuli as a proxy measure of affective valence in rats. In general, animals are trained to associate two different cues, which usually lie at the ends of a continuum (high note vs. low note, black vs. white, left vs. right, etc.), with either a positive or negative outcome and to show different responses to these cues (e.g., Mendl et al., 2009). The animals are then confronted with probes, i.e., cues that lie between the trained reference cues. Animals in a positive affective state are more likely to show a response resembling the response given to the positive reference cue in this test situation, whereas a "pessimistic-like choice" (i.e., a response resembling the response given to the negative trained cue) is interpreted as an indicator of a more negative affective state. This approach, which has previously been used in human psychology only (Eysenck et al., 1991), has since been integrated into animal welfare research. Although designs such as the spatial judgement task (Burman et al., 2008) have been applied to various vertebrate and even invertebrate species, one of the most important farm animal species in Europe, the domestic pig, has rarely been studied, and the existing studies encountered several methodological difficulties. One of the first studies in pigs (Douglas et al., 2012) used complex acoustic cues; hence, the probe could not be considered "intermediate" so much as novel. Furthermore, this study was performed with a small sample size that could not be analysed with a full statistical model appropriate for this type of dataset (Gygax, 2014; Douglas et al., 2012); thus, conclusions drawn from these data should be considered with caution. Another study by our group (Düpjan et al., 2013) used the spatial judgement task on a larger sample size; however, no effects of repeated social isolation could be found, which was contradictory to expectations. Notably, all probes were treated the same as the rewarded reference cue (i.e., the goal box on the rewarded location) with no gradual differences along the continuum. Additionally, animals could only be tested with each probe once because they soon learned about their outcomes (Düpjan et al., 2013; also see Doyle et al., 2010 and Scollo et al., 2014 for similar effects in sheep and pigs,

respectively, and Roelofs et al. 2016 for a broader discussion). Both the lack of differentiation along the continuum of cues and the fast learning of probe outcomes are problematic in the application of the cognitive bias test in pigs. Firstly, a clear differentiation enables the testing of affective states in both negative and positive treatments (if all probes are treated as positive, how could you measure a change to a more positive affective state?). Secondly, testing animals repeatedly enables testing before and after treatment (thereby reducing the influence of individual differences often present in pigs' behaviour) as well as research on optimism and pessimism as (personality) traits instead of states induced by a given treatment.

Given the enormous number of domestic pigs on farms across Europe and the growing public concern about their welfare, we sought to develop a reliable design for measuring cognitive bias in pigs. We have therefore modified our earlier design of the spatial go/no-go task by adding a mild punishment and partial reinforcement, i.e., omission of both the positive and the negative reinforcer in a subset of cue presentations. In our previous study, we concluded that the animals did not consider our 'negative' unrewarded cue as something to be avoided (i.e., inaccessible food with nearly no cost of a false response to a (putatively) negative cue apart from wasting time). Hence, we increased this cost of a false response to a (putatively) negative cue by introducing a negative reinforcer, an approach widely used in cognitive bias studies, choosing the punishment introduced by Douglas et al. (2012) in their study on cognitive bias in pigs. Learning of probe outcome can be suppressed by an increase in uncertainty. Hence, we introduced partial reinforcement, i.e., subjects would not always be rewarded or punished when on reference locations (Bateson and Matheson, 2007; Matheson et al., 2008). In combination with the negative reinforcer, this led to three different outcomes: the pigs could be rewarded (positive cue only), punished (negative cue only), or neither (positive, negative and probe cues). The latter could happen on both the positive and negative locations; this meant that subjects would not consider any of the probe cues equal to one of the reference cues. Previously (rewarded vs. unrewarded), the probe outcome was the same as for the unrewarded cue; thus, the animals quickly learned that these were alike.

Our hypothesis was that after introducing a stronger negative reinforcer and partial reinforcement, pigs would (1) show increasing approach latencies from the rewarded to the punished cue because there was now a cost of responding incorrectly to a potentially punished cue and (2) show stable responses when tested repeatedly because the unreinforced probes resembled both unreinforced reference cues, causing uncertainty. Lastly, we hypothesized that (3) the analysis

of additional behaviours provides a deeper insight into the animals' state than approach latency alone.

Animals, material and methods

2.1 Ethical note

This study was approved by the ethics committee of the federal state of Mecklenburg-West Pomerania (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei LALLF, AZ: 7221.3-1.1-105/12). All efforts were made to ensure minimal animal number and suffering.

2.2 Animals and housing

The study was conducted with 16 female German Landrace pigs aged 4-10 weeks in two replicates between July and September in 2013 at the experimental pig unit of the Leibniz Institute for Farm Animal Biology (FBN) in Dummerstorf, where all pigs were born and housed with their littermates and mother until weaning. At weaning (28 d of age), the animals were regrouped and subsequently housed in larger, stable social groups of ten (two groups per replicate, four full siblings from five mothers split up so that there were five pairs of full siblings in each group). At an age of 36 days, the animals were moved to the experimental room. The pens measured 1.7 x 2.5 metres each and were enriched with commercial and custom-made pig toys plus a mix of chaffed straw, hemp pellets and sawdust (approx. 50 g) twice per day. Per replicate, eight subjects (four pairs of full siblings, with one sibling per group) were chosen pseudorandomly, excluding animals not eating the food reward in a standardized reward consumption-test on two subsequent days before training. Subjects remained in their groups to maintain stable social conditions. During experiments, the subjects had acoustic contact with their group mates.

Commercial piglet feed (Trede und von Pein, Itzehoe, Germany) and water were available ad libitum. The room temperature was automatically controlled (EuroMatic climate computer, SKOV A/S, Roslev, Denmark), starting at 28°C on the day of weaning and decreasing stepwise to 19°C on the final experimental day.

All animals were subjected to further behavioural tests before experiments and underwent heart rate measurements using a non-invasive system on test days (data not presented here).

2.3 Experimental setting

The experimental setting consisted of an experimental arena that could be accessed from a start box (0.6 x 1.1 m) through a guillotine door and a movable goal box (Fig. 1). The far wall opposite the start box provided permanent fixtures

for the goal box on five locations with the right and the left corner being the rewarded (S^+) and the punished (S^-) location (reference location) and the three in the middle being the probe locations (near rewarded: nS^+, middle: M, near punished: nS^-). The arena had the shape of a circle sector (radius 3.2 m) so that distances between the start box and all goal box locations were the same. Distances between different probes were 1.26 m and 1.15 m between the reference location and the nearest probe. The goal box was custom-built from wood and plastic and contained a metal food bowl behind a flap that could be opened by the pigs with a gentle push of the snout.

Rewards (5 ml of applesauce "Kaufland Classic") were provided in this food bowl and could be made inaccessible (during punished and probe trials) by adding a perforated plastic plate (enabling olfactory cues). Punishment at S^- was applied by waving a plastic bag above or in front of the subjects until these showed a clear avoidance response. The bag was shaken by an experimenter hidden outside the arena next to the goal box so that the bag was encountered unexpectedly.

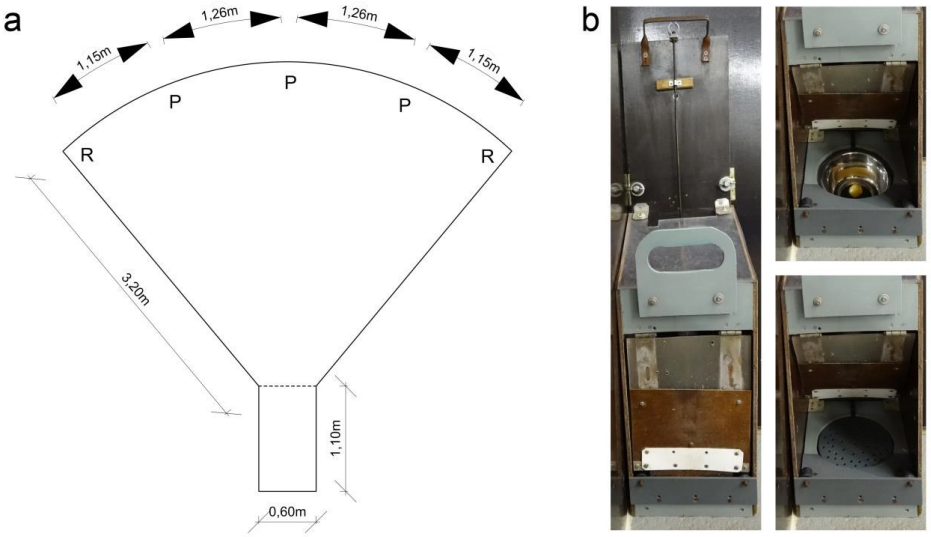

Figure 1: Schematic of the experimental arena with the start box and the five locations of the goal box (a) and photographs of the goal box itself (b). Reference locations (i.e., S+or S−) and probe locations (nS+, M, nS−; depending on where S+and S−were for the given individual) are marked "R" and "P", respectively. The photos show the box closed (left), open with the reward accessible (upper right) and open with the reward inaccessible (lower right).

2.4 Training

The animals were trained individually on a go/no-go task to discriminate between a rewarded (S^+) and unrewarded (S^-) location of a goal box. Half of the animals were rewarded on the left corner of the arena and punished on the right corner and vice versa for the other half. Training was conducted on seven consecutive days (d1-d7). To avoid fatigue in the subjects, training was divided into three sessions per day with a minimum of one-hour break between. Each session consisted of four consecutive trials per animal (i.e., 12 trials in total per day per animal), and each trial lasted one minute after the subject entered the arena with all four feet. In total, the goal box was presented on each reference location six times per day. Once per day, each of the reference locations was neither rewarded ($S^{(+)}$) nor punished ($S^{(-)}$) but unreinforced; thus, there were five rewarded and five punished trials and one non–rewarded and one non-punished trial. On training day 1, the order of the goal box locations and outcomes was fixed (session 1: S^+, S^+, S^+, $S^{(-)}$; session 2: $S^{(-)}$, S^+, $S^{(-)}$, S^+; session 3: $S^{(-)}$, $S^{(-)}$, S^-, S^+), and during session 1, the goal box was not fully closed for the rewarded trials so that the animals could learn to open the flap. From day 2 on, goal box locations were presented in a pseudorandomized order with a maximum of three trials on the same location. Animals had one day of rest between training and testing.

2.5 Testing

Testing started on day 9 and was performed on three consecutive days per week (Tuesday, Wednesday and Thursday) for four weeks. On the days in between, no training or testing took place to avoid habituation to the punishment. Probes were on equidistant locations between S^+ and S^-. Probes were unreinforced and hence resembled unreinforced trials on either reference location. Probe trials were added to the normal training sequences before session 2 and session 3 (i.e., two per day). All three probes were presented twice per week (once in session 2, once in session 3) and eight times in total in a pseudorandomized order (avoiding presenting the same probe twice in a day).

2.6 Behavioural observation

Animals were observed by video recordings. The camera was installed centrally above the arena. Videos were recorded and stored on a digital recorder (Everfocus HD-4H4) and analysed using the Observer XT version 12 (Noldus Information Technology, Wageningen, The Netherlands).

Recorded behaviours, their definitions and type of recording are listed in Table 1. If a subject did not open the goal box, latency was set to 60 s for further analyses.

Latency from entering the arena until opening the goal box was measured for each trial, including the reinforced trials. For further analyses, relative latencies were calculated for the test days (all locations), setting the latency of the choice behaviour in the test trials in relation to the individual daily mean of the rewarded training trials (mean≙0) and the punished training trials (mean≙1) using this formula:

$$relative\ latency = \frac{absolute\ latency - mean\ latency\ (S^{+}, S^{(+)})}{mean\ latency\ (S^{-}, S^{(-)}) - mean\ latency\ (S^{+}, S^{(+)})}$$

In this manner, the potential effects of age/body weight/size or feeding motivation were compensated for.

2.7 Statistical analyses

Statistical analyses were performed using the SAS software for Windows, version 9.3 (Copyright, SAS Institute Inc., Cary, NC, USA). The GLIMMIX procedure for generalized mixed models was used with goal box location, test week and the interaction goal box location*test week as fixed effects in the model, and repeated measurements on the same animal were taken into account. Pairwise multiple comparisons of the least square means were performed using Tukey Kramer tests. For comparisons of the goal box locations within test weeks and comparisons of the test weeks within goal box locations the slicediff option of the lsmeans statement of the GLIMMIX procedure was applied. Data of the reference locations (d1-d32) and test days (d9-d32) were analysed separately. Additionally, latencies to reference locations were analysed for each individual separately. Test days with non-significant differentiation of S+ and S- were excluded from the following analyses. Spearman rank correlations were calculated for behaviour and relative latency using the CORR procedure.

Table 1: Overview of recorded behaviours, giving the name, type of event (according to The Observer), measured parameter (D = duration, F = frequency, L = latency), and definition. Behaviours in the grey section were mutually exclusive.

Behaviour	Type	Measurement	Definition
open	point event	L	opening the goal box
start	point event	reference point	four feet in the arena
locomotion	state event	D	moving; minimum one forward step
standing	state event	D	no locomotion, all four legs stretched out, no floor contact of the torso; minimum 3 feet on the ground
lying	state event	D	lying down with the whole belly or the whole lateral part of the animal touching the ground
exploration	state event	D	physical contact (nose) with the goal box, sniffing, licking
scratching	point event	F	pig is scratching itself, either with the foot, or scrubbing the body on either wall of the arena or the goal box
abrupt movements	state event	F	one of the following components or a combination of them: sudden cantering, jumping and twisting
defecation	point event	F	defecate
urination	point event	F	urinate

Results

Data from 15 animals were included in the statistical analyses. One subject had to be excluded because it stopped eating the reward after the training phase. Data from the second test week of one animal had to be excluded because it refused to eat the reward. Further trials had to be excluded due to technical problems. In the following, each paragraph will first provide the percentage of data that were included in the reported analyses.

3.1 Discrimination learning

Data from 98% of the trials could be used for the analyses. In the training phase, a significant effect of location ($F=5840.8$, $p<0.001$) on absolute latencies could be found, as well as a significant effect of day ($F=2.6$, $p<0.001$) and their interaction ($F=26.1$, $p<0.001$). Pairwise comparisons within days showed that latencies to S^+ were shorter than to S^- on d1 already ($t=-3.6$, $p<0.001$). From day 4 (S^-) and day 5 (S^+) onwards, there were no significant differences within location between days.

3.2 Cognitive Bias Test

3.2.1 Opening the goal box

In total, 97% of the trials were included in the analyses. The analysis of relative latencies showed a significant effect of the relative location ($F=1226.6$, $p<0.001$) with gradually increasing values from S^+ to S^- (Fig. 2 A). Additionally, a significant effect of the test week ($F=13.1$, $p<0.001$) and a significant interaction between the week and the relative goal box location was found ($F=4.2$, $p<0.001$). Latencies at nS^+ and nS- were shorter in week 1 than weeks 2 to 4 (*nS+*: week 2: $t=-2.5$, $p=0.055$; week 3: $t=-2.9$, $p<0.05$; week 4: $t=-3.9$, $p<0.001$; *nS-*: all $t<-3.4$, all $p<0.01$). For M, latencies significantly increased from week 1 to week 2 ($t=-3.0$, $p<0.05$), then decreased to week 4 ($t=2.7$, $p<0.05$). However, there were no such differences for S^+ and S^-, and values remained stable at all locations but M in weeks 2-4 (Fig. 2 B).

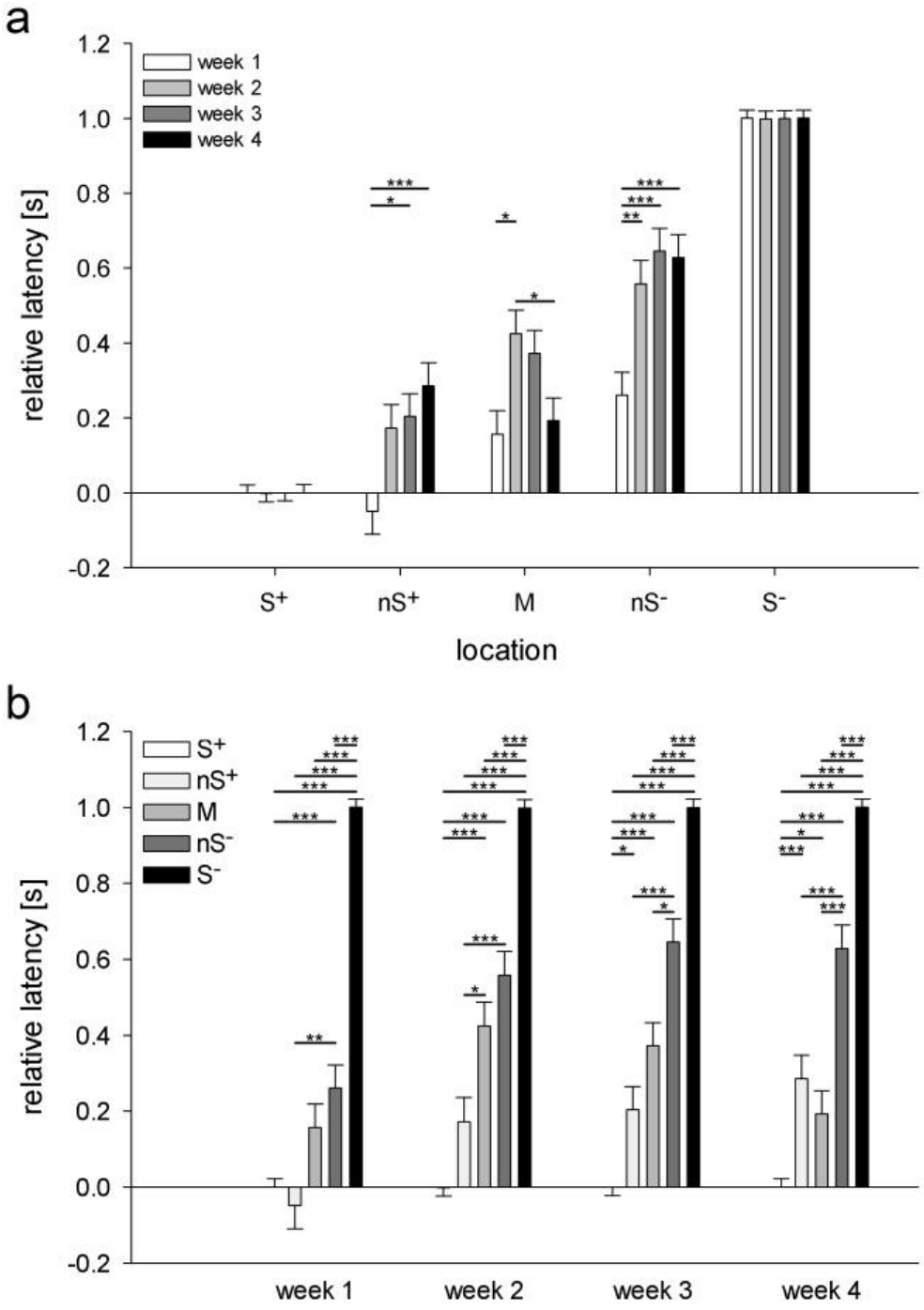

Figure 2: Relative latency to open the goal box (LSM±S.E.) plotted for location (S+=rewarded, nS+=near rewarded, M=middle, nS-=near punished, S-=punished) by week (a) and week by location (b). Pairwise comparisons were made either within location (a) or within week (b) only and are depicted in the respective figure (*=p<0.05, **=p<0.01, ***=p<0.001).

3.2.2 Behaviour

In total, 93% of the observations were included in the analyses. The relative location of the goal box affected the duration of locomotion ($F=9.1$; $p<0.001$), standing ($F=9.4$; $p<0.001$) and exploration ($F=28.9$, $p<0.001$; Fig. 3), with animals performing less locomotion and standing but more exploration when the box was on S+ or the probes. There was a significant effect of week on locomotion ($F=35.2$, $p<0.001$) and standing ($F=12$, $p<0.001$), whereas it had no effect on exploration ($F=0.6$, $p>0.05$). Time spent with locomotion decreased over time, whereas standing increased. There was no significant effect for location or week on the duration of lying.

Location ($F=12.1$, $p<0.001$), week ($F=14.6$, $p<0.001$) and their interaction ($F=1.9$, $p<0.05$) showed a significant effect on the frequency of abrupt movements. Abrupt movements occurred more often on the probe locations than on the reference locations and were most frequent in week 3 (Fig. 4). Furthermore, location revealed a significant effect on the frequency of defecation ($F=4.1$, $p<0.05$) and scratching ($F=3.6$, $p<0.05$), as did week (defecation: $F=3.0$, $p<0.05$; scratching: $F=4.4$, $p<0.05$).

Locomotion, standing and exploration were correlated with each other and with relative latency (Table 2). Most correlations were weak; however, exploration of the goal box and latency to open it showed a moderate correlation, as did standing with both exploration and abrupt movements (Table2).

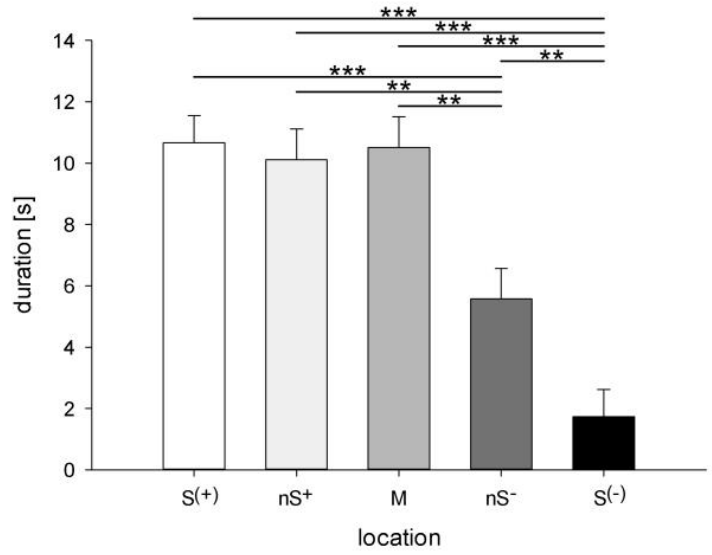

Figure 3: Duration of exploring the goal box at the different locations (LSM ± S.E.; S(+)=unrewarded, nS+=near rewarded, M=middle, nS-=near punished, S(-)=unpunished) and results of pairwise comparisons (**=p<0.01, ***=p<0.001). Note that for the reference locations, only data from unreinforced trials were analysed to exclude the effects of the respective reinforcer.

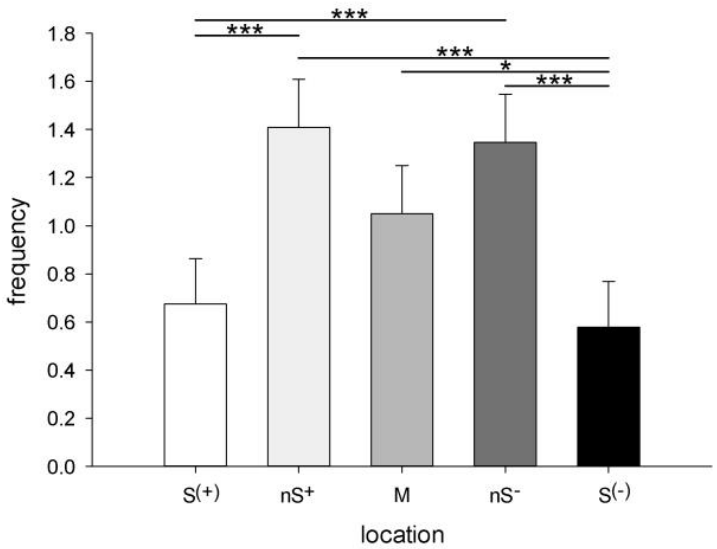

Figure 4: Frequency of abrupt movements behaviour on the different locations (LSM ± S.E.; S(+)=unrewarded, nS+=near rewarded, M=middle, nS-=near punished, S(-)=unpunished) and results of pairwise comparisons (*=p<0.05, ***=p<0.001). Note that for the reference locations, only data from unreinforced trials were analysed to exclude the effects of the respective reinforcer.

Table 2: Spearman rank correlations between relative latency to open the goal box and other relevant behaviours (top value = r; bottom value = p; n = 704 for correlations with latency and 713 for all others). Significant correlations are highlighted in bold letters.

	relative latency	locomotion	standing	lying	exploration	abrupt movement	scratching
relative latency		0.11 **<0.01**	**0.26** **<0.001**	−0.07 <0.1	**−0.52** **<0.001**	**−0.11** **<0.01**	−0.02 n.s.
locomotion			**−0.32** **<0.001**	**−0.19** **<0.001**	**−0.19** **<0.001**	**−0.24** **<0.001**	**0.08** **<0.05**
standing				**−0.30** **<0.001**	**−0.53** **<0.001**	**−0.47** **<0.001**	−0.06 n.s.
lying					0.03 n.s.	**0.19** **<0.001**	0.04 n.s.
exploration						0.05 n.s.	**0.12** **<0.001**
abrupt movement							0.04 n.s.
scratching							

Discussion

This study aimed to establish a reliable design for testing cognitive judgement bias in domestic pigs. By introducing partial reinforcement and a negative reinforcer, we were able to elicit stable responses for six repeated presentations of the probe cues, with latencies gradually increasing from the rewarded to the punished location. Hence, our design appears suitable for future studies on affective valence in this species.

All of our subjects could learn the discrimination task, with decreasing latencies to open the goal box on the rewarded location and increasing latencies to the punished location. This supports previous results that pigs can learn the location of food rewards (Arts et al., 2009; Düpjan et al., 2013). In contrast with other studies (e.g., Bateson and Nettle, 2015; Rygula et al., 2015a), we chose not to define a learning criterion based on which pigs would enter the testing phase on an individual schedule. This was due to practical considerations for our future studies that will employ this design. In those, we will test the effects of treatments aimed at inducing more positive or negative affective states, and some of the treatments may have to be applied to the entire housing group, or at least to influence group members. Hence, all animals within a group should be at the same stage of the experiment. Individual housing is not typical for domestic pigs (with the exception of the temporary confinement of breeding sows); therefore, it should be avoided in experiments. Nevertheless, the performance of individual animals in the training trials was checked each testing day before analysis. Tests of animals that failed to distinguish the reference locations were excluded; however, such cases were rare. As in our own previous study (Düpjan et al., 2013), there was a significant difference between locations on day one, which is surprising. In the previous design, we suspected that this occurred because the selection of the subjects from the larger groups took place in the experimental arena, i.e., the animals could have learned a conditioned place preference for the rewarded location before beginning the training. For this reason, we decided to perform selection in yet another area of the experimental room in this study; however, few (three) presentations of the goal box on the rewarded location at the beginning of training on day one were sufficient to develop this place preference. This demonstrates the rapidity at which pigs learn about food sites. Alternatively, we could have randomized the order of goal box locations from the beginning onwards. However, in our experience, pigs tend to be frustrated quickly, meaning that starting with an unrewarded or punished trial could have resulted in the pigs never exploring the goal box at the rewarded location. Furthermore, developing

conditioned place preferences is not necessarily detrimental to our study design because the subjects remained able to learn the discrimination task (this study and Düpjan et al., 2013) and even showed the expected response patterns in the test situations (see below). Hence, we assume that having a fixed order of cues on training day one is suitable in pigs. From training day five onwards, latencies did not significantly change for either reference location; thus, we can assume that seven days of training, which translate to 42 presentations of each location, are sufficient to guarantee stable discrimination between stimuli.

Furthermore, absolute latencies were stable over time. This can be considered a prerequisite for using relative latencies during the test period. If only relative latencies were reported for this period, one could not determine whether the subjects have already reached a stable level of discrimination, i.e., deficiencies in learning would be masked. However, given the nature of the judgement bias task, the reference system must be set before the testing begins so that the animals have certainty regarding the possible response outcomes on the rewarded and punished locations. However, absolute latencies can be affected by various factors that are both internal and external to the subjects. These may affect the balance between the systems underlying reward seeking on the one hand and punishment avoidance on the other hand (Mendl et al, 2010; Rygula et al. 2015b). For example, in animals fed *ad libitum*, the duration since the last feeding may influence feeding motivation, as could temperature (pigs usually eat less as temperatures increase). Furthermore, there may be individual differences in whether the punished location is avoided altogether or 'just' approached after a longer latency, perhaps because of differences in impulse control or perceived aversiveness of the negative reinforcer. An alternative for controlling for differences in motivation or general activity would have been to use an active choice task (Murphy et al., 2013; Parker et al., 2014). In this task, subjects are trained to make active responses to both training cues (e.g., by pressing different levers), which works best with two rewards of different value instead of a reward vs. punishment. Unfortunately, in pilot studies, we were unable to establish two rewards of different value for our pigs, at least not across individuals. Hence, we used the go/no-go paradigm. Provided that absolute latencies are stable, relative latencies measured in such a design profit from setting a precise, individual reference framework while not suffering from potential drawbacks.

During the test phase, subjects showed graded responses to the five goal box locations, which confirms that the changes we made to our earlier design (Düpjan et al. 2013) were suitable for inducing the intended differentiation of probe cues (Gygax 2014). Notably, there were probe trials in which subjects would not open

the goal box at all (probability highest on the near punished location and lowest on the near reward location); however, even responses to the probe locations closest to the reference locations were significantly different from 'their' reference location. This means that the improved design would now allow for the testing effects of positive or negative treatments, as latencies to probes can significantly decrease or increase, respectively. The choice of a suitable reinforcer is of utmost importance in cognitive bias tests. Having an imbalance between positive and negative reinforcement could result in a bias towards more optimistic/pessimistic behaviour in the cognitive bias test due to low costs and high benefits (Mendl et al., 2009). However, punishments cannot be too aversive because this may scare subjects off altogether. While waving a plastic bag was sufficient to induce an avoidance response, it did not lead to avoidance of the goal box in general (or at least not for long). Notably, this reinforcement may not be suitable in other species or even in more anxiety-prone pig breeds.

The results for the temporal stability of probe responses were somewhat surprising in that animals showed lower latencies to nS+ and nS- in the first of four test weeks, whereas response latencies remained on a stable level afterwards. The lower latencies in test week one may have been interpreted as an indicator of learning the probe outcome between week one and two; however, this explanation can be excluded because there was no further increase in latencies after week two (and even a decrease from week two to week four at the middle probe location). Probe outcome was neutral in that there was neither a reward nor a punishment; hence, learning should have led to increasing latencies as observed in previous pilot studies (unpublished data, reported in Düpjan et al., 2013) and in the literature (Doyle et al., 2010; Scollo et al., 2014; Carreras et al. 2015). Possible reasons for the short latencies in week one may also have been interpreted as novelty effects. While this seems to be supported by our finding that all three probe locations are approached more quickly in week one, duration of exploring the goal box showed no such effect, which contradicts this interpretation. Anyway, this aspect should be considered in future studies, at least by counterbalancing the order of probes (as we did in both the present and our earlier study) so that none of the probe locations are overrepresented in the first probe presentations.

In this study, we decided to investigate the latency in opening the goal box and other behaviours shown during the trials. In designs in which trials end with the decision of the subject (or, more precisely, after the application of the reinforcement), such an analysis is not possible because of highly variable trial durations. We decided to use standardized durations primarily because we wanted

to prevent the animals from learning to end the trials by showing a go-response no matter the stimulus; however, we also implemented these durations because we were interested in other behaviours. We hypothesized that other behaviours, especially exploration of the goal box, could also be indicative of which outcome the subject anticipates. This hypothesis was founded on our previous study (Düpjan et al., 2013) in which latencies did not differ between probes (and rewarded location), whereas exploration showed a gradient from rewarded to unrewarded. In this study, we confirmed our previous results and showed such a graded response. Furthermore, locomotion and standing changed over time, and latencies differed between the first and all following weeks; however, exploration remained stable over time. Additionally, there was a significant negative correlation between exploration and latency. This may seem trivial because earlier subject arrival at the goal box leads to more time left for its exploration. However, exploration may represent an interesting measure because (unlike latency) it was stable over time. Another interesting behaviour appears to be abrupt movements, which resembled play behaviour in pigs described in other studies (Donaldson et al., 2002; Held and Špinka, 2011). According to Burghardt (2005), play behaviour is only initiated when animals are in a relaxed state. This is not likely the case in the present study because animals were separated from their group and challenged with an experimental situation (Kanitz et al., 2009; Kanitz et al., 2004). We therefore assume the described behaviour to be a displacement behaviour rather than play behaviour, illustrating a conflict situation between two motivations, one being the motivation for food and the second the motivation for escape. Displacement behaviour has been described as deriving from the normal motor patterns of an animal but appearing out of context with the situation and the following behaviours (Tinbergen, 1952). Displacement behaviour is typical for conflict situations, especially when motivation for two different directed behaviours is high. This study supports such an interpretation because abrupt movements were more frequent in probe trials than in reference trials, which should not have been the case for play behaviour.

Conclusions

In conclusion, we suggest that the experimental design presented here for testing judgement bias in pigs can be used in future studies to reliably assess their affective state or even optimism/pessimism as individual, temporally stable traits. There, our model's sensitivity to differences in affective states is to be validated. Hence, our study provides a sound basis for future research on the welfare of this intensively farmed species.

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2.2 Study 2: Serotonin depletion induces pessimistic-like behavior in a cognitive bias paradigm in pigs

Redrawn from Physiology and Behavior: Serotonin depletion induces pessimistic-like behavior in a cognitive bias paradigm in pigs

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Abstract

Cognitive and affective processes are highly interrelated, having implications for neuropsychiatric disorders such as major depressive disorder in humans but also for the welfare of non-human animals. The brain serotonergic system might play a key role in mediating the relationship between cognitive functions and affective regulation. The aim of our study was to examine the influence of serotonin depletion on the affective state and cognitive processing in pigs, an important farm animal species but also a potential model species for biomedical research in humans. For this purpose, we modified a serotonin depletion model using para-chlorophenylalanine (pCPA) to decrease serotonin levels in brain areas involved in cognitive and affective processing (part 1). The consequences of serotonin depletion were then measured in two behavioral tests (part 2): the spatial judgement task (SJT), providing information about the effects of the affective state on cognitive processing, and the open field/novel object (OFNO) test, a mostly affective test paradigm. In part 1, 40 pigs were treated with either pCPA or saline for six consecutive days. Serotonin levels were assessed in seven different brain regions 4, 5, 6, 11 and 13 days after the first injection. Serotonin was significantly depleted in all analyzed brain regions (all $F>37$ all $p<0.001$) up to 13 days after the first application. In part 2, the pCPA model was applied to 48 animals in behavioral testing. Behavioral tests, the OFNO test and the SJT, were conducted both before and after pCPA/saline injections. While pCPA-treated animals showed no clear behavioral changes in the OFNO tests (all $p>0.05$), an effect of treatment ($F=6$, $p<0.05$) as well as an effect of the phase (before and after treatment) ($F=45.2$, $p<0.001$) was observed in the SJT. Animals treated with pCPA showed more pessimistic-like behavior, suggesting a more negative affective state due to serotonin depletion. Thus, our results confirm that the serotonergic system is a key player in cognitive-emotional processing. Hence, the serotonin depletion model and the spatial judgement task can increase our understanding of the basic mechanisms underlying both human neuropsychiatric disorders and animal welfare.

Highlights

- Para-chlorophenylalanine depletes the brain of serotonin for at least 13 days.
- The effects of serotonin depletion were observed in behavioral tests.
- Serotonin depletion induced pessimistic-like behavior indicating negative affect.
- Serotonin depletion did not induce consistent changes in behavioral reactivity.

Introduction

Modern theories describe the brain as a complex network with a high connectivity between different brain regions jointly regulating affective or cognitive functions Pessoa, 2013). Emotions, therefore, can modulate cognitive processes and vice versa. A variety of neuropsychiatric disorders are reflected by changes in both emotion and cognition. For example, patients suffering from major depressive disorder show a so-called 'cognitive bias', meaning that they tend to interpret ambiguous stimuli as more pessimistic than healthy individuals (Gotlib et al., 2004). However, the mechanisms underlying such interactions between both emotion and cognition are largely unknown (Shackman et al., 2015). One prominent transmitter system in affective and cognitive research is the serotonergic system. Serotonin (5-HT) plays a crucial role in cognitive functions, for example in memory consolidation (Cowen et al., 2013) and learning (Harvey, 2003). On the other hand, 5-HT was found to regulate affective states Cools et al.,2008), such as anxiety (Zhao et al., 2006) and aggression (Jacobsen et al., 2012), and is known for its relevance in major depressive disorder (Jans et al., 2007). Serotonergic neurons originate from the raphe nuclei of the brainstem, and its projections innervate multiple brain structures related to mood disorders and processes of attention towards stimuli, stimulus evaluation and (selection of) behavioral responses to these stimuli, such as the amygdala (Murray, 2007), anterior cingulate cortex (Pezawas et al., 2005), striatum (Dalley et al., 2011), hippocampus (Alenina et al., 2015), hypothalamus (Drevets et al., 2008), and prefrontal cortex (Puig et al., 2011). Therefore, a pharmacological 5-HT depletion model using para-chlorophenylalanine (pCPA) has been developed to study the biobehavioral effects of serotonin [Koe et al., 1966; for review see Ruhé et al., 2007). PCPA is an inhibitor of tryptophan hydroxylase, the rate-limiting enzyme in 5-HT biosynthesis. It was successfully used for selective 5-HT depletion in different species such as rats (Kornum et al., 2006), sheep (Doyle et al., 2011) and, recently, in pigs (Ettruo et al., 2011).

A number of behavioral tests have been applied to evaluate the affective or emotional state in non-human animals (for review see Murphy et al., 2014)]. Here, the focus lies on the implications for animal welfare. This calls for experimental designs to specifically test the valence dimension of affective states (e.g., Mendl et al., 2010)]. The most promising approach is the cognitive bias test known in humans (see above), which measures affective valence indirectly by observing its effect on cognitive processing. Harding et al. (2004) first established a nonverbal paradigm for testing cognitive judgement bias for use in animal experiments.

Their approach is based on a discrimination task, where animals learn to distinguish two stimuli with outcomes of different valence. The information on the stimulus-response-outcome contiguity then has to be transferred to judge the most likely outcome of novel, ambiguous stimuli. Current research implies that affective states influence this judgement (Mendl et al., 2009). The approach has been used in a variety of non-human animals, for example in rats (Burman et al., 2008), monkeys (Bethell et al., 2012) and, recently, in pigs (Douglas et al., 2012, Düpjan et al., 2013, Düpjan et al., 2017, Murphy et al., 2013). Recently, pharmacological manipulation of the 5-HT system was shown to alter the behavioral response in the cognitive bias task in sheep (Doyle et al., 2011) and rats (Rygula et al., 2014). McHugh et al. (2015) showed that the serotonin transporter in mice, similar to in humans (2009), modulates the behavioral response in cognitive bias tasks. As opposed to these approaches, the combined open field/novel object (OFNO) test, where animals are first confronted with a novel environment and then with an unfamiliar object, involves less (or less complex) cognitive processing. The OFNO test rather measures behavioral reactivity (e.g., Mormède et al., 1994; Puppe et al., 1999; Puppe et al., 2007), i.e. it scores affective behavior patterns in response to an emotionally challenging situation. Thus, it has been suggested that this kind of emotional reactivity primarily relates to the arousal dimension (Donald et al., 2011).Alterations in 5-HT availability in the brain have been shown to change behavior in the OFNO test in different species. In rats, a knockout of the 5-HT2 receptor resulted in less anxiety-like behavior (Heisler et al., 2007). Moreover, 5-HT depletion decreased the vocalization rate in sheep, supporting the suggestion of diminished emotional reactivity of the animals (Doyle et al., 2011).

The aim of the present study was to investigate the effects of central 5-HT depletion on affective states and cognitive processing in pigs. Thus, we established and applied a 5-HT depletion model using pCPA to decrease 5-HT levels in various brain regions involved in processes of attention towards stimuli, stimulus evaluation and (selection of) behavioral responses to these stimuli as well as emotional processing. Using this model, we then investigated the impact of 5-HT depletion on two behavioral tasks, one of them providing information about behavioral reactivity (OFNO) and one measuring the interaction of emotional and cognitive processing (cognitive bias). For the latter, we have previously developed a reliable design for eliciting stable behavioral responses of pigs in a repeated spatial judgement task (SJT; Düpjan et al., 2017). We hypothesized that 5-HT depletion would result in a behavioral alteration in both tests, indicating a negative affective state. In the OFNO test, we predicted an increased latency in

approaching the novel object as well as a decreased rate of behavioral activity, which in conclusion could be interpreted as decreased emotional reactivity (Puppe et al., 1999). In the SJT, we hypothesized a more pessimistic-like interpretation of ambiguous stimuli due to 5-HT depletion. We suggest that our approach will provide valuable insights into neurobehavioral processes underlying animal welfare in an important farm animal species, but, these results from pigs, as an animal model (e.g.; Vodiča et al., 2005; Lind et al., 2007), also have implications for biomedical research in humans.

Materials and Methods

2.1 Subjects and housing

The study was conducted in two parts: first, the verification of the 5-HT depletion model (n=40) and second, the application of the model in two behavioral tests, the spatial judgement task and the open field/novel object test (n=48). Both parts were conducted in the experimental pig unit of the Leibniz Institute for Farm Animal Biology with female German Landrace piglets (36 days of age at the start of the experiments) bred there for experimental purposes. The studies were conducted in several replicates (two for part one, six for part two). Within each replicate, two groups of 10 animals each were selected from a larger pool of animals to guarantee similar group composition (each group consisted of 5 pairs of full siblings, with each pair having a full-sibling pair in the second group within the replicate). Groups were established after weaning (28 days of age) and remained stable throughout experiments.

Pens measured 1.7 x 2.5 m. Commercial piglet food (Trede und von Pein, Itzehoe, Germany) and water were provided ad libitum. Room temperature was automatically controlled, starting at 28°C and decreasing stepwise to 19°C on the final experimental day to meet the changing requirements of the animals with age (Euromatic temperature curve). Pens were enriched with commercial and custom-made pig toys, and the animals received a mix of chopped straw, hemp pellets and sawdust (approx. 50 g) twice a day.

2.2 Part 1: Verification of the serotonin depletion model

2.2.1 Experimental design

The study was conducted in two replicates between November 2013 and January 2014 with 20 animals per replicate (n_{total}=40). Starting at 56 days of age, pigs were injected i.p. with either 50 mg/kg pCPA (4-Chloro-DL-phenylalanine, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) dissolved in approx. 10 ml saline (pCPA group) or 10 ml saline (control group) at 14:00 h for up to six consecutive days (Fig. 1). Concentrations of 5-HT were analyzed in seven brain areas (see 2.2.2) at five time points corresponding to testing days in part two of this study (day 4, 5, 6, 11 and 13 after the first injection). Per time point, four animals were sampled, balanced for treatment, housing group and kinship.

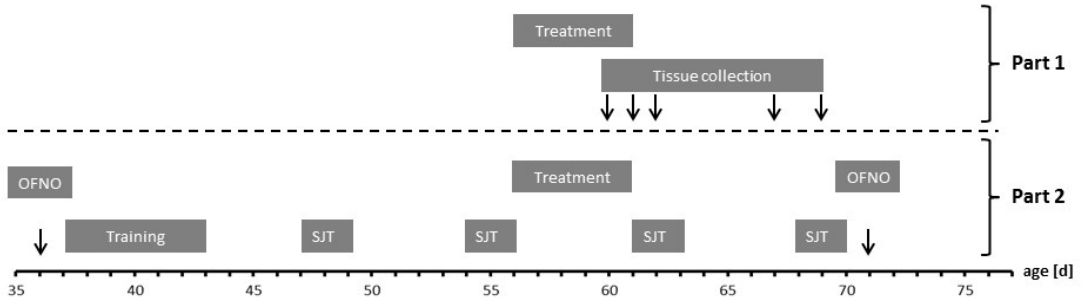

Figure 1: Experimental schedule for part 1 (above) and part 2 (below) of the study. OFNO, open field/novel object test; SJT, spatial judgement task (test period).

2.2.2 Brain tissue collection

Animals were narcotized with Ursotamin (ketamine, 10 mg/ml; Serumwerk Bernburg AG, Bernburg, Germany) and Stresnil (Azaparone, 40 mg/ml; Janssen-Cilag GmbH, Neuss, Germany) and afterwards, were sacrificed with i.v. injections of T61 (embutramide, 200 mg/ml; mebezonium iodide, 50 mg/ml; tetracaine hydrochloride, 5 mg/ml; Intervet Deutschland GmbH, Unterschleißheim, Germany). Doses were adjusted to body weight as instructed by the manufacturers. Brains were quickly removed, and the following regions were dissected by qualified personnel (Kanitz et al., 2011) using a stereotaxic atlas of the pig brain as a reference (Felix et al., 1999): rostral anterior cingulate cortex, prefrontal cortex, striatum (including putamen, nucleus caudatus and nucleus accumbens), amygdala, hippocampus, hypothalamus and brain stem. Tissue samples were frozen in liquid nitrogen and stored at −80°C until analysis.

2.2.3 High-performance liquid chromatography analysis

Concentrations of 5-HT were determined in brain tissue samples using high-performance liquid chromatography (HPLC) with fluorescence detection.

Brain samples were weighed and homogenized on ice with an Ultra Turrax (T25, IKA-Werke GmbH & Co. KG, Staufen, Germany) at 9,500 rpm in 0.5-10 ml of 0.2 M perchloric acid, depending on tissue weight. After incubation for 5 min on ice, homogenates were centrifuged at 5,100 x g for 10 min at 4°C. This procedure was repeated after collection of the supernatants. Pooled supernatants of the repeated extractions were again centrifuged at 37,000 x g for 10 min at 4°C. Immediately after extraction, aliquots of 10 µl were injected directly into the

HPLC system (Shimadzu Deutschland GmbH, Duisburg, Germany). Duplicates were assayed from each extract. The HPLC system was equipped with a 125 x 4 mm reversed-phase column packed with Prontosil C18 AQ (Bischoff Analysentechnik, Leonberg, Germany). The mobile phase consisted of 58 mM sodium hydrogen phosphate buffer containing 1.2 mM octanesulfonic acid, 0.3 mM EDTA, and 13.5% methanol at pH 3.6 and was used at a flow rate of 0.8 ml/min.

The 5-HT concentration was analyzed with fluorescence detection (RF-20Axs, Shimadzu Deutschland GmbH, Duisburg, Germany) using an excitation wavelength of 280 nm and a detection wavelength of 335 nm. The elution time was 16.0 min. Peak identification was accomplished by spiking samples with a reference compound (5-HT, H9523, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Calibration curves were calculated using the peak area vs. the analyzed concentration at two concentrations (dilution factor: 8). Standard curves were conducted routinely before and after analysis of approximately 10 samples. The intra-assay coefficient of variation for 5-HT was 0.6%, and the inter-assay coefficient of variation was 0.6%. The concentrations were expressed as ng/g tissue.

2.3 Part 2: Serotonin depletion and behavior

2.3.1 Experimental design

The study was conducted in six replicates from March 2014 to December 2014. All 20 animals per replicate underwent a test aimed at identifying and excluding the animals that would not eat the food reward. Per replicate, eight animals (four per housing group, balanced for kinship) were then chosen pseudorandomly (i.e., n_{total}=48). Subjects remained in their respective housing group. All subjects underwent both OFNO and SJT testing.

2.3.2 Treatment

In total, 24 animals received injections of pCPA (pCPA group), and 24 animals received injections of saline (control group), balanced for housing group and kinship. Both treatments followed the procedures established in part one of this study (cf. 2.2.1).

2.3.3 Open field/novel object (OFNO) test

All pigs underwent the OFNO twice, once before (d 36) and once after (d 71) treatments and SJT training/testing (Fig. 1).

Subjects were tested individually. The test arena (3 x 3 x 1.25 m) was located in a sound attenuated room, and the behavior of the pigs was observed by video recordings. Subjects were led from their home pen to the test room as gently as possible to avoid stress prior to testing. The open field test lasted for five minutes, after which the novel object was introduced manually by a windlass in the middle of the open field, and subjects remained in the arena with the object for another five minutes. Two different objects were used in the two OFNOs, differing in shape, size and color (a purple plastic dumbbell in the first novel object test (d 36) and a blue plastic container in the second novel object test (d 71)). Behavior was analyzed using the Observer XT version 12 (Noldus Information Technology, Wageningen, The Netherlands). All recorded behaviors along with their definitions and type of recording are listed in Table 1.

2.3.4 Spatial judgement task (SJT)

Details on the experimental procedure can be found in (Düpjan et al., 2017). The SJT consists of two phases: in the first, subjects learn to discriminate between a positively and a negatively associated location of a goal box in an arena. They must learn to open the goal box ('go' response) when it is in the positive location to achieve a reward and to not open the box ('no go' response) when it is in the negative location to avoid punishment. All subjects were familiarized with the goal box and the food reward before training started. The experimental setting consisted of an arena that could be accessed from a start area through a guillotine door (to be opened by an experimenter). The arena was shaped like a quarter circle (radius 3.2 m), and the goal box could be fixed to the back wall at five locations. The right and left corner were the rewarded (S^+) and the punished (S^-) location (reference locations, counterbalanced between subjects/groups/treatments), with three probe locations equidistant between them (near rewarded (nS^+), middle (M), near punished (nS^-)). The goal box had a flap door that could be opened by the subjects with a gentle push of the snout to gain access to a metal food bowl. The food bowl contained 5 ml of applesauce, which could be either freely accessible (rewarded trials; see 2.3.5) or inaccessible by the placement of a perforated plastic plate on top (unrewarded, unreinforced, and probe trials; see 2.3.5 and 2.3.6). Punishment was provided by waving a plastic bag in the pig's face as soon as they opened the goal box (but not at further goal box contacts). Probes were neither rewarded nor punished.

Table 1: Recorded behaviors. D, duration, F, frequency, L, latency, SJT, spatial judgement task, OFNO, open field/novel object test; behaviors in the grey sections mark groups of mutually exclusive state events.

Behavior	Type	Measurement	Definition
Choice (SJT)	Point event	L	Opening the goal box
Start (SJT/OFNO)	Point event	Reference point	Four feet in the arena or open field/object in place
Locomotion (SJT/OFNO)	State event	D	Moving; minimum one forward step
Standing (SJT /OFNO)	State event	D	No locomotion, all four legs stretched out, no floor contact of the torso; minimum 3 ft on the ground
Lying (SJT /OFNO)	State event	D	Lying down with the whole belly or the whole lateral part of the animal touching the ground
Jumping (OFNO)	State event	D	Jumping at the walls
Abrupt movements (SJT)	State event	F	One of the following components or a combination of them: sudden cantering, jumping and twisting
Exploration goal box	State event	D/F	Physical contact (nose) with the goal box, sniffing, licking
Scratching (SJT)	Point event	F	Pig scratching itself, either with the foot, or by scrubbing the body on either wall of the arena or the goal box
Defecation (SJT /OFNO)	Point event	F	Defecate
Urination (SJT /OFNO)	Point event	F	Urinate
Contact object (OFNO)	State event	D/L	Exploring the object using the nose
Exploration floor	State event	D	Manipulating the floor using the nose
Exploration wall (OFNO)	State event	D	Manipulating the wall using the nose
No exploration (OFNO)	State event	D	No exploration of wall, floor or object

2.3.5 Training

The animals were trained individually on the spatial go/no go discrimination task. Training was conducted on seven consecutive days, with 6 trials per location and day (Fig. 1). The sequence of goal box locations was pseudorandomized for each individual and day. Half of the animals were rewarded in the left corner of the arena and punished in the right corner and vice versa. Subjects had one minute after entering the arena with all four feet until they had to return to the start box. From day two of training onwards, one trial per location was neither rewarded ($S^{(+)}$) nor punished ($S^{(-)}$) but neutral (corresponding to the probes), a procedure that should counteract learning of the outcome of the probe locations [29]. To summarize, each training day had five rewarded, five punished, one non–rewarded and one non-punished trial.

2.3.6 Testing

After training, animals had a three-day break before testing started. Testing was performed on three consecutive days a week (Tuesday through Thursday) for four weeks (Fig. 1), with no trials on the days in between to avoid habituation to the punishment. Test trials (two per day) were integrated in the familiar training procedure as additional, unreinforced trials. Probe locations were, as described previously (2.3.4), on equidistant locations between S^+ and S^- (nS^+, M, nS^-). The sequence of probes was pseudorandomized, presenting each location twice per animal and week but never twice on the same day for the same subject.

2.3.7 Behavioral observations

Subjects were observed by video recordings, and their behavior was analyzed using the Observer XT version 12 (Noldus Information Technology, Wageningen, Netherlands).

Recorded behaviors with their definitions and type of recording are listed in Table 1. All behaviors except for choice behavior (see Table 1) were scored for a total of 60 seconds after the subject had entered the arena with all four feet and only for the unreinforced trials, i.e., probe trials as well as the unreinforced reference location trials, to exclude an influence of the reinforcements (reminder: rewards were available in S^+-trials only, which is likely to increase the time spent at the goal box due to reward consumption and lower the time spent on any other behavior; punishments were only applied in S^--trials, which is likely to decrease the time spent at the goal box, increasing the time available for other behaviors).

The latency from entering the arena (Start, see Table 1) until opening the goal box ('choice behavior') was measured for each trial in the testing phase, this time including the reinforced trials. Relative latency was calculated by setting the latency of choice behavior in the probe trials in relation to the daily mean of trials on the rewarded location (S^+ and $S^{(+)}$; mean≙0) and the trials on the punished location (S^- and $S^{(-)}$; mean≙1):

$$relative\ latency = \frac{absolute\ latency - mean\ latency\ (S^+,\ S^{(+)})}{mean\ latency\ (S^-,\ S^{(-)}) - mean\ latency\ (S^+,\ S^{(+)})}$$

This formula was used to eliminate the effects of overall differences in speed between subjects but also within subjects between days, caused for example by differences in size, feeding motivation, and time of day (see [29] for a more elaborate discussion).

In case one animal did not show one of the mentioned behaviors, latency was set to the maximum time (60 s) for further analysis.

2.4 Statistical analysis

Data were analyzed using the MIXED procedure (SAS Version 9.4, SAS Institute Inc., Cary, NC, USA). For the analysis of the 5-HT concentration in the seven brain areas (part 1), ANOVAs were calculated for each area separately, with treatment (control/pCPA), replicate, day (day after first application) and all of their interactions as fixed factors and mother and father as random effects. Multiple pairwise comparisons were calculated using Tukey-Kramer tests.

In the SJT (part 2), we first analyzed the absolute latencies in a repeated measurement ANOVA model including location, treatment, day and their interactions as fixed factors and then compared the absolute latency to the rewarded vs. the punished location for both treatment groups on each testing day (t-tests with Bonferroni correction for multiple testing) to make sure that the animals discriminated between the reference locations. All other data were subsumed in two phases, before treatment (test week 1 and 2) and after treatment (test week 3 and 4). For the analysis of relative latency and behavior, goal box location, treatment, phase (before/after treatment), and all of their interactions were considered as fixed effects, and mother and father were considered as random effects. Repeated measurements (within location and phase) were considered. Pairwise multiple comparisons of the least square means were done

using the Bonferroni-procedure to correct for multiple testing, reducing comparisons to those within treatment/between locations and within location/between treatments only.

The behavioral variables in the OFNO tests (2.3) were analyzed as the differences between the two tests (test 2 – test 1), with treatment and replicate as fixed effects and mother and father as random effects in the model.

Effects and differences were considered significant when $p<0.05$.

2.5 Ethical statement

This study and all of its procedures were approved by the LALLF (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei) Mecklenburg Vorpommern (AZ:7221.3-1-066/13). All efforts were made to ensure minimal animal number and suffering.

Results

3.1 Verification of the serotonin depletion model

In total, 38 animals (control=19, pCPA=19) were included in the statistical analysis. Two animals had to be excluded from experiments due to health problems unrelated to the treatments.

Treatment had a significant effect on 5-HT concentrations in all of the analyzed brain regions (amygdala $F=46.9$, $p<0.001$; hypothalamus $F=175.2$, $p<0.001$; hippocampus $F=171.1$, $p<0.001$; striatum $F=103.6$, $p<0.001$; rostral anterior cingulate cortex $F=66.8$, $p<0.05$; brain stem $F=37.3$, $p<0.001$; prefrontal cortex $F=63.7$, $p<0.001$) (Fig. 2). In the pCPA group, 5-HT concentrations were reduced by 26% (brain stem) to 50% (hippocampus) compared to the control group. There was also a significant effect of treatment*replicate on 5-HT concentration in the prefrontal cortex ($F=5.6$, $p<0.05$), but the pairwise comparisons revealed that there were significant differences between treatments in both replicates (replicate 1: $t=4.0$, $p<0.05$; replicate 2: $t=7.3$, $p<0.001$), while there were no differences between replicates within treatments (pCPA: $t=2.1$, $p=0.2$; control: $t=-0.6$, $p=0.9$). No effect of the different time points of brain sampling could be found in any of the brain regions (all $F<2.7$, all $p>0.4$). There was also no significant interaction effect between day and treatment (all $F<2.4$, all $p>0.09$). Thus, our results indicate a consistent depletion of 5-HT in all brain regions across the experimental period.

3.2 Serotonin depletion and behavior

3.2.1 SJT

Three animals had to be excluded from the experiments because they stopped eating the food reward after the training phase. Two more animals were excluded throughout the experiments, one after the second test week and one in the last test week, both due to illness unrelated to the treatment. Data of these two animals are included until the appearance of first symptoms. Therefore, in total 45 animals were included in the statistical analysis, 24 in the pCPA group and 21 in the control group. Please be reminded that the term "location" always refers to the relative location (S+, S-, etc.), not left/right.

On all testing days, animals showed significantly longer absolute latencies to open the goal box in the punished location than in the rewarded location (all p b 0.001), meaning that they discriminated the reference locations throughout the testing phase. Treatment had no effect on the absolute latency to reach the reference locations, neither alone or in interaction with another factor.

The relative latency revealed a significant threefold interaction effect between location, phase and treatment ($F=4.1$, $p<0.01$) (Fig. 3). The pCPA group showed a significantly longer relative latency for all three probe locations after treatment than before treatment (nS+: $t=5.2$, $p<0.001$; M: $t=4.7$, $p<0.001$; nS-: $t=3.7$, $p<0.01$). In control animals, no such differences could be found (all $|t|<1.7$, all $p>0.05$). Additionally, our analyses revealed a significant difference between the control group and pCPA group after treatment for the most ambiguous location in the middle (M: $t=-4.0$, $p<0.001$), but not for any of the other locations or before the treatment (all $|t|<2.3$, all $p>0.05$).

Only standing showed a treatment-related effect, namely a significant treatment*phase interaction ($F=27.2$, $p<0.001$) with control animals standing significantly less after treatment than before treatment ($t=-5.8$, $p<0.001$) and the pCPA group after treatment ($t=-2.6$, $p<0.05$). Otherwise, treatment had no effect on the measured behavioral variables (all $F<2.8$, all $p>0.05$). Apart from that, location*phase interactions were found in the frequency and duration of exploring the goal box (frequency: $F=3.4$, $p<0.01$; duration: $F=3.1$, $p<0.05$), duration of locomotion ($F=3.3$, $p<0.05$), and displacement activity ($F=2.5$, $p<0.05$). Across treatments and phases, the duration of exploring the goal box was significantly lower in the S- location than in all other locations (all $|t|>3.5$, all $p< 0.01$). Phase had a significant effect on scratching ($F=8.3$, $p<0.01$), with more frequent scratching before than after treatments.

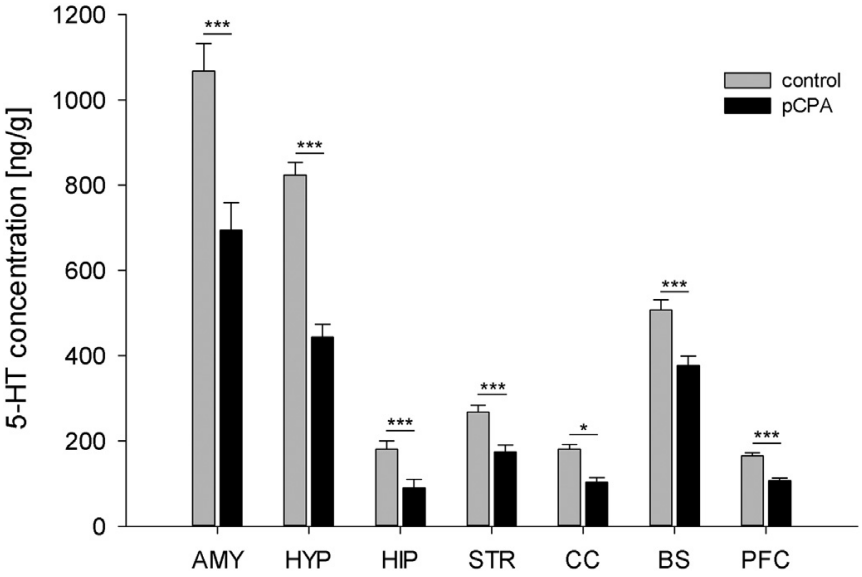

Figure 2: 5-HT concentration in different brain regions averaged across the five time points (LSM ± SEM; n = 38 (19 per treatment), Tukey-Kramer t-tests, *p b 0.05, ***p b 0.001); AMY, amygdala; HYP, hypothalamus; HIP, hippocampus; STR, striatum; CC, rostral anterior cingulate cortex; BS, brain stem; PFC, prefrontal cortex; gray represents the control group; black represents the para-chlorophenylalanine (pCPA)-treated group.

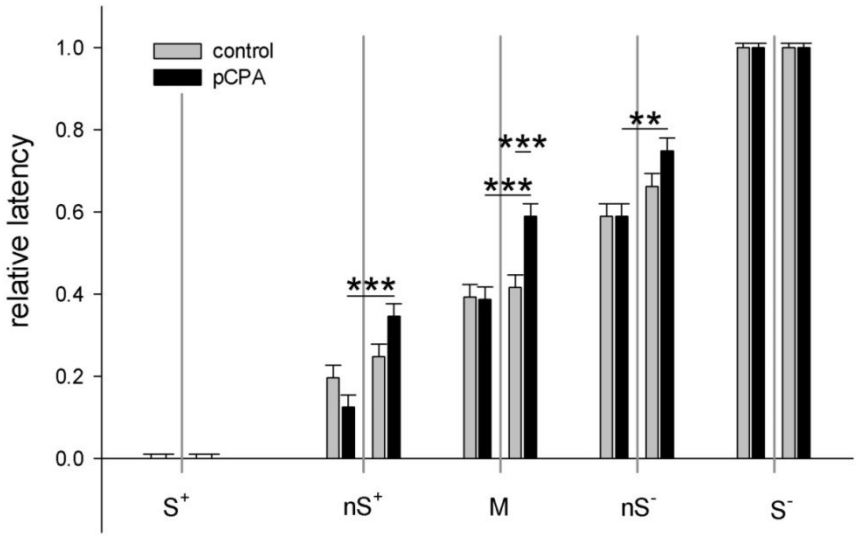

Figure 3: Relative latency to open the goal box in the spatial judgement task (SJT) (LSM±SEM, **p<0.01, ***p<0.001). S^+, rewarded; nS^+, near rewarded; M, middle; nS^-, near punished; S^-, punished; gray represents the control group; black represents the para-chlorophenylalanine (pCPA)-treated group; gray vertical lines indicate the time point of the treatment (left, before treatment; right, after treatment).

3.2.2 OFNO

Due to technical problems, one replicate could not be included in the statistical analysis, so in total, data from 37 animals (control=17; pCPA=20) are included in these results. The open field (OF; 5 min) and novel object (NO; 5 min) tests were analyzed separately.

Analyzing the differences between test 2 and test 1, a treatment effect could be found for exploring the wall in the open field ($F=5.2$; $p<0.05$), with pCPA-treated animals showing a more pronounced increase from test 1 to test 2. For the remaining variables, no effects of treatment could be found (all $F<4.5$, all $p>0.05$) (Fig. 4). An effect of replicate was found for defecation ($F=3.4$, $p<0.05$).

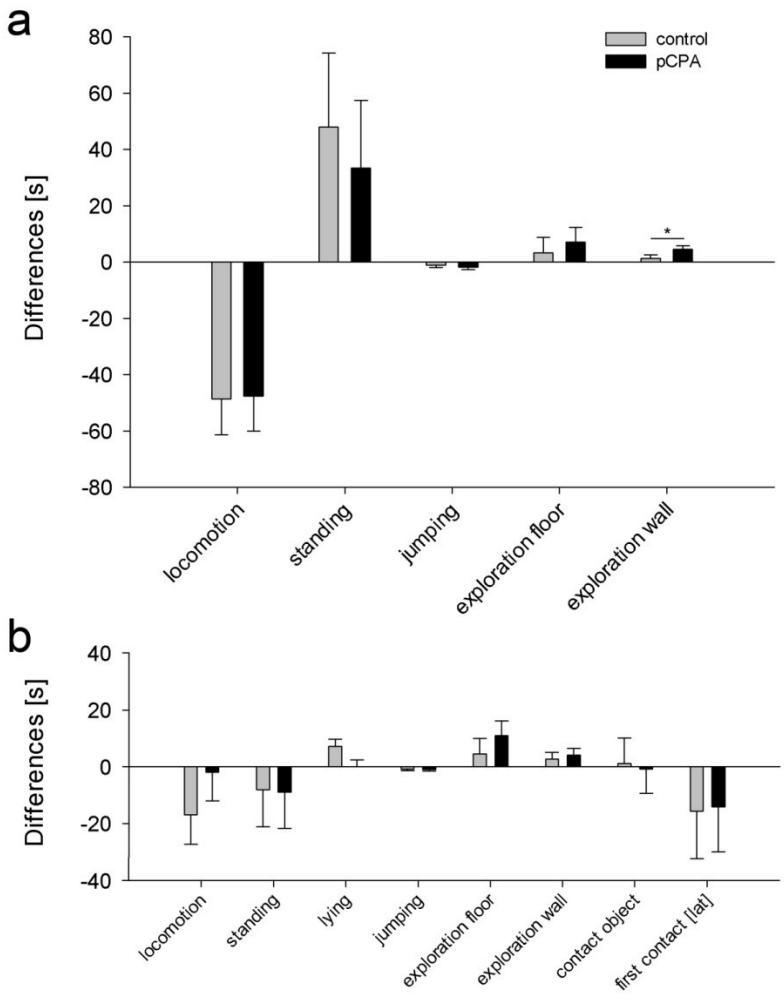

Figure 4: Differences in the behavior in the open field/novel object (OFNO) test between test 2 and test 1 (LSM±SEM, *$p<0.05$); a: open field test; b: novel object test; gray represents the control group; black represents the para-chlorophenylalanine (pCPA)-treated group.

Discussion

In part 1 of our study, we found that treatment with pCPA significantly depleted 5-HT in all examined brain areas over the experimental period of 13 days after the first treatment. In part 2, we then showed that this induced a shift to more pessimistic judgements on ambiguous stimuli but not in behavioral reactivity in general.

4.1 Serotonin depletion model

Animals treated with pCPA showed significantly lower concentrations of 5-HT across the experimental period. The effect of pCPA was ubiquitous; therefore, all brain areas were affected. In our study, we modified the protocol of Ettrup et al. (2011). They did four days of i.m. injections with pCPA followed by brain tissue sampling on day 5, where they found reduced 5-HT concentrations. However, our aim was to achieve a long-term 5-HT depletion rather than a short-term change, because the behavioral tests, especially the SJT, require more time (Düpjan et al., 2017). To adjust the model for use in a 4-week testing paradigm in the SJT (two weeks before and two weeks after treatment), we injected the pCPA i.p. for six days, followed by tissue sampling in seven brain regions up to 13 days after the first injection to confirm the 5-HT depletion. The selected brain regions are involved in processes of attention towards stimuli, stimulus evaluation and (selection of) behavioral responses to these stimuli and, therefore, should be involved in different aspects of cognitive bias. Because we were able to induce the long-term 5-HT depletion that we had aimed for, we were then able to apply this model in behavioral tests. However, we cannot say how long the 5-HT depletion lasted because we observed no effect of day or interaction of day and treatment, meaning that there was no significant decrease, even on the last sampling day, 7 days after the last injection of pCPA. Therefore, for future studies, the duration of the central 5-HT depletion by pCPA should be investigated.

Up until now, there has been no method for monitoring brain 5-HT levels during the behavioral testing of freely moving animals. Doyle et al. (2011) assumed a relationship between increased corticosteroid levels and decreased 5-HT levels as described by Porter et al. (2004). Working with 5-HT depletion in an SJT in sheep, they could not confirm this relationship, as they found no change in the corticosteroid system in response to depleted 5-HT. Our testing showed that the kinetics of serum 5-HT levels do not reflect 5-HT depletion in the brain (data not shown). This means that it is not possible to get reliable information on 5-HT brain concentrations in animals in behavioral testing, but this information can

only be confirmed invasively in a separate sample. By using subjects of the same breed in the same housing conditions and of the same age, we suggest that we can safely assume that the effects found in part 1 of our study would also have been found in the subjects in part 2, i.e., the behavioral tests.

4.2 Serotonin depletion and behavior

We here provide the first evidence that 5-HT depletion in pigs induces a pessimistic response (relative latency in opening the goal box) to ambivalent stimuli in the SJT, which provides evidence that 5-HT in the brain is a key factor of cognitive-emotional processing.

4.2.1 SJT

In the SJT, the influence of affective states on cognitive processing are observed, combining cognition and emotion/mood. As a prerequisite for cognitive bias testing, we had to confirm that animals discriminated between the reference locations, which proved to be true. This discrimination (as shown in the absolute latencies to open the goal box) was not affected by treatment. These results confirm the applicability of our experimental design established in an earlier study (Düpjan et al., 2017). In the SJT, we could then confirm the hypothesis that 5-HT depletion induces a more pessimistic evaluation of ambivalent stimuli, which is indicative of a shift towards a more negative mood. Similar effects could be demonstrated in sheep (Doyle et al., 2011). However, there was no experimental confirmation of 5-HT depletion in the pCPA model used. Hence, our results are the first to both prove the physiological effect of 5-HT depletion and its effect on affective state. In mice, McHugh et al. (2015) showed that an increased expression of the 5-HT transporter results in an optimistic response in judgement bias. Studies in humans demonstrate that allelic variation in the promotor region of the 5-HT transporter gene (affecting transporter synthesis and therefore 5-HT reuptake from the synaptic cleft) is associated with differential biases for positive and negative affective pictures (Shackman et al., 2015). Along this line, Ruhé et al. (2007) found that 5-HT depletion had no effect on mood in healthy individuals but did have an effect in patients with a family history of major depressive disorder, implying a role for the serotonergic system in vulnerability to depression.

5-HT depletion had no influence on the judgement of the reference locations, demonstrating that it does not affect memory retrieval or decision making in unambiguous situations. This assumption is supported by findings of Hritcu et al. (2007), who found that 5-HT depletion in rats has no effect on long term

memory, as well as by another study of Petrásek and Stuchlík (2009), who found that 5-HT depletion is not necessary for allothetic learning, such as SJT. Along this line, Robinson and Sahakian (2009) found that 5-HT depletion does not affect non-affective cognitive performances such as planning, which would be the counterpart to the reference cues in our study, but can alter cognitive tasks that require the processing of affective stimuli, such as the ambiguous cues in our SJT.

The changes in affective state indicated by the latency to open the goal box were, however, not reflected in the additional behavioral parameters. In an earlier study, we suggested that exploration of the goal box might be an additional indicator of the subjects' expectation of rewards (Düpjan et al., 2013). However, in this study we could neither confirm a linear relationship between the goal box location and exploration of this goal box nor find any effects of treatment on this behavior. Only standing was affected by treatment in that pCPA-treated animals stood significantly more after treatment than the control animals, suggesting that the control animals were more active.

4.2.2 OFNO test

Only exploration of the wall in the open field phase of the OFNO was affected by treatment in that there was a bigger increase in this behavior from test 1 to test 2 in the pCPA group than in the control group. However, the affective impact of exploratory behavior in the pig has been controversial: exploratory behavior can be interpreted as an indicator of either lower anxiety to the new surrounding or rather a higher arousal (Donald et al., 2011). Hence, our results do not provide sound support for the hypothesis that 5-HT depletion induces changes in behavioral reactivity. The open field test was developed to measure anxiety-like behaviors in rodents, but it is also used in a variety of species such as primates, sheep and pigs (e.g., Prut et al., 2003; see Murphy et al., 2014 for a review). In pigs, the open field test is often combined with a novel object test (Murphy et al., 2014; Puppe et al., 2007; Kornum et al.,2011), measuring the reactivity to/fear of an unfamiliar stimulus. The use of these tests as general fear tests is controversial (Forkman et al.,2007), and results need to be interpreted cautiously. For example, Andersen et al. (2000) found no effects of anxiolytics (diazepam), whereas Donald et al. (2011) found an effect of a tranquilizer (azaperone). Still, as decreased 5-HT function promotes different behavioral phenotypes of depression, for example, fear of novelty and immobility in mice and rats (Fernandez and Gaspar, 2012), we predicted changes in these behaviors in the pCPA-treated animals in the OFNO test. However, during SJT training and testing, they became well habituated to being temporarily separated from their social group and confronted with novel

stimuli. Murphy and colleagues (2014) pointed out that social isolation and novelty (of the environment and object) are the key features contributing to the overall averseness of the OFNO test. This averseness might have been reduced by our experimental procedure, habituating the subjects to isolation and novelty. Therefore, we conclude that OFNO behavior might not be suitable to test emotional reactivity and underlying (negative) affective states in the overall experimental design used in our study.

4.3 Conclusion

Using pCPA, we could show that 5-HT depletion shifted the behavioral response of pigs to ambivalent stimuli towards a more pessimistic stimulus evaluation. This supports research on 5-HT availability in farm and laboratory animal behavior but also on cognitive-emotional processing mechanisms in human neuropsychiatric disorders. The cognitive component in the spatial judgement task seems to be crucial, as we could find no effects of 5-HT depletion in the OFNO test, a behavioral test lacking this cognitive element. On the other hand, 5-HT depletion did not affect memory retrieval, a purely cognitive task. Therefore, 5-HT depletion does not appear to alter either emotions or cognition alone but rather affects the interactions of these two components. This study increases our understanding of the basic mechanisms regulating cognition and emotion and provides a method for measuring affective states in pigs, which is essential for the assessment of animal welfare in this species.

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2.3 Study 3: Dietary tryptophan supplementation and affective state in pigs

Redrawn from Journal of Veterinary Behavior: Dietary tryptophan supplementation and affective state in pigs

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Jenny Stracke designed and performed the experiment, analysed the data and wrote the manuscript with the support of and in agreement with her supervisor Dr. Sandra Düpjan and the co-authors of this manuscript.

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Abstract

The assessment and provision of welfare in farm animals has become a major issue in animal science. A key element for providing good welfare is the enabling of positive affective states in the animals. As the serotonergic system plays a central role in regulating affective behavior, an increase in centrally available serotonin (5-HT) via dietary supplementation of its precursor, tryptophan (TRP), might be an approach to induce positive affective states. Therefore, the aim of our study was to investigate the effects of dietary TRP supplementation on brain TRP metabolism and 5-HT levels, but also on affective state and behavioral reactivity in pigs. All subjects were fed a standard diet until eight weeks of age, then feed was changed for all animals, with half the animals (control) receiving a diet with the recommended TRP content (2.5 g/kg), while the other half (TRP+) received a TRP enriched diet (10.2 g/kg). In part 1 of our study, we investigated the effects of the dietary TRP supplementation on TRP metabolism in brain areas related to affective and cognitive processing. We found significantly increased concentrations of TRP (all $F>82.7$ all $p<0.001$) and its metabolites in nearly all analyzed brain tissues. In part 2 of our study, we analyzed the effects of these alterations on the affective state as measured in a cognitive bias test, namely the spatial judgement task (SJT), but also on behavioral reactivity as measured in a combined open field/novel object test (OFNO).The TRP enrichment revealed no significant behavioral changes in the OFNO tests (all $p>0.05$). In the SJT, the TRP+-group showed more pessimistic behavior after dietary change than before. Thus, our results do not support the suggestion that TRP supplementation induces positive affective states and thus improves animal welfare in pigs.

Keywords: spatial judgement task; cognition; emotion; brain; animal welfare

Highlights

- We investigated effects of dietary tryptophan supplementation on affective state
- Dietary tryptophan supplementation enhanced centrally available serotonin in pigs
- Tryptophan supplementation did not induce positive affective states in pigs

Introduction

Over the last decades, the provisioning of good animal welfare has gained more and more importance in animal farming. It has been recognized that to achieve good welfare, animals should experience positive affective states (Boissy et al., 2007). The brain serotonergic system is involved in the regulation of different affective behaviors like anxiety (Zhao et al., 2006) or aggression (Jacobsen et al., 2012) and in the regulation of mood in general (Castanon, 2015; Young and Leyton, 2002). Serotonergic neurons originate from the raphe nuclei of the brainstem, and projections innervate multiple brain structures (Charnay and Léger, 2010) related to cognitive processing, regulation of affective states and mood disorders (Rogers, 2011), like the amygdala (Murray, 2007), anterior cingular cortex (Pezawas et al., 2005), striatum (Dalley et al., 2011), hippocampus (Alenina and Klempin, 2015), hypothalamus (Drevets et al., 2008), and prefrontal cortex (Puig and Gulledge, 2011). In a previous study, we could demonstrate that pharmacologically induced serotonin (5-HT) depletion in these regions induces a shift towards a more negative affective state in pigs (Stracke et al., submitted). The rate of 5-HT synthesis in the brain depends on the availability of its precursor tryptophan (TRP), an essential amino acid, which needs to be transported actively through the blood brain barrier. In the domestic pig, one of the most important farm animal species in Europe, dietary TRP is known to affect feed intake, body weight, health state, and behavior. Eder et al. (2001) could show that TRP deficiency in the diet has a negative effect on appetite, which is also mediated by 5-HT, as well as on weight gain in pigs. TRP supplementation, on the other hand, has been suggested to increase feed intake because of its enhancing effect on brain 5-HT levels (Le Floc´h and Sève, 2007). Le Floc´h et al. (2004) showed that adequate TRP supply is crucial to provide weight gain when the immune system is challenged, and suggested that dietary levels required for optimal health might exceed those required for optimal growth (Le Floc'h and Sève, 2007). Ettle and Roth (2004) even demonstrated that pigs are able to select diets based on their TRP content, which might be mediated by ghrelin in the gut (Zhang et al., 2007). In humans, dietary TRP supplementation has been shown to reduce stress responses and to increase positive mood (Firk and Markus, 2009). In pigs, supplemental dietary TRP can reduce salivary cortisol responses to social stress (Koopmans et al., 2006) and aggressive behavior (Liu et al., 2013; Li et al., 2006; but see also Koopmans et al., 2005 for contradictory results). Taken together, we suggest that dietary supplementation of tryptophan beyond the levels suggested

for optimal feed intake and weight gain, might increase pig welfare by inducing positive affective states via the activation of the serotonergic system.

Affective states, like short-term emotions and long-term moods, can be described by a two dimensional model, measuring the state of arousal (low/high) on the one hand and the valence (negative/positive) on the other hand (Russell, 2003; Mendl et al., 2010). Valence is the more relevant dimension when it comes to animal welfare, but is far more elusive. It can, however, be measured indirectly by observing so-called cognitive biases, i.e. the shifting of cognitive processes under the influence of affective states. This phenomenon is well known in human psychiatric research, and has been adapted for testing non-human animals by Harding et al. (2004). Specifically, their experimental approach measures the influence of affective states on the judgement of ambiguous stimuli. It has been shown in several species that animals judge ambiguous stimuli more pessimistic when in a negative affective state and vice versa (Mendl et al., 2009). We have previously developed a reliable design for eliciting stable behavioral responses of pigs in a variation of the cognitive bias test, namely the repeated spatial judgement task (SJT; developed for rats by Burman et al., 2008; adapted for pigs by Düpjan et al., 2013, 2016). With this experimental design, we could demonstrate that 5-HT depletion induces pessimistic-like behavior in the SJT (Stracke et al., submitted; see Douglas et al., 2012, for similar results in sheep). An additional behavioral test, often used in the context of affective states (Donald et al., 2011) would be the combined open field/novel object test (OFNO) basically measuring behavioral reactivity (Puppe et al., 1999, 2007). Ursinus et al. (2013) found that hippocampal concentrations of 5-Hydroxyindoleacetic acid (5-HIAA), a 5-HT metabolite, correlate positively with exploratory behavior in such a test.

Our aim was to investigate a. the effect of TRP supplementation on concentrations of TRP, of 5-HT and their metabolites in brain areas related to affective and cognitive processing in pigs (part 1), and b. potential positive effects of dietary TRP supplementation on the affective state in pigs as measured in the spatial judgement task but also on behavioral reactivity as measured in the combined open field/novel object test (part 2).

Materials and Methods

The study comprised two parts: first, we examined the effects of dietary tryptophan supplementation on concentrations of TRP and its metabolites in several brain areas relevant for cognitive-emotional processing; then, we investigated the effects of these physiological consequences on behavior and affective state.

2.1 Subjects and housing

Both parts of this study were conducted in the experimental pig unit of the Leibniz Institute for Farm Animal Biology with female German Landrace piglets bred there for experimental purposes. Experiments were conducted in several replicates (one in part 1, four in part 2). For each replicate, two groups of 10 animals each were assembled (four piglets from five litters per replicate); these groups were established after weaning (28 days of age) and remained stable throughout experiments.

Pens measured 10.6m² with partly slatted concrete floor (approx. half solid, half slatted floor). Water was provided ad libitum. Food (see 2.2.1 for details) was provided via an automatic feeder (Hoko Farm, IVOG System, INSENTEC, The Netherlands), and individual feed intake was monitored using HDX ear tags (Allflex Transponder Bro, Allflex, Hamburg, Germany) for individual recognition. Room temperature was automatically controlled and adjusted with age, starting at 28 °C and decreasing stepwise to 19°C on the final experimental day (Euromatic temperature curve). Pens were enriched with commercial and custom-made pig toys and the animals received a mix of chopped straw, hemp pellets and sawdust (approx. 50 g) twice a day.

Animals were weighed on experimental days between 10:30 and 12:00 a.m.

2.2 Part 1: Tryptophan, serotonin and their metabolites in the brain

2.2.1 Experimental design

The study was conducted in February and March 2015 with 20 animals (one replicate/two housing groups) in total. All animals received the same standard piglet diet (Trede und von Pein, Itzehoe, Germany) until the 56th day of life. On day 56, diet was changed for both groups. There was only one automatic feeder per housing group, hence groups were randomly allocated to the control or the TRP enriched diet. The control group received food containing a tryptophan

concentration comparable to the standard diet (control: 2.5 g/kg TRP; Trede und von Pein, Itzehoe, Germany), whereas the TRP enriched group (TRP+) received a diet with approx. four times as much TRP (TRP+: 10.2 g/kg TRP; Trede und von Pein, Itzehoe, Germany). For detailed information on the experimental diets see Appendix 1.

2.2.2 Brain tissue collection

Animals were pseudorandomly allocated to two different sampling time points (day 6 or day 13 after dietary change). Animals were narcotized with Ursotamin (ketamine, 10 mg/ml; Serumwerk Bernburg AG, Bernburg, Germany) and Stresnil (azaparone, 40 mg/ml; Janssen-Cilag GmbH, Neuss, Germany) and afterwards sacrificed with i.v. injections of T61 (embutramide, 200 mg/ml; mebezonium iodide, 50 mg/ml; tetracaine hydrochloride, 5 mg/ml; Intervet Deutschland GmbH, Unterschleißheim, Germany). Doses were adjusted to body weight as instructed by the manufacturers. Brains were quickly removed and the following regions were dissected by qualified personnel (Kanitz et al., 2011) using a stereotaxic atlas of the pig brain as reference (Félix et al., 1999): rostral anterior cingulate cortex, prefrontal cortex, striatum (including putamen, nucleus caudatus and nucleus accumbens), amygdala, hippocampus, hypothalamus and brain stem. Tissues were frozen in liquid nitrogen and stored at −80°C until analysis.

2.2.3 High-performance liquid chromatography analysis

Concentrations of TRP and its major metabolites 5-HT, 5-HIAA and kynurenine (KYN), both from the 5-HT and KYN pathway, were determined in brain tissues using high performance liquid chromatography (HPLC) with fluorescence and UV detection.

Brain samples were weighed and homogenized on ice with an ultra turrax (T25, IKA-Werke GmbH & Co. KG, Staufen, Germany) at 9,500 rpm in 1-20 ml of 0.2 M perchloric acid, depending on tissue weight. After incubation for 5 min on ice, homogenates were centrifuged at 5,100 x g for 10 min at 4°C. This procedure was repeated after collection of the supernatants. Pooled supernatants of the repeated extractions were again centrifuged at 37,000 x g for 10 min at 4°C. Immediately after extraction, aliquots of 10 µl were injected directly into the HPLC system (SHIMADZU, Duisburg, Germany). Duplicates were assayed from each extract. The HPLC system was equipped with a 125 x 4 mm reversed-phase column packed with Prontosil C18 AQ (Bischoff Analysentechnik, Leonberg, Germany). The mobile phase consisted of 58 mM sodium hydrogen phosphate buffer

containing 1.2 mM octanesulfonic acid, 0.3 mM EDTA, and 12 % methanol at pH 3.2, and was used at a flow rate of 0.8 ml/min.

TRP, 5-HT and 5-HIAA concentrations were analyzed with fluorescence detection (RF-20Axs, SHIMADZU, Duisburg, Germany) using an excitation wavelength of 280 nm and a detection wavelength of 335 nm. The elution times for 5-HIAA, 5-HT and TRP were 8.7, 17.5 and 23.6 min respectively. KYN concentration was analyzed with UV detection (SPD-10AV, SHIMADZU, Duisburg, Germany) at a wavelength of 363 nm with an elution time of 7.7 min. Peak identification was accomplished by spiking samples with reference compounds. Calibration curves were calculated using peak area versus analyzed concentration at two concentrations (dilution factor: 8). Standard curves were conducted routinely before and after analysis of approximately 10 samples. The reference compounds used were TRP (T0254), 5-HT (H9523), 5-HIAA (55360), and KYN (K8625), all purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). The intra-assay coefficients of variation for TRP, 5-HT, 5-HIAA and KYN were 0.4, 0.7, 0.8, and 12.8 %, and the inter-assay coefficients of variation were 0.6, 2.1, 4.2, and 10.9 % respectively. The concentrations were expressed as ng per gram tissue.

2.3 Part 2: Tryptophan supplementation and behavior

2.3.1 Experimental design

The study was conducted in four replicates from March 2015 to August 2015. Per replicate, eight subjects (balanced for housing group, home pen and kinship) were chosen pseudorandomly, excluding animals not eating the food reward in a standardized reward consumption test (Düpjan et al., 2016). All 32 subjects underwent both behavioral tests (Fig. 1).

In each replicate, the two housing groups were pseudorandomly allocated to either the control or the TRP enriched diet, balanced for the home pen. Feeding schedule was the same as in part 1 (2.2.1), with the change at 56 days of life (at the end of test week two in the SJT; see below). In total, 16 animals received the control food (control) and 16 animals received the TRP enriched diet (TRP+).

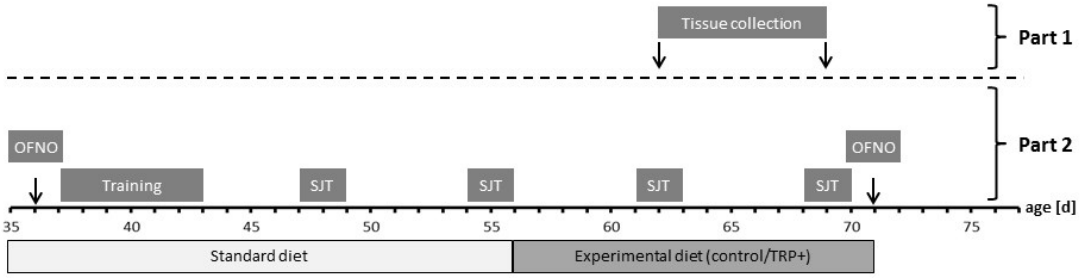

Figure. 1: Experimental schedule for part 1 (above) and part 2 (below) of the study. OFNO, open field/novel object test; SJT, spatial judgement task (test period).

2.3.2 Spatial judgement task (SJT)

The spatial judgement task (SJT) is a variation of the cognitive bias test, which has been adapted for the use in domestic pigs in our group. Details on the experimental procedure can be found in Düpjan et al. (2016). The SJT consists of two phases: in the first, subjects learn to discriminate between a positively and a negatively associated location of a goal box in an arena. They must learn to open the goal box ('go' response) when it is in the positive location to achieve a reward and to not open the box ('no go' response) when it is in the negative location to avoid punishment. All subjects were familiarized with the goal box and the food reward before training started. The experimental setting consisted of an arena which could be accessed from a start area through a guillotine door (to be opened by an experimenter). The arena was shaped like a quarter circle (radius 3.2 m), and the goal box could be fixed to the back wall at five locations. The right and left corners were the rewarded (S^+) and the punished (S^-) locations (reference locations, counterbalanced between subjects/groups/diets), with three probe locations equidistant between them (near rewarded (nS^+), middle (M), near punished (nS^-)). The goal box had a flap door which could be opened by the subjects with a gentle push of the snout to gain access to a metal food bowl. The food bowl contained 5 ml of applesauce, which could be either freely accessible (rewarded trials; see below) or inaccessible by placing a perforated plastic plate on top (unrewarded, unreinforced, and probe trials; see below). Punishment was

provided by waving a plastic bag into the pig's face as soon as they opened the goal box (but not at further goal box contacts). Probes were neither rewarded nor punished.

2.3.2.1 Training

The animals were trained individually on a go/no-go task to discriminate between S^+ and S^-. Half of the animals were rewarded on the left corner of the arena and punished on the right corner and vice versa.

Training was conducted on seven consecutive days, starting at 37 days of age (Fig.1). Each animal had three training sessions per day, each comprising four consecutive trials per animal (i.e. 12 trials in total per day per animal). The goal box was presented on each of the reference locations six times per day, but from day two of training onwards the opening of the goal box was reinforced (rewarded on S+, punished on S-) only five of these six times. This so-called partial reinforcement ($S^{(+)}/S^{(-)}$) prevents learning of probe outcome (for details see Düpjan et al., 2016). The sequence of goal box locations was pseudorandomized for each individual and day. Animals stayed in the arena for one minute per trial, during which they could make their decision.

2.3.2.2 Testing

Testing started at 47 days of age and was performed on three consecutive days a week (Tuesday to Thursday) for four weeks (Fig.1). Two test trials per day were integrated in the normal training schedule as additional trials before session 2 and session 3. Probes were, as described in experimental settings, on equidistant locations between S^+ and S^- (nS^+, M, nS^-). The probes were unreinforced and here again animals had one minute time to make their choice. Probes were pseudorandomized with each being presented twice per week but never the same probe twice a day.

2.3.3 Open field/Novel Object test (OFNO)

All pigs underwent the OFNO twice, first on d 36 and twice on d 71 of life (Fig.1).

The pigs were tested individually. The test arena (3x3x1.25 meters) was located in a noise reduced room and behavior of the pigs was observed by video recordings. Animals stayed in the arena for ten minutes in total, after five minutes the novel object was introduced manually by a windlass in the middle of the open field. As

novel objects we used a plastic dumb bell in the first novel object test (d 36) and a plastic container in the second novel object test (d 71).

2.3.4 Behavioral observation

Animals were observed by video recordings. The camera was installed centrally above the arena. The videos were analyzed using the Observer XT version 12 (Noldus Information Technology, Wageningen, The Netherlands).

Recorded behaviors with their definitions and type of recording are listed in Table 1. All behaviors except opening of the goal box were scored for the test trials as well as for each reference location ($S^{(+)}$, $S^{(-)}$) for a total of 60 s. This was done for better comparability of ambivalent goal box locations (nS^{+}, M, nS^{-}) (unreinforced) and extreme goal box locations (S^{+}, S^{-}) (reinforced).

The latency from entering the arena ('start', see Table 1) until opening the goal box was measured ('open') for each trial, including the reinforced trials. Relative latency was calculated by setting the latency to open the goal box in the probe trials in relation to the daily mean of trials on the rewarded location (S^{+} and $S^{(+)}$; mean≙0) and the trials on the punished location (S^{-} and $S^{(-)}$; mean≙1):

$$relative\ latency = \frac{absolute\ latency - mean\ latency\ (S^{+},\ S^{(+)})}{mean\ latency\ (S^{-},\ S^{(-)}) - mean\ latency\ (S^{+},\ S^{(+)})}$$

This formula was used to eliminate effects of overall differences in speed between subjects, but also within subjects between days, caused for example by different size, feeding motivation, time of day (see Düpjan et al., 2016 for a discussion).

In case one animal did not show one of the mentioned behaviors, latency was set to the maximum time (60s) for further analysis.

Table 1: Recorded behaviors. D, duration, F, frequency, L, latency, SJT, spatial judgement task, OFNO, open field/novel object test; behaviors in the grey sections mark groups of mutually exclusive state events.

Behavior	Type	Measurement	Definition
Choice (SJT)	Point event	L	Opening the goal box
Start (SJT/OFNO)	Point event	Reference point	Four feet in the arena or open field/object in place
Locomotion (SJT/OFNO)	State event	D	Moving; minimum one forward step
Standing (SJT /OFNO)	State event	D	No locomotion, all four legs stretched out, no floor contact of the torso; minimum 3 ft on the ground
Lying (SJT /OFNO)	State event	D	Lying down with the whole belly or the whole lateral part of the animal touching the ground
Jumping (OFNO)	State event	D	Jumping at the walls
Abrupt movements (SJT)	State event	F	One of the following components or a combination of them: sudden cantering, jumping and twisting
Exploration goal box	State event	D/F	Physical contact (nose) with the goal box, sniffing, licking
Scratching (SJT)	Point event	F	Pig scratching itself, either with the foot, or by scrubbing the body on either wall of the arena or the goal box
Defecation (SJT /OFNO)	Point event	F	Defecate
Urination (SJT /OFNO)	Point event	F	Urinate
Contact object (OFNO)	State event	D/L	Exploring the object using the nose
Exploration floor	State event	D	Manipulating the floor using the nose
Exploration wall (OFNO)	State event	D	Manipulating the wall using the nose
No exploration (OFNO)	State event	D	No exploration of wall, floor or object

2.4 Statistical analysis

Data were analyzed using the MIXED procedure (SAS Version 9.4, SAS Institute Inc., Cary, NC, USA). For the analysis of the TRP metabolite concentrations for each brain area (2.2) ANOVAs were calculated with diet (control/TRP+), time point (day of the brain tissue sampling), and their interaction as fixed factors and mother and father as random effects in the model. Multiple pairwise comparisons were made using Bonferroni corrections. The increase between control and TRP+-groups were calculated as percentages.

In the SJT (2.3.2), data were combined for the test weeks before dietary change (test weeks 1 and 2) and the test weeks after dietary change (test weeks 3 and 4).

For the analysis of latency to open the goal box (absolute and relative) and for each measured behavior variable in the SJT, repeated measurement ANOVAs were used with goal box location (S^+/nS^+/M/nS^-/S^-), diet, time point (before/after dietary change), replicate, and their interactions as fixed effects, mother and father as random effects in the model. Repeated measurements for the goal box location and time point were taken into account. Again, pairwise comparisons of the least square means were done with Bonferroni correction for multiple testing.

In the OFNO tests, differences were calculated from second and first OFNO test to correct for possible time effects. Diet, replicate and their interaction were considered as fixed effects, mother and father were considered as random effects in the repeated measurement ANOVA model. Repeated measurements for the same animal were taken into account.

The average daily gain (ADG) of the subjects was calculated for the phase before and after dietary change. A repeated measures ANOVA was calculated with diet, time point, replicate and their interactions as fixed effects, and mother and father as random effects in the model.

For all tests, effects or differences were considered significant when $p<0.05$.

Results

3.1 Tryptophan and its metabolites in the brain

One animal had to be excluded from the analysis due to illness unrelated to the treatment. In total, data of 19 animals could be used for statistical analysis (brain tissue sampling on day 6 after dietary change: TRP+, N=4; control. N=5; brain tissue sampling on day 13 after dietary change: TRP+, N=5; control, N=5). A significant effect of diet could be found for most analyzed parameters and brain tissues (all $F>8.4$; all $p<0.05$). TRP supplementation significantly increased concentrations of TRP, 5-HT. 5-HIAA and KYN in all brain regions, with the exception of 5-HT concentration in the hippocampus ($F=4.2$; $p=0.058$). Detailed information on all parameters and brain tissues can be found in Table 2.

There were significant effects of the interaction of diet and time point on the concentrations of TRP in the amygdala ($F=4.6$; $p<0.05$), the hippocampus ($F=6.5$; $p<0.05$) and the prefrontal cortex ($F=5.9$; $p<0.05$). These interactions resulted from significantly higher TRP levels on day 6 compared to day 13 for the TRP+-animals (all $|t|>2.9$; all $p<0.05$), while the diet groups differed significantly on both days (d6: all $|t|>8.1$; all $p<0.001$; d13: $|t|>4.9$; all $p<0,001$). The same effect was found for the concentration of KYN in the striatum ($F=16.1$; $p<0.01$). Here again, the interaction resulted from significantly higher values on day 6 compared to day 13 for the TRP+-animals ($t=5.2$, $p<0.001$), while significant differences were maintained between the diet groups on both days (d6: $t=7.8$, $p<0.001$; d13: $t=2.4$; $p<0.05$)). In the hypothalamus, a significant effect of time point could be found for 5-HT ($F=9.1$; $p<0.01$), with generally lower 5-HT concentrations on day 6 after dietary change compared to day 13 after dietary change, independent of diet. Time point also revealed a significant effect on 5-HIAA in the amygdala ($F=5.4$; $p<0.05$) and in the brain stem ($F=9.3$; $p<0.01$) and on KYN in the brain stem ($F=6.6$; $p<0.05$), cingulate cortex ($F=10.0$; $p<0.01$) and striatum ($F=13.0$; $p<0.01$). Here, in all analyzed tissues the concentrations of the measured parameters were higher 6 days than 13 days after dietary change, independent of diet.

Table 2: Concentrations (ng/g) of tryptophan (TRP) and its metabolites serotonin (5-HT), 5-Hydroxyindoleacetic acid (5-HIAA), and kynurenine (KYN) in different brain regions of animals from the two feeding groups (control and tryptophan enriched (TRP+)). The increase between control and TRP+-groups are presented as percentages. Additionally, ANOVA results (F-test) for the main effect "diet" are presented (DF=1.15).

	Amygdala	Brain stem	Cingulate cortex	Hypothalamus	Hippocampus	Prefrontal cortex	Striatum
TRP	+178%	+151%	+165%	+156%	+174%	+184%	+151%
Control	2978 ± 308	2384 ± 225	2859 ± 323	3061 ± 359	2845 ± 365	2833 ± 374	3323 ± 302
TRP+	8275 ± 327	5980 ± 239	7584 ± 342	7831 ± 381	7785 ± 387	8042 ± 397	8335 ± 320
F-test	$F = 138.8$; $P < 0.001$	$F = 120.2$; $P < 0.001$	$F = 100.8$; $P < 0.001$	$F = 82.8$; $P < 0.001$	$F = 86.4$; $P < 001$	$F = 91.1$; $P < 0.001$	$F = 130.0$; $P < 0.001$
5-HT	+18%	+35%	+22%	+15%	+19%	+36%	+29%
Control	1065 ± 45	608 ± 22	145 ± 6	838 ± 28	153 ± 10	133 ± 7	163 ± 6
TRP+	1256 ± 48	819 ± 24	177 ± 6	963 ± 30	182 ± 10	181 ± 7	210 ± 6
F-test	$F = 8.4$; $P < 0.05$	$F = 41.5$; $P < 0.001$	$F = 12.8$; $P < 0.01$	$F = 9.5$; $P < 0.01$	$F = 4.2$; $P = 0.058$	$F = 23.2$; $P < 0.001$	$F = 29.7$; $P < 0.001$
5-HIAA	+99%	+140%	+69%	+70%	+72%	+66%	+72%
Control	306 ± 19	367 ± 24	108 ± 8	475 ± 30	128 ± 10	62 ± 4	186 ± 13
TRP+	608 ± 20	881 ± 25	183 ± 8	809 ± 32	220 ± 10	103 ± 4	320 ± 13
F-test	$F = 120.5$; $P < 0.001$	$F = 220.0$; $P < 0.001$	$F = 44.8$; $P < 0.001$	$F = 57.0$; $P < 0.001$	$F = 40.9$; $P < 0.001$	$F = 48.1$; $P < 0.001$	$F = 53.9$; $P < 0.001$
KYN	+317%	+213%	+204%	+406%	+197%	+146%	+417%
Control	84 ± 30	103 ± 29	101 ± 24	103 ± 53	129 ± 33	247 ± 54	59 ±
TRP+	350 ± 32	322 ± 30	307 ± 25	522 ± 56	383 ± 35	607 ± 58	305 ± 25
F-test	$F = 36.8$; $P < 0.001$	$F = 27.6$; $P < 0.001$	$F = 35.2$; $P < 0.001$	$F = 29.6$; $P < 0.001$	$F = 27.4$; $P < 0.001$	$F = 20.7$; $P < 0.001$	$F = 53.1$; $P < 0.001$

3.2 Tryptophan supplementation and behavior

One animal had to be excluded from the analysis due to illness unrelated to the experimental procedure. In total, 31 animals were included in the statistical analyzes, 16 from the TRP+-group and 15 from the control. In the SJT, lying and scratching were too rare for analysis. In the OFNO, lying, sitting, jumping and urination had to be excluded for the same reason.

The analysis of the ADG revealed no significant effects of diet, time point, replicate or any of their interactions (all $F<2.3$; all $p>0.05$).

3.2.1 Spatial judgement task (SJT)

There was a significant effect of the goal box location (S^+/S^-) on absolute latency to open the goal box ($F=32191.6$; $p<0.001$), resulting from significant differences between the reference locations S^+ and S^- on all test days (all $|t|>49.3$; all $p<0.001$). Diet had no effect on absolute latency ($F=0.0$; $p>0.05$). Additionally, a significant interaction effect between goal box location and replicate ($F=13.8$; $p<0.001$) as well as a threefold interaction effect of goal box location, replicate and diet could be found ($F=7.0$; $p<0.001$).

The analysis of relative latency to open the goal box showed a threefold interaction effect of goal box location, diet and time point ($F=3.0$; $p<0.05$). Before dietary change, diet groups showed significant differences in relative latency on all three probes (nS^+, M, nS^-) (all $|t|>3.2$; all $p<0.05$), with TRP+ animals showing shorter relative latencies than control animals. After dietary change, no significant differences between the diet groups could be detected (all $|t|<2.3$; all $p>0.05$). On test location nS^+, the TRP+ group showed significantly longer latencies after dietary change than before ($t=3.3$; $p<0.01$) (Fig. 2).

Replicate showed significant effects on relative latency alone, in interaction with location, in interaction with diet and in interaction with both diet and location (all $F>6.4$; all $p<0.01$).

No effect of diet or any of its interactions could be found on any behavioral parameters recorded during the SJT (all $F<3.5$; all $p>0.05$). The location of the goal box had a significant effect on locomotion ($F=8.7$; $p<0.001$), standing ($F=19.7$; $p<0.001$) and exploration of the goal box ($F=7.5$; $p<0.001$). These parameters also revealed a significant effect of the time point (locomotion: $F=37.2$; $p<0.001$; standing: $F=145.9$; $p<0.001$; exploration of the goal box: $F=9.3$; $p<0.01$), with animals showing more locomotion, less standing and more exploration of the goal box after dietary change. Additionally, an interaction effect

of location of the goal box and time point could be found for the parameter standing ($F=2.4$, $p<0.05$).

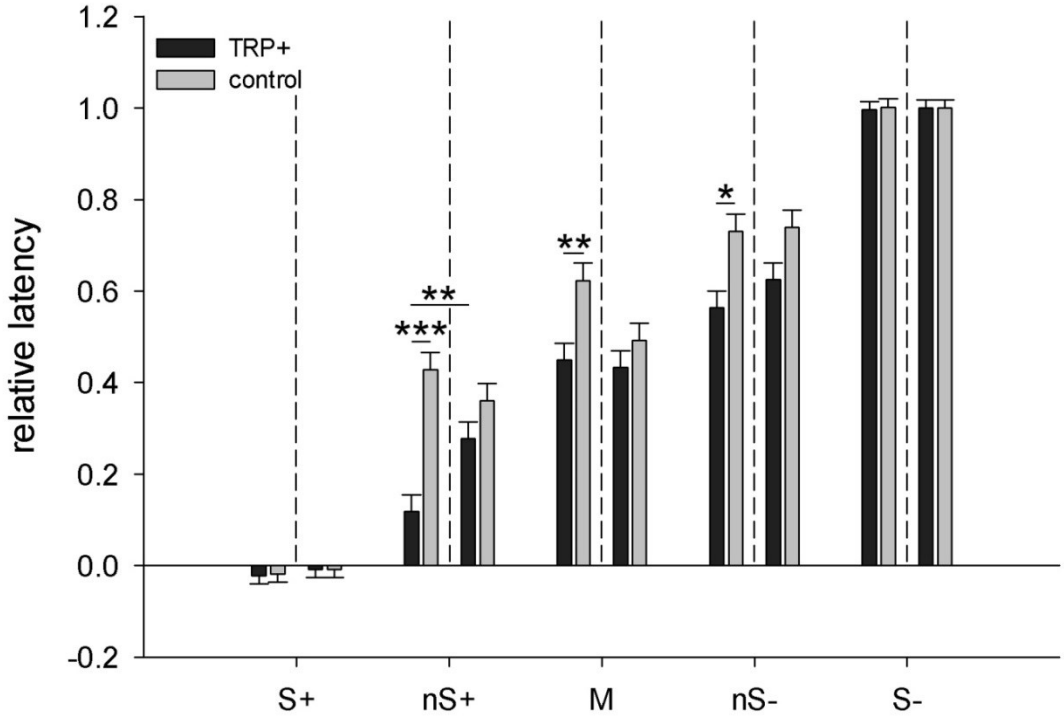

Figure 2: Relative latency to open the goal box in the spatial judgement task (SJT) (least square mean± standard error of the means, *p<0.05, **p<0.01, ***p<0.001). S+, rewarded; nS+, near rewarded; M, middle; nS-, near punished; S-, punished; gray bars represent the control group; black bars represent the tryptophan enriched feeding group (TRP+); vertical broken lines indicate the time point of the dietary change (left, before; right, after).

3.2.2 Open field/novel objects test (OFNO)

Times in the open field (OF; 5min) and with the novel object (NO; 5min) were analyzed separately. Diet revealed no significant effect on any of the analyzed behavioral parameters in the OF or NO (all $F<3.8$; all $p>0.05$) (Fig.3). A significant effect of replicate ($F=3.5$; $p<0.05$) and its interaction with diet ($F=3.5$; $p<0.05$) was found on exploring the wall in the OF.

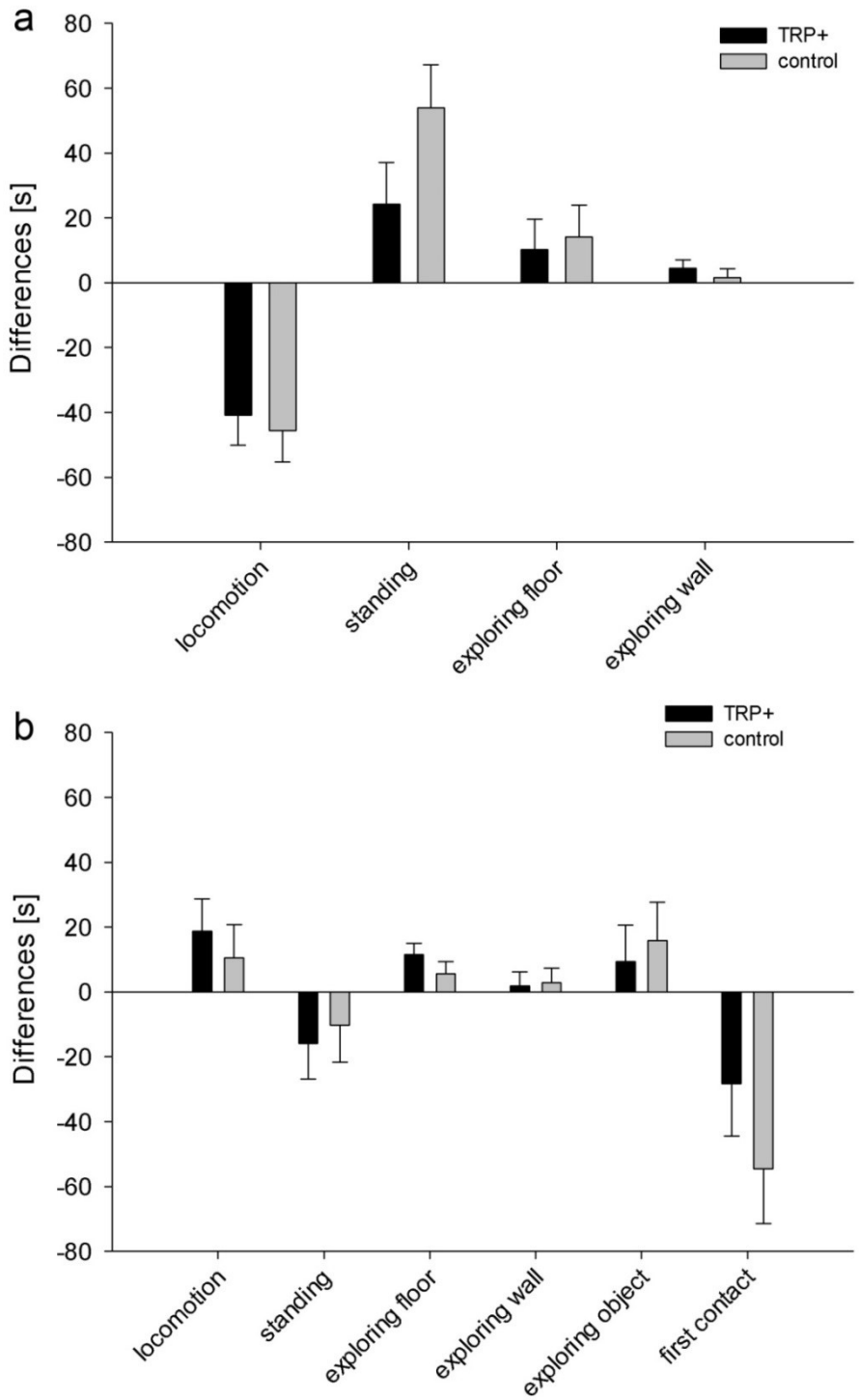

Figure 3: Differences in the behavior in the open field/novel object (OFNO) test between test 2 and test 1 (least square mean±standard error of means); a: open field test; b: novel object test; gray bars represent the control group; black bars represent the tryptophan enriched feeding group (TRP+).

Discussion

Our study examined the effects of dietary TRP supplementation on central concentrations of TRP and its metabolites, as well as on affective state and behavioral reactivity in the domestic pig. In part 1 of our study, we found that a fourfold increase in dietary TRP content significantly increased TRP and 5-HT concentrations in most examined brain areas. Contrary to our suggestion, there was no shift towards a more optimistic judgement on ambiguous stimuli in the SJT and no effect on general behavioral reactivity as measured in the OFNO associated with these physiological changes in part 2. Instead, TRP supplemented animals even showed more pessimistic behavior after dietary change than before.

4.1 Tryptophan and its metabolites in the brain

In our study, TRP concentrations in the brain were increased by 151% to 184% in the TRP+ group compared to the control. These physiological effects could already be found on day 6 after dietary change (representing test week 3 in the SJT) and persisted at least to 13 days after dietary change (representing test week 4 in the SJT). TRP concentrations in amygdala, hippocampus and prefrontal cortex were higher on day 6 compared to day 13 for the TRP+ group whereas they remained the same in the control animals. These differences might reflect mechanisms of adaptation to elevated TRP availability, e.g., by increased degradation. However, we found no evidence of increased degradation along the 5-HT and KYN pathways. Instead, the observed changes in TRP levels over time might have been mediated for example by reduced uptake of TRP into the brain, changes in intestinal morphology (Koopmans et al., 2006; Tossou et al., 2016) or interactions with other pathways. TRP in the brain is used in the biosynthesis of proteins, functions as a precursor for 5-HT and melatonin and can be metabolized along the KYN pathway (Le Floc'h et al., 2011). However, in the regulation of affective states, the 5-HT and the KYN system play a major role (Castanon, 2015; Cools et al., 2008). In our study, TRP supplementation resulted in an overall activation of the 5-HT system (increase of 5-HT ranging between 18% and 35%; increase of 5-HIAA ranging between 66% and 140%). It also resulted in a higher degradation of TRP along the KYN pathway (increase of KYN from 197% to 417%).

We selected brain areas which are known to play a role in affective/cognitive regulation and the processing of environmental stimuli (i.e., attention to, evaluation of, and (selection of) behavioral reaction to stimuli), and therefore relate to the behavioral tests in part 2 of our study.

4.2 Tryptophan supplementation and behavior

Although we found enhanced brain 5-HT concentrations in part 1 of our study, the same dietary TRP supplementation did not result in a more optimistic evaluation of the ambivalent probe cues in the SJT. On the contrary, animals receiving the TRP+ diet showed significantly longer latencies to the nS+ probe after dietary change than before, which rather indicate a shift towards a more pessimistic evaluation of this ambivalent position, comparable to results found after 5-HT depletion (Stracke et al., submitted). However, TRP+ animals showed more optimistic behavior than the control group before the different diets were fed, and only reached response levels comparable to the control in both phases. We found no evidence for an effect of dietary TRP content on average daily gain; hence, we can assume that both diet groups had similar feeding motivation in the SJT. All subjects were tested repeatedly in both SJT (using a design which has proven to be suitable for repeated testing (Düpjan et al., 2016)) and OFNO, so that each animal was its own control. Therefore, we argue that the measured shift in the behavioral response to ambiguity revealed a shift in the affective state even though results have to be considered with caution.

Our results on weight gain, which was not affected by the experimental diets, confirm previous findings by authors investigating the effect of dietary tryptophan content on feed intake. Our control diet fulfilled the recommendations for optimal growth in pigs, while the TRP enriched diet exceeded this level by far and cannot be expected to increase feed intake and weight gain (e.g., Eder et al., 2001). Whether such a surplus of TRP has a relevant impact beyond feed intake, especially on the affective state of animals, was the focus of our study.

Higher TRP levels in the diet are reported to have positive effects on the behavior in different species. In pigs, TRP administered via drinking water or feed can reduce behavioral responses to transport stress (Peeters et al., 2004), increase lying behavior and decrease aggression (Li et al., 2006). Decreases in aggression could also be found in dogs and Atlantic cod (DeNapoli et al., 2000, Höglund et al., 2005). Our data do not confirm effects of TRP supplementation on general reactivity in the OFNO, a test measuring emotional reactivity in pigs (Puppe et al., 1999, 2007). Instead, while animals were found to be more active in the second test (meaning after dietary change), diet groups did not differ significantly. The same trend in behavior could be found in the SJT. In laying hens, TRP supplementation decreases feather pecking, one of the most important abnormal behaviors indicating impaired welfare in chicken (Brunberg et al., 2016). Murphy et al. (2006) even showed that TRP supplementation can induce a positive

cognitive bias in humans. However, results on the effects of dietary TRP supplementation are controversial. For example, similar to our own results Koopmanns et al. (2005) found physiological effects of TRP supplementation (control: 1.4 g/kg TRP, TRP enriched: 7 g/kg TRP at 11.2 g/kg lysine (LYS)), namely a reduction in basal plasma cortisol and noradrenaline concentrations, but no significant differences in the behavior of pigs. It has to mentioned, however, that the control diet used in that study can actually be considered to be TRP deficient (up to 2.6 g/kg TRP at 12.5 and 12.7 g/kg LYS as suggested by Eder, 2001), and that both their diets had higher LYS:TRP ratio than our experimental diets (control: 10.0 g/kg LYS, TRP+: 9.9 g/kg LYS). In horses, where TRP is used as a commercially available calmative (i.e. intended to calm down the animals), such a treatment can also lead to an increase in behavioral reactivity (Malmqvist and Christensen, 2007; Grimmett and Sillence, 2005). Discrepancies across findings are discussed to be related to factors impinging on TRP passing the blood brain barrier via active transporters selective for long neutral amino acids (LNAAs), like for example high protein diets rich in LNAAs, or maturation effects on the permeability of the blood brain barrier (Shen et al., 2012; Tang and Melethil, 1995). In our study, we found that TRP supplementation significantly increased TRP levels in all analyzed brain regions, indicating that such limitations were not relevant in our model.

Many of the effects of TRP supplementation or depletion are assumed to be based on its role as the precursor of 5-HT. Different studies show that a manipulation of 5-HT availability might influence affective behavior as well as cognitive abilities (Mosienko et al., 2012; Lapiz-Bluhm et al., 2008). A pharmacologically induced 5-HT depletion results in significantly more pessimistic behavior in the SJT, indicating a negative affective state in pigs (Stracke et al., submitted) and sheep (Douglas et al., 2012). Studies in humans show that acute TRP depletion (usually induced by a combination of a TRP free and LNAA rich diet) induces depressive-like states in individuals genetically prone to major depression (i.e., there is a gene by environment interaction; e.g., Caspi et al., 2003). To our knowledge, similar genetic factors are not known in pigs, but the differences between diet groups before the actual dietary change hint at individual differences caused by other than environmental factors (environments were the same for both groups of animals).

Of course, serotonergic pathways are complex; not only are the involved brain regions highly connected (Pessoa, 2013), but they interact with other structures regulating affective behavior, like the dopaminergic system (Boureau and Dayan, 2011) or the extended amygdala (Marcinkiewcz et al., 2016). From human studies,

it is known that a surplus of 5-HT can also have a negative impact on the organism. This phenomenon is discussed as the "serotonin syndrome", which might result in autonomic, cognitive and behavioral dysfunctions and (in the worst case) can be lethal (Boyer and Shannon, 2005). Comparable symptoms could be found in rats (Haberzettl et al., 2013). However, the serotonin syndrome described in humans is only achieved with pharmacologic intervention; hence we do not assume that we reached a comparable effect with our dietary TRP supplementation model.

When investigating effects of TRP availability in the brain, one should also consider the KYN pathway, because the majority of TRP is metabolized in that pathway (approx. 90% according to Le Floc'h and Sève, 2007), which is reflected in the data from part 1 of our study. Activation of the KYN pathway is known to impair mood and cognitive functions due to inflammation-induced activation of indoleamine 2,3-dioxygenase (Castanon, 2015; Wirthgen et al., 2014). Research indicates that such activation might be strongly correlated with depressive-like behaviors (Fu et al., 2010) independent of 5-HT levels (O'Connor et al., 2008; Castanon, 2015). As we found excess levels of KYN in the TRP+ group, we cannot exclude behavioral effects of KYN metabolites. However, behavior differed between diet groups before dietary change, already. Additionally, these effects were found in the evaluation of the ambivalent stimuli in the SJT alone, and could not be observed in any other behavioral parameter measured in the SJT or the OFNO. Hence, it seems to be unlikely that the behavioral differences can be explained by changes in the KYN pathway.

4.3 Conclusion

Our study shows that dietary TRP supplementation significantly enhances concentrations of TRP and its metabolite KYN, as well as 5-HT and its metabolite 5-HIAA, indicating serotonergic activation and increased TRP degradation along the KYN pathway in several brain areas relevant for affective/cognitive processing. However, contrary to our suggestion, these physiological changes were accompanied by a shift towards more pessimistic judgements on ambiguous stimuli, indicating a more negative affective state, and no changes in behavioral reactivity. Hence, our results do not confirm the assumption that TRP supplementation can induce positive affective states and therefore improves animal welfare in pigs.

Appendix 1:

Table: Ingredients and composition (%) of the two experimental diets (as fed basis). Analyses followed the VDLUFA III protocol.

	control		TRP+ diet	
ingredients	%		%	
corn	65.63		65.55	
soybean meal	28.00		28.00	
vitamin/mineral mixture[1]	0.50		0.50	
molasses	2.00		2.00	
monocalcium phosphate	0.70		0.70	
limestone (Vitacarb 15)	1.00		1.00	
sunflower oil	1.00		1.00	
salt NaCl	0.30		0.30	
L-tryptophan			0.60	
L-alanine	0.87		0.35	
	calculated	analyzed	calculated	analyzed
composition	%	%	%	%
dry matter	88.2	88.3	88.2	87.8
crude protein	19.5	19.6	19.5	19.3
crude fat	4.4	3.5	4.4	3.7
starch	41.2	42.8	41.2	43.6
sugar	4.7	4.0	4.7	3.8
crude fiber	3.2	2.4	3.2	2.6
crude ash	5.3	4.7	5.3	4.5
Trp	0.23	0.25	1.23	1.02
Lys	0.96	1.00	0.96	0.99
Meth	0.29	0.27	0.29	0.27
Cys	0.33	0.32	0.33	0.31
Thr	0.70	0.71	0.70	0.69
Ile		0.79		0.78
Leu		1.61		1.61
Ala		0.92		0.90
Tyr		0.63		0.61
Val	0.26	0.86	0.26	0.84

[1] Vitamin and mineral mixture (Premix Vormast 0.5%, Provimi B.V., Rotterdam, The Netherlands; contains (g/kg): zinc, 20.0; manganese, 12.5; selenium, 0.05; iodine, 0.25; iron, 20.0; copper, 33.0; vitamin A, 3000.0; vitamin D3, 300.0; vitamin E, 16.0; vitamin K3 0.40; vitamin B1, 0.44; vitamin B2 0.89; vitamin B12, 6.0; vitamin B3, 6.0; vitamin B5, 3.0; vitamin B6, 0.67)

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Ethical statement

The study was approved by the LALLF (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei) Mecklenburg Vorpommern (AZ:7221.3-1-066/13). All efforts were made to ensure minimal animal number and suffering.

Conflict of interest statement

The authors wish to declare that there are no known conflicts of interest associated with this publication. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Chapter 3:

General Discussion

3.1 Discussion of the results of studies 1-3

The goal of the thesis was to provide a test design suitable for testing cognitive bias in pigs and meeting all criteria relevant for affective behavioural testing listed in the introduction (1.5.1; see also next section). This design had to be validated and transferred to practically relevant approaches. As overall subjective, this should provide insight into the mechanisms underlying cognitive – affective appraisal processes.

Recapitulating the main aspects relevant for affective behavioural testing, tests should be (a) ethological-relevant in the behavioural response (b) robust against side effects, (c) should detect subtle differences already, (d) be robust when tested repeatedly, (e) should be standardized and (f) able to be automatable (Murphy et al., 2014). Cognitive bias tests in particular should (1) provide a no-go reaction to the negative associated cue and a go reaction to the positive associated cue, (2) they should show a monotonic graded response (from negative to positive) to ambiguous cues and (3) the behavioural response to the reference cues should not change due to manipulation of the affective state while (4) the response to the ambiguous cues should be affected (Gygax, 2014).

The studies included in my thesis present a test paradigm to measure the cognitive bias in pigs in a spatial judgement task (SJT). This test design was found to be appropriate in testing pigs repeatedly with presenting each of three ambivalent probes up to eight times in a test period of four weeks. It was also found that animals show graded responses to graded probes. The test design was validated pharmacologically, proving that animals are more pessimistic in the SJT when under 5-HT depletion treatment. In a practical approach 5-HT availability in the brain was increased via dietary TRP supplementation. The surplus of 5-HT resulted in a pessimistic evaluation of ambivalent probes and could not induce an optimistic behavioural response in the SJT. Both manipulations of the 5-HT system could not be found to alter behaviour in a standard behavioural test (OFNO). The following parts will discuss the presented results of each study in detail, referring to their appropriateness according to the above mentioned criteria.

3.1.1 Study 1: Modification of a spatial judgement bias task for pigs

The test design used throughout my thesis is based on a spatial discrimination task as originally introduced by Burman et al. (2008). With respect to the first assumption for behavioural tests (i.e. being ethological relevant to the specific species: criterion (a), Murphy et al. (2014)), using spatial cues seems to be promising, as the ability of pigs to discriminate spatial information is well developed (Arts et al., 2009; Marino and Colvin, 2015). The ability of processing spatial information is deeply embedded in the behavioural repertoire of pigs, as the 'normal behaviour' requires a well-established spatial memory for places which provide food versus places which might be dangerous. This also could be confirmed by my results as the animals learned the discrimination task already after five days.

Criterion (b), demanding a measurement which is robust against side effects and specific to target the affective state, could also be fulfilled in the presented test design. In cognitive bias approaches using the go/no-go task, most studies consider the latency or the frequency to approach the goal as the parameter to measure. This might be problematic as this behavioural reaction is depending on different factors, like the motivation for food, the ability to move or a preference for the positive associated location (e.g. in spatial designs; see Murphy, 2015 for a discussion). In my thesis I use relative latency which was calculated by setting the latency at ambiguous probes in relation to the daily means of latency to both references. Differences which result from a lack in food motivation, individual differences in impulsive control (Melotti et al., 2013) or age relating factors therefore can be excluded, as they should be reflected on the reference cues as well. Therefore, the behavioural reaction is specifically directed to the evaluation of the probe. I additionally analysed different behavioural parameters which, besides others, included locomotion, standing, exploration and abrupt movements. This was done to provide additional information on the animal's state (e.g. a decrease of locomotion could reflect a general loss of motivation), but most importantly those analyses were conducted in order to find substitutes for the relative latency. Here I found the exploration of the goal box to be the most promising parameter to indicate information concerning the internal expectation of the animal. Exploration behaviour might be due to extrinsic factors (e.g. finding food, finding a place to rest) [Wood-Gush and Vestergaard, 1989], but on the other hand might be due to intrinsic factors, commonly stated as curiosity

(Studnitz et al., 2007). Sometimes it is assumed to reflect a state of uncertainty (i.e. if the outcome of a situation is not clear this could be reflected in an increased rate of exploration behaviour (Wood-Gush and Vestergaard, 1991; Rushen, 1993). Therefore, finding alternative parameters to measure the intrinsic purpose of the animals in the cognitive bias test might not only meet criterion (b) of Murphy et al. (2014) but as well might help to detect subtle difference in affective states already, which would be the third criterion on valid affective behavioural tests. However, behaviour often leaves room for speculation. Possibly the most prominent example would be 'play behaviour' which most often is presented as an indicator of positive affective state (de Passillé et al., 1995; Spinka et al., 2001). In contrast to this, a study of Mintline et al. (2012), measuring play behaviour of calves in an OF, found calves to show more play behaviour during the first exposure to a novel environment than in subsequent exposures. In this case play might be indicating a rather negative valence. An increase of play behaviour in a negative affective context could also be found in the thesis of Arelis (2006) who exposed rats to social deprivation which is known to be a mild stressor in these animals. Play behaviour might have a 'preventive' effect to reduce subsequent stress, which is discussed to be a reason for such unexpected effects (Palagi et al., 2004; Horváth et al., 2008). In the presented study, pigs as well showed behaviour which resembles characteristics of play behaviour. This behaviour was only occurring occasionally and in individual animals and therefore could not be analysed statistically. However, the behaviour predominantly occurred in the early training phase and during the test situation, and therefore might rather reflect a state of uncertainty than a positive affective state. This would coincide with the definition of "displacement activities" (Tinbergen, 1952) which occur when motivation for two different directed behaviour patterns is equally high. Such "displacement activities" are described in other species as well, as for example in Rhesus monkeys (scratching in a conflict situation: Diezinger and Anderson, 1986). Thus, in the context of measuring affective states, conclusions drawn from this behaviour should be interpreted with caution, even if this parameter might be interesting evaluating the grade of "uncertainty" of an animal judging ambiguous probes.

However, another tool in order to detect subtle difference in affective states would be the usage of different valued probes. The presented design uses three different probes – near rewarded, middle and near punished. Using various probes enables to find differences which may be missed when only using one (centered) probe. One interesting example in affective science would be the fact that affective states, even if having the same valence (e.g. depression and anxiety)

might reveal different behaviour in cognitive bias testing, with depressive states showing a higher tendency to decrease the expectation for positive events while states of anxiety are more prone to increase the anticipation of a negative event (Paul et al., 2005; Mendl et al., 2009). Therefore, the test design has the prerequisites to test subtle differences already. However, as results of study 3 could not confirm an optimistic behaviour due to an increase of 5-HT concentration via TRP supplementation in the diet, I will come back to this issue later on, when discussing these results in more detail.

Focus of study 1 was laid on presenting probes repeatedly (i.e. criterion (d): being robust when tested repeatedly, Murphy et al. (2014)) as this turned out to be a major problem in cognitive bias approaches in pigs (Carreras et al., 2015) and other species (Doyle et al., 2009). Some animals seem to be able to learn the outcome of the ambiguous cues when they remain unreinforced (which is mainly the case) (Mendl et al., 2009). This leaves the results of repeated cognitive bias tests highly unreliable, as results rather reflect learning curves than differences in cognitive bias. In preliminary studies pigs were found to be highly sensitive to this problem. For some animals the representation of only one unreinforced cue seems to be sufficient enough, that the animals do not consider them ambiguous anymore. Apart from test repetitions being statistically desirable per se, testing repeatedly can be inevitable when analysing the efficacy of treatments under specific circumstances. This might be the case if for example testing before and after treatment, as in study 2 and 3 of my thesis. Additionally it is useful when correcting for individual differences. Here again, it is worthwhile testing each individual at least twice, each before and after treatment. Furthermore, there are indications that optimism/pessimism might be characterised as special personality trait, with 'personality' or 'temperament' being defined as individual differences which are stable over time and situations (Gosling, 2008; Réale et al., 2007). Results of the presented study indicate at least one of those criteria (being stable over time) being fulfilled – if optimism/pessimism are stable in different situations and therefore might be considered as personality trait needs further studies. However, this question is not only basically interesting, but could affect treatments, too: In human studies there is evidence of the personality interacting with antidepressant treatment (Åkerblad et al., 2008). For optimism/pessimism there are indications directing to differences in neurophysiology as for example a hemispheric asymmetry in mediating both (Hecht, 2013). Here again, testing animals repeatedly, providing a baseline for each individual and testing shifts in cognitive bias rather than absolute values, would counterbalance the risk of effects due to interactions between above mentioned differences in

neuromodulation and treatment. Furthermore, there is strong evidence in human studies that there is a linkage between personality traits and mental disorders like depression (see Clark et al., 1994 and Kotov et al., 2010 for a review). As studies with non-human animals provide evidence of personality traits affecting various factors like health, behaviour and cognitive processes (Gosling, 2001, Gosling, 2008), too, in pigs such interactions shall be assumed as well. As already reviewed in chapter 1.5.1, there are different approaches to enable repeated testing in cognitive bias tests. In my thesis I decided to use the partial reinforcement (Bateson and Matheson, 2007; Matheson et al., 2008). To recapitulate, here, some of the training cues remain unreinforced, therefore an unreinforced test cue in the cognitive bias might still reveal a valued outcome when presented repeatedly, which could be confirmed by my results.

As already mentioned above, the test design presented in my thesis uses a spatial go/no-go task which was originally established in rats (Burman et al., 2008) and then adapted to the use in pigs by Dr. Düpjan at the Department of Behavioural Physiology, Leibniz Institute for Farm Animal Biology (Düpjan et al., 2013). Besides the introduction of the partial reinforcement, the original design, which used a 'reward' as positive reference cue while using 'no reward' as negative reference cue, was modified by introducing a mild punishment, which I will discuss more in detail later. This combination of modifications enabled testing judgement bias in pigs repeatedly with the test design being suitable to test each of three probes up to eight times in a four week period without the animals learning of the probe outcome and therefore could confirm the hypothesis made. However, it remains to remark that the first test week revealed shorter latencies compared to the following three test weeks. In the study we discussed this deviation to be independent from learning of the probe outcome and therefore a loss of ambiguity of the probes, as this would have been expected to be due to a further increase in latencies after week two. We also discussed this effect be due to a novelty effect (first presentations of the ambiguous probes); an explanation which was contradicted by the findings of the exploratory behaviour. Here, the duration of exploring the goal box showed no differences between testing weeks. However, keeping the deviation of week one in mind, the test design allowed consistent results in the last three test weeks. I can make no conclusions about how long this consistency persists – extended studies (e.g. testing finishing pigs or sows later again) would be highly interesting. Beside introducing a partial reinforcement and the mild punishment, here again the parameter of the 'relative latency' plays an important role to enable repeated testing as it rules out

differences due to temporal influences (e.g. weather, health, motivation and individual differences) [see Mendl et al., 2010 for a comparable approach].

The affective state in the presented study was not manipulated externally. Therefore, the study might not provide a measurement of cognitive bias in a strict sense. But as effects of multiple testing may be confused with any experimental treatment when testing before and after treatment (for a comparison see Doyle et al., 2010b), assessing the behaviour of animals independently from affective manipulation is unavoidable in establishing a valid test design which can then be used in further studies, especially in designs where animals have to be tested repeatedly. Concentrating on the development of the test design rather than on the effect of a specific treatment also facilitated the fulfillment of criterion (e) [i.e. being standardized, Murphy et al., 2014]. With regard to this criterion, I chose to use a fixed scheme for discrimination learning, meaning each animal was trained for seven days. As already discussed in study 1, this is in contrast with other studies (e.g., Bateson and Nettle, 2015; Rygula et al., 2015a), where individuals enter the testing phase on an individual schedule, according to their learning success.

Still, the last criterion of Murphy et al. (2014) [criterion (f): being automatable] cannot be fulfilled using the proposed test design. However, in chapter 3.2.1 I will discuss approaches to comply this important requirement.

Gygax (2014) defines requirements which are tailored to test designs testing the judgement bias. The presented test design fulfills the assumption (1) of Gygax (2014) [distinction and specific behavioural response to each reference cue]. As mentioned above, we found that animals were able to distinguish clearly between positively and negatively associated cues after 5 days of training already. Spatial go/no-go designs in pigs are based on the behavioural reaction to only one goal box with the distinction between negative and positive being expressed via a go or a no-go behaviour. As a clear differentiation of the reference cues is crucial for testing cognitive bias at a later stage, choosing the appropriate reinforcement is extremely important. Testing cognitive bias in pigs, literature proposes different references: different values of rewards; rewarded vs. unrewarded and reward vs. punishment. Murphy et al. (2013a) proposes using different valued rewards as pigs can distinguish between various food locations based on differing amounts of rewards. This was also shown in a study by Held et al. (2005). We tried to replicate their achievements in pre-studies but were not able to reproduce the results. This may be due to the different ages of the test animals used in the different studies (comparing the study of Murphy et al., 2013b: five months of

age; versus my thesis using juvenile pigs of five to nine weeks of age). It therefore might be due to this juvenile phase as the pigs in my study were still developing their individual taste preferences and cognitive abilities to discriminate between different quantities. However, there are indications in the literature that pigs per se are highly variable in their penchant for different rewards (Archer et al., 2003). Therefore, as already mentioned above, in my thesis I modified the approach of Düpjan et al. (2013) and - in order to enhance the cost due to a false choice - I decided to additionally introduce a mild punishment (Mendl et al., 2009). Using reward versus punishment as proposed in my thesis not only enabled repeated testing as discussed above, but in consequence resulted in a graded response to the ambiguous test positions. Here again this contributes to criterion (c) of Murphy et al. (2014) [differentiating subtle differences in affective states]. But more importantly this fulfills the assumption (2) of Gygax, (2014) [i.e. monotonic graded response to the probes] and therefore enables testing both, positive and negative treatments as well as biases. This gradation of the probes stayed stable throughout the four weeks of testing as was the case for the differentiation of the reference cues. This supports the above stated assumption that the output of probes was not distorted by learning due to repeated presentation. Apart from a gradation in relative latency, I found the duration of exploratory behaviour not only to show a gradient from rewarded to punished goal box location as well, but this gradation could be reproduced in the behavioural response to the ambiguous cues (gradient from near rewarded cue to near punished cue). This gradation in behaviour, here as well, was consistent over all of the four test weeks.

Up to now a rising number of studies use the cognitive bias approach to measure affective states in pigs (see Chapter 1 for an introduction of the different studies). These studies are dealing with different methodical difficulties of the cognitive bias approach. The first study of my thesis systematically eliminated sources of methodical difficulties, following the suggestions to the above mentioned criteria for affective behavioural tests (Murphy et al., 2014; Gygax, 2014). In conclusion the presented test design (Chapter 2; study 1) provides the opportunity of testing animals repeatedly for up to four weeks, with animals showing graded behavioural responses to three ambiguous probes. This standardized test design was used for the both subsequent studies (2 and 3), presenting a validation and testing applicability of the test design. Results of those will be discussed in the following chapters.

3.1.2 Study 2: Pharmacological validation of the spatial judgement bias task for pigs

According to Gygax (2014) judgement bias testing should fulfill following requirements (besides criteria discussed in chapter 3.1.1 already): (3) the behavioural response to the reference cues should not change due to manipulation of the affective state while (4) the response to the ambiguous cues should be affected.

To verify those, the first issue would be to manipulate the affective state. This implicates the major problem of finding the appropriate stimulus to change behaviour in a predictable direction. As there is no valid possibility to measure affective state yet, both the affective input of the chosen stimulus and the affective output remain interpretable. Challenges regarding the affective output were discussed in chapter 3.1.1 already (risk of misinterpretation of the observed behaviour). One prominent example for challenges of the affective input (i.e. finding the appropriate stimulus to induce a specific affective state), would be the use of food rewards. Most studies - using a reward - are working with food, as food, from an anthropomorphic view, is thought to be positively associated. This is not always the case as a study of Krause et al. (2017) found pigs to show a sympathetic activation with accompanying vagal decrease during a feeding situation which indicates an aroused state with a rather negative valence. In cognitive bias studies manipulations of the animal's external environment are commonly used to manipulate the animal's state. Here again the affective impact of treatments remain interpretable. In contrast to those external stimuli Mendl et al. (2009) proposes to use pharmacological agents to internally manipulate the animal's state, a proposal which was implemented in the presented study of my thesis.

In human studies affect-dependent shifts in cognitive performance are well reported (Blanchette and Richards, 2010). Even if the detailed mechanisms underlying these phenomena are not well studied yet, there are strong hints of the 5-HT system to be involved on different levels (see 1.3.2 for a detailed introduction of 5-HT). Therefore, the aim of study 2 was to deplete the concentration of available 5-HT in different affect regulating brain regions as a first step of validating the SJT.

5-HT depletion was achieved by using pCPA, an inhibitor of the 5-HT synthesis in the brain. According to a study in sheep (Doyle et al., 2011) this was expected

to manipulate affective state negatively. Doyle et al. (2011) could not assure this method to reliably deplete 5-HT in the brain. They expected to indirectly get access to 5-HT concentrations via monitoring cortisol profiles which are supposed to be linked to decreased serotonin in clinical depression. However, they could find no treatment effects affecting the cortisol concentration. Therefore, in my thesis the 5-HT depletion was validated beforehand. As described in the general introduction, different brain regions are integrated in the regulation of affective state, all of them interacting as a complex network. With regard to this complexity attention was laid on the overall concentration in the analysed tissues rather than on each specific tissue in detail. Indeed, results of study 2 found significant depletion of 5-HT concentration in all analysed brain regions.

Depletion of 5-HT concentration is assumed to manipulate the affective state negatively (e.g. pessimistic answer in the cognitive bias paradigm). Examples could be shown in mice (Mosienko et al., 2012), sheep (Doyle et al., 2011) and humans (Robinson et al., 2013). I therefore expected a pessimistic shift in the cognitive bias, a hypothesis which could be confirmed. Animals treated with pCPA showed a significantly longer relative latency to the middle probe, meaning their behaviour resembled the behaviour at the negative reference. Moreover, behavioural response of the animals concerning the reference cues (rewarded/punished) remained unaffected. This coincides with statement 3 and 4 of Gygax (2014) which therefore could be approved by the presented study.

The pCPA-induced 5-HT depletion in this study is an invasive treatment. Comparable studies in humans are rare, as there are concerns of pCPA being toxic (Young, 2013). Doses tested in humans therefore are quite low (for an example see Shopsin et al., 1976: 0.5-2.5g/day in humans vs. 0.05g/kg body weight in pigs in our study). However, there are examples in non-human animal studies using pCPA to manipulate 5-HT synthesis. Up to my knowledge, none of these studies report toxic effects, doses provided in study 2 therefore are derived from proposed dose levels in literature (Ettrup et al., 2011 (pigs): 0.05-0.1 g/kg; Doyle et al., 2011 (sheep): 0.04 g/kg; Tran and Keele, 2011 (rat): 0.3g/kg). Despite the low numbers of studies in humans, the method is reported to being associated with a lowering in moods (Young, 2013). Therefore, I argue this to be transferable to pigs, an assumption which could be confirmed by the pessimistic behaviour in the SJT.

Nevertheless even if finding a significant effect in the SJT, no effects could be observed in the OFNO test. Even if the interpretation of behaviour in the

OFNO is discussed controversially (see chapter 1.4.2 and 1.5.2 for more details), a manipulation like presented here (with decrease of 5-HT concentration up to 50%) was expected to alter the behaviour in the OFNO. More specifically, I expected the latency to the novel object to increase (maybe reflecting an increase in anxiety, or a loss in curiosity – both indicating an affective state with negative valence) or changes in locomotor behaviour. In the presented study, we discuss the OFNO might not be suitable to test emotional reactivity and underlying (negative) affective states in the overall experimental design used in our study. Animals participating in the study were well habituated to novel stimuli (due to training and testing in the SJT) and were familiarized with being separated from conspecifics, therefore the second testing might not reflect a novel situation. However, to correct for these influences (including time effects, age effects, etc.) differences between second and first OFNO testing were calculated and used for analysis. Still, side effects as mentioned above cannot be excluded completely.

As I found effects of 5-HT depletion in the judgment of ambiguous stimuli, this could indicate different underlying mechanisms in cognitive processing between both behavioural tests (SJT/OFNO). More specifically, whereas the SJT uses information of the negative/positive value of reference cues, which than have to be transferred to evaluate the impact of ambiguous cues I here expect a clear influence of affective state (i.e. depleted 5-HT synthesis), presented by the shift in cognitive judgement (see chapter 1.2/1.4.1 for a review). In contrast, the OFNO test rather tests the immediate reaction to a novel object or the open field, i.e. to unfamiliar, potentially dangerous stimuli. Even if this (according to appraisal theories) requires cognitive processes as well, behaviour in the OFNO might not be the appropriate parameter to detect the underlying subjective component of affective states.

Anyway, no effects could be found in alternative behaviour parameters during cognitive bias tests either. It therefore might as well be stated that decreased 5-HT concentration - even if having an impact on the cognitive process while evaluating the ambiguous probe - might cause different counterregulation processes which in consequence inhibits processes which would cause other behavioural changes. For example, there is evidence that there is an upregulation of cortical 5-HT2 receptors associated to reduced 5-HT availability in the synaptic cleft (e.g. due to impairments in the 5-HTT)[Owens and Nemeroff, 1994; Hermann, 2009]. Other examples for such feedback regulation would be findings showing that the administration of selective serotonin reuptake inhibitors (designed to increase 5-HT concentration at the synaptic terminals) also trigger the upregulation of inhibitory autoreceptors, which in consequence inhibit 5-HT neurons from firing

(Artigas et al., 1996). In antidepressant studies counterregulations are thought to reason an effect-latency in the usage of anti-depressive pharmaceutics (effect latencies of 2-3 weeks are the standard) [Ballesteros and Callado, 2004]. Behavioural changes due to the 5-HT depletion in both, SJT and OFNO, might occur with an effect latency as well. Here again, an extended test phase would be highly interesting, as I cannot exclude such delayed responses with my test design.

As already mentioned in the introduction (1.3.2) the 5-HT system is quite complex, neuromodulation itself being flexible. Therefore, further evidence of the underlying mechanisms by other methods depleting 5-HT should also be considered. One example, commonly used in humans, would be the acute tryptophan depletion (ATD). Here, a TRP reduced diet is combined with administration of a mix of large neutral amino acids, competing with TRP at the blood brain barrier (Hood et al., 2005). According to Cools et al. (2008) this method produces a mild and only transient depletion of 5-HT. Even if Young et al. (2013) states that in humans there are many conditions where ATD does not coincide with a negative affective state, this method could be interesting to test the sensitivity of the cognitive bias paradigm in pigs. Additionally, changes of mood (or a lack of any changes respectively) are not regulated by the concentration of 5-HT alone but may be due to adaptive processes of other parts in the 5-HT system. Studies concerning 5-HT receptors or the 5-HTT and 5-HT related transmitter systems like the dopaminergic system might clarify the complex interrelation of mood changes and behaviour in the cognitive bias paradigm.

3.1.3 Study 3: Application of the spatial judgement bias task

In study 3, I focused on inducing positive affective states which are of rising importance concerning animal welfare. To induce a positive affective state, here again the 5-HT concentration in the brain was manipulated. In contrast to the pharmacological manipulation as used in the second study a practice-related approach was implemented in this study by supplementing the pig´s diet with additional TRP. TRP - being the precursor of 5-HT - is an essential amino acid and has to be ingested via the diet. Unlike 5-HT it can pass the blood-brain barrier, a surplus of TRP in the diet should therefore increase 5-HT levels in the brain, which it did in our experimental approach. In human studies there is evidence showing that a surplus of 5-HT availability does enhance mood (Young, 2007). Hence, analogous to the results in study 2, we expected the increase in 5-HT concentration to be reflected in the SJT (i.e. optimistic behaviour due to a positive affective state) and the OFNO (e.g. increased time spent exploring, changes in locomotor behaviour). However, results did not confirm these expectations in pigs as the animals did not show a primarily optimistic answer in the cognitive bias and no behavioural changes could be detected.

However, here again the 5-HT manipulation was validated beforehand by measuring 5-HT concentration in cognitive-affective relevant brain areas (see. Chapter 1.3.1). Results approved the hypothesis that TRP supplementation in the diet would increase 5-HT concentrations in the brain and therefore provide a physiological basis for positive affective states. As the SJT did not result in an optimistic behaviour, I here again refer to criterion (c) of Murphy et al. (2014) as already mentioned above (3.1.1). The presented study could not provide evidence for the assumption of the SJT being capable to detect subtle differences already. Even if preconditions were met (e.g. graded response to ambiguous cues) the thesis could not provide evidence of the sensitivity of the test design to the positive extreme in the valence dimension. It remains open if this is due to the test design itself or rather due to failures in regards to induce positive affective state.

Other than in study 2 differences between TRP supplemented group and control group were less pronounced, even if being statistically significant. Whereas pCPA treatment could decrease 5-HT concentrations in the amygdala and the hypothalamus to 35% and 45% respectively, TRP supplementation only achieved an increase of 18% and 15%. As already reviewed in the introduction (1.3.1) both

amygdala and hypothalamus are highly interconnected structures, regulating the output to autonomic, endocrine and behavioural processes. The achieved increase in 5-HT concentration therefore might not be sufficient enough to trigger the expected change in behaviour in both, OFNO and SJT, or to induce a positive affective state at all. In the hippocampus no significant difference in 5-HT concentrations between treatment groups could be detected at all (increase of 19%) whereas study 2 could provide a significant decrease (48%). As the hippocampus is highly relevant in affective disorders, here again the increase of 5-HT concentrations might be insufficient to induce changes in the behavioural response.

In contrast to above made explanations, results of study 3 could show a behavioural shift in behaviour, but, contrarily to the hypothesis, TRP supplemented animals reacted more pessimistically on one of the ambivalent test positions. This rather indicates other factors being relevant for interpretation of the results. However, as treatment groups (control/TRP+-diet) differed significantly in their behavioural reaction in cognitive bias before diet already (e.g. TRP+ group showed more optimistic behaviour), results of the presented study have to be considered with caution. Nevertheless, results do raise some interesting issues which may play a superior role in designing cognitive bias studies.

Especially for group housed animals, group effects might strongly influence results. In this study we decided not to separate the animals for feeding as social isolation is known to be a stressor to pigs (Herskin and Jensen, 2000). This could possibly influence the cognitive bias, resulting in pessimistic behaviour. As our technical supply did not allow an individual-specific diet per animal, we allocated the supplements to one of the two housing groups/replicate and animals were fed in the group. Group composition itself was balanced carefully: Four piglets per litter were chosen randomly out of five litters, two siblings of each litter were assembled to either the control or the TRP+-group. Therefore genetical influences were balanced over groups, even if 5-HT-associated allelic variation comparable to human findings (Caspi et al., 2003) [not studied in pigs yet] cannot be entirely excluded. However, the environment, the management and the general treatment remained the same for all animals. Therefore, environmental influences are most likely to be excluded. Effects that could not be excluded in the presented study are intra-group effects. Group effects are quite complex, depending on different dominance structures, communication capabilities and the group composition. Treatment groups in the presented study differed significantly in their behavioural response in cognitive bias even before the dietary change, so an induced group effect due to the treatment seems to be unlikely. However, there

are strong indications that pigs are capable of empathy, or at least emotional contagion, which would be shown in a study by Reimert et al. (2013). They trained pigs to anticipate a rewarding or an aversive event following a combination of auditory and visual cues. In a test situation, they found that naive pigs show the same behavioural indicators as their trained pen mates when those were confronted with these conditioned cues. Groups in the presented study were compiled directly after weaning and remained stable throughout the whole experimental setup, potential effects might have developed at that stage already. Weaning is known to be a severe stressor to pigs (Moeser et al., 2007). As previously shown by Ruis et al. (2001), there are individual differences in the capability of coping with stress. More precisely, authors found significant differences in cortisol response, body temperature and behaviour between animals which were classified as 'high reactive' or 'low reactive' using the backtest, a behavioural test identifying coping styles in pigs. Comparable results (i.e. differences between coping types) could be found for cardiovascular parameters and sympathetic/parasympathetic activation as Krause et al. (2007) could show. Therefore, assuming emotional contagion influencing the affective state of the whole group, an imbalance in low reactive and high reactive animals might cause such unexpected group effects. Additionally, optimism or pessimism themselves might display stable personality traits although further studies are needed in order to confirm this assumption. If so, emotional contagion might play a role in giving an overestimation of optimistic or pessimistic animals changing the affective state of the whole group here as well. A bigger sample size could minimize such subtle influences, as would be balancing the treatment over groups, as done in study 2. Additionally these results emphasize the importance of studies, giving evidence of optimism/pessimism to be basic personality traits.

Disregarding differences in affective state between treatment groups before treatment, study 3 found a significant change in behaviour of the TRP+-group after dietary change. Unexpectedly, animals displayed more pessimistic behaviour to the ambivalent stimulus in the SJT. There may be different reasons for this as discussed above (3.1.2; e.g. counter-regulation by other pathways). Referring to the regulation of positive affective states, the dopamine pathway plays a major role (Burgdorf and Panksepp, 2006; Sharot et al., 2012). Hashiguti et al. (1993) found 5-HT and dopamine to interact, more precise they found that intraperitoneal injections of 5-hydroxytryptophan (5-HTP) increased 5-HTP, but decreased L-3,4-dihydroxyphenylalanine (L-DOPA) in a dose-dependent manner. A study by Rygula et al. (2015b) manipulated the affective state pharmacologically to induce an optimistic cognitive bias in rats. They could show the manipulation

of both, 5-HT and dopamine system enhancing optimistic behaviour – further studies highlighting dopamine-5-HT interactions might give evidence of mechanisms regulating positive affective state.

Much has to be done in order to clarify the mechanisms behind positive emotions, as studies concentrating on negative affective state/pessimism are overbalanced in scientific literature. From an evolutionary view this seems to be rational, as negative affective states might be far more important. As Fredrickson (1998) states, costs due to failures responding to a life threat might carry more weight than failures responding to an opportunity for a positive event. As known from human psychology studies, there seems to be an asymmetry between positive and negative evaluation, a concept which is called "negativity effect" (Peeters and Czapinski, 1990). Physiological mechanisms enhancing negative affective states might therefore be more pronounced than mechanisms regarding positive affective states.

However, as Boissy et al. (2007) state: "…good welfare is not simply the absence of negative experiences, but rather is primarily the presence of positive experiences such as pleasure.", therefore studying positive affective states remains an important field of science.

Even if some of the results were unexpected, the presented study therefore contributes to a better understanding of positive affective states as our results contradict the hypothesis that TRP supplementation via the diet can induce positive affective states in pigs. However, further studies and other methods to induce positive affective states (e.g. pharmacologically, by environmental enrichment etc.) are required to study the applicability of the cognitive bias paradigm here.

3.2 Implications

The theory behind the cognitive (judgement) bias paradigm is simple – the outcome of an ambiguous situation is judged pessimistically when being in a negative affective state while the expectations in the same situation would be rather optimistic when being in a positive affective state. However, as mentioned before, the practical implementation has its pitfalls and limits: therefore cognitive bias tests have to be designed deliberately. Study 1 of my thesis presents a standardized test design which is well suited for testing cognitive bias in pigs (repeatedly) and is validated in study 2 which demonstrates its sensitivity to detect a negative affective state after 5-HT depletion via pCPA. Other than expected, study 3 could not detect optimistic behaviour in the SJT after increasing the 5-HT concentration in the brain but rather indicated a pessimistic behavioural response. Therefore, further studies are needed to (a) reveal underlying mechanisms of negative affective states and (b) to determine the sensitivity of the SJT to detect positive affective states likewise.

Finding valid indicators of animal welfare is a central goal of modern animal science. Puppe et al. (2012) introduced a definition comprising the different aspects relevant for the assessment of animal welfare, defining welfare as "the state of physical and mental health resulting from the process of behavioural and physiological adaptation when coping with environmental challenges, and the associated subjective experiences and emotional evaluation, all in the light of individual and/or cognitive needs and abilities". This clearly includes that indicators measuring good welfare should not stop at measures of "health" or "species specific normal behaviour" but should implicate the component of the affective state and more specifically the subjective evaluation of these intrinsic mechanisms (also see Dawkins, 1990; Mendl, 2001; Mendl and Paul, 2004; Boissy et al., 2007; also see Dawkins, 2017 for a controversial view). Referring to the statement of Boissy et al. (2007) mentioned above, this also implicates the relevance of positive experiences. However, study 3, increasing the 5-HT concentration in order to induce a positive affective state, already hints at the complexity of finding a practical and simple way to induce positive affective states in modern pig husbandry (i.e. via food additives). Therefore, further studies validating the cognitive bias approach for its sensitivity to detect positive affective states (e.g. pharmacologically) are required. There are only a few studies manipulating the affective state positively, most use external manipulations like for example environmental enrichment (Brydges et al., 2011; Richter et al., 2012; Bethell and Koyama, 2015). To my knowledge, the study of Douglas et al. (2012)

is the only study measuring optimistic cognitive bias in pigs (apart from the presented study in my thesis). Here again authors use environmental enrichment and therefore an external manipulation. They could find an optimistic cognitive bias, but due to the methodical difficulties already mentioned in the introduction (part 1.4.1), results remain doubtful. However, enrichment, e.g. cognitive enrichment (Puppe et al., 2007; Zebunke et al., 2013) or structuring of the housing pens (Fraser et al., 1986; Simonsen, 1990; van de Weerd and Day, 2009) was found to improve welfare. Studies revealed changes in behaviour, as measured in behavioural tests like the novel object test, preference tests, or in physiological parameters like for example the autonomic control. Therefore, it would be interesting to see whether those treatments also induce a shift in the cognitive bias and hence give evidence of the affective (subjective) component.

However, the results presented in this thesis also reflect the complex mechanisms and again confirm flexibility and plasticity to be major features of affective states. Whereas in study 2 a decrease of 5-HT resulted in a pessimistic behaviour in the SJT, the reverse treatment (increase of 5-HT) did not trigger an analogous reverse answer in the cognitive bias task (optimistic answer) but also resulted in pessimism. Furthermore, behaviour in the OFNO did not reflect results found in the SJT, indicating divergent basal mechanisms for both behavioural tests. Further studies combining different components of affective states analogous to the studies presented in my thesis (e.g. SJT=affective cognitive component; OFNO=behavioural component; 5-HT concentration in the brain= neurophysiological component) would be beneficial to get a better impression of the big picture.

3.2.1 Applicability of the test design

The main focus of this thesis was to find a suitable and valid test design testing cognitive bias in pigs. However, the superior goal in cognitive bias studies per se is to find reliable and valid indicators of affective states in order to provide and to judge animal welfare. The cognitive bias design as presented in my thesis is not (yet) suitable to judge animal welfare on farm. Even if the proposed test design tried to optimize the required time needed for training, the SJT still is time-consuming and requires space for an experimental arena. Considering these practical issues and including the numerous side effects influencing results, the test design is restricted to experimental conditions at this stage.

One last criterion of a suitable behavioural test is its possibility to be automated (Murphy et al., 2014; see Introduction 1.5.2). Automatization of the cognitive bias approach would be highly advantageous. It would not only eliminate practical issues as mentioned above but gives the opportunity to measure behaviour in the familiar environment of the animals without disturbance by a human observer or a test situation (van der Staay et al., 2009). Even if this will not be relevant in pig production from an economically view directly, an automated system could be used to test animals under practical relevant conditions. It then might be used to either validate other ("easy to apply") indicators of animal welfare (Hintze et al., 2017) or to reliably test different conditions (e.g. environmental enrichment) regarding their positive/negative impact. Therefore, working at the interface between fundamental and applied animal welfare research, the cognitive bias test might influence animal welfare in production systems indirectly.

There are first attempts to modify the cognitive bias task to be automated. In starlings, Brilot et al. (2009) suggests an interesting approach by using responses to eyespot stimuli that are naturally aversive in many bird species. This would enable the relinquishment of associative training and therefore would hold the opportunity to use the approach in field. However, the study revealed no effect of the treatment (auditory cues which were supposed to generate a state of anxiety) on the behavioural response in the cognitive bias task presented.

In laboratory rodents the group around Mendl currently develops and validates an automated test system (Mendl, National Centre for the Replacement, Refinement and Reduction of Animals in Research).

However, up to now, no automated test paradigm is available for pigs yet. Pigs have variable cognitive availabilities. An approach by Ernst et al. (2005) revealed

their capability in learning complex tasks in their home environment. In their study, they developed an automated feeding system (call-feeding-station) for pigs. Within the study animals had to discriminate and recognize an individual acoustic signal to localize and approach a particular feeding station to access a food reward. It is conceivable that a comparable learning paradigm might replace the time consuming learning phase of the SJT.

3.3 Conclusion

Understanding cognitive-affective appraisal processes (represented by the SJT) is of elemental interest, not only for fundamental research but for applied science as well. They provide the potential to judge the affective state as one essential part of animal welfare objectively. However, these processes are complex and flexible. They are not only originating in the brain, which itself is characterised by highly connected structures, circuits and networks, but its phenotype is multifaceted, comprising physiological, behavioural and subjective components. The presented thesis therefore not only presents a standardized, validated test design, but addresses the above mentioned complexity by integrating different components of affective states, represented by measurements of 5-HT in the brain (neurophysiological), behavioural tests (OFNO/SJT) and the cognitive-affective appraisal in the SJT (subjective).

Affective states are a relevant part of animal welfare and studying these affective states offers a broad range of unsolved questions. These concern the underlying mechanisms, the processing, the perception and the feedback to cognition and behaviour. The judgement bias test offers a promising tool to provide answers in this complex field. Therefore, it is of significant importance to both current and future animal welfare studies as knowledge on animals' affective abilities may reveal new perspectives to improve and to judge husbandry systems. Finally, awareness of affective (subjective) abilities may shift the current perception of farm animals from being simple products to being complex individuals, with each having their own individual needs.

Summary

Modern definitions of animal welfare (in farm animals) explicitly underline the relevance of affective states. The focus of the presented thesis is the cognitive bias, an approach originally developed in human psychiatric research and more recently used in various species (farm) to measure the valence of affective states based on optimistic (positive valence) or pessimistic (negative valence) evaluations of ambivalent stimuli. The goal of the presented studies was to establish a cognitive bias paradigm for pigs (*Sus scrofa domestica*). To reach this aim a test design - originally developed in the group of Behavioural Ethology of the Leibniz Institute of Farm Animal Biology (Düpjan et al., 2013) - was modified to enable repeated testing and graded responses on ambiguous stimuli. This modified test paradigm (SJT) was used to study the physiological base of cognitive-affective evaluation and to validate the test design. Focus was laid on the serotonin (5-HT) system, testing two main approaches. First the 5-HT availability was decreased by depleting the 5-HT synthesis and secondly the 5-HT availability was increased supplementing tryptophan (TRP) via the diet.

The thesis is divided into three chapters.

Chapter I provides a general introduction. Here, the relevance of affective states according the evaluation of animal welfare is illustrated and different definitions are introduced. The principle of the cognitive bias is explained, the role of the 5-HT system is described and different brain areas relevant for affective-cognitive processing are presented.

The goals of the thesis were defined and the background of the studies, presented in this thesis, is specifically highlighted and credited. Chapter I proposes five hypotheses concerning the three studies presented:

- The modified design, training pigs in a spatial go/no-go task using a reward as positive and a mild punishment as negative reinforcer and additionally introducing a partial reinforcement to the training process, enables repeated testing of the ambivalent probes and induces graded responses to graded probes.
- Depletion of the 5-HT synthesis decreases the 5-HT concentration in brain areas relevant for cognitive-affective processes whereas the supplementation of TRP increases 5-HT concentration respectively.

- Pharmacologically induced 5-HT depletion results in a pessimistic evaluation of the ambivalent probes in the SJT, indicating a negative affective state.
- A surplus of 5-HT acquired by dietary TRP supplementation results in an optimistic evaluation in the judgement bias task.
- Both manipulations of the 5-HT system (5-HT depletion, TRP supplementation) were expected to alter behaviour in the SJT and an open field/novel object test.

Chapter II comprises the studies published as part of this thesis.

Study 1 - "*An improved design for the spatial judgement task in domestic pigs*" was published in the journal *Applied Animal Behaviour Science*. The study proposes a modified test design in order to test pigs repeatedly and induce graded responses to graded probes. A total of 16 female juvenile German Landrace pigs were tested in a spatial go/no-go paradigm to discriminate a positive associated position of a goal box (reward) from a negative associated position (mild punishment) and to respond with a specific behavioural reaction (go/no-go). In addition, partial reinforcement was introduced. Afterwards the behavioural reaction of the animals to three intermediate, unreinforced goal box positions was tested by presenting each of three test positions eight times in a four-week period. Relative latency till opening the goal box and different behavioural parameters (e.g. exploration, locomotion) were analysed. Animals learned to discriminate between the two reference locations and showed graded responses (latency to open the goal box) to the probes. The subjects showed no signs of learning with respect to the outcome of probes but exhibited stable response levels during test weeks two to four. In conclusion, the design presented in this paper is suitable for spatial judgement tasks in domestic pigs and therefore forms the basis for further studies concerning affective states in pigs. This is fundamental in studies 2 and 3 of this thesis.

Study 2 – "*Serotonin depletion induces pessimistic-like behaviour in a cognitive bias paradigm in pigs*" was published in *Physiology and Behaviour*. Goal of this study was to contribute to the clarification of mechanisms underlying affective regulation and to validate the test design presented in study 1. Focus was laid to the 5-HT-system by depleting 5-HT concentration pharmacologically. In a first part, 40 pigs were treated with either para-Chlorophenylalanine (which inhibits the 5-HT-synthesis; pCPA) or saline for six consecutive days. 5-HT levels were assessed in seven

different brain regions. Results found 5-HT to be significantly depleted in all analysed brain regions. In a second part, the consequences of serotonin depletion were measured in two behavioural tests: the spatial judgement task (SJT; as established in study 1), and the open field/novel object (OFNO) test. The pCPA model (as described above) was applied to 48 animals, conducting both behavioural tests before and after pCPA/saline injections. While results from the OFNO tests were inconclusive, an effect of treatment as well as an effect of the phase (before and after treatment) was observed in the SJT. Animals treated with pCPA showed more pessimistic-like behaviour, suggesting a more negative affective state due to 5-HT depletion. Thus, our results validate the test design and confirm that 5-HT system is a key player in cognitive-emotional processing.

Study 3 - "*Dietary tryptophan supplementation and affective state in pigs*" was published in the *Journal of Veterinary Behaviour: Clinical applications and Research*. The aim of this study was to investigate the effects of dietary TRP supplementation on brain, TRP metabolism and the influence on the affective state in pigs. All subjects were fed a standard diet until eight weeks of age. Then, the feed was changed for all animals with half the animals (control) receiving a diet with the recommended TRP content (2.5 g/kg), while the other half (TRP+) received a TRP enriched diet (10.2 g/kg). In part 1, 20 animals were tested, ten receiving the control diet while ten were receiving the TRP-enriched diet. Results found significantly increased concentrations of TRP and its metabolites in nearly all analysed brain tissues for the TRP-enriched diet group. In part 2 the effects of these alterations on the affective state were measured in the SJT and the OFNO test. Whereas the TRP enrichment revealed no significant behavioural changes in the OFNO tests, in the SJT, the TRP+-group showed more pessimistic behaviour after dietary change than before.

Chapter III comprises a comprehensive discussion of the results. After discussing the results of the three studies in specific detail, a general discussion of the cognitive bias is provided and set in relation to current scientific research. Finally, chapter III provides the conclusion of the thesis.

Therefore the thesis presents a judgement bias test suitable for testing cognitive bias in pigs repeatedly (up to four weeks) and which meets a majority of assumptions required for testing affective states. The test design is sensitive to detect a negative affective state following depletion of the serotonin concentration in the brain whereas animals, contrary to expectations, revealed a negative evaluation of ambivalent stimuli after dietary tryptophan supplementation. Altogether the judgement bias test offers a promising tool to provide answers in

the complex field of affective states, which is of significant importance to both current and future animal welfare studies.

Zusammenfassung

Aktuelle Definitionen von „Animal Welfare“ bei Nutztieren stellen insbesondere die Bedeutung affektiver Zustände in den Vordergrund. Im Mittelpunkt der vorliegenden Dissertation steht der „Cognitive Bias“, ein ursprünglich aus der Humanpsychologie stammender Ansatz, welcher aktuell in zahlreichen Studien an (Nutz-) Tieren eingesetzt wird, um die Valenz affektiver Zustände anhand optimistischer (positive Valenz) oder pessimistischer (negative Valenz) Bewertungen ambivalenter Reize zu messen. Ziel der hier präsentierten Arbeit war es, einen solchen Test für die Nutztierart Schwein (*Sus scrofa domestica*) zu modifizieren und im Hinblick auf die Regulation affektiven Verhaltens zu validieren. Ein in der Arbeitsgruppe Nutztierethologie des Leibniz Institut für Nutztierbiologie entwickelter Versuchsansatz wurde modifiziert, um ein zeitlich stabiles Reaktionsmuster der Tiere über mehrere Testwiederholungen darstellen zu können. Zusätzlich sollte eine graduell abgestufte Verhaltensantwort auf drei ambivalente Testreize erzielt werden.

Um sowohl physiologische Grundlagen kognitiv-affektiver Bewertungsmechanismen zu klären, als auch die Methodik des modifizierten Versuchsansatzes zu validieren, wurde am Serotonin (5-HT)-system angesetzt. Vorgestellt werden hier zwei Versuchsansätze: Zum einen wurde die 5-HT-Synthese pharmakologisch blockiert, zum anderen wurde die 5-HT-Verfügbarkeit im Gehirn über eine Tryptophan (TRP)-Supplementierung deutlich erhöht.

Die Dissertation ist aufgeteilt in drei Kapitel:

Kapitel I beinhaltet eine generelle Einführung in die Thematik. Hier wird zunächst die Bedeutung affektiver Zustände im Hinblick auf „Animal Welfare“ erläutert und unterschiedliche Definitionsansätze vorgestellt. Weiterhin wird das Prinzip des Cognitive Bias erläutert, die Rolle des Serotoninsystems wird beschrieben und ausgewählte Gehirnareale, welche eine Rolle bei der Verarbeitung affektiv-kognitiver Prozesse spielen, werden vorgestellt. Die Zielsetzungen des Dissertationsprojektes werden dargestellt und die Hintergründe der im Rahmen dieser These durchgeführten Studien werden im Speziellen eingeführt. Außerdem werden Hypothesen entwickelt, welche sich wie folgt darstellen:

Das modifizierte Design, (SJT) welches sowohl eine Belohnung als positive und eine milde Bestrafung als negative Referenz nutzt, als auch das Prinzip des ‚partial

reinforcement' einführt, erlaubt wiederholtes Testen ambivalenter Testpositionen in einem räumlichen go/no-go Paradigma bei Schweinen und induziert graduell abgestufte Verhaltensreaktionen.

Eine pharmakologische 5-HT-Depletion reduziert die 5-HT-Konzentration in ausgewählten Gehirnarealen, während eine TRP-Supplementierung im Futter diese erhöht.

Die pharmakologische Blockade der 5-HT Synthese führt zu einer pessimistischen Bewertung ambivalenter Testreize im SJT.

Eine deutliche Erhöhung der 5-HT-Verfügbarkeit über eine TRP-Supplementierung im Futter führt zu einer optimistischen Bewertung ambivalenter Reize.

Bei beiden Manipulationen des 5-HT-Systems wurde eine Verhaltensänderung sowohl im SJT als auch in einem open field/ novel object Test (OFNO) erwartet.

Kapitel II beinhaltet die im Rahmen dieser Dissertation veröffentlichten Studien.

Studie 1 ("*An improved design for the spatial judgement task in domestic pigs*") wurde in der Zeitschrift *Applied Animal Behaviour Science* veröffentlicht. In dieser Studie wird ein modifiziertes Testdesign vorgestellt, welches es ermöglicht den ‚Judgement Bias' von Schweinen über einen Zeitraum von vier Wochen wiederholt zu testen. Dazu wurden 16 weibliche juvenile Schweine in einem räumlichen go/no-go Paradigma trainiert, eine positiv assoziierte Position einer Zielbox (Belohnung) von einer negativ assoziierten Position (milde Strafe) zu unterscheiden und mit einer spezifischen Verhaltensantwort zu belegen (go/no-go). Zusätzlich wurde im Training das Prinzip der partiellen Verstärkung angewandt. Anschließend wurde die Verhaltensantwort der Tiere auf drei intermediäre, unverstärkte Positionen getestet, wobei jede Testposition in einem Zeitraum von vier Wochen achtmal präsentiert wurde. Als Zielvariablen wurden die Latenzzeit bis zum Öffnen der Zielbox, sowie zusätzliche Verhaltensparameter (z.B. Erkundungsverhalten, Lokomotion etc.) erfasst. Ergebnisse zeigten, dass die Tiere nach einer siebentägigen Trainingsphase die positiv bzw. negativ assoziierten Reize sicher voneinander unterscheiden konnten. In der Testphase stellte sich die Reaktion der Tiere (relative Latenz bis zum Öffnen der Zielbox) auf die intermediären Positionen als differenziert und graduell abgestuft dar, wobei diese Abstufung über die Testwiederholungen stabil blieb. Die Methodik ermöglicht somit wiederholtes Testen und schafft damit die Basis für weiterführende Studien zur

Beurteilung affektiven Verhaltens beim Hausschwein, als auch die Basis für die in Studie 2 und 3 vorgestellten Versuchsansätze.

Studie 2 („*Serotonin depletion induces pessimistic-like behaviour in a cognitive bias paradigm in pigs*") wurde in der Zeitschrift *Physiology and Behaviour* veröffentlicht. Ziel dieser Studie war es zugrunde liegende Mechanismen der Regulation affektiven Verhaltens aufzuklären, sowie die in Studie 1 vorgestellte Methodik pharmakologisch zu validieren. Dazu setzt die vorgestellte Studie am 5-HT-System an, wobei durch pharmakologische Manipulation der 5-HT-Synthese die 5-HT-Verfügbarkeit im Gehirn signifikant reduziert wurde. Im ersten Versuchsteil der Studie konnte dies experimentell nachgewiesen werden: Hier wurden 20 Tieren über einen Zeitraum von sechs aufeinanderfolgenden Tagen para-Chloropenylalanin (pCPA; Inhibitor der Tryptophanhydroxylase, eines essentiellen Enzyms der 5-HT-Synthese) injiziert, 20 Tiere wurden mit einer Kochsalzlösung behandelt (Kontrolle). Anschließend wurde die 5-HT-Konzentration in sieben verschiedenen Gehirnarealen untersucht, welche maßgeblich an der Regulation affektiv-kognitiven Verhaltens beteiligt sind. Ergebnisse ergaben eine signifikante 5-HT-Depletion bei den pCPA-behandelten Tieren. Die Auswirkungen einer solchen 5-HT-Depletion auf das affektive Verhalten wurde anschließend an 48 Tieren in jeweils zwei Verhaltenstests getestet: dem in Studie 1 etablierten „spatial judgement test" (SJT) sowie einem „open field/novel object"-Test (OFNO). Die Ergebnisse im OFNO-Test waren nicht eindeutig, Ergebnisse im SJT konnten dagegen zeigen, dass Tiere nach einer 5-HT-Depletion signifikant pessimistischeres Verhalten aufwiesen als vor der Behandlung, ein Effekt, der sich bei den Kontrolltieren nicht fand. Die vorgestellte Studie identifiziert das 5-HT-System somit als einen Schlüsselfaktor, sowohl in der Regulation affektiven Verhaltens, als auch dessen Projektion im SJT.

Studie 3 ("*Dietary tryptophan supplementation and affective state in pigs*") wurde in der Zeitschrift *Journal of Veterinary Behaviour: Clinical applications and Research* veröffentlicht. In dieser Studie wurde untersucht, inwiefern eine TRP-Supplementierung über die Nahrung die 5-HT-Konzentration und in Folge den affektiven Zustand von Schweinen positiv beeinflussen kann. Hierbei wurden Schweine zunächst mit einer Standarddiät gefüttert. Ab dem 56. Lebenstag fand eine Futterumstellung statt, die Kontrollgruppe erhielt eine Diät mit der üblicherweise empfohlenen TRP-Konzentration (2,5 g/kg), die Versuchsgruppe erhielt eine Diät mit deutlich erhöhten TRP-Konzentrationen (10,2 g/kg). Auch hier war die Studie zweigeteilt. In einem ersten Versuchsansatz wurden je zehn Tiere mit der TRP-angereicherten Diät gefüttert, zehn Tiere bekamen dagegen die

Kontrolldiät. Anschließend wurden sowohl 5-HT- und TRP-Konzentrationen als auch die Konzentrationen unterschiedlicher TRP-Metaboliten in sieben verschiedenen Gehirnregionen gemessen. Ergebnisse zeigten signifikante erhöhte Konzentrationen von TRP, 5-HT als auch anderen Metaboliten bei der TRP-angereicherten Futtergruppe. Die Auswirkungen einer solchen TRP-Supplementierung auf das affektive Verhalten wurden im zweiten Versuchsteil sowohl im SJT als auch im OFNO getestet. Die Behandlung ergab keine signifikanten Verhaltensänderungen im OFNO. Entgegen den Erwartungen führte die TRP-Supplementierung nicht zu einer positiveren Bewertung ambivalenter Reize im SJT, die Ergebnisse wiesen sogar auf eine signifikant pessimistische Verhaltensantwort der Tiere hin.

Kapitel III enthält eine übergreifende Diskussion der Ergebnisse. Nach einer Diskussion der Ergebnisse der drei vorgestellten Studien im Speziellen, wird die Anwendung des Cognitive Bias zur Erfassung affektiver Zustände im Allgemeinen diskutiert und in Relation zu aktuellen wissenschaftlichen Erkenntnissen gesetzt. Abschließend enthält Kapitel III das Fazit der Dissertation.

In dieser Dissertation wird ein ‚Judgement Bias' Test präsentiert, welcher es ermöglicht, den Cognitive Bias von Schweinen wiederholt (in einer Testphase von vier Wochen) zu testen. Das Testdesign erlaubt die Detektion von negativen affektiven Zuständen, ausgelöst durch eine Serotonindepletion im Gehirn, wohingegen die Tiere nach einer Tryptophan Supplementierung über die Nahrung, entgegen den Erwartungen, eine pessimistische Evaluation der Testreize aufwiesen. Um die Haltung von Schweinen zu verbessern, sind zuverlässige Methoden zur Bewertung des Wohlbefindens unumgänglich. Die hier vorgestellte Dissertation leistet einen wichtigen Beitrag zur Bewertung affektiver Zustände welche einen essentiellen Aspekt bei der Beurteilung von Wohlbefinden darstellen.

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Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorgelegte Arbeit selbstständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Curriculum Vitae

Jenny Stracke

Date of birth: 29.03.1981

Place of birth: Chemnitz / Karl-Marx-Stadt, Germany

Nationality: German

Education

PHD student in Behavioural Physiology

Since 2013 Institute of Behavioural Physiology; Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany

Project: Measuring cognitive-affective appraisal processes in pigs.

Supervisor: Prof. Dr. Birger Puppe, Dr. Sandra Düpjan

M.Sc. in Neurobiology and Behaviour

2008 - 2010 Freie Universität Berlin, Germany

Project: Assessment of stress in laboratory beagle dogs constrained by a Pavlov sling.

Supervisor: Prof. Dr. Heidrun Fink

B.Sc. in Biology

2005 - 2008 Leibniz Universität Hannover, Germany

Project: Messung der Stressbelastung von Junghengsten beim Dülmener Wildpferdefang

Supervisor: Dr. Willa Bohnet

Qualification for University (Abitur)

2000 Schiller Gymnasium Hameln, Germany

Academic Positions

2013 – 2016	Scientific Assistent, Institute of Behavioural Physiology, Institute for Farm Animal Biology, Dummerstorf, Germany
Since 2016	Scientific Assistent, Institute for Animal Hygiene, Animal Welfare and Farm Animal Behaviour, Hannover, Germany

Further Education

2017	Workshop: Sensible statistics in animal welfare research; WAFL, 2017; ReeHorst, Ede, Netherlands
2016	Workshop: Statistical Analysis in R, Veterinary University Hannover, Germany
2016	Workshop: DFG-Proposal-Processes, Hannover, Germany
2016	Workshop: Project-Management, Hannover, Germany
2014	Workshop: Statistics with SAS, Institute for Farm Animal Biology, Dummerstorf, Germany
2014	Workshop: Graduiertenkurs Ethology, University Hohenheim, Germany
2013	Workshop: Scientific Writing: Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany
2011 – 2013	Research Project: Paternal Investment of Males in a semi-feral population of horses, Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany
2011	Internship, Animal Sanctuary, Animal Welfare Consulter, Berlin, Germany
2010 – 2013	Correspondence course; Degree “Physiotherapy for Horses and dogs”, IfT (Institut für Tierheilkunde), Berlin, Germany
2010 – 2011	Internship, Institute for Animal Welfare, Behaviour, Animal Hygiene and Animal Husbandry, Veterinary University Munich

2005	Internship, Veterinary practice, Dr. Pox, Hannover, Germany
2005	Internship Sheep Farm, Moorhausen, Elsfleth, Germany
2002 – 2005	Internship and professional education „Pferdewirt, Schwerpunkt Reiten“ , Reiterhof Stuft and Ausbildungsstall Schulze-Wierlin, Münster, Germany
1997	Internship, Unit of Reproductive Medicine of the clinics, University of Veterinary Medicine, Hannover, Germany
1996	Internship, Veterinary practice, Dr. Herm-Meyer, Bad Münder, Germany

Academic and non-academic Working Experience

2012	Lecturer ATN (Akademie für Tier- und Naturheilkunde)
Since 2011	Freelancer, Physiotherapy and Behavioural Therapy for Horses and Dogs
2011 – 2012	Dog training, Behavioural Therapy, Anubys Hundeverhaltenszentrum, Berlin
2011	Freelancer at Freie Universität Berlin
Since 2005	Freelancer, Training and Riding of Horses, Riding Instructor
2001 – 2002	Professional Rider, Stud Kate Beaumont, Kill Co. Kildare, Ireland
2001	Professional Rider, Cork, Ireland

List of Publications

Peer-reviewed journal articles

Stracke, J., Otten, W., Tuchscherer, A., Witthahn, M., Metges, C.C., Puppe, B., Düpjan, S. 2017. Dietary tryptophan supplementation and affective state in pigs. Journal of Veterinary Behaviour 20:82-90.

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Tierhaltung, KTBL-Schrift 511; 48. Internationale Arbeitstagung Angewandte Ethologie bei Nutztieren der Deutschen Veterinärmedizinischen Gesellschaft e.V.

Stracke, J., Otten, W., Tuchscherer, A., Puppe, B., Düpjan, S. 2016. Serotonin depletion in a cognitive bias paradigm in pigs. Oral presentation, Proceedings of the 50th Congress of the International Society for Applied Ethology, Edinburgh, United Kingdom

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Stracke, J., Düpjan, S., Tuchscherer, A., Puppe, B. 2014. Wiederholtes Testen in einem cognitive bias Paradigma beim Hausschwein. Oral presentation, Aktuelle Arbeiten zur artgemäßen Tierhaltung, KTBL-Schrift 505, 46. Internationale Arbeitstagung Angewandte Ethologie bei Nutztieren der Deutschen Veterinärmedizinischen Gesellschaft e.V.

Stracke, J., Berger, A. 2012. Project on paternal effects to the cognitive and social development of the offspring on feral horses (Equus caballus). Poster presentation, International Equine Science Meeting, Regensburg, Germany.