The cognitive bias test as a measure of emotional state in pigs

Tesis doctoral presentada per RICARD CARRERAS UBACH

Ricard Carreras Ubach

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FACULTAT DE VETERINÀRIA

EVA MAINAU BRUNSÓ, Investigadora del Departament de Ciència Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona (com a directora),

XAVIER MANTECA VILANOVA, Catedràtic del Departament de Ciència Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona (com a director i tutor acadèmic) i

ANTONIO VELARDE CALVO, Director del Subprograma de Benestar Animal de l'Institut de Recerca i Tecnologia Agroalimentàries (com a director)

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Dra. Eva Mainau Brunsó Dr. Xavier Manteca Vilanova Dr. Antonio Velarde Calvo

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Abstract

The assessment of animal emotions is a crucial goal in the study of animal welfare science. Therefore several physiological and behavioural measures have been proposed and although they offer a great deal of information its interpretation is often controversial and they are mainly focused to assess negative emotional states. The cognitive bias (CB) test has been proposed as a measure to overcome some of these issues, since is presumed to assess the valence (positive vs. negative) and the intensity of the emotion. The term CB has been used as a general label for the effects of the emotional state on the cognitive processes, including memory, attention, learning and decision making or judgment. Indeed, most of the studies assessing CB in animals use the judgment bias, which is based on the premise that subjects with negative emotional state will judge an ambiguous stimulus more negatively than subjects with positive emotional state. A total of four experiments were carried out, involving between 36 and 56 pigs each. Pigs were crosses of Large White × Landrace with Piétrain and aged between two and six months. The aims of our first study were to assess the applicability and the consistency of the CB test in pigs and the proposal of an individual CB classification. Our results showed that pigs were able to learn the spatial discrimination task (associate a bucket in on side of the test pen with food as a positive reinforcer and a bucket in the other side of the pen without food as a negative reinforcer) necessary to subsequently perform the CB test (CBT) by assessing the animal response in front of an ambiguous stimulus (a bucket in a intermediate position between the two previous and with a small amount of food). However, there was lack of consistency between the response of the CBT performed twice, leaving 5 weeks between them. This result suggests that pigs changed the perception (or judgement) of the ambiguous stimulus due to its ability to remember the outcome of the ambiguous stimulus during the second CBT or due to uncontrolled factors such as their age or hunger state over time. Moreover, most of the pigs were classified with a positive CB, suggesting that the design of the CBT induced the pigs to respond more positively to the ambiguous stimulus or pigs tended to be optimistic as an adaptive response. The aims of our second study were 1) to assess the effect of the gender and the halothane genotype on CB (using the CBT) and on the level of fear (using a novel object test, NOT), 2) to assess the relationship between the CB and the level of fear and 3) assess the role of several brain neurotransmitters on the CB and level of fear. No differences were found between genders and genotypes regarding the CB and regarding the level of fear but a positive correlation was found between the CBT and the NOT results, suggesting that fear plays an important role in the decision taken by the pig dealing with ambiguous stimuli. Moreover, more fearful pigs had lower concentration of dopamine on

the prefrontal cortex, supporting the relationship between this neurotransmitter and the fear response. The aims of the third study were 1) to assess the effect of handling on the CB (assessed by a CBT), on the fear (assessed by NOT) and on the defence cascade response (assessed by the defence cascade test; DCT), 2) to assess the effect of handling on serum, saliva and hair cortisol concentration and 3) to assess the relationship between behavioural tests (CBT, NOT and DCT) and with cortisol concentrations. No differences between positive and negative handling were found regarding the behavioural tests and cortisol concentrations, suggesting that the handling treatment was not powerful enough to induce such differences or that the measures used were not valid or not sensitive enough to assess such differences. Nevertheless, positive correlations were found between behavioural tests supporting that individual factors, beyond the handling treatment, such as the fear level, the motivation or the coping style had an effect on pigs' affective state. Moreover, more fearful pigs had higher concentrations of serum cortisol at slaughter, supporting the positive correlation between this hormone concentration and the fear response. The fourth study carried out was aimed to assess the effect of housing conditions on the CBT, on the qualitative behaviour assessment (QBA), on the serum cortisol concentration and on the number of wounds on pigs' carcass. The results showed that pigs raised in enriched housing conditions showed better QBA scores, lower serum cortisol concentration and lower number of carcass lesions than pigs raised in barren housing conditions. However, the results of the CBT did not show those differences suggesting that the test is not a valid or not sufficiently sensitive to detect emotional variation in pigs raised in different housing conditions. In conclusion, it is feasible to apply the CBT in pigs, as they performed correctly the required learning process, however, according to our results, the test showed no consistency and no validity questioning its utility to assess the emotional state in pigs.

Resum

L'avaluació de les emocions en animals representa un objectiu crucial en l'estudi de la ciència del benestar animal. És per això que s'han proposat diverses mesures fisiològiques i de comportament, que tot i que ofereixen una gran quantitat d'informació, la seva interpretació és sovint controvertida i estan principalment enfocades a avaluar estats emocionals negatius. El test de biaix cognitiu (CB) s'ha proposat com una mesura amb l'objectiu de solucionar alguns d'aquests aspectes, ja que presumptament permet avaluar la valència (positiva vs. negativa) i la intensitat de les emocions. El terme CB s'ha utilitzat per englobar els efectes que té l'estat emocional sobre els processos cognitius, incloent la memòria, l'atenció, l'aprenentatge i la presa de decisió o judici. En efecte, la major part dels estudis que avaluen el CB en animals utilitzen el judici esbiaixat, que es basa en la premissa que els subjectes amb un estat emocional negatiu jutjaran un estímul ambigu de forma més negativa que els subjectes amb un estat emocional positiu. Es van realitzar un total de quatre experiments, utilitzant entre 36 i 56 porcs en cadascun d'ells. Els porcs eren creuaments de Landrace x Large White amb Pietrain i amb edats compreses entre dos i sis mesos. Els objectius del nostre primer estudi eren avaluar l'aplicabilitat i la consistència del test de CB en porcs i el de proposar una classificació individual de CB. Els nostres resultats van mostrar que els porcs eren capaços d'aprendre la tasca de discriminació espaial (associar la menjadora a un costat del corral del test amb menjar com a reforçament positiu i associar la menjadora a l'altre costat del corral amb l'absència de menjar com a reforçament negatiu) necessària per, posteriorment, poder dur a terme el test de CB (CBT) on s'avaluava la resposta de l'animal davant un estímul ambigu (menjadora intermèdia a les posicions anteriors i amb una petita quantitat de menjar). Malgrat tot, es va observar una falta de consistència entre la resposta al CBT dut a terme dues vegades, deixant 5 setmanes entre ells. Aquest resultat suggereix que els porcs van canviar la percepció (o judici) de l'estímul ambigu degut a la seva habilitat de recordar el resultat de l'estímul ambigu durant el segon CBT o degut a factors no controlats com ara l'edat dels porcs o la seva sensació de gana al llarg del temps. D'altra banda, la majoria dels porcs es van classificar amb un CB positiu, suggerint que el disseny del CBT va induir als porcs a respondre de forma més positiva a l'estímul ambigu o que els porcs tendien a ser més optimistes com una resposta adaptativa. Els objectius del segon estudi van ser 1) avaluar l'efecte del gènere i del genotip halotà en el CB (utilitzant el CBT) i en la resposta de por (utilitzant el test d'objecte novedós, NOT), 2) avaluar la relació entre el CB i la resposta de por i 3) avaluar el rol d'una sèrie de neurotransmissors cerebrals en el CB i en la resposta de por. No es van trobar diferencies entre gèneres i genotips respecte el CB i respecte la resposta de por, però es va observar una

correlació positiva entre els resultats del CBT i del NOT, suggerint que la por juga un paper important en la presa de decisió del porc davant d'estímuls ambigus. A més a més, els porcs amb més por van presentar concentracions més baixes de dopamina en el còrtex prefrontal, constatant la relació entre aquest neurotransmissor i la resposta de por. Els objectius del tercer estudi van ser 1) avaluar l'efecte del maneig en el CB (avaluat pel CBT), en la resposta de por (avaluat per el NOT) i en la resposta de cascada defensiva (avaluat per el test de cascada defensiva, DCT), 2) avaluar l'efecte del maneig en la concentració de cortisol en sèrum, saliva i pel i 3) avaluar la relació entre els testos de comportament (CBT, NOT i DCT) i amb les concentracions de cortisol. No es van trobar diferències entre porcs amb maneig positiu i negatiu respecte als testos de comportament i les concentracions de cortisol, suggerint que el tractament de maneig no va ser prou intens per induir diferències o que les mesures utilitzades no eren valides o prou sensibles per avaluar aquestes diferències. Malgrat tot, es van trobar correlacions positives entre els resultats dels diferents testos de comportament constatant que factors individuals, més enllà del tractament de maneig, com ara la resposta de por, la motivació o el "coping style" van tenir un efecte en l'estat afectiu dels porcs. A més a més, els porcs amb més por van tenir concentracions més altes de cortisol sèric a l'escorxador, confirmant la correlació positiva entre la concentració d'aquesta hormona i la resposta de por. El quart estudi dut a terme tenia per objectiu avaluar l'efecte de les condicions d'allotjament en el CBT, en l'avaluació qualitativa de comportament (QBA), en la concentració sèrica de cortisol i en el número de ferides en les canals dels porcs. Els resultats van mostrar que els porcs criats en condicions enriquides d'allotjament tenien millors puntuacions en el QBA, concentracions de cortisol sèric més baixes i un número més baix de ferides a la canal que els porcs criats en condicions d'allotjament no enriquides. Malgrat tot, els resultats del CBT no van mostrar aquestes diferències suggerint que el test no és vàlid o prou sensible per detectar les alteracions emocionals en porcs induïdes per les diferents condicions d'allotjament. En resum, és factible aplicar el CBT en porcs, ja que van realitzar correctament la tasca d'aprenentatge requerida, tot i així, d'acord amb els nostres resultats, el test no va presentar ni consistència ni validesa qüestionant-ne la utilitat per avaluar l'estat emocional en porcs.

Resumen

La evaluación de las emociones en animales representa un objetivo crucial en el estudio de la ciencia del bienestar animal. Por este motivo se han propuesto diversas medidas fisiológicas y de comportamiento, que a pesar de que ofrecen una gran cantidad de información, su interpretación es a menudo controvertida y están principalmente enfocadas a evaluar estados emocionales negativos. El test de sesgo cognitivo (CB) se ha propuesto como una medida con el objetivo de solucionar algunos de estos aspectos, ya que presuntamente permite evaluar la valencia (positiva vs. negativa) y la intensidad de las emociones. El término CB se ha utilizado para englobar los efectos que tiene el estado emocional sobre los procesos cognitivos, incluyendo la memoria, la atención, el aprendizaje y la toma de decisión o juicio. En efecto, la mayor parte de los estudios que evalúan el CB en animales utilizan el juicio sesgado, que se basa en la premisa de que los sujetos con un estado emocional negativo juzgarán un estímulo ambiguo de forma más negativa que los sujetos con un estado emocional positivo. Se realizaron un total de cuatro experimentos, utilizando entre 36 y 56 cerdos en cada uno de ellos. Los cerdos eran cruces de Landrace x Large White con Pietrain y con edades comprendidas entre dos y seis meses. Los objetivos de nuestro primer estudio eran evaluar la aplicabilidad y la consistencia del test de CB en cerdos y el de proponer una clasificación individual de CB. Nuestros resultados mostraron que los cerdos eran capaces de aprender la tarea de discriminación espacial (asociar el comedero a un lado del corral del test con comida como refuerzo positivo y asociar el comedero al otro lado del corral con la ausencia de comida como refuerzo negativo) necesaria para, posteriormente, poder llevar a cabo el test de CB (CBT) evaluando la respuesta del animal ante un estímulo ambiguo (comedero intermedio a las posiciones anteriores y con una pequeña cantidad de comida). Sin embargo, no se observó consistencia entre los resultados del CBT llevado a cabo dos veces, separados 5 semanas entre ellos. Este resultado sugiere que los cerdos cambiaron la percepción (o juicio) del estímulo ambiguo debido a su habilidad de recordar el resultado del estímulo ambiguo durante el segundo CBT o debido a factores no controlados como la edad de los cerdos o su sensación de hambre a lo largo del tiempo. Por otro lado, la mayoría de los cerdos se clasificaron con un CB positivo, sugiriendo que el diseño del CBT indujo a los cerdos a responder de forma más positiva al estímulo ambiguo o que los cerdos tendían a ser más optimistas como una respuesta adaptativa. Los objetivos del segundo estudio fueron 1) evaluar el efecto del género y del genotipo halotano en el CB (utilizando el CBT) y en la respuesta de miedo (utilizando el test de objeto novedoso, NOT), 2) evaluar la relación entre el CB y la respuesta de miedo y 3) evaluar el rol de una serie de neurotransmisores cerebrales en el CB y la respuesta

de miedo. No se encontraron diferencias entre géneros y genotipos respecto al CB y respecto a la respuesta de miedo, pero se observó una correlación positiva entre los resultados del CBT y el NOT, sugiriendo que el miedo juega un papel importante en la toma de decisión del cerdo ante estímulos ambiguos. Además, los cerdos con más miedo presentaron concentraciones más bajas de dopamina en el córtex prefrontal, constatando la relación entre este neurotransmisor y la respuesta de miedo. Los objetivos del tercer estudio fueron 1) evaluar el efecto del manejo en el CB (evaluado por el CBT), en la respuesta de miedo (evaluado por el NOT) y en la respuesta de cascada defensiva (evaluado por el test de cascada defensiva, DCT), 2) evaluar el efecto del manejo en la concentración de cortisol en suero, saliva y pelo y 3) evaluar la relación entre los test de comportamiento (CBT, NOT y DCT) y las concentraciones de cortisol. No se encontraron diferencias entre cerdos con manejo positivo y negativo respecto los test de comportamiento y las concentraciones de cortisol, sugiriendo que el tratamiento de manejo no fue lo suficientemente intenso para inducir diferencias o que las medidas utilizadas no eran válidas o suficientemente sensibles para evaluar estas diferencias. Sin embargo, se encontraron correlaciones positivas entre los resultados de los diferentes test de comportamiento constatando que factores individuales, más allá del tratamiento de manejo, tales como el nivel de miedo, la motivación o el “coping style” tuvieron un efecto en el estado afectivo de los cerdos. Además, los cerdos con más miedo presentaron concentraciones más altas de cortisol sérico a matadero, confirmando la correlación positiva entre la concentración de esta hormona y la respuesta de miedo. El cuarto estudio realizado tenía por objetivo evaluar el efecto de las condiciones de alojamiento en el CBT, en la evaluación cualitativa de comportamiento (QBA), en la concentración sérica de cortisol y en el número de heridas en las canales de los cerdos. Los resultados mostraron que los cerdos criados en condiciones enriquecidas de alojamiento tenían mejores puntuaciones en el QBA, concentraciones de cortisol sérico más bajas y un menor número de heridas en la canal que los cerdos criados en condiciones de alojamiento no enriquecidas. Sin embargo, los resultados del CBT no mostraron estas diferencias sugiriendo que el test no es válido o suficientemente sensible para detectar las alteraciones emocionales en cerdos inducidas por las diferentes condiciones de alojamiento. En resumen, es factible aplicar el CBT en cerdos, ya que realizaron correctamente la tarea de aprendizaje requerida, sin embargo, de acuerdo con nuestros resultados, el test no presentó ni consistencia ni validez cuestionando su utilidad para evaluar el estado emocional en cerdos.

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General Introduction

1. Concept and assessment of animal welfare

Social concern about animal welfare has grown steadily during the last decades and consumers increasingly demand some major changes in animal production systems (Broom, 2010). This public pressure has promoted the approval of animal protection legislation, especially in European Union, and this legislation must be based on scientific evidence (Moynagh, 2000). Animal welfare arouses from this necessity and one of its main targets is to find objective measures to enable us to assess welfare itself.

Before assessing animal welfare, an agreement should be reached on how it should actually be defined. Indeed, there is not an unanimous definition, but there is a growing consensus that it should include three aspects (Fraser et al., 1997): the emotional state of the animal (animals have to feel well) (e.g. Mendl and Paul, 2004; Dawkins, 2006), its biological functioning (animals have to be healthy and produce well) (Broom, 1986) and its ability to express normal patterns of behaviour (animals have to live according to their nature) (Kiley-Worthington, 1989). These three elements are, by no means, contradictory; in fact they are closely interrelated (Mendl, 2001).

The Five Freedoms developed in 1992 by the Farm Animal Welfare Council (FAWC) of the UK provided a framework to take into account all these aspects. It includes freedom from hunger and thirst, freedom from discomfort, freedom from pain, injury and disease, as well as freedom to express normal behaviour and freedom from fear and distress. The Five Freedoms have been used as the basis for animal protection legislation in the European Union as well as in other parts of the world. Despite its clear usefulness, it is too generic and there is a certain overlap between some of the Five Freedoms (Hewson, 2003; Duncan, 2005). For this reason, slightly different approaches based on the same concepts have been proposed. One of the most relevant is Welfare Quality®, a five-year European Union research project launched in 2004 that focuses on the development of a protocol to assess animal welfare that prioritizes the measures evaluated on the animal itself, named animal based measures. Traditionally, farm animal welfare assessment has focused on measuring the resources supplied to animals, named resource based measures. These measures have the advantage that are objectively assessed, however, the provision of such resources does not guarantee that an animal is healthy and fit and with high welfare standard (Whay et al., 2003). Besides, since welfare is a condition of the animal, animal based measures are likely to be the most direct reflection of its welfare state. Animal based measures reflect the combined effects of resources and management.

Welfare Quality project proposed four principles: good housing, good feeding, good health and appropriate behaviour (Velarde and Dalmau, 2012) being the last principle the most innovative and controversial. All in all, it refers to the fact that animals should not experience fear, pain, distress or any other negative emotional state, at least in a chronic or very intense way. Moreover, they should experience positive emotional states such as, calmness and comfort.

2. Concepts of emotion and emotional state

As described above, emotional states (e.g. happy or angry) and emotions (e.g. pleasure, fear, pain) are an essential aspect to consider when talking about animal welfare. Emotions and emotional states are the causes, mediators, or effects of other psychological processes such as attention, memory, and perception (Barrett, 2006). An emotional or affective state is long-lasting and has lower intensity than an emotion, while an emotion is focused on an object and tends to be short-lasting and higher in intensity (Schnall, 2010). Moreover the terms emotional state and emotion are often used interchangeably, and so will be used in this document.

Human and animal emotions can be defined in terms of two fundamental underlying dimensions (Stanley and Meyer 2009). One dimension is the valence of the emotion, which means that an emotion can be perceived as positive or negative, rewarding or punishing, pleasant or unpleasant. The other dimension is the intensity or arousal of the emotion, for example, the states of depression and sadness are both negatively valenced, but the first is more intense than the second (Mendl et al., 2010b).

3. Factors affecting emotional state in pigs

The presence of specific emotions, such as fear, stress, pain, calmness and comfort are determined by several animal welfare aspects, including internal animal characteristics, such as the genetics and the gender of animals, and environmental conditions, among which social interactions, management and housing play a central role.

During the last years, pig genetic selection has been used to obtain more lean and productive lines. Halothane positive and halothane carrier pigs, i.e. pigs with a mutation on the halothane gene, are known to have a superior lean carcass content and conformation compared with pigs free of this mutation (Gispert et al., 2000). However, those genotypes have also been associated with a greater stress sensitiveness in pigs (Fàbrega et al., 2002). Indeed, halothane genotypes are related with malignant hyperthermia, which leads to a state known as porcine stress syndrome (PSS) and to an increased probability of palid, soft and exudative (PSE) meat (Gispert et al., 2000).

Gender differences have been found in terms of behaviour and welfare. Entire male pigs have higher levels of testosterone than castrated males and females. Testosterone is synthesized in the testes, and has a high influence on animal behaviour (Signoret, 1976). Entire males are more likely than castrates (Cronin et al., 2003) and females (Rydhmer et al. 2006) to show aggressive and sexual behaviour, such as mounting (Fredriksen et al., 2004; Rydhmer et al., 2006). As a result, entire male pigs or pigs shearing the environment with them are more prone to get injured and, therefore, suffer more negative affective states than castrates and females housed alone. Moreover, social interactions, including both positives and negatives, are not only affected by gender hormones being other factors such as housing conditions and other individual factors (i.e. breed) also very important to be considered.

The way stockpeople interact with animals also affect the animal's level of fear or confidence to humans and subsequently the animals' behaviour, their stress responses and productivity (Hemsworth and Coleman, 1998). Therefore, fear of humans can be considered detrimental for welfare when pigs are handled frequently. In fact, one of the most potentially frightening events that farm animals are likely to experience is exposure to human beings (Waiblinger et al., 2006). Negative interactions with stockpeople are linked to greater avoidance of humans in pigs and can lead to acute and chronic stress responses and impaired growth and reproductive performance. On the opposite side, positive interactions may decrease the general level of stress. For example, Pedersen et al. (1998) found that gentling of sows during pregnancy reduced their physiological and behavioural stress-reactions to tethering. Besides, the presence of a familiar human, contingently providing gentle handling, may calm the animals in potentially aversive situations (e.g., isolation, tethering, rectal palpation, insemination) thereby reducing distress and the risk of injury to the animal and the human (Waiblinger et al., 2006).

Housing conditions may also have an effect on animal emotions. For example, straw has three distinct effects: as bedding it improves the thermal comfort and the physical comfort of the floor; it results in greater filling of the gut; and it provides with a "recreational" function since it may act as a stimulus and outlet for the rooting and chewing activities that are natural to pigs (Fraser et al., 1991). According to European Union legislation ‘‘pigs must have permanent access to a sufficient quantity of material to enable proper investigation and manipulation activities, such as straw, hay, wood, sawdust, mushroom compost, peat or a mixture of such, which does not compromise the health of the animals''. Another characteristic of the housing conditions is the type of floor, especially the presence of slats, which is also important when determining the impact of housing conditions on welfare. Floor characteristics are known to have effects on some behavioural patterns, the type and prevalence of body lesions, and the health of pigs affecting ultimately its affective state (Kilbride, 2009). It is well known that under thermoneutral conditions pigs prefer to rest on solid floor, however the percentage of pigs lying on a slatted area will increase with temperature when they are housed under partially slatted conditions (Aarnink et al., 2006). Furthermore the requirement for space of a pig covers both the space that is occupied by the animal when standing or lying and during posture changes and the space that allows them to show normal behaviour and interactions (Weng et al., 1998). In addition, there is evidence that reduced space availability per animal has a negative impact on their behaviour, physiology, health and productive performance (Meunier-Salaun et al., 1987; Street and Gonyou, 2008). Providing sufficient space reduces negative behaviour such as persistent nosing of pen mates and tail biting (Beattie et al., 1996). Moreover, there is evidence that decreasing space allowance leads to increased agonistic behaviour (Ewbank and Bryant, 1972; Meunier-Salaun et al., 1987).

4. Assessment of animal emotions

Animal emotion assessment has long been dismissed by science due to its subjective nature. In order to study emotions and emotional states scientifically, the development of objective measures is an essential first step. Emotions cannot be measured directly in animals, as they cannot verbally self-report their subjective emotional experience, therefore it has to be relayed in other measures, mainly physiological and behavioural measures but also in clinical or production ones (Mendl et al., 2009).

4.1 Physiological measures to assess emotions

Animal welfare studies have usually used the term 'stress' when using physiological measures to assess emotions in animal welfare, as the term 'emotion' was considered as too subjective. Indeed, as noted by Dantzer (2002), research on farm animal emotions has generally been limited to indicators of stress and has not linked these indicators to the existence of other emotions.

The hypothalamic-pituitary-adrenal (HPA) axis and the sympatho-adrenomedullary (SAM) system are the two main elements of the physiological stress response. Moreover, serum cortisol is the most used physiological marker to assess animal stress response (Moberg, 2000). However both HPA and SAM are activated similarly in aversive and rewarding situations being able to assess the intensity but not the valance of the emotional response. Furthermore, the relationship between the amount of cortisol and the apparent intensity of stress in animals is not linear (Mormède et al., 2007). Indeed, there are a number of other factors which influence the concentrations of this indicator such as the time of day/night, physical activity, health, feeding or temperature and humidity. In addition, some studies in humans (e.g. Lane, et al., 1997; Stone and Nielson, 2001) failed to find a correlation between the golden standard of verbal report and cortisol concnetrations.

Beyond the relationship between cortisol and stress, cortisol is associated with a large number of brain normal functions and dysfunctions. It has been demonstrated that activation of the HPA axis and chronic alterations in the circulating levels of cortisol modulate numerous behaviours and brain plasticity, including depression and hippocampal neurogenesis (Yehuda et al., 1996). Furthermore, it has been reported that in depression state there is hypercortisolemia and HPA axis dysfunction (Holsboer, 2001).

Although cortisol hormone is commonly measured in blood plasma, alternative methods such as the measurement of cortisol levels in saliva, urine or faeces have been developed to overcome the stress induced by blood sampling itself (Mormède et al., 2007). More recently, hair cortisol analysis have been proposed as a complementary measure for monitoring stress, revealing systemic cortisol exposure over longer periods of time (Russell et al., 2012).

Other physiological measures have also been used to assess stress and other emotions, such as HPA function (e.g. levels of adrenocorticotropic hormone (ACTH), response to dexamethasone, ACTH, and CRH challenge tests), sympathetic and autonomic function (variation in heart rate, blood pressure, catecholamine levels, skin conductance and skin temperature), and neuroendocrine activity, including levels of blood circulating oxytocin, vasopressin, prolactin, and opioid peptides.

Nevertheless, other molecules, such as acute phase proteins (APPs) have also been proposed as stress biomarkers in farm animals. APPs have been described as stress biomarkers, besides their well-known role as indicators of infection and inflammation (Petersen et al., 2004). Haptoglobin, C-reactive protein and Pig-MAP are the main APPs in porcine. For example, it has been found that Haptoglobin and Pig-MAP increases in pigs subjected to a long road transport (Saco et al., 2003) and Marco-Ramell et al. (2011) reported an increase in Pig-MAP in pigs housed at high density.

Catecholamines (noradrenaline (NA), adrenaline (A), dopamine (DA) and their metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenyl acetic acid (DOPAC)) and serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), particularly in the hypothalamus and hippocampus are also involved in stress (Möstl and Palme, 2002). In fact, SAM, which produces NA and A, is one of the fastest systems activated during stress. Catecholamine levels may be measured in brain as well as in blood, taking into account that they are very rapidly released. In pigs, similarly to laboratory animals, catecholamines are involved in the neurochemical background to stress sensitivity (Piekarzewska et al., 2000). As for the serotonergic system, it has been found to be altered in various brain areas of prenatally stressed piglets, as shown by a significantly increased 5-HIAA/5-HT ratio (Otten et al., 2010).

4.2 Behavioural measures to assess emotions

Behavioural measures are the most used indicators to assess emotions in animals since the behavioural component of the emotion allows the subject to respond to the emotion eliciting stimulus and therefore measurement of this may give some indication of the intensity and the valence of the underlying emotion (Murphy et al., 2014b). A great concern for animal welfare related with emotional state appears to be centred on behaviour related with negative emotions. For example, four of the Five Freedoms are freedoms from negative outcomes and the fifth freedom to express normal behaviour does not explicitly value positive experiences. More recently, there has been an increased interest on a welfare science paradigm that attempts to include positive outcomes (Yeates and Main, 2008).

Specific behaviours such as playing, vocalizations, social and exploratory behaviour, freezing or turning back or even urination and defecation are studied to assess animal emotions (Murphy et al., 2014b). Behaviours such as playing or tail movement are focused on assessing positive emotional states, others such as social behaviour, vocalizations and facial expressions can offer information of both positive and negative emotional states. Furthermore there is a large number of behaviours focused to assess negative emotional states, such as reluctant to move, escape attempts or defecation (Murphy et al., 2014b).Behaviours which occur consistently in a variety of contexts that seem to have the same affective valence, and which do not appear in oppositely valenced situations are likely to be more reliable indicators (Paul et al., 2005). For example, playing behaviour always appears in positive but never in negative affective valence contexts.

A method that deserves a special mention is the qualitative behaviour assessment (QBA) developed by Wemelsfelder and co-workers which is included in the Welfare Quality® protocol (Wemelsfelder, 2007). This method relies on the human observer's ability to integrate perceived details of an animal's behaviour and its context, using 'whole animal' descriptors such as calm, playful, content, indifferent or frustrated (Wemelsfelder et al., 2000). Such descriptors aim to reflect the animals' emotional experience of a situation (Wemelsfelder, 2007), and such emotions have been suggested to be directly relevant to its welfare (Wemelsfelder, 1997, 2007). QBA was first explored in growing pig (Wemelsfelder et al., 2000). After this study, several studies have been carried out in different species, by observers ranged from having none to extensive experience with animals. The outcomes of these studies show QBA to have generally good inter-observer reliability, and to correlate significantly with measures of individual animals' behaviour and/or physiological stress responses (Wemelsfelder et al., 2001; Rousing and Wemelsfelder, 2006). However, other studies reported low inter-observer reliability between observers of different countries (Temple et al., 2011). Moreover, Andreasen et al. (2013) did not find evidence of a meaningful pattern of correlation between QBA and welfare assessments.

Beyond specific behaviours and the QBA, several behavioural tests have been proposed to assess emotions. Fear or anxiety tests, such as novel object, open field and elevated plus maze tests are the most used methods to assess behaviour (Forkman et al., 2007). In these studies the conflict between the motivation to explore and the motivation to avoid (i.e. fear) the unfamiliar stimulus is assessed. The main measure to assess the animal response in front a novel object test is the latency to explore an unfamiliar object. Furthermore, the duration and frequency of object exploration is usually measured. Avoidance of the stimulus, indicated by longer latencies to approach/investigate and less time spent investigating the stimulus, is taken to indicate higher levels of anxiety or fear (Murphy et al., 2014b). Moreover, preference and avoidance tests have been commonly used to establish what an animal wants and how hard it will work to get at or avoid resources, such as flooring or nesting substrates (Blom et al., 1996; Sherwin and Glen, 2003).

The vast majority of behavioural tests to assess emotions are focused on assessing negative emotional states (e.g. open field test and novel object test), which means that they are not able to assess the valence of the emotions (Mendl et al., 2009). Given the above issues there is a need to develop new methods to assess animal emotions, which can consider the two dimensions of emotions, the valence and the intensity.

5. Relationship between emotions and cognition

Cognition is defined as a range of processes, including attention, learning, memory and decision-making involved in the acquisition, storage and manipulation of information (Shettleworth, 1999). The study of both emotions and cognition had been considered separate disciplines for years. In more recent decades several studies, first in humans later in animals, have been carried out focusing on the relationship between both disciplines. For example, Mason (1971) showed that it is the animal's representation of an event (e.g. the perception of deprivation of having access to food) rather than the event itself (e.g. the absence of food deprivation) that determines its emotional reaction. A recent example is the study performed by Lang et al. (1998) that showed that the response in rodents induced by exposure to a sudden event tends to be faster and with greater amplitude under negative emotional states. Therefore, it was proposed that emotional processes compressed not only behavioural, physiological and subjective components but also a cognitive component (Lerner and Keltner, 2000).

Initially the relationship between cognition and emotion was focused on the effect of cognitive processes on emotions and affective state. Furthermore, there is increasing evidence that links between emotions and cognition occur in both directions, being also emotional manipulations influencing cognitive processes (Paul et al 2005).

5.1 The effect of cognition on emotions: appraisal theories

Appraisal theories, first described by Arnold (1960) suggest that emotions are extracted from the evaluations (i.e. appraisals) of events that cause specific reactions. Thus, it is the evaluation of the event that determines the valence and the intensity of the specific subjective emotion experienced. The same emotional response is elicited when the outcome of the appraisal process is the same, but not necessarily when the stimulus is the same. That is, appraisal theorists propose that emotions arise, not just as a result of the characteristics of the stimulus (e.g. see spider—feel fear), but also due to the evaluation of the stimuli by the subject at that moment in time. Based on this principle different approaches have been proposed. For example, according to Scherer (1987) in humans this evaluation is based upon a number of elementary criteria, including the intrinsic characteristics of the event (its suddenness, familiarity, predictability and pleasantness), the consequences of the event relative to the individual's expectations (e.g. the expected rewarding or punishment and the intensity of the event), the coping potential of the individual, and then the relevance of the response to social norms. Later on, Dantzer (2002) and Désiré et al. (2002) suggested that a similar criterion proposed by human appraisal theorists is likely to be used by animals in the generation of emotions. Moreover, Rolls (2000) proposed a simplified approach in order to better adapt the framework to animals. He proposed that the appraisal of an event depends on whether the event is perceived as a reward or a punishment. Appraisal theories offer a framework to classify specific physiological and behavioural responses to particular emotional states, including positive ones (Paul et al., 2005).

5.2 The effect of the emotions on cognition: cognitive bias

While cognitive processes can influence emotions, emotions can in turn affect cognitive processes. The term cognitive bias has been used as a general label for the effects of emotional state on cognitive processes. A wide range of studies in humans have focused on how emotional states can bias the cognition. For instance, a consistent finding from those studies is that the valence of an individual's emotional state appears to influence different cognitive processes such as attention, memory and judgment (Mendl et al., 2009). For example, people with anxiety are more prone to pay attention towards potential threatening information (e.g. Mathews and MacLeod, 1994; Bradley et al., 1997). Likewise, optimistic people are more likely to remember positive memories and unhappy or depressed people more likely to remember negative ones (e.g. Bower, 1981; Mineka et al., 1998). Furthermore, emotionally charged events are more easily remembered than neutral ones (Reisberg and Daniel, 1995). Judgment processes are also affected by current emotions (i.e. judgment bias): people in a negative affective state are more likely to make negative judgements about future events or ambiguous stimuli than people in positive affective states (e.g. Eysenck et al., 1991; Wright and Bower, 1992). For a review of the emotion-induced cognitive biases see Paul et al. (2005).

Once established the effect of the emotions in cognitive processes in humans, it is conceivable that such cognitive biases also exist in animals. Indeed, in humans and rodents it has been described a suite of defensive responses when disturbed by an altering stimulus, named the defensive cascade (Lang, 1990; 2000) that may be altered by the emotional state of the subject. The defensive cascade includes an initial response (startle), evaluation of the situation and a final response of fleeing or resuming ongoing behaviour. These studies demonstrated that the emotional state of subjects can modulate their defensive cascade response. For example in rodents, startle responses may be attenuated in individuals experiencing positive affective state (Koch, 1999). Recently, Statham et al. (2015) proposed a test to assess the defence cascade response (DCT) in pigs and found that barren conditions induced negative emotional state, and pigs decreased their magnitude of startle in the initial response compared with pigs raised in enriched conditions.

Another approach to assess emotions in animals through the cognitive processes is the anticipatory behaviour (Spruijt et al., 2001): animals in more negative affective states exhibit a reduced anticipatory behaviour between the presentation of a conditioned stimulus predicting a reward and the arrival of that reward. For example, Van Den Berg et al. (1999) showed that rats isolated during 4-5 weeks decreased the anticipated motivation of sucrose-drinking compared with the no isolated ones.

However, there are inevitably some uncertainties in these research findings. For example, it is not always the case that similarly valenced states, such as depression, anxiety and fear, have the same effects on cognitive function (Lerner and Keltner, 2000). Additionally, some effects may be the result of trait, as well as or instead of, state differences in emotions (Mineka et al., 1998; Mogg and Bradley, 2005). Nevertheless, taken together, these findings suggest that measures of attention, memory and judgment biases can be indicative of an animal emotional state (Mendl et al., 2009).

5.3 The judgment bias

Judgment bias is the most common affect-induced cognitive bias measure used in animals. Therefore in the present document the terms judgment bias and cognitive bias are used interchangeably. Judgements and decisions on how to respond to a particular stimulus or situation are complex processes, often involving attention, memory and other aspects of cognition and perception (Paul et al., 2005). According to Mendl et al. (2009) judgment bias refers to the propensity of a subject to show behaviour indicating anticipation of either relatively positive or relatively negative outcomes in response to affectively ambiguous stimuli. It is supposed that subjects with more positive affective state (optimistic) will be more likely to show positive outcomes than subjects with negative affective state (pessimistic) in front ambiguous stimuli. Animal judgment bias studies are based on previous results in humans showing that people reporting negative emotions appear to make negative judgements about ambiguous stimuli while optimistic people show the opposite bias.

Harding et al. (2004) first designed a methodology to determine the emotional state of nonhuman animals by using a judgment bias test. In this study, rats were trained to perform one response (lever press) when presented with one stimulus (a tone of a particular frequency) in order to receive a positive reinforcer (food pellet), and to perform a different response (no lever press) when presented with a different stimulus (a tone of a different frequency) in order to avoid a negative reinforcer (burst of white noise). Once trained on this discrimination task, rats were tested by presenting them with three ambiguous stimuli (tones of intermediate frequency between the one associated with a positive reinforcer and the one associated with the negative reinforcer). The hypothesis was that animals with a more positive affective state would assess the ambiguous stimuli as predicting the positive reinforcer, that is, the response would be similar to the one in front the positive stimulus and vice versa for the animals with more negative affective state. To induce presumed differences in affective state, rats were housed in either unpredictable housing, widely demonstrated that induce a mild stress/depression-like state, or in predictable, control conditions. As predicted, rats under unpredictable housing treatment showed a tendency to perform a lower proportion of positive responses to the ambiguous stimuli and were significantly slower in making these responses to these stimuli compared to rats in the predictable conditions.

Since this initial judgment bias study was performed, a large number of studies have been carried out in different species comparing a wide variety of treatments (for a review see Mendl et al., 2009; Gygax, 2014; Baciadonna and McElligott, 2015). For example, judgment bias studies have been carried out in laboratory animals (e.g. rats and mice), in farm animals (e.g. calves, sheep, pigs and laying hens), in companion animals (e.g. dogs, cats and horses) but also in birds, primates, bears and even in honeybees. Treatments include housing conditions, such as density, enrichment material and types of floor; human animal interactions such as handling management, training reinforcement and husbandry procedures; administration of drugs such as anxiolitics and antidepressants; specific genotypes such as congenitally helpless rats or halothane genotype in pigs; isolation from the congeners and differences between breeds and genders. The treatments of the cognitive bias studies carried out in pigs are shown in Table 1.

As described above, the methodology to assess the judgment bias in those studies is based on Harding et al. (2004) but several differences can be found between them, for example, regarding the type of the stimuli, the type of trained responses and the type of positive and negative reinforces. The most used type of stimuli are auditory including different tones and noises; visual, including a scale of greys and different colours and finally based on spatial location in a test pen. Moreover, other stimuli such as olfactory and tactile have also been used. The trained response can be a go/no go response (the animal is trained to respond to the positive stimulus and refrain from responding to the negative stimulus) or an active choice response (the animal has to perform a similar response to receive the reinforcer of the positive stimulus and avoid the reinforcer of the negative stimulus). Finally, food is nearly always the positive reinforcer while different options, such as absence of food, unpalatable food, electric shock or air puff have been used as a negative reinforcer.

The maximum time of training and tests sessions, the measures assessed (e.g. the mean latency to respond to the stimuli, percentage of animals that respond to the stimuli), the criterion to include and eliminate the animals from the test (e.g. eliminate the animals that do not learnt after several sessions or train each animal until achievement of the required discrimination learning process) and the number of types of test (usually three, but also one or more than three) are other aspects to consider to plan a design for a judgment bias study.

Studies carried out so far offer a wide range of results. Some of them confirm the expected hypothesis, that animals in a presumed negative affective state respond more pessimistically in front the ambiguous stimuli (e.g. Douglas et al., 2012; Enkel et al., 2010; Mendl et al., 2010a). Others only confirm the hypothesis partially, that is, animals respond as predicted just in some of the ambiguous stimuli (e.g. Brajon et al., 2015; Harding et al., 2004). There are also several studies that failed to find differences between treatment groups (e.g. Düpjan et al., 2013; Scollo et al., 2014). Finally, some of the studies found differences between groups but in the opposite direction than expected, that is, animals with a presumed negative affective state respond more optimistically in front the ambiguous stimuli (e.g. Destrez et al., 2012; Briefer Freymond et al., 2014).

Six studies of judgment bias have been carried out in pigs so far. A summary of these studies is presented in table 1. Beyond the aspects showed on table 1, it should be recalled that there are some other differences in the test design, such as the presence (Brajon et al., 2015; Scollo et al., 2014) or absence (Douglas et al., 2012; Düpjan et al., 2013) of habituation sessions before starting the training sessions. Moreover a huge variability in the number of training sessions is present between studies, ranging from 52 (Murphy et al., 2014a) to 380 (Douglas et al., 2012). Some studies performed as much training sessions as necessary to ensure that all pigs learned the discrimination task (e.g. Douglas et al., 2012; Düpjan et al., 2013) while others established a criterion to exclude the pigs that did not learn after several training sessions (e.g. Brajon et al., 2015).

5.3.1 Cognitive bias classification

As mentioned above most of the cognitive bias studies carried out so far compare two treatment groups. Therefore the outcomes of the subjects of each group are analyzed together despite the fact that the animals are tested individually. However, animals can perceive the treatment differently due to individual differences widely described in animal species (Spoolder et al., 1996). As suggested by Wichman et al. (2012) individual factors may have a greater effect on the cognitive bias results than the treatments per se. An individual cognitive bias classification could be useful to detect individual emotional changes on animals due to the treatments carried out in order to induce such changes. For example, it is supposed that an animal classified with negative emotional state will change to positive emotional state after improving the environmental conditions and vice versa.

5.3.2 Cognitive and neural mechanisms underlying judgment bias

Judgements of ambiguous stimuli are the final result of several processes: attention and sensory registration of the stimulus, evaluation of the stimulus, decision-making and selection of a response (Mendl et al., 2009) (Fig.1). Ambiguous stimulus is perceived as objective information and is then processed into a neural 'representation' at the level of the primary sensory cortices (Rolls, 2005). Then the subjectivity is attributed to the processed information, and may occur in brain areas such as the amygdala and orbitofrontal cortex, involving changes in dopaminergic (DA) activity in the mesocorticolimbic system (Cardinal et al., 2002; Rolls, 2005). Once the subjectivity is attributed, the information may be evaluated. According to the expected utility theory (Loewenstein, 2008), when decisions are made under uncertainty about the outcome they are based on a combination of the anticipated magnitude and probability of different known and similar outcomes (Loewenstein et al., 2008). Afterwards the basal ganglia is involved in the selection of the appropriate behavioural action (Redgrave et al., 1999; Rolls, 2005; Bogacz and Gurney, 2007). Although it is helpful to think about those processes as occurring sequentially (see fig.1), they are implemented by multiple interconnected and parallel-processing circuits distributed throughout the brain (Mendl et al., 2009).

Other brain areas are also related in this process as they are involved in emotional processes. For example, the hypothalamus is involved in functions related to pleasure, pain or stress responses or the hippocampus is related with memory, anxiety and depression. In fact, several studies reported that negative affective states, particularly anxiety, can direct attentional resources to stimuli which are associated with danger or threat (e.g. Mogg and Bradley, 1998; Bishop, 2007). The neural basis of such effects may involve modulatory signals from the amygdala enhancing threat-processing, and overriding those from prefrontal cortex that support ongoing 'neutral' task-related processing. Moreover, the affective state such as anxiety or depression can lead to a biased evaluation of the stimulus. Indeed, activity of the DA system appears to be sensitive to environmental stressors and to be associated with mood disorders such as depression (Spruijt et al., 2001; Southwick et al., 2005; Cabib, 2006; Dunlop and Nemeroff, 2007), thus providing a potential link between reward evaluation and affective state.

5.3.3 Validity and consistency of the judgment bias test

When assessing how well behaviour is measured, two principal issues must be considered: reliability and validity. Reliability concerns the extent to which the measure is consistent (i.e. do repeated measurements of the same thing produce the same results?) and validity refers to the relation between what a parameter is supposed to measure and what really measures (Martin and Bateson, 1993).

Consistency, as part of the reliability, is often measured as a correlation coefficient between two samples of the same measurement. To our knowledge, consistency over time of a cognitive bias test has been assessed by using the mean value of time that animals of each treatment group took to interact with ambiguous stimulus in several studies (Doyle et al., 2010; Murphy et al., 2013). Moreover, it has never been assessed individually, despite the fact that the cognitive bias is assessed individually. In those studies, no consistency of the cognitive bias test was found over time, suggesting that animals can be habituated to the test-situation (Forkman et al., 2007), that is, animals are able to remember the reinforcer of the first CB test during the following CB tests.

Moreover, behavioural measures can be validated by other behavioural, physiological or pharmacological indicators (Murphy et al., 2014b). Studies of cognitive bias have been using other behavioural tests in order to compare results, such as, the novel object test (Chaby et al., 2013; Destrez et al., 2012; Doyle et al., 2011b; Wichman et al., 2012), the tonic immobility test (Seehuus et al., 2013; Wichman et al., 2012) , the open field test (Doyle et al., 2010; 2011a), or other less common tests such as the elevated plus maze (Brydges et al., 2012), the anticipation test (Wichman et al., 2012), the suddenness test (Destrez et al., 2012; Doyle et al., 2011b) and the human approach test (Destrez et al., 2012). Most of those tests are commonly carried out to assess fear, which seems to have a closer relationship with what is assessed in the cognitive bias tests (Wichman et al., 2012). Other studies have been assessed if the treatment performed have similar effect in cognitive bias test and in other behaviour patterns, such as, negative social behaviour (Wichman et al., 2012), exploratory behaviour (Chaby et al., 2013), locomotor activity (Verbeek et al., 2014), vocalizations (Rygula et al., 2012) and stereotypic behaviours (Brilot et al., 2010; Pomerantz et al., 2012).

Cortisol concentration is the most widely physiological indicator used in cognitive bias studies. It has been measured in plasma (e.g. Sanger et al., 2011; Verbeek et al., 2014), in urine (Titulaer et al., 2013), in faeces (Pomerantz et al., 2012) and in saliva (e.g. Düpjan et al., 2013; Murphy et al., 2014a). In most of these studies (e.g. Scollo et al., 2014; Titulaer et al., 2013; Verbeek et al., 2014) cortisol is used to assess if the treatment has an effect on physiological parameters and if this effect is similar on the results of the cognitive bias test. Moreover, just one study (Murphy et al., 2014a) has assessed the correlation between cortisol concentrations and the results of the cognitive bias test. Other physiological parameters have also been measured in judgment bias studies, such as, hematological parameters (e.g. Guldimann et al., 2015; Sanger et al., 2011), cardicac activity (Destrez et al., 2012; Doyle et al., 2011b) and body weight (Scollo et al., 2014; Wichman et al., 2012).

Finally several studies have used pharmacological treatments to validate the judgment bias thecnique. Anxiolitic treatments have been administered in rats (Anderson et al., 2013) and in lambs (Destrez et al., 2012) as well as antidepressants (Anderson et al., 2013; Enkel et al., 2010) which are suposed to reduce negative affective states. Moreover, Doyle et al. (2011a) used a serotonin antagonist to determine if it had an effet on the response of sheep in the judgment bias test. Other mesasures than cognitive bias carried out in pigs' cognitive bias studies are described in table 1.

Table 1. Some characteristics of the six studies published on cognitive bias before the redaction of the thesis.

Study	Treatment	N recruited	N tested	Gender	Breed	Maximum time of sessions (s)	Cue type	Positive reinforcer	Negative reinforcer	Required response	N types of tests	N test replications	Measures to assess cognitive bias	Other measures	Cognitive bias effect
Douglas et al. (2012)	Environmental enrichment	10	10	F	LW × L	30	Auditory	Apples	A plastic bag waved in the face	Go/no go	3	10	Proportion of go responses and latency	-	Yes
Düpjan et al. (2013)	Repeated social isolation	32	30	F	L	120	Location	Piglet feed, oat flakes, sugar and water	No food	Active choice	3	3	Latency and duration of exploration	Saliva cortisol	No
Murphy et al. (2013)	Restraint of movement and breeds	15	15	F	G minipigs and D x Y and D x L	60	Auditory	4 M&M® chocolates	1 M&M® chocolate	Go/no go	3	1	Proportion of go responses and latency	-	No
Murphy et al. (2014)	Birth weight	16	16	EM	D x Y and D x L	30	Auditory	4 M&M® chocolates	1 M&M® chocolate	Go/no go	3	4	Proportion of go responses and latency	-	(Yes)
Scollo et al. (2014)	Stocking density	40	40	EM and F	LW × L	120	Location	Pellets	No pellets	Go/no go	3	9	Latency	Behavioural observations, skin lesions, saliva cortisol and α-amylasa, weight	No
Brajon et al. (2015)	Handling management	54	32	EM and F	(Y x L) x D	35	Auditory	Cereals	Tennis ball fell on the back, fresh water spilled on the back, water spray spouted in the face…	Go/no-go	3	4	Proportion of go responses and latency	-	(Yes)

Gender: F = female, EM = entire male; Breed: LW = Large White, L = Landrace, G = Göttingen, D = Duroc; Y = Yorkshire, P = Pietrain; Cognitive bias effects: (Yes) = indicates that differences between cognitive bias were identified only in some type of test.

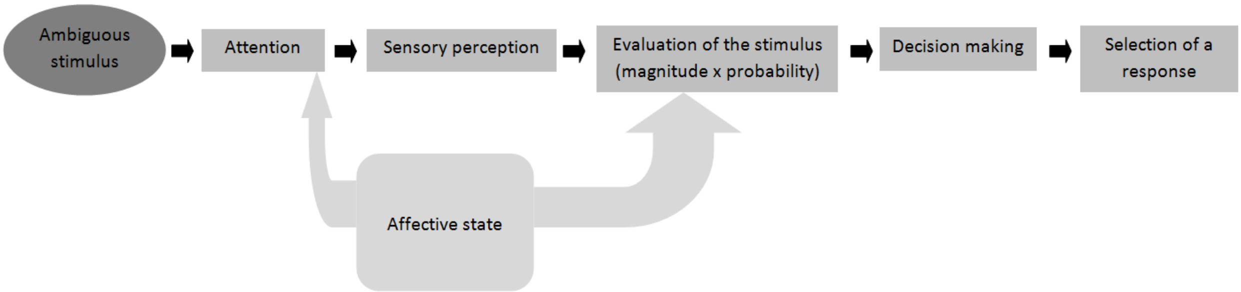

Fig.1. Schematic diagram based on Mendl et al. (2009) of some of the cognitive and neural processes that may underlie the judgement bias.

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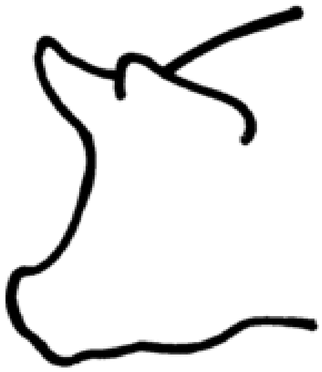
Objectives



The general objective of this thesis is to assess the validity and consistency of the cognitive bias test comparing the affective state of pigs under different treatment conditions.

The specific objectives are as follows:

1. To assess the applicability and the consistency of the cognitive bias test over time. (Chapter 2)

2. To propose an individual classification of the animals according to the results of the cognitive bias test. (Chapter 2)

3. To assess if the halothane genotype, the gender and the management and housing conditions have an effect on the cognitive bias in pigs. (Chapter 3, 4 and 5)

4. To validate the cognitive bias test using behavioural measures: novel object test, defence cascade test and qualitative behaviour assessment. (Chapter 3, 4 and 5)

5. To validate the cognitive bias test using physiological and clinical measures: serum, saliva and hair cortisol concentrations, neurotransmitters concentrations and the number of wounds on pigs' carcass. (Chapter 3, 4 and 5)

Chapter 1

Cognitive bias in pigs: Individual classification and consistency over time

Chapter based on a paper published in Journal of Veterinary Behavior 2015 10, 577-581

Abstract

The aim of the study was to ascertain if the cognitive bias (CB) test can be used to assess pigs' emotional state and classify them individually. Moreover, the test was repeated over time to assess its consistency. Thirty-six male pigs were individually trained during 14 training sessions to discriminate between a bucket with (A) or without (NA) access to chopped apples depending on its position (left or right) in a test pen. Once pigs were able to discriminate between both positions, each animal was subjected to 2 A and 2 NA reminder sessions before performing the CB test session, where the bucket was placed on a central position with access to 2 pieces of apple. The trial was repeated after 5 weeks, reducing the number of training sessions to 4. Time to contact the bucket, time to eat (or try to eat in the case of NA sessions), number of vocalizations, number of times pigs were reluctant to move, number of escape attempts, and number of urination and defecation events were recorded. In the first trial, time to contact the bucket and time to eat was significantly lower in A than in NA from session 10 ($P < 0.0012$), indicating that pigs were able to discriminate between both positions. In the second trial, both variables were significantly lower in A compared to NA from session 2 ($P < 0.005$) onward, confirming the pigs' capacity to remember the task. Pigs were individually classified as having positive, negative, or neutral CB, according to the time to contact the bucket during the CB test session in comparison with the time taken during the remainder sessions. A large percentage of pigs were classified as positive CB in both trials (84.85% and 94.29%, respectively). However, there was no consistency between the results of both trials, suggesting that during the second CB test session animals were able to remember the content of the bucket of the first CB test session. Alternatively, other factors such as the time of the day that pigs were tested, the age of the animals, or their hunger state could have an effect on the results.

Keywords: cognitive bias, emotional state, pig welfare

1. Introduction

Measures based on animal emotion are of increasing interest in animal welfare science (Panksepp, 1998; Mendl and Paul, 2004; Rolls, 2005; Lawrence, 2008). Because nonhuman animal cannot self-report their feelings, the assessment of an animal's emotional state has to rely on measures of physiology, neurology, and behavior, including cognitive bias (CB) tests.

The term "cognitive bias" refers to the influence that the emotional state has on cognitive processes such as attention, learning, memory, and decision making (Mendl et al., 2009). There is an extensive bibliography in human psychology that discusses how the affective state influences the cognitive function (e.g., Eysenck et al., 1991; Williams et al., 1997).

In recent years, several studies have explored CB in nonhuman animals. Harding et al. (2004) first designed a methodology to determine the emotional state of nonhuman animals by using a "judgment bias" task. The concept of judgment bias refers to the response of an individual to an ambiguous cue and is based on the premise that individuals in negative affective states make negative judgments about ambiguous cues more frequently than individuals in positive affective states. After this experiment, a large number of studies of CB have been carried out in different species (Gygax, 2014).

Most studies on CB have used the judgment bias task to compare two or more groups of animals subjected to different treatments, such as different housing (e.g., Douglas et al., 2012; Wichman et al., 2012) or management (e.g., Papciak et al., 2013; Düpjan et al., 2013) conditions. For instance, Douglas et al. (2012) showed that pigs kept in an enriched environment made more positive judgment in front of an ambiguous cue than pigs kept in a barren environment. Düpjan et al. (2013), however, did not find differences in judgment bias between pigs kept isolated and pigs kept in groups.

It is widely accepted that animal welfare indicators should have a high reliability, for example, they should be repeatable and consistent over time (Temple et al., 2013). The consistency has been assessed individually in several studies of different behavior test such as novel arena test (e.g., Jensen et al.,1995) or novel object test (e.g., Spoolder et al., 1996). To the best of our knowledge, consistency over time of CB has been assessed by using the mean value of a group of animals (e.g., Doyle et al., 2010 and Murphy et al., 2013), but has never been tested using individual values despite the fact that the CB is assessed individually.

The objective of the present study was to classify pigs individually and to assess the consistency over time of individual CB by using a go or no-go spatial judgment task (Burman et al., 2009; Mendl et al., 2009) in pigs from the same sex and genotype, exposed to the same housing and management conditions.

2. Material and methods

2.1 Animals and housing conditions

Thirty-six male pigs aged 8 weeks coming from a commercial farm were moved to the experimental facilities in IRTA (Monells, Spain) and randomly allocated to 4 groups of 9 animals each. The experimental pigs were crosses of Large White x Landrace halothane gene -RYR1-free (NN) sows with Pietrain heterozygous (Nn) boars. Animals were housed in slatted pens (5 x 2.7 m) and under natural light conditions at a constant environmental temperature of 22°C ± 3°C. Each pen was provided with 1 steel drinker bowl (15 x 16 cm) connected to a nipple and a concrete feeder (58 x 34 cm) with 4 feeding places. Pigs had water and food ad libitum. The pigs were inspected daily and no health problems were observed during the experimental period.

2.2 Cognitive bias test

Pigs were subjected to the CB test twice, at the age of 10 (first trial) and 15 (second trial) weeks. Pigs were individually moved from the housing pen to a 31.5 m^2 test pen (4.7 length x 6.7 m width) separated from the housing pens by a corridor (6.4 x 2.2 m). The temperature of the test pen was kept at 22°C. The floor was partially slatted with a corridor of concrete (6.4 x 2.3 m) in the middle of the test pen. Pigs entered and left the test pen through the same door. A bucket (30 x 40 cm) was placed close to the wall opposite to the door, on the right or left side of the pen during the training sessions and in a central position during the test session (Fig. 1).

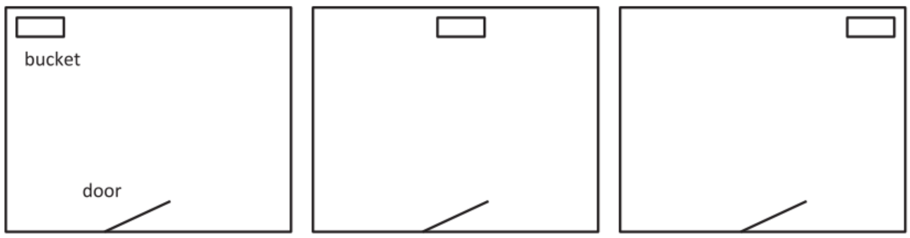


Fig. 1. The test pen with the three positions of the bucket according to the session: right or left side for the training and reminder sessions and the central position for the cognitive bias test session.

One video camera was placed above the door and connected to a video recorder and a screen located outside the test facilities. Each trial procedure included training, reminder, and test sessions. The procedure was the same in both trials except the number of training sessions (14 in the first trial and 4 in the second trial).

2.2.1 Training sessions

Pigs were trained individually on a go or no-go discrimination task (Mendl et al., 2009). Pigs had to learn to discriminate between a bucket with or without free access to chopped apples according to its position in the test pen (left or right side). In each training session, only one bucket was presented as a cue either with free access (A) to the chopped apples (rewarded reinforcer) or with a wire mesh covering the apples to prevent the animals from eating them (NA) (unrewarded reinforcer). The wire mesh was placed inside the bucket to ensure that pigs could only see it when they were in contact with the bucket. To avoid a lateralization effect, half of the pigs had the rewarded reinforcer on the right side and the unrewarded reinforcer on the left side, and vice-versa. The training sessions finished 30 seconds after the pig ate or tried to eat the chopped apples, or 10 minutes after entering the test pen if the pig did not contacted the bucket. Afterward, the test pen door was opened, and the animals were taken back to the housing pen. In the first training session, all pigs had access to chopped apples for 10 minutes. If the pig did not eat the chopped apples during the first 2 A sessions, the apples were put half a meter in front of the bucket for 2 minutes to encourage the pig to eat them and learn the association between the bucket and the food.

In consecutive training sessions, the bucket was randomly placed (no more than 2 consecutive sessions on the same side) on one side of the test pen according to the accessibility of the apples. The animals were subjected to 2 training sessions per day for 7 days (from 08:00 to 14:00 hours randomly). Before the CB test, all pigs were exposed to a total of 7 A and 7 NA training sessions.

2.2.2 Reminder sessions and test session

After the 14 training sessions, pigs were subjected to 4 reminder sessions (2 A and 2 NA randomly placed as in training sessions), 2 sessions per day for 2 days. Pigs that did not learn to discriminate between the 2 cues during the reminder sessions were excluded from the study. The criterion used to exclude pigs from the CB trial was the time to contact the bucket during the reminder sessions: if the mean time during the 2 A sessions was higher or equal than in the 2 NA sessions, the pig was excluded from the study.

The day after the 4 reminder sessions, each pig was individually subjected to the CB test session, where the bucket was placed in a central position in contact with the wall opposite the door. The bucket contained 2 pieces of apple that were not visible until the animal contacted the bucket. The CB test session finished when the pig ate the chopped apples or 10 minutes after the pig had entered the pen. Afterward, the test pen door was opened, and the animal was returned to the housing pen. Both reminder and test sessions were conducted during the same time frame as in the training sessions. After 5 weeks, pigs were subjected to a second trial which was conducted as the first trial, including training, reminder, and test sessions, but the number of training sessions were reduced to 4 (2 A and 2 NA).

2.2.3 Measures

Behavioral data were directly recorded by 4 observers, and video recordings were used to confirm the observations in case of doubt.

The following variables were recorded during all the sessions:

(1) time to contact the bucket
(2) time to eat or try to eat the chopped apples
(3) number of times the pig was reluctant to move [when the pig stopped for at least 2 seconds without showing exploratory behavior (Dalmau et al., 2009)]
(4) presence or absence of escape attempts (when the pig hit the door of the test pen)
(5) presence or absence of defecation and urination
(6) number of vocalizations

Individual cognitive bias classification

Pigs were classified according to the time to contact the bucket during the CB test session in relation to the reminder sessions following the formula described by Mendl et al. (2010):

Adjusted score = $[(x-y)/(z-y)] \times 100$

where x is time to contact the bucket during the CB test session; y is mean time to contact the bucket during the A reminder sessions; and z is mean time to contact the bucket during the NA reminder sessions.

If x = y the score was 0%, if x = z it was 100%. Categorical classification: if the adjusted score was ≤33.33%, the animal was classified as having a positive CB (PosB); if the result was between 33.33% and 66.66%, the animal was classified as having a neutral CB (NeuB); and if the score was ≥66.66%, the animal was classified as having a negative CB (NegB).

2.3 Statistical analyses

The statistical analysis was carried out with the Statistical Analyses System (SAS V9.2; software SAS Institute Inc., Cary, NC; 2002-2008). The significance levelwas established at $P < 0.05$, and a tendency was considered at $0.05 \leq P \leq 0.1$.

Data were analyzed separately in the first and the second trial. Each pig was introduced in the models as the experimental unit. The means of time in seconds are always presented with the standard error (mean ± SE).

For the training sessions, a normality test of data and residuals was performed for every variable. Time to contact the bucket, time to eat or try to eat, number of times the pig was reluctant to move, and number of vocalizations showed a Poisson distribution according to Cameron and Trivedi (1998). Presence or absence of escape attempts, and defecation and urination events, had a binomial distribution. All variables were analyzed using the GENMOD procedure. The fixed effects included in the models were as follows: observer (observer 1 to 6), session (session 2 to 14 in the first trial and session 2 to 4 in the second trial), and access to food (access or no access) and all pairwise interactions. The first training session in the first and the second trial was not included in the analyses because all pigs had access to chopped apples.

Time to contact the bucket in A and NA reminder sessions and in CB test session was compared between trials using a GENMOD procedure. Animals were included in the model as a repeated measure.

To assess the consistency of the CB test, Spearman correlation coefficient was determined for the time to contact the bucket during the CB test session of the first and second trial, and the same coefficient was determined for the adjusted score. Kappa coefficient (k) was determined for the categorical classification.

The guidelines developed by Landis and Kock (1977) were used to interpret the k-values: poor agreement ($k < 0.00$), slight agreement ($k = 0.00$-0.20), fair agreement ($k = 0.21$-0.40), moderate agreement ($k = 0.41$-0.60), substantial agreement ($k = 0.61$-0.80), and almost perfect agreement ($k = 0.80$-1).

3. Results

3.1 First CB trial

The mean time to contact the bucket was significantly lower in A than in NA from session 10 (S10) onward (49.63 ± 5.58 seconds and 119.00 ± 9.08 seconds, respectively; χ^2 = 10.44, P < 0.0012). Therefore, it was considered that pigs learn to discriminated between A and NA from this session (Fig. 2A). The time to eat or try to eat was also affected by the interaction between accessibility of food and session. The time to eat or try to eat was significantly lower during A sessions compared with NA sessions (68.69 ± 17.01 seconds and 184.67 ± 15.94 seconds, respectively; χ^2 = 15.13, P < 0.0001) from S10 onward (Fig. 2B).

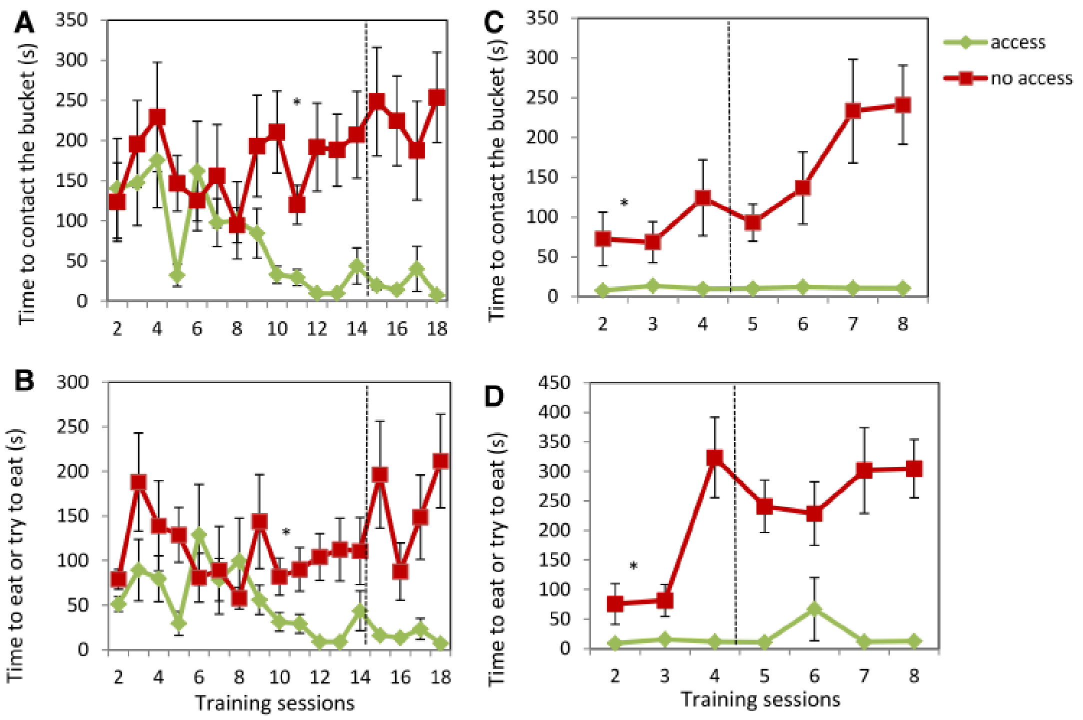


Fig. 2. Results of the training sessions for the first trial (A) and (B) and for the second trial (C) and (D). Panels A and C show the time to contact the bucket (mean and SE; in s), and panels B and D show the time to eat or try to eat (mean and SE; in s) of all animals according to the accessibility of food and sessions. The discontinuous line delimits between the training sessions and the reminder sessions. The asterisk shows the session from which the differences of the time to contact the bucket between access and no access were significantly different.

Comparing NA with A training and reminder sessions, pigs showed a higher number of vocalizations (9.83 ± 1.05 and 3.25 ± 0.68, respectively; χ^2 = 2452.06, P = 0.0024) and a higher prevalence of escape attempts (16% and 3%, respectively; χ^2 = 304.15, P = 0.0039) and defecation events (23% and 10%, respectively; $\chi^2 = 429.13$, P = 0.0039). The number of times pigs were reluctant to move and the number of urination events were not affected by session either accessibility of food. According to the results of the reminder sessions, 3 of the 36 pigs did not learn to discriminate between A and NA position, and were removed from the study. Regarding the CB test session, the mean time to contact the bucket and to eat or try to eat was 19.69 ± 8.01 seconds and 58.3 ± 23.30 seconds, respectively. Five animals were classified as NegB (15.15%) and 28 as PosB (84.85%). No pig was classified as NeuB. The mean time to contact the bucket was 49.40 ± 19.77 seconds for NegB and 15.96 ± 3.13 seconds for the PosB pigs. The mean ± SE of the adjusted score of CB of the animals classified as NegB and PosB was 146.80% ± 29.73 and -10.18% ± 5.01, respectively.

3.2 Cognitive bias test: Second trial

One of the 33 pigs failed to learn and was removed from the study. The time to contact the bucket was affected by the interaction between accessibility of food and session. The mean time to contact the bucket was lower in all A sessions compared with all NA sessions (10.40 ± 3.30 seconds and 132.23 ± 16.57 seconds, respectively; $\chi^2 = 12.35$, P < 0.005). Animals were able to discriminate between A and NA from training session 2 onward (P = 0.005) (Figure 2C). The time to eat or try to eat was lower during A sessions compared with NA sessions (19.62 ± 9.92 seconds and 222.22 ± 49.91 seconds, respectively; $\chi^2 = 12.66$, P < 0.0005) (Figure 2D).

During the CB test session of the second trial, the mean time to contact the bucket and to eat or try to eat of all animals was 13.27 ± 3.65 seconds and 17.52 ± 4.54 seconds, respectively.

One of the 32 pigs was classified as NegB (3.13%), one as NeuB (3.13%), and 30 as PosB (93.95%). The animal classified as NegB took 22 seconds to contact the bucket, the animal classified as NeuB took 14 seconds, and the mean time to contact the bucket ± SE of all PosB animals was 13.20 ± 3.65 seconds. The adjusted scores of the CB test were 254.55% for the animal classified as NegB, 36.00% for the animal classified as NeuB, and the mean ± SE was -0.85% ± 12.51 for the animals classified as PosB.

3.3 Consistency between first CB trial and second CB trial

No correlation was found between the time to contact the bucket (r = - 0.139; P = 0.448) and the CB adjusted score r = - 0.009; P = 0.957) of each animal during the first and the second trials. A poor agreement (k = - 0.072; P = 0.276) was found between the results of the CB classification obtained in both trials. Otherwise, 78.13% of the pigs remained in the same category in both trials. Comparing the results of all the pigs together between trials, the mean time (in seconds) to contact the bucket for the CB test session was 19.70 ± 5.93 for the first trial and 12.70 ± 3.35 for the second trial (χ^2 = 2.83, P = 0.123), for the A reminder sessions was 15.16 ± 6.86 for the first trial and 10.51 ± 1.76 for the second trial (χ^2 = 3.17, P = 0.082) and for the NA reminder sessions was 180.07 ± 50.04 for the first trial and 237.04 ± 57.38 for the second trial (χ^2 = 0.06, P = 0.358).

4. Discussion

4.1 Training sessions

This study shows that most pigs are able to learn to discriminate between rewarded and unrewarded cues based on their location after several training sessions (at least 10) in accordance with the results of Düpjan et al. (2013). In addition, after 5 weeks (second trial) without training, pigs were able to remember (training session 2) the location task (Arts et al., 2009).

The number of vocalizations and attempts to escape and defecation events were only affected by the accessibility of food and therefore were not valid behavioral measures to determine in which session pigs were able to discriminate both cues. Time to contact the bucket and time to eat or try to eat showed an interaction effect between accessibility of food and session. Therefore, these 2 measures were useful to determine in which session the animals were able to discriminate between the 2 cues. Time to contact the bucket showed less variability between pigs than time to eat or try to eat, probably because time to eat or try to eat was affected not only by the location of the cue but also by the content of the bucket (chopped apples or a wire mesh covering the apples).

Therefore, we decided to use time to contact the bucket as a measure to assess the learning process and the CB.

Assessing the time to contact the bucket, a tendency was found between trials regarding the A reminder sessions, being the time of the second trial lower than the first one; in contrast, no differences were found regarding NA reminder sessions. These results suggest that pigs improve the learning process to associate a cue with a rewarded reinforcer over the trials, although this improvement does not appear in front an unrewarded reinforcer. A possible explanation is that the magnitude of the reward of the rewarded cue is probably higher than the punishment associated with the unrewarded one (Mendl et al., 2009).

4.2 Reminder sessions and cognitive bias test session

The formula described previously provides an adjusted score that expresses the latency of the CB test session as a percentage of the difference between each pig's baseline mean latencies to the rewarded and unrewarded locations (Mendl et al., 2010). Regarding the results of the CB test, a high percentage of pigs were classified as PosB (84.85% and 94.29% during the first and the second trial, respectively). Moreover, latencies for the CB test session were closer with latencies for A reminder sessions than with latencies for NA reminder sessions in both trials. Haselton and Nettle (2006) suggest that when faced with an ambiguous cue, animals are better off if they respond as if the cue was rewarded provided the benefit of success outweighs the cost of failure. In this study, the mild severity of the unrewarded reinforcer might predispose the animal to assume a certain risk when exposed to the ambiguous cue (Ortony et al., 1988; Rolls, 2005; Mendl et al., 2009). Moreover, it is possible that pigs tend to be optimistic as an adaptive response (Metcalfe, 1998) to cope with difficult environments, such as the intensive farming systems. Furthermore, those pigs were reared on a commercial farm during the first 8 weeks of life and moving them to the experimental facilities could result in an improvement of the conditions in which they were confined. This fact is supported by the hypothesis that previous environment, even if it is better or worse than the current one, can alter the perception of the animal and hence its CB (Bateson and Matheson, 2007; Douglas et al., 2012).

A second trial was performed to assess the consistency of the CB over time. In the second trial, a higher percentage of pigs showed a positive bias in front of the ambiguous cue compared with the first trial. In fact, there were no correlation and poor agreement between the results of the first and the second trials, which accounts for the very low repeatability of the test.

Assessing repeatability for validating some behavior tests such as CB could be difficult because animals can be habituate to the test-situation (Forkman et al., 2007). Therefore, we decided to test the animals once per trial and with a period of 5 weeks between them. Moreover, although most of the CB studies have used more than one location for the CB test (e.g., Düpjan et al., 2013 and Murphy et al., 2013), we refused this option to remain, as much as possible, the ambiguity of the test. For the ambiguous cue we used a neutral stimulus (i.e., 2 pieces of apple) between the reward (chopped apples) and the punishment (chopped apples with a wire mesh covering them). A possible explanation could be that animals in the second CB test were able to remember the content of the bucket of the first CB test; therefore, the animals responded more positively, and no consistency was found between tests. In fact, other CB studies that retested the animals but using unreinforced ambiguous cues (Doyle et al., 2010;

Murphy et al., 2013), showed the opposite results, that is, displayed an increased reluctance to approach the ambiguous locations with repeated testing over time. In contrast, Douglas et al. (2012) suggested that since the go or no-go discrimination task was relatively hard for the pigs to learn, they learnt about the ambiguous cue equally slowly.

The perception of the ambiguous cue may also be changed due to uncontrolled factors such as the time of the day that pigs were tested (randomly selected from 08:00 to 14:00 hours), their age or even by their hunger state over time. This difference may have been due to the potential greater food motivation that older pigs have compared with younger ones (Murphy et al., 2013).

5. Conclusions

Our results suggest that pigs are able to discriminate between rewarded and unrewarded cues based on their location and that they remember even after 5 weeks. Most of the pigs were classified with a PosB. Consistency of the CB technique over time was not found. Further research is needed to understand the reasons that explain such lack of consistency.

Acknowledgments

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Chapter 2

Effect of gender and halothane genotype on cognitive bias and its relationship with fear in pigs

Chapter based on a paper published in Applied Animal Behaviour Science 2016 177, 12-18

Abstract

Cognitive bias (CB) has been recently proposed as a tool to study emotions by assessing the cognitive function through behaviour observation. It is based on the premise that subjects in a negative affective state perform more negative judgements about ambiguous stimuli than subjects in positive affective state. This study aimed at investigating if halothane genotype (homozygous Hal-free, NN vs. heterozygous Hal carrier, Nn) and gender (gilts, G vs. entire males, EM) affect the CB in pigs. Moreover, the results of the CB test (CBT) were compared with the results of a novel object test (NOT) in order to assess the influence of fear in the decision taken by pigs during the CBT. The results of both tests were contrasted with the concentration of brain neurotransmitters in four different brain areas in order to analyse the involvement of the dopaminergic and serotonergic pathways on the pigs' affective state and fear. A total of 48 pigs, in terms of 12 Hal-free gilts (NNG), 12 Hal carrier gilts (NnG), and 12 Hal-free entire males (NNEM) and 12 Hal carrier entire males (NnEM) were put on the CBT at the age of 20 weeks and on the NOT four days later. After two days, pigs were slaughtered and four brain structures (amygdala, prefrontal cortex, hippocampus and hypothalamus) were dissected for the analysis of brain neurotransmitters. The CBT and NOT results did not show any effect of the genotype and gender or their interaction on pigs' emotional response ($p > 0.10$). However, the CBT correlated positively with the NOT ($r = 0.49$; $p = 0.0005$), with pigs classified with a negative CB tending to be more fearful in front of the novel object than those with a positive CB ($p = 0.05$). Moreover, the pigs that took longer to get in contact with the novel object in the NOT also had lower ($p = 0.013$) concentration of dopamine in the prefrontal cortex and increased DOPAC/dopamine ratio in the hypothalamus ($p = 0.003$). These results suggest that fear level plays an important role in the decision taken by the pig dealing with ambiguous stimuli.

Keywords: Cognitive bias, halothane genotype, gender, fear, neurotransmitters

1. Introduction

The assessment of the affective state in non-human animals is a crucial goal in the study of animal welfare which has to be unequivocally achieved through behavioural and physiological measures. Several authors have reported a relationship between cognition and emotional state in animals (Boissy and Lee, 2014; Dantzer, 2002; Paul et al., 2005). The concept of cognitive bias (CB) refers to the influence of emotional state on cognition. Whereas, judgement bias, which is the most widely used technique to assess cognitive bias in animals, refers to the predisposition of an individual to show behaviours indicating anticipation of relatively either positive or negative outcomes in response to ambiguous stimuli (Mendl et al., 2009). Individuals in a negative affective state tend to perform more negative judgements about ambiguous stimuli than individuals in a positive affective state (Eysenck et al., 1991).

Comparing the CB test (CBT) with other techniques used for the assessment of the emotional state it provides a better measure of the valence of the emotion and allows distinguishing between positive and negative emotional states. Moreover, helps avoid the confounding effects of the novel environment on animal behaviour during the test as animals are habituated in a test arena before being tested, and is not invasive (Mendl et al., 2009; Murphy et al., 2014b). However, the CBT is not easily repeatable over time since its ambiguity for pigs is short-lived (Doyle et al., 2010), is time consuming and is difficult to perform in non-experimental, uncontrolled conditions (Murphy et al., 2014b; Seehuus et al., 2013).

A large number of studies have been carried out on CB in several species (Gygax, 2014) based on the methodology for its assessment in non-human animals developed by Harding et al. (2004). As yet, the CB has been used to assess the affective state of farm animals in response to extrinsic factors, such us environmental enrichment (Douglas et al., 2012; Wichman et al., 2012), isolation (Düpjan et al., 2013; Salmeto et al., 2011), restraint (Doyle et al., 2010; Murphy et al., 2013) and handling practices (Daros et al., 2014; Neave et al., 2013; Sanger et al., 2011), and to intrinsic factors, such as genotype, breed or gender.

Several studies have assessed if genotype that is related to anxiety or depression like behaviour have an effect in the response to CBT in rodents (Boleij et al., 2012; Enkel et al., 2010; Kloke et al., 2014; Richter et al., 2012). Other studies assessed the response to CBT between different genders of goats (Briefer and McElligott, 2013) and dogs (Müller et al., 2012). In pigs, the CBT was used to assess the effects of breed and birth weight on their emotional response (Murphy et al., 2013, 2014a). However, to our knowledge, the effects of the gender and the Hal gene on the pigs' response to the CBT are unknown.

The Halothane gene, referred to as the porcine stress syndrome gene, causes malignant hyperthermia, which is usually triggered by stress being more sever in pigs both Nn (heterozygous Hal carrier) and nn (homozygous Hal carrier) than in NN (homozygous Hal-free) (Rosenvold and Andersen, 2003). Indeed, the Hal carrier genotypes have been associated with greater stress sensitiveness in pigs. Moreover, Fàbrega et al. (2004) studied the effect of the Hal genotype in an open field test and demonstrated that Nn pigs performed more locomotive activity compared with NN pigs.

Regarding the gender, van Erp-van der Kooij et al. (2000) found that female piglets were more active than males in a backtest. Moreover other studies have related the gender with differences in emotional behaviour (Gray, 1971) and in the propensity to take risks (Felton et al., 2003).

According to Wichman et al. (2012) and Seehuus et al. (2013), CBT results should be interpreted with caution as they may be confounded by individual parameters, such as fearfulness. Fearfulness may, in fact, have an effect on the perception of the individual animal towards the test, thus influencing the final result of the CBT. An individual in an anxiety or fear-inducing situation may benefit from making 'safety first' judgements when facing ambiguous stimuli (Mendl et al., 2009), which may lead to the interpretation that the response of fearful and anxious individuals to such stimuli may be excessive.

The novel object test (NOT) is commonly used to assess fear or anxiety responses to unfamiliarity (Murphy et al., 2014b). Wichman et al. (2012) found a positive correlation between the results of the CBT and the level of fear assessed with a NOT in laying hens. To our knowledge, such a comparison was never made in pigs.

Brain areas, such as the prefrontal cortex and the amygdala, and the dopaminergic and serotonergic activities are involved in the judgement of ambiguous stimuli (Berridge, 2007; Schultz, 1997) and in the fear response (D'Angio et al., 1988; Davis et al., 1994). The animal's affective state or mood may modulate some or all activities of these brain areas (Mendl et al., 2009; Ruhé et al., 2007). For example, in mice studies, high brain dopamine levels have been associated with positive affective state (Ashby et al., 1999; Burgdorf and Panksepp, 2006), while the absence of D4Rs, a dopamine receptor, has been related to increased avoidance behaviour to novel stimuli (Dulawa et al., 1999; Falzone et al., 2002). To our knowledge, the involvement of brain neurotransmitters (NT) in the pigs' emotional state in response to the CBT or NOT has never been studied.

The overall objective of this study was to determine the CB in pigs of different gender and carrying or not carrying the Hal gene. In addition, the study aimed to assess to what extent pigs consider ambiguous the stimulus triggered by the contact with a novel object (known object but situated in a new location) in the CBT and the relationship between affective state and fear. Another goal was to assess the involvement of the dopaminergic and serotonergic pathways in the response to judgement and fear.

2. Material and Methods

2.1 Animals and housing conditions

In this study, 48 crossbred pigs (Landrace x Large White sows sired with Piétrain boars) were divided into four groups of 12 pigs each. Each group either consisted of Hal-free gilts, Hal-free entire males (NNG and NNEM), Hal carrier gilts and Hal carrier entire males (NnG and NnEM).

At 9 weeks of age, pigs were transported from a commercial farm to the experimental facilities of IRTA (Monells, Spain) and housed separately by gender and genotype in 8 pens (6 pigs per pen). Pigs were kept in pens (5 x 2.7 m) on fully slatted floor under natural light conditions and at a constant environmental temperature of 22 ± 3°C. Each pen was provided with one steel drinker bowl (15 x 16 cm) connected to a nipple and with a concrete feeder (58 x 34 cm) with four feeding places. Pigs had water and feed ad libitum. Pigs were inspected daily and no health problems were observed during the experimental period. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of IRTA.

2.2 Cognitive bias test

At 19 weeks of age, pigs were trained and tested individually for the CB according to the methodology described by Carreras et al. (2015). Twelve training sessions were performed, 6 with access and 6 without access to chopped apples (A and NA sessions, respectively). During the first training session all pigs had access to chopped apples for 10 min. If the pig did not eat them during the first and second A sessions, the apples were put 0.5 m in front of the bucket for 2 min in order to encourage the pig to eat them and learn the association between the bucket and the food. The remaining sessions finished 30 s after the pig ate (in the A sessions) or tried to eat the chopped apples, i.e. contacted the wire mesh with the snout (in the NA sessions) or 90 s after entering the test pen if the pig did not eat or try to eat. Pigs were subjected to two training sessions per day (from 07:00 a.m. to 04:00 p.m.) during 6 consecutive days. During the following two days, two additional A and NA reminder sessions were performed before the test session. The latency to contact the bucket, defined as the time pigs took from entering the test pen to the contact with the bucket, was recorded in all sessions. The pigs that did not learn to discriminate between the two cues after the reminder sessions were excluded from the CBT. The criterion used to exclude pigs from the CBT is described in Carreras et al. (2015). Briefly, pigs were excluded from the study if the mean time during the two A sessions was higher or equal than in the two NA sessions.

After the reminder sessions, each pig was individually subjected to a CBT that finished when the pig ate the chopped apples or 90 s after the pig had entered the test pen. At the end of the CBT, the test pen door was opened and the pig returned to the housing pen. The latency to contact the bucket was also recorded as in the previous sessions.

Pigs were classified according to the latency to contact the bucket during the CBT in relation to the reminder sessions according to the following formula (adapted from Carreras et al., 2015):

$x/[(y + z)/2]$ x 100 = adjusted score

where:

x = latency to contact the bucket during the CBT.

y = mean latency to contact the bucket during the A reminder sessions.

z = mean latency to contact the bucket during the NA reminder sessions.

If the adjusted score was ≤ 75%, the animal was classified with a positive CB (PosCB); if the score was between 75 and 125%, the animal was classified with a neutral CB (NeuCB); and if the score was ≥ 125% the animal was classified with a negative CB (NegCB).

2.3 Novel object test

The novel object test (NOT) was performed in the same test pen four days after the CBT. In this test the novel object was represented by a traffic cone located in the centre of the pen. The traffic cone was filled with concrete in order to prevent pigs from knocking it down for the contact. Each pig was individually kept in the test pen during 5 min. The latency to contact the novel object, defined as the time pigs took from entering the test pen to contact the traffic cone, was recorded. The number of times the pig contacting the novel object (maximum of five times), vocalizations and reluctant to move events (maximum of 20) were also counted. A reluctant to move event was defined as a pig stopping for at least 2 s without showing exploratory behaviour (Dalmau et al., 2009).

These behaviours were both directly noted by one trained observer and video recorded to ensure consistency in the results.

2.4 Brain neurotransmitters

Two days after the NOT pigs were fasted for 8 h, transported to the experimental slaughterhouse of IRTA (1.2 km trip), stunned by exposure to 90 % CO_2 at atmospheric air for 3 min and exsanguinated. Immediately after slaughter, the brain was removed and the selected brain structures (amygdala, hippocampus, hypothalamus and prefrontal cortex) were dissected and frozen immediately at -80°C until analysis.

Samples were weighed and homogenized (1:10 w/w) in an ice-cold 0.25 M perchloric acid containing 0.1 M $Na_2S_2O_5$ and 0.25 M ethylenediaminetetraacetate (EDTA). Dihydroxybenzylamine (DHBA) and Nω–metil-5-hydroxytryptamine (Nω) were added as internal standards for catecholamines and indolamines, respectively. The mixture was homogenized by sonication (Branson Digital Sonifier, model 250, Branson Ultrasonics Corp., Danbury, CT) followed by centrifugation at 3,000 g for 10 min at 4°C and the supernatant was kept frozen at -80°C. After centrifugation at 12,000 g for 10 min at 4°C, the concentrations of noradrenaline, dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and serotonin and its metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) were determined in 20 µL aliquots using high performance liquid chromatography (HPLC; Elite LaCHrom, Merck, Hitachi, Japan) equipped with a Chromolith Rp-18e 100 x 4.6mm column (Merck KgaA, Darmstadt, Germany) with electrochemical detection (ESA Coulochem II 5200, Bedford, MA). The mobile phase consisted of 0.5 M citrate buffer pH 2.8, 0.05mM EDTA, 1.2 mM sodium octyl sulphate (SOS) and 1% acetonitrile. The applied voltage was set at 0.4 mV and the flow rate was 1mL/min.

2.5 Statistical analyses

The statistical analysis was carried out with the Statistical Analyses System (SAS V9.2; SAS Institute Inc., Cary, NC, 2002-2008). The significance level was established at $p < 0.05$ and a tendency was considered at $0.05 \leq p \leq 0.10$. Descriptive data are presented with the means of time in s and the standard error (mean ± SE).

Normality test of data and residuals was performed for each measure. If data were not parametric, they were log transformed to correct the distribution and when possible parametric statistics were used. The latency to contact the novel object was normally distributed after a log transformation, whereas the latency to contact the bucket during the CBT and the latency to contact the bucket during the training sessions showed a Poisson distribution. The CB classification showed a multinomial distribution. Normally distributed measures were analyzed using the MIXED procedure with Tukey adjustment. Measures with Poisson or multinomial distributions were analyzed using the GENMOD procedure. In all models, each pig was considered as the experimental unit. The fixed effects included in the models were gender (entire male and gilt), genotype (NN and Nn) and their interaction. When the latency to contact the bucket during the training sessions was analyzed, the accessibility to the food (A and NA sessions), the session order (from 2 to 12) and their interaction were also treated as fixed effects. The first training session was not included in the analyses because in this session pigs had access to chopped apples. In the MIXED models, the housing pen was considered as a random effect.

The correlation between the latency to contact the novel object and the latency to contact the bucket during the CBT and the latency to contact the bucket during the A and NA reminder sessions was analysed with the CORR Spearman procedure. The effect of the CB classification (PosCB, NegCB and NeuCB) regarding the latency to contact the novel object was analyzed using the MIXED procedure. The numbers of contacts of the novel object (which followed a multinomial distribution) and the number of vocalizations and reluctant to move events during the NOT (which followed a negative binomial distribution) were analyzed using the GENMOD procedure. Each pig was the experimental unit and in the MIXED model the housing pen was considered as a random effect.

The levels of the NT in each brain area, expressed in ng of NT per g of tissue, were correlated with the latency to contact the bucket during the CBT using the CORR Spearman procedure. The correlations between the NT concentrations and the latency to contact the novel object during the NOT were calculated using the CORR Pearson procedure when NT concentrations followed a normal distribution, before or after a log transformation, and a CORR Spearman procedure when they were not parametric. The MIXED procedure was used to compare the NT concentrations between pigs classified as PosCB, NeuCB and NegCB when they followed a normal distribution. Whereas, the GENMOD procedure was used when NT concentrations did not follow a normal distribution and followed a negative binomial distribution.

3. Results

3.1 Cognitive bias test

The latency to contact the bucket during the A training sessions was lower ($p < 0.0001$) than in the NA sessions starting from training session 10 (30.38 ± 2.58 s vs. 58.41 ± 2.38 s; data not shown) meaning that pigs had learned to discriminate between A and NA at the end of the training programme. However, 9 pigs (3 NNG, 1 NnG, 2 NNEM and 3 NnEM) were not able to discriminate between A and NA positions during the reminder sessions and were, therefore, excluded from the CBT. No differences between genders ($p = 0.384$) and Hal genotypes ($p = 0.950$), and no effect of their interaction ($p = 0.456$) were found regarding the latency to contact the bucket during the training and reminder sessions.

The proportion of pigs classified as PosCB, NeuCB and NegCB for each treatment group is shown in Fig. 1. The proportion of CB classes was not affected by gender (p = 0.278), genotype (p = 0.358) and their interaction (p = 0.560).

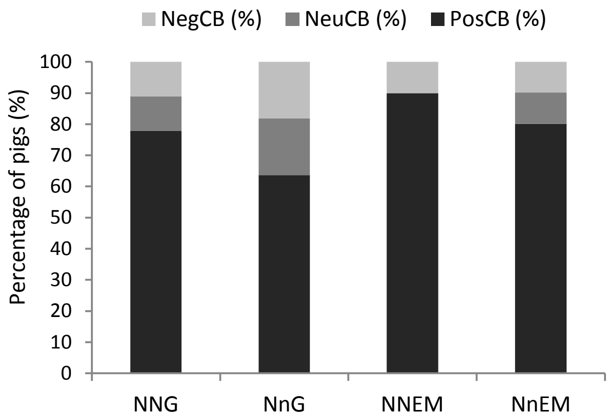

Fig.1. Percentage of pigs classified as negative (NegCB), neutral (NeuCB) and positive (PosCB) to cognitive bias according to gender (G = gilts and EM = entire males) and Hal genotype (NN = halothane-free, Nn = halothane carrier genotype)

The mean latency to contact the bucket during the CBT was 19.08 ± 3.23 s and was neither affected by gender (p = 0.610) nor genotype (p = 0.789). No significant interaction between these two factors was found either (p = 0.701). This latency was not different from the mean latency to contact the bucket during the A reminder sessions either (19.05 ± 3.64 s; p = 0.994), whereas it was different from the mean latency to contact the bucket during the NA reminder sessions (53.55 ± 4.34 s; p < 0.0001).

3.2 Novel Object test

The mean latency to contact the novel object during the NOT was 26.10 ± 2.98 s and was neither affected by the gender (p = 0.611) nor genotype (p = 0.228), no significant interaction between them was found either (p = 0.614).

The latency to contact the novel object during the NOT was positively correlated with the latency to contact the bucket during the CBT (r = 0.49; p = 0.0005). However, there was neither correlation between the latency to contact the novel object and the mean latency to contact the bucket during the A reminder sessions (r = 0.244; p = 0.1) nor with the mean latency to contact the bucket during the NA reminder sessions (r = 0.167; p = 0.256).

Regarding the CB classification, NegCB pigs tended to take longer (p = 0.05) to contact the novel object than PosCB pigs. Pigs of the NeuCB class did not differ in the latency to contact the novel object compared with NegCB and PosCB pigs (Fig. 2). Moreover, no differences were found in relation to the number of times that pigs contacted the novel object, the number of vocalizations and the number of reluctant to move events during the NOT (Table 1).

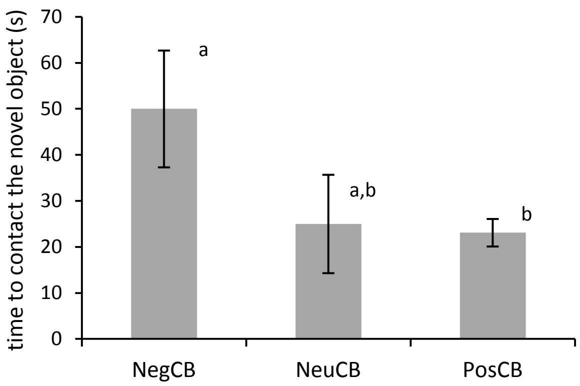

Fig.2. The mean time ± Standard Error in s of the latency to contact the novel object during the novel object test of the animals classified as negative (NegCB), neutral (NeuCB) and positive (PosCB) to cognitive bias. Different letters show significant differences ($p < 0.05$).

Table 1. Variation of behaviours (mean ± S.E; in s) between pigs classified as negative (NegCB), neutral (NeuCB) and positive (PosCB) to the cognitive bias test during the novel object test

	NegCB	NeuCB	PosCB	P value
Contacts to the novel object, n	3.25 ± 0.48	4.60 ± 0.24	3.63 ± 0.24	0.704
Vocalizations, n	5.75 ± 3.33	6.4 ± 3.66	5.63 ± 1.27	0.839
Reluctant to move events, n	1.75 ± 0.63	1.00 ± 0.77	2.20 ± 0.63	0.470

3.3 Brain neurotransmitters

The NT levels did not vary between CB classes (Table 2). Moreover, there was no significant correlation between the time to contact the bucket in the CBT and any NT in any of the four brain areas (amygdala, prefrontal cortex, hippocampus and hypothalamus; $p > 0.10$). Whereas, in the NOT, significant correlations were found between the latency to contact the novel object and some brain NT, with pigs showing a longer latency to contact the novel object presenting lower concentration of dopamine in the prefrontal cortex ($r = -0.374$; $p = 0.013$) and higher DOPAC/dopamine ratio in the hypothalamus ($r = 0.466$; $p = 0.003$).

Table 2. Concentrations of the neurotransmitters (mean ± S.E.; in ng/g tissue)) in each brain region of negative (NegCB), neutral (NeuCB) and positive (PosCB) to cognitive bias test pigs.

	Variable[a]	NegCB	NeuCB	PosCB	p value
Amygdala	NA	213.7 ± 23.09	146.8 ± 9.60	210.9 ± 11.71	0.106
	DA	416.7 ± 66.42	366.3 ± 39.77	464.1 ± 27.79	0.673
	DOPAC	416.7 ± 66.42	84.3 ± 7.69	90.2 ± 4.97	0.718
	HVA	294.5 ± 28.44	364.4 ± 26.07	358.9 ± 16.02	0.445
	5-HT	1229.4 ± 226.41	942.0 ± 80.78	1153.1 ± 57.46	0.532
	5-HIAA	315.3 ± 226.42	294.4 ± 80.78	316.8 ± 57.46	0.866
Prefrontal cortex	NA	174.1 ± 14.95	164.1 ± 17.29	161.4 ± 4.78	0.531
	DA	13.0 ± 1.18	18.7 ± 6.39	25.4 ± 4.10	0.337
	DOPAC	8.2 ± 2.14	15.2 ± 4.82	15.3 ± 1.77	0.266
	HVA	28.6 ± 4.58	67.1 ± 20.26	71.1 ± 6.99	0.112
	5-HT	343.9 ± 28.05	310.1 ± 60.57	323.0 ± 8.13	0.264
	5-HIAA	108.9 ± 28.05	111.7 ± 60.57	114.8 ± 8.13	0.636
Hippocampus	NA	170.2 ± 20.57	193.2 ± 20.57	177.9 ± 7.10	0.621
	DA	28.5 ± 5.25	27.9 ± 4.07	27.2 ± 1.08	0.984
	DOPAC	3.1 ± 1.36	4.0 ± 0.90	3.4 ± 0.29	0.641
	HVA	26.8 ± 2.26	30.8 ± 5.09	31.1 ± 1.87	0.747
	5-HT	355.8 ± 39.07	388.6 ± 18.80	336.3 ± 13.18	0.396
	5-HIAA	126.6 ± 39.07	150.2 ± 18.80	133.0 ± 13.18	0.548
Hypothalamus	NA	2092.9 ± 559.29	2268.4 ± 524.60	2495.9 ± 182.75	0.669
	DA	695.1 ± 59.69	537.9 ± 158.12	540.7 ± 57.62	0.687
	DOPAC	130.7 ± 50.95	77.0 ± 26.57	93.6 ± 9.87	0.636
	HVA	429.6 ± 55.01	382.3 ± 75.76	419.9 ± 36.91	0.894
	5-HT	821.8 ± 151.80	620.4 ± 131.74	771.2 ± 60.97	0.601
	5-HIAA	647.7 ± 151.79	304.4 ± 131.74	447.5 ± 60.98	0.299

[a]NA: noradrenaline; DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; 5-HT: serotonin; 5-HIAA: 5-hydroxyindole-3-acetic acid

4. Discussion

A positive correlation between the latency to contact the bucket during the CBT and the latency to contact the novel object during the NOT was found in this study. Wichman et al. (2012) also observed a similar correlation between the latency to peck at the bowl near a rewarded ambiguous cue in the CBT and the latency to contact the novel object in laying hens. In agreement with Wichman et al. (2012), our results suggest that fear plays an important role in the CBT outcomes. The pigs classified as NegCB in this study took more time to contact the novel object during the NOT, which indicates that they were more fearful in front of a novel stimulus than PosCB pigs.

Although CBT and the NOT results are correlated, no correlation was found between the latency to contact the novel object during the NOT and the latency to contact the bucket during the A and NA reminder sessions of the CBT. These results suggest that the ambiguous stimulus during the CBT is perceived by pigs as more novel than the known rewarded and unrewarded stimuli during the reminder sessions (Mendl et al., 2009). This correlation, thus, can be explained by the individual differences in the fear state while judging the ambiguous stimuli. Therefore, our results suggest that responses in front of ambiguous stimuli are more affected by the level of fear rather than other intrinsic factors, such as gender or genotype.

Interestingly, pigs showing a longer latency to contact the novel object in the NOT also presented decreased dopamine concentration in the prefrontal cortex and increased DOPAC/dopamine ratio in the hypothalamus. This physiological response may be associated with the greater level of fear in front of the unknown in these pigs. The greater DOPAC/dopamine ratio results from the relatively decreased dopamine levels with respect to its metabolite DOPAC may suggest that the dopamine degradation pathway to DOPAC and HVA is more active than the degradation pathway to noradrenaline (Kandel et al., 2013). Dopamine is involved in the mechanisms modulating states of fear and anxiety (Espejo, 2003), and, more specifically, dopaminergic activity plays a role in fear development, expression and extinction. Dopamine activity in the prefrontal cortex is involved in the neuromodulation of cognitive functions and its role is critical for fear extinction (Abraham et al., 2014). Indeed, prefrontal cortex dopamine is increased following fear extinction (Espejo, 2003). Dulawa et al. (1999) found similar results in mice comparing the behavioural response in front a NOT between D4R-knock-out (inhibition of a dopamine receptor) and wild-type mice. They found that D4R-knock-out mice showed decrease exploration in front novelty. Moreover, Falzone et al. (2002) also found that D4R-knock-out mice showed heightened avoidance to the more fearprovoking areas of the elevated plus maze and the light/dark preference exploration test compared with the control group.

Likewise Carreras et al. (2015), at the end of the training period of this study, pigs were able to learn to discriminate between a positive and a negative cue based on its location. However, this learning capability was independent from their gender and genetic background. Müller et al. (2012) also failed to find an effect of the gender on the learning process of dogs in the CBT. Whereas, Briefer and McElligott (2013) reported a faster learning skill in female than in male goats during the training for the judgement bias task. Sex differences in spatial abilities have been also largely reported in rodent species, with males giving better results than females (Roof, 1993; Williams and Meck, 1991). Furthermore, the lack of difference of learning capability between NN and Nn pigs was also reported by Dantzer and Mormede (1978). The low level of handling stress during the training sessions and the habituation of the pigs to the test conditions during the first training sessions of the CBT may have levelled the differences between the two genotypes in this study.

No difference was found between genotypes and genders in CB during the CBT in this study. Murphy et al. (2013) did not find differences in judgement bias between pigs and minipigs either. The lack of the effect of the Hal gene on pigs' CB as assessed in the CBT is surprising based on the results of a number of behaviour studies indicating a greater locomotive activity and exploratory behaviour in pigs carrying the Hal gene (nn or Nn) compared with non-carrier genotypes (Fàbrega et al., 2004; Robert and Dallaire, 1986; Schaefer et al., 1989). These results either suggest that Nn and NN pigs do not differ in the affective state or that the CBT efficiency is species-specific and thus may not be an appropriate method for the assessment of CB in pigs. Indeed, the CBT successfully showed differences between genotypes in rat studies. Enkel et al. (2010) and Richter et al. (2012) using the "learned helplessness" genetic rat model of depression in which the experience of uncontrollable stress leads to a helpless state with depression-like symptoms found a more negative response in front of an ambiguous stimulus in helpless rats than non-helpless rats.

The lack of difference in the affective state as assessed by the CBT between entire males and gilts found in this study agrees with other previous behavioural studies involving these two genders of pigs (Hessing et al., 1993; van Erp-van der Kooij et al., 2000). Studies on dogs and goats using CB techniques did not find gender differences either (Briefer and McElligott, 2013; Müller et al., 2012).

In this study, 76.9 % of pigs responded positively in front of the ambiguous cue. Moreover, the mean latency to contact the bucket during the CBT did not differ from the mean latency to contact the bucket during the A reminder sessions, while it did during NA reminder sessions. These results suggest that the mild severity of the unrewarded reinforcer may predispose the pig to assume little risk when exposed to the ambiguous cue (Mendl et al., 2009). Therefore, the motivation for exploration exceeds the potential fear aversion of the stimulus (Asano, 1986). If a more severe unrewarded reinforcer, such as an air puff (Brajon et al., 2015; Svendsen et al., 2012) or an electric shock (Enkel et al., 2010; Rygula et al., 2012), was applied fewer pigs may have been classified as PosCB in this study.

In agreement with the results of the behavioural tests, pigs showing different CB classification did not present differences in NT in any of the brain areas analysed. These results are opposite to the results of Doyle et al. (2011) that showed that sheep with administration of serotonin inhibitor (p-Chlorophenylalanine) had a more negative CB compared with the control group. Moreover, Sharot et al. (2012) showed that the administration of a drug that enhances dopaminergic function (dihydroxy-L-phenylalanine; L-DOPA) increased the positive bias in humans, reducing negative expectations regarding the future. The lack of consistency between the CBT and NT levels in the present study may indicate that, as stated above, the mild severity of the unrewarded reinforcer or the overlapping effect of the slaughter could mask or prevent conspicuous changes in NT.

5. Conclusions

The results of this study showed that pigs' emotional response as assessed using the cognitive bias and the novel object tests is neither affected by the gender nor by the halothane genotype. A positive correlation was found between CBT and NOT suggesting that fear level plays an important role in the decision taken by the pig in dealing with an ambiguous stimuli. Pigs showing longer latency to contact the novel object also presented decreased dopamine concentration in the prefrontal cortex, supporting the evidence of the paper of dopamine in the fear response. However further research on both behaviour and related brain NT activity is necessary to confirm this interpretation.

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Chapter 3

The effect of previous handling experience on cognitive bias, novel object and defence cascade tests, and on cortisol concentration in pigs

Chapter based on a paper submitted to Animal Cognition

Abstract

So far, research on human-animal relationship in animal production has been mainly focused on its effect on stress, productivity and meat quality. Only few studies have assessed its effects on the animals' affective state. In the present study, the influence of positive and negative handling (PH and NH, respectively) on affective state and fear as assessed by the cognitive bias test (CBT), the novel object test (NOT) and the defences cascade test (DCT) was studied in 56 female pigs. Handling treatments were preceded a pre-treatment CBT and were applied for 6 weeks before a second CBT, a NOT and a DCT. Serum, saliva and hair were sampled during the behavioural tests and serum was collected at exsanguination for the analysis of cortisol concentration. Results showed no differences between PH and NH pigs in the behavioural tests ($p > 0.05$), which may be either due to the lack of previous handling effect on the test results, the lack of validity or the low sensitivity of these tests or a combination of them. Moreover, no differences were found in serum, salivary and hair cortisol concentration between handling groups ($p > 0.05$). However, correlations between tests were found ($p < 0.05$) suggesting that there are other individual factors beyond the handling treatment, such as the fear level, the motivation or the coping style, that have a similar effect on the response to these tests. Moreover, more fearful pigs, as assessed in the NOT, had higher ($r = 0.37$; $p = 0.014$) levels of serum cortisol at slaughter.

Keywords: handling, cognitive bias, affective state, fear, cortisol, pigs

1. Introduction

Exposure to humans is one of the potentially most frightening events that many farm animals experience in their life (Waiblinger et al., 2006). It has been reported that human-animal relationship does not only affect animal welfare, as showed by increased corticosteroids concentrations (Hemsworth and Barnett, 1991; Probst et al., 2013), but also productivity (Day et al., 2002; Hemsworth, 2003; Paterson and Pearce, 1992; Rushen et al., 1999) and meat quality (Geverink, et al., 1998). However, to our knowledge, only one study focused on the assessment of its effect on the animal affective state (Brajon et al., 2015).

Recently, methods based on the study of cognitive changes have been used to assess emotion and mood states in non-human animals. One of these methods is the cognitive bias test (CBT), which is based on the premise that subjects in negative affective states make more negative and pessimistic judgements about ambiguous stimuli than subjects in positive affective states (Mendl et al., 2009). Since Harding et al. (2004) work, a large number of studies on cognitive bias have been carried out in different species (i.e. dogs, monkeys and rats; Gygax, 2014; Mendl et al., 2010; Müller et al., 2012; Rygula et al., 2012), including several farm livestock species (Baciadonna and McElligott, 2015). Brajon et al. (2015) compared the influence of previous positive and negative handling (PH and NH, respectively) on the affective state of piglets using a CBT and found that PH induced a more optimistic response in front of the ambiguous stimulus than NH. Whereas, Briefer Freymond et al. (2014) found that mares trained with negative reinforcement were more optimistic when facing an ambiguous stimulus than the ones trained with positive reinforcement. Authors suggested that mares trained with negative reinforcement may have been more motivated to obtain a food reward than those trained with positive reinforcement as the latter ones were used to be rewarded throughout the treatment phase.

The term “defence cascade response” has been defined as a continuum of innate, hard-wired, automatically activated defence behaviours (Kozlowska et al., 2015). However, the behaviours included in the assessment of the defence cascade response vary between studies. Gratton et al. (2007) included freezing, flight or fight, and tonic immobility, while Kozlowska et al. (2015) included arousal, flight or fight, freezing, and tonic, collapsed and quiescent immobility. Recently, defence cascade response has been proposed as a tool to assess the emotional state in pigs (Statham et al., 2013), as it has been suggested to reflect affective state in both humans and rodents (Lang et al., 1990; 2000). Statham et al. (2015) proposed a test to assess the defence cascade response (DCT) in pigs which involves the assessment of an initial response (magnitude of startle), an evaluation phase (duration of freeze) and a final response (defensive or resume behaviour) and found that pigs raised in barren conditions decreased the magnitude of startle in their initial response compared with pigs raised in enriched conditions.

The novel object test (NOT) is a widely used technique to assess fear or anxiety responses to unfamiliarity (Murphy et al., 2014). Hemsworth et al. (1996) found that positively handled pigs were quicker to approach the novel stimulus in the NOT than minimally handled pigs. However, the same study failed to find differences in the NOT in cows with different handling experience.

A positive correlation (r = 0.39; p = 0.05) between the latency to contact the bowl in an ambiguous location during the CBT and the latency to contact the novel object during the NOT showing the role of fear in the decision making was reported in laying hens by Wichman et al. (2012).

The effects of negative handling and human-animal interaction on serum and saliva cortisol concentrations in serum and saliva has been studied extensively (Bergamasco et al., 2010; Hemsworth et al., 1986a,b; Paterson and Pearce, 1992). However, both serum and saliva samples provide a measurement of the cortisol concentrations at a single point in time (Russell et al., 2012). Although a multi-sampling over time is possible for saliva and serum, animals have to be subjected to invasive collection techniques and thus it can alter the results. Hair cortisol it has recently been proposed as a biomarker of chronic stress to overcome this issue (Stalder and Kirschbaum, 2012). The effect of human animal interaction in serum (e.g. Hemsworth et al., 1986a,b; Paterson and Pearce, 1992) and saliva (e.g. Bergamasco et al., 2010) samples has been measured in several studies. However, to our knowledge, the effect of human animal interaction has not been assessed in hair cortisol in any animal species.

The hypothesis of this study was that pigs with a PH experience resulting in positive affective state have a more optimistic response in the CBT, are less reactive to a sudden stimulus and resume the activity faster in the DCT, are less fearful in the NOT and have lower concentrations of cortisol than pigs with a NH experience.

Thus, the present study was aimed to assess the effect of PH vs. NH on the pigs' affective state, as assessed by the CBT, the fear response, as assessed by the NOT and a DCT was performed to assess the fear response presumably affected by the affective state. Another objective of this study was to study the effects of the handling on the variation of cortisol concentration through the tests and correlate it with the CBT, NOT and DCT behavioural observations.

2. Material and Methods

2.1 Animals and housing conditions

In this study, 56 female pigs ((Landrace x Large White) x Piétrain) were used. At 9 weeks of age, pigs were transported from a commercial farm to the experimental facilities of IRTA (Monells, Spain) and housed in two rooms of four pens with seven pigs each. Pens (5 x 2.7 m) had fully slatted floor and were under natural light conditions at a constant environmental temperature (22 ± 3 °C). Each pen was provided with one steel drinker bowl (15 x 16 cm) connected to a nipple and a concrete feeder (58 x 34 cm) with four feeding places. Pigs had water and food *ad libitum*. Pigs were inspected daily and no health problems were observed during the experimental period. At 27 weeks of age, pigs were transported to the experimental slaughterhouse of IRTA (1.2 km trip). On arrival, pigs were immediately driven to a CO_2 stunner and exposed to 90% CO_2 for 3 min before exsanguination.

The study was approved by the Institutional Animal Care and Use Committee (IACUC) of IRTA.

2.2 Treatments

Treatments started when pigs aged 19 weeks and lasted until 27 weeks of age (slaughter age). The pigs in one of the rooms received positive handling (PH) and the pigs in the other room received negative handling (NH).

During these 9 weeks, one experimenter entered five days per week for approximately 30 min each day in each room between 9:00 am and 5:00 pm. The time to perform the handling treatment and the order to enter the room and the pens was randomly distributed each day.

The PH treatment consisted in entering the room calmly and letting the pigs realize about the presence of the experimenter in the room before entering the pens. After 1 min, the experimenter entered the first pen and walked slowly around its perimeter. Then, the experimenter stopped at one corner of the pen and adopted a steady squat posture during 5 min, touching and interacting gently with the approaching pigs. Once finished, the experimenter stood up and tried to have a contact with the remaining pigs as long as they did not escape. This procedure was carried out in each pen of the room. The positive interaction consisted in gently stroking pigs on the nose and behind the ears and, whenever possible, on the back from head to tail in a uniform manner.

The NH treatment consisted in entering the room roughly and talking loudly. Inside the pen, after having walked around it and stopped at its centre, the experimenter performed one of the five types of negative interactions (Table 1) with all pigs of the pen. To prevent pigs from getting used to negative handling, the experimenter did one different negative interaction each day of the week following a random order each week.

Table 1. Description and duration of negative interactions applied to pigs in the NH treatment

Interaction	Description	Duration/pig (s)
Water pressure	Hurling pressure water onto pigs' body with a hose	20
Air pressure	Hurling pressure air onto pigs' body with an air gun	20
Loud noise	Using a horn in front of pigs' faces	20
Immobilization	Holding the neck of pigs with both hands	20
Restraint	Restraining pigs between a board and a corner of the pen	40

2.3 Behavioural tests

Pigs were subjected to three different behavioural tests, namely the cognitive bias test (CBT), the novel object test (NOT) and the defence cascade test (DCT). The CBT was carried out twice: at 16 weeks of age before starting the treatments in order to have a pre-treatment measure (CBT1) and at 26 weeks of age (CBT2). The NOT and DCT were performed 3 and 4 days after the CBT2, respectively (Table 2). Before carrying out each CBT some training and remaining sessions were necessary. In all tests, training and remaining sessions, pigs were individually moved from the housing pen to a 31.5 m^2 test pen (4.7 x 6.7 m) separated from the housing pens by a corridor (6.4 x 2.2 m). The floor of the test pen was partially slatted with a corridor of concrete (6.4 x 2.3 m) in the middle. In the test pen pigs were kept at a temperature of 22 ± 3 °C.

Table 2. Experimental timeline. The first row shows the age of the pigs in weeks, each week being represented with a box. The second row shows the pre-treatment and treatment phase. The third row shows all procedures carried out

Age (weeks)												
15	16	17	18	19	20	21	22	23	24	25	26	27
Pre-treatment Phase				Treatment Phase								
T1[a]	R1[b] CBT1[c]	S1[f]								T2[a] R2[b] CBT2[c]	NOT[d] DCT[e] S2[f]	SLA[g] S3[f]

[a]T1: first training sessions; T2: second training sessions

[b]R1: first reminder sessions; R2: second reminder sessions

[c]CBT1: first cognitive bias test; CBT2: second cognitive bias test

[d]NOT: novel object test

[e]DCT: defence cascade test

[f]S1: first hair, saliva and serum sampling; S2: second hair, saliva and serum sampling; S3: third serum sampling

[g]SLA: slaughter

2.3.1 Cognitive bias test

Pigs were trained and assessed through the CBT individually according to the methodology described by Carreras et al. (2016). In brief, pigs were trained to discriminate between a bucket with or without access (A and NA, respectively) to chopped apples and positioned in the left or right side of the test pen. Ten training sessions (five A and five NA) were performed during the CBT1 and this number was reduced to four training sessions (two A and two NA sessions) for the CBT2. Pigs were subjected to two training sessions per day (from 08:00 am to 14:00 pm) during 5 days in the case of the CBT1 and during 2 days in the case of the CBT2. During the next 2 days, two additional A and NA training sessions, called reminder sessions, were performed before the CBT1 and CBT2. The latency to contact the bucket, defined as the time taken by pigs from the entrance into the test pen to contact the bucket, was recorded in all sessions.

The pigs that did not learn to discriminate between the two cues (the bucket with and without access to chopped apples) after the reminder sessions of the CBT2 were excluded from the experiment. Pigs were also excluded from the study if the mean time spent in the two A sessions was equal or longer than in the two NA sessions (Carreras et al., 2015).

At the end of the reminder sessions, each pig was individually subjected to the CBT where a bucket containing two apple chops was placed in the middle of the pen. The test ended once the pig had finished eating the chopped apples or 90 s after the pig had entered the test pen. The latency to contact the bucket was recorded as in the previous sessions.

2.3.2 Novel object test

In this test a traffic cone filled with concrete and positioned in the middle of the test pen was used as a novel object and a line was drawn on the floor at 1 m distance around it. The latency to cross this line and the latency to contact the novel object, defined as the time taken by the pig from the entrance into the test pen to crossing the line around the novel object and to contact the traffic cone, were recorded. The number of times the pig touched the novel object (maximum of five times) was also assessed. At the end of the test (5 min) each pig was moved back to the housing pen.

2.3.3 Defence cascade test

As in the CBT sessions, for the defence cascade test, a bucket with chopped apples was placed in the middle of the pen against the wall opposite to the gate and a line was drawn on the floor at 1 m distance around it. A ball filled with bells and tied to a rope was hung inside the bucket. When the pig crossed the line with the two front legs, the hanging ball withdrew upward above the animal and behind the wall (Fig. 1). The behavioural response of pigs was divided into three stages: an initial response, an evaluation response and a final response. The initial response was assessed as presence or absence of a flight response to the movement of the ball, which was defined as a change of orientation of the pig body in relation to the position of the bucket. The evaluation response was defined as the time lapsed from the first time the pig crossed the line around the bucket (i.e. before the ball appeared) until it crossed the line for a second time. If the pig did not cross the line a second time, a maximum time of 5 min was assigned. In case the pig stayed inside the semi-circle after the movement of the ball it was considered that it had taken 1 s to cross it. The final response considered if the pig had crossed (resume response) or not (defensive response) the line around the bucket for a second time. The pig that had not crossed the line any time after 5 min was excluded from the DCT.

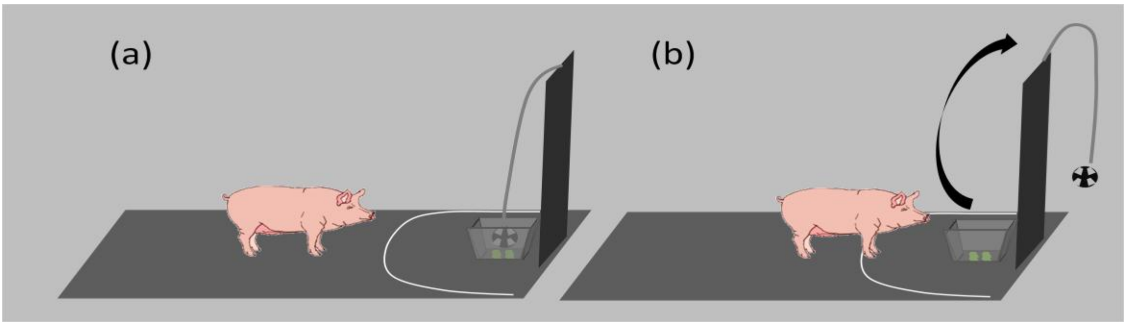

Fig. 1. Sketch of the test pen during the defence cascade test (DCT). (a) Before the pig crossed the line around the bucket, i.e. the ball filled with bells was inside the bucket. (b) After the pig had crossed the line around the bucket, i.e. the ball filled with bells was withdrawn and moved upward behind the wall. The arrow shows the trajectory of the ball.

2.4 Cortisol samples

For the analysis of cortisol concentration, serum, saliva and hair samples were collected twice during the experiment, while a third sample of serum was collected at exsanguination (Table 2). On farm, saliva samples were collected by allowing pigs chew a cotton swab during approximately 30 s. The saliva was stored in Salivette tubes (Sarstedt, Nümbrecht, Germany) and later centrifuged at 3,000 x g for 10 min. Saliva samples were then collected and stored at -80°C until analysis. Blood samples were collected by cava venepuncture in 10-mL tubes without anticoagulant using a vacutainer needle. In this case two experimenters were necessary, one taking the blood and the other restraining the pig using a snare. Blood was allowed to clot and serum was obtained by centrifugation at 2,000 x g for 10 min and immediately frozen at -80°C until analysis. Hair was collected by shaving the lumbar area of pigs restrained in a scare. At the slaughterhouse, blood samples were collected at exsanguination from each pig in 10-mL tubes without anticoagulant. Serum were obtained by centrifugation at 2,000 x g for 10 min and immediately frozen at -80°C until analysis. Cortisol concentrations were determined using commercial ELISA kits, namely DRG Cortisol ELISA (DRG Diagnostics, Marburg, Germany) for serum samples, DRG Salivary Cortisol ELISA (DRG Diagnostics, Marburg, Germany) for salivary samples and High Sensitivity Salivary Cortisol EIA Kit (Salimetrics, State Collage, PA, USA) for hair samples.

2.5 Statistical analyses

The Statistical Analyses System (SAS V9.2; software SAS Institute Inc., Cary, NC; 2002-2008) was used to analyse data and the significance threshold was 0.05. Descriptive data is presented with the means of time in seconds and the standard error (mean ± SE).

The latency to contact the bucket during the CBT1 and CBT2, the latency to cross the line around the novel object and the latency to contact the novel object during the NOT showed a normal distribution after a log transformation. Similarly, cortisol concentrations of serum, saliva and hair samples were normally distributed after a log transformation and removal of the outliers.

The evaluation phase of the DCT showed a negative binomial distribution, while the number of times that pigs contacted the novel object presented a multinomial distribution. A binomial distribution was applied for the learning process during the reminder sessions (yes or not) of the CBT and for the initial and final response in the DCT.

2.5.1 Effect of handling

Multiple Generalized Linear Mixed Models were performed separately for each measure using the GLIMMIX procedure. The least square means of fixed effects (LSMEANS) achieved using Tukey adjustment HSD test for pair-wise comparisons were used when the analysis of variance indicated significant differences.

In all the statistical analyses, the pig was the experimental unit, the treatment (PH vs. NH) was included as a fixed effect and the housing pen as a random effect. Moreover, for the latency to contact the bucket during the CBT1 and CBT2, the interaction effect between handling treatments and between CBT trials (CBT1 vs CBT2) was also analysed. The latency to contact the bucket during the CBT1 was included in the model as a co-variable. For cortisol concentrations, the sampling, the treatment and their interaction was introduced in the model as fixed effect.

2.5.2 Relationship between behavioural tests and cortisol concentration

Correlations were analysed using a Pearson correlation procedure between the latency to contact the bucket in the CBT2 and the latency to cross the line around the novel object in the NOT, as both measures followed a normal distribution. All other measures assessed in the behavioural tests were correlated using the Spearman correlation procedure as at least one of the correlated measures not followed a normal distribution.

The effect of the initial and the final responses of the DCT was analysed based on the latency to contact the bucket during the CBT2 and the latency to cross the line around the novel object, and the latency to contact the novel object in the NOT using a MIXED procedure with Tukey adjustment. The latency to contact the bucket during the CBT and the evaluation phase of the DCT were compared between the pigs that contacted different times to the novel object using a GLIMMIX procedure.

Saliva, serum and hair cortisol concentrations were correlated with the latency to contact the bucket during the CBT, the latency to cross the line around the novel object and the latency to contact the novel object during the NOT using the CORR Pearson procedure, while their correlation with the evaluation phase of the DCT was calculated using the CORR Spearman procedure.

The effect of the initial and final response of the DCT on the cortisol concentrations was analysed using a MIXED procedure with Tukey adjustment.

3. Results

3.1 Effect of handling

3.1.1 Cognitive bias test

Of the 56 pigs, 19 pigs (8 NH and 11 PH pigs; 33.9% of the total) did not learn to discriminate between the two cues during the CBT1. During the CBT2 this number was reduced to 14 pigs (6 NH and 8 PH pigs; 25% of the total). The proportion of pigs that completed the training sessions correctly during the CBT2 did not differ between the handling groups (p = 0.541).

No differences in the latency to contact the bucket were found between the handling groups during the CBT2 (Table 3). However, when comparing the CBT1 and CBT2, the time to contact the bucket was lower (p = 0.0007) in CBT2 than in CBT1 in both groups (11.8 ± 1.78 s vs. 22.6 ±

4.14 s for the NH group and 16.9 ± 3.93 s vs. 24.4 ± 3.39 s for the PH group; data not shown). Moreover, no interaction was found between the handling treatment and the CBT test (CBT1 and CBT2) results.

3.1.2 Novel object test

In this study, the handling treatment had no effect on the behaviour of pigs during the NOT ($p > 0.05$; Table 3).

3.1.3 Defence cascade test

Three pigs were excluded from the DCT because they did not cross the line around the bucket during the 5 min test. No effect of the handling treatment on the behaviour of pigs as assessed during the DCT was found ($p > 0.05$; Table 3).

Table 3. Comparison of behaviours as assessed during the second cognitive bias test (CBT2), the novel object test (NOT) and the defence cascade test (DCT) between the positive and the negative handling (NH and PH) groups.

Test	Measure	n	NH	PH	P -value
CBT2	Latency to contact the bucket[a], s	42	11.8 ± 1.78	16.9 ± 3.93	0.852
NOT	Latency to cross the line around the novel object[b], s	56	17.7 ± 4.47	9.5 ± 4.47	0.187
	Latency to contact the novel object[c], s	56	37.4 ± 11.33	26.8 ± 6.84	0.994
	Number of contacts to the novel object[d], n	56	4.0 ± 0.24	4.0 ± 0.22	0.888
DCT	Flight response[e], %	53	81.48	61.54	0.119
	Evaluation response[f], s	53	96.4 ± 22.98	78.8 ± 20.23	0.467
	Defensive response[g], %	53	22.22	19.23	0.734

[a]Latency to contact the bucket: mean time pigs took from entering the test pen to contact with the bucket in seconds
[b]Latency to cross the line around the novel object: mean time pigs took from entering the test pen to cross the line 1 meter around the novel object in seconds
[c]Latency to contact the novel object: mean time pigs took from entering the test pen to contact with the novel object in seconds

[d]Number of contacts to the novel object: number of contacts the pigs performed during the 5 min test
[e]Flight response: percentage of pigs that change the orientation of the body in relation to the position of the bucket after the movement of the ball
[f]Evaluation response: mean time lapsed from the first time the pigs crossed the line around the bucket until they crossed the line for a second time in seconds
[g]Defensive response: percentage of pigs that not resumed the activity, that is, that not crossed the line around the bucket for a second time during the 5 min test

3.1.4 Cortisol concentration

Either due to sensitivity of the analytical technique or insufficient sample quantity, two samples obtained on the second serum sampling, seven samples on the first saliva sampling, five on the second saliva sampling, four on the first hair sampling and one on the second hair sampling were missed.

As shown in Table 4, the handling treatment did not affect salivary, serum and hair cortisol concentrations in this study ($p > 0.05$).

However, within each handling treatment groups, the serum cortisol concentration was higher ($p < 0.0001$) during the third sampling than in the first and the second sampling. Whereas, hair cortisol concentrations decreased between the first and the second samplings ($p = 0.007$). No differences in saliva cortisol were found between the first and the second samplings ($p > 0.05$).

Table 4. Comparison of the serum, saliva and hair cortisol concentrations (mean ± SE) at each sampling between negative and positive handling (NH and PH) groups

Cortisol samples	Sampling	n	NH	PH	P-value
Serum (ng/mL)	First	55	23.5 ± 2.22	17.6 ± 3.80	0.686
	Second	49	24.2 ± 2.01	21.2 ± 1.89	1.000
	Third	55	51.3 ± 3.70	53.5 ± 3.42	0.688
Saliva (ng/mL)	First	48	3.5 ± 0.29	4.3 ± 0.52	0.633
	Second	46	3.6 ± 0.18	3.6 ± 0.25	0.998
Hair (pg/mg)	First	52	24.7 ± 1.94	19.8 ± 3.02	0.446
	Second	55	20.8 ± 2.00	17.6 ± 1.51	0.500

3.2 Relationship between behavioural tests

3.2.1 Relationship between cognitive bias test and novel object test

The latency to contact the bucket during the CBT were positively correlated with the latency to cross the line around the novel object (r = 0.37; p = 0.013) and the latency to contact the novel object in the NOT (r = 0.40; p = 0.0075) (Table 5).

During the CBT the pigs that contacted the novel object only one time took more time to contact the bucket (42.00 ± 11.45 s; data not shown) than those that contacted it four and five or more times (14.47 ± 2.82 s and 17.32 ± 4.43 s, respectively; p = 0.0045 and p = 0.0046, respectively; data not shown).

3.2.2 Relationship between cognitive bias test and defence cascade test

During the CBT, no difference in the time to contact the bucket was found between the pigs that performed or not a flight response (18.30 ± 3.60 s vs. 19.87 ± 5.12 s; p = 0.865; data not shown). The correlation between the time to contact the bucket during the CBT and the evaluation response during the DCT was of low magnitude and non-significant (r = 0.13; p = 0.42; Table 5). However, those pigs that showed a resume response during the DCT took less time to contact the bucket during the CBT than those that performed a defensive response (13.55 ± 2.17 s vs. 38.55 ± 9.64 s; p = 0.0008; data not shown).

3.2.3 Relationship between novel object test and defence cascade test

No differences were found between the pigs that performed or not a flight response regarding the latency to cross the line around the novel object (p = 0.724), and the latency and the number of times to contact it (p = 0.261 and p = 0.398, respectively).

No significant correlation was found between the evaluation response during the DCT and the latency to cross the line and the latency to contact the novel object during the NOT (p = 0.93 and p = 0.06, respectively; Table 5). The evaluation response did not have an effect on the number of contacts to the novel object either (p = 0.832). The pigs that performed a resume response took less time to contact the novel object than pigs that performed a defensive response (23.89 ± 5.72 s vs. 62.64 ± 13.88 s; p = 0.016). Moreover, the time to cross the line around the novel object and the number of contacts to the novel object was not affected by the final response in the DCT (p = 0.137 and p = 0.10, respectively).

Table 5. Pearson correlations between measures of the cognitive bias test (CBT), the novel object test (NOT) and the defence cascade test (DCT)

Test	Measure		CBT	NOT		DCT
			Latency to contact the bucket	Latency to cross the line around the novel object	Latency to contact the novel object	Evaluation response
CBT	Latency to contact the bucket	r P n	-	0.37 0.013 42	0.40 0.0075 42	0.13 0.42 41
NOT	Latency to cross the line around the novel object	r P n		-	-	0.01 0.93 53
	Latency to contact the novel object	r P n			-	0.26 0.06 53
DCT	Evaluation phase					-

3.3 Relationship between behavioural tests and cortisol concentrations

Any of the behaviours assessed in the CBT, NOT and DCT was significantly correlated with the cortisol concentrations in the serum, salivary and hair collected in the second sampling. However, significant, although rather weak, correlations were found between serum cortisol concentrations of the third sampling and the latency to cross the line around the novel object (r = 0.37; p = 0.014) and the latency to contact the novel object (r = 0.31; p = 0.02) during the NOT.

4. Discussion

The present study failed to find differences in the behavioural response as assessed in the CBT, DCT and NOT between PH and NH pigs. This result can be either explained by the lack of effect of the previous handling experience on the behaviours assessed in these tests or by the lack of validity or the low sensitivity of the behavioural tests to assess such effect (Martin and Bateson, 1993).

The effect of handling on pig behaviour is well known (Hemsworth, 2003). However, its effects can be modulated by the type and intensity of the handling treatment, with long lasting and more intense handling treatment producing the greater effects on the animals (Hemsworth et al. 1986a). Our results may thus suggest that in this study the handling treatments were not applied over a sufficiently long period (6 weeks) or were not sufficiently intense to have an effect on the behavioural response to the following tests in these pigs. Furthermore, in this study, the pigs' response to these tests may have been also biased by the frequent and gentle handling applied on these pigs during the training sessions in preparation of the CBT.

The lack of effect of different treatments on the affective state of pigs as assessed by the CBT is in agreement with several previous studies in pigs (Carreras et al., 2016; Düpjan et al., 2013; Murphy et al., 2013; Scollo et al., 2014), which makes the effectiveness of the CBT for the assessment of the affective state of pigs questionable. Brajon et al. (2015) reported a more positive judgment bias as assessed by the CBT in PH piglets compared with NH and minimal handling (MH) ones. The discrepancy in the results may be explained by the different age of pigs used in the two studies, i.e. 25 days piglets in Brajon et al. (2015) study vs. 19 weeks old pigs in ours. Greater effects of management have been, in fact, reported on pigs during their first period of life than later (Hemsworth et al., 1986b; Hemsworth and Barnett, 1992).

The handling treatments applied in this study did not alter the defence cascade response in pigs either. These results are in contrast with Statham et al. (2015) who found that the magnitude of the first response and the freezing time was lower in pigs raised in barren than in enriched conditions. The diversity of enrichment factors they included as a treatment: bedding, enrichment material, lighting, feeding and human interactions compared with the only effect of handling carried out in the present study could explain such differences on the results.

The NOT has been already validated and used to assess the effect on behaviour of pigs subjected to different types of treatment in previous studies (Dalmau et al., 2009; Murphy et al., 2014). However, to our knowledge, only Hemsworth et al. (1996) specifically used it to assess the effects of handling on the affective state in pigs showing a quicker approach to the novel object in PH pigs than in MH ones. The present study failed to find similar results, probably because pigs were trained and tested, receiving a positive reinforcement, in the cognitive bias test (CBT1 and CBT2) twice before being exposed to the NOT in the same test pen, which may have biased the pigs' response during this test.

Similarly to Brajon et al. (2015), no differences were found between PH and NH during the training before the CBT2, which indicates the lack of effect of the handling treatment on the pigs' learning process likely due to the previous experience and the memory of the training during the CBT1.

The effects of handling stress on the physiological response of pigs have been reported in a number of studies (Hemsworth, 2003; Hemsworth et al. 1986a, 1987). However, as for behaviours in this study, serum, saliva and hair cortisol concentrations were not affected by the handling treatment, suggesting that the handling treatment applied in the present study was not sufficiently intense or long to alter the pigs' physiological response. Paterson and Pearce (1992) also failed to find differences in serum cortisol concentrations between pigs subjected to different handling treatments.

Serum cortisol concentrations were higher in the third sampling compared with the first and second one in both handling treatments groups, probably because the third sampling was performed at slaughter, which is a more stressful environment than the farm (Gregory, 1998). In the present study, in fact, more fearful pigs (based on the NOT results) had higher levels of serum cortisol at slaughter.

Similarly to previous studies on humans (Dettenborn et al., 2012; Fourie and Bernstein, 2011) and cows (González-de-la-Vara et al., 2011), this study shows that hair cortisol concentration decreases over a period of 9 weeks (between 17 and 26 weeks of age).

A positive correlation was found between the latency to contact the bucket during the CBT and the latency to cross the line and to contact the novel object during the NOT. Similar correlations were also found in a study on laying hens (Wichman et al., 2012). These results suggest that fear level plays an important role in the decision taken by the animal dealing with ambiguous stimuli (Wichman et al., 2012). Furthermore, the pigs that contacted more times the novel object also took less time to contact the bucket during the CBT, which suggests that motivation and curiosity prevail over avoidance and fear in pigs with more positive affective state when confronted with a novel object (Archer, 1973). Regarding the relationship between the CBT and DCT, animals with more positive affective state tend to resume the activity in the DCT and take less time to contact the bucket in the CBT. These results suggest that individual factors, beyond the handling treatment, such as fearfulness or coping style, may affect the animal affective state as reflected in these behaviours.

5. Conclusions

The present study failed to find an effect of the previous handling experience (positive vs. negative) on the affective state and fear of pigs as assessed through the CBT, NOT and DCT tests, probably due to 1) a real lack of effect of handling on the affective state of pigs; 2) too short and/or insufficiently intense handling sessions; 3) poor validity or the low sensitivity of these behavioural tests to assess such effect; 4) a combination of all these factors. The first and second hypothesis would be supported by the cortisol concentrations, as no differences between handling treatments were found, and by the correlations between tests suggesting that individual factors, other than the handling treatment, such as the fear level, the motivation or the coping style, may have an effect on the behavioural and physiological response in pigs. Further research on the effect of individual factors, such as fear or motivation, should be carried out to confirm their role in the pigs' behavioural responses.

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Chapter 4

Housing conditions do not alter cognitive bias but affect serum cortisol, qualitative behaviour assessment and wounds on the carcass in pigs

Chapter based on a paper submitted to Applied Animal Behaviour Science

Abstract

Measures of animal emotions are essential to assess animal welfare. Recently, the cognitive bias technique has been proposed as a measure of animal affective state. This technique is based on the premise that subjects in negative affective states make more negative judgements about ambiguous stimuli than subjects in positive affective states. In the present study, 44 female pigs were divided into two groups of equal size (22 pigs each): one group was allocated in enriched housing conditions (more space allowance, presence of straw and solid floor) and the other in barren housing conditions (lower space allowance and slatted floor) in order to induce differences in the affective state. Three cognitive bias tests (CBT) were performed: 1 week before starting the housing conditions (CBT1) and 1 and 5 weeks (CBT2 and CBT3, respectively) after it. Moreover, 3 and 4 days after each CBT, a qualitative behaviour assessment (QBA) and a serum sampling for the assessment of cortisol concentration were carried out. Finally, the number of wounds was counted on the pig carcass at slaughter. The results showed that the cognitive bias did not differ between treatment groups in any of the two CBT carried out after starting the housing conditions ($p > 0.05$). However, during the CBT2 and CBT3 when compared with the barren group the enriched group presented a lower concentration of serum cortisol ($p = 0.008$ and $p = 0.011$, respectively), a higher QBA score ($p = 0.022$ and $p = 0.027$, respectively) and a lower number of wounds on the carcass ($p = 0.05$). Considering the QBA, serum cortisol and skin wounds results, the CBT used in this study was not valid or sensitive enough to assess the variation in the affective state between pigs raised in different housing conditions.

Keywords: cognitive bias, pigs, housing conditions, qualitative behaviour assessment, cortisol, carcass' wounds

1. Introduction

Assessing the affective state in animals has become one of the most important challenges for the animal welfare scientific community during the last decades. As animals cannot report their feelings, scientists have developed and tested several behavioural and physiological measures in order to assess animal emotions. Although these measures offer a great deal of information, to fully understand animals' affective state the cognitive component must be also considered (Paul et al., 2005). The term cognitive bias has been used as a general label for the effects of the affective state on the cognitive processes (Mendl et al., 2009). A large number of studies in humans have reported how emotional states can bias different cognition processes, such as attention, memory or judgement (Bradley et al., 1997; Mineka et al., 1998). Based on these findings, Hardling et al. (2004) first reported the effect of the affective state on judgment in rats being exposed to unpredictable housing using a cognitive bias test (CBT). Their results showed that rats exposed to unpredictable housing, widely demonstrated that induce a mild stress/depression-like state, perceived the ambiguous stimulus as more negatively than rats being exposed to predictable housing. Since this first experiment, a large number of studies have been carried out in different species (for a review see Baciadonna and McElligott, 2015; Gygax, 2014; Mendl et al., 2009). Among the different treatments that have been performed to induce changes in affective state, the manipulation of housing conditions is one of the most used. Indeed, the effect of different housing conditions on the cognitive bias has been assessed in several species, such as rats (Brydges et al., 2011; Burman et al., 2008), European starlings (Bateson and Matheson, 2007; Matheson et al., 2008), sheep (Vögeli et al., 2014), laying hens (Wichman et al., 2012), horses (Löckener et al., 2016) and pigs (Douglas et al., 2012; Scollo et al., 2014). Some of these studies confirmed the hypothesis that animals raised in better housing conditions have a more positive cognitive bias (Bateson and Matheson, 2007; Brydges et al., 2011; Douglas et al., 2012; Löckener et al., 2016; Matheson et al., 2008), while others failed to report so (Burman et al., 2008; Scollo et al., 2014; Wichman et al., 2012).

Providing the housing pen with enrichment, and particularly with rooting material, such as straw, is known to have a positive effect on pigs' welfare (De Jong et al., 1998; Scott et al., 2006). Indeed, straw offers a recreational and nutritional stimulus, and bedding (Fraser et al., 1991). Moreover, floor characteristics are known to have an effect on some behavioural patterns, the type and the prevalence of body lesions, and the health of pigs (Kilbride et al., 2009). For example, pigs housed on slatted floor reduced significantly the time devoted to floor exploration, redirecting this behaviour towards other pen features and their pen mates (Averós et al., 2010a). Higher stocking density also has a negative effect on pigs based on both behavioural and physiological indicators (Averós et al., 2010a,b; Jensen et al., 2010). Indeed, stocking density is known to be an important risk factor for tail-biting (Moinard et al., 2003).

Scientific evidences on the effects of stocking density on cognitive bias in pigs are unclear, ranging from no effect (Scollo et al., 2014) to a more positive cognitive bias in pigs provided with more space, solid floor, straw and objects to manipulate compared with those raised at lower space allowance and on partially slatted floor (Douglas et al., 2012). Some authors suggest that changing housing conditions may have an immediately effect on affective state, although this effect appears to disappear over time (Löckener et al., 2016). Whereas, other authors suggest that to alter the affective state through the housing conditions, animals must experience a drop or an improvement in the environmental quality (Bateson and Matheson, 2007).

The qualitative behaviour assessment (QBA) is a method based on the ability of human observers to integrate perceived animal behaviours using 20 descriptors such as 'relaxed', 'tense', 'frustrated' or 'active'. Previous research has shown that this technique has high inter- and intra- observer reliability and to be coherent with other behaviour and welfare measures (Rousing and Wemelsfelder, 2006; Wemelsfelder et al., 2001). Temple et al. (2011) found that Iberian pigs raised in intensive conditions were in more positive mood than Iberian pigs raised in extensive conditions according to the QBA results.

Animals raised in barren housing conditions are supposed to have higher concentrations of serum cortisol since they are potentially exposed to more stressful situations (Möstl and Palme, 2002). However, the results about the effects of the environmental enrichment in the pen on serum cortisol concentration in pigs are rather inconclusive, ranging from a positive effect (Janssens et al., 1994), to none (Pearce and Paterson, 1993; Scollo et al., 2014) or negative (De Jong et al., 1998). Indeed, there are a number of factors which can influence the concentration of this indicator such as the time of day/night, physical activity, health, feeding or temperature and humidity, hindering the assessment of a specific treatment effect on this hormone.

Barren housing conditions are known to promote harmful social behaviour, aggressions and higher stress levels in pigs, leading to alter carcass and meet quality (van de Weerd and Day, 2009). One of the measures that can be used to assess such effects is the number of wounds on the carcass, however other factors, such as, the way pigs are transported from the farm to the slaughterhouse and the mixture of the pigs during the lairage at the slaughterhouse can mask the effects caused by the type of housing conditions.

The aim of the present study was to assess if pigs raised in enriched housing conditions, i.e. lower stocking density, solid floor and presence of straw, showed a more positive affective state as assessed by the CBT and QBA and had lower serum cortisol concentration and number of wounds on the carcass than those raised in more barren housing conditions, i.e. higher stocking density, slatted floor and no provision of enrichment material.

2. Material and methods

2.1 Animals and housing conditions

A total of 44 female pigs aged 8 weeks originating from the same commercial farm were housed in four pens of 11 animals each at the experimental facilities of IRTA (Monells, Spain). Pigs were crosses of Large White × Landrace with Piétrain heterozygous. During 7 weeks, pigs were kept in the same housing conditions, which consisted in a full slatted floor with a density of 1.2 m^2/pig. During the following 8 weeks, in two pens this density was maintained (enriched treatment), but the floor changed to concrete and 700 g of straw/pig were provided every 2-3 days, while in the other two pens space allowance was reduced to 0.7 m^2/pig (barren treatment). During the experiment, pigs were housed under natural light conditions at a constant environmental temperature of 22 ± 3°C. Each pen was provided with one steel drinker bowl (15 x 16 cm) connected to a nipple and a concrete feeder (58 x 34 cm) with four feeding places. Pigs had water and food *ad libitum* and were inspected daily. At 23 weeks of age, pigs were transported to the experimental slaughterhouse of IRTA (1.2 km trip). Afterwards, a 1 h lairage was carried out maintaining the housing pen groups and pigs were stunned by exposure to 90 % CO_2 at atmospheric air for 3 min and exsanguinated afterwards. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of IRTA.

2.2 Cognitive bias test

Three cognitive bias tests (CBT) were carried out: one 2 weeks before changing the housing conditions (CBT1) and the other two after 1 and 5 weeks (CBT2 and CBT3, respectively) while pigs were in the new housing conditions. Pigs were trained and tested individually for the CBT according to the methodology described by Carreras et al. (2016).

2.2.1 Habituation and training sessions

Before carrying out the training and the test sessions, pigs were habituated to the test pen (habituation sessions), first in groups, that is, all the pigs of each pen were moved from the housing pen to a 31.5 m^2 test pen (4.7 length x 6.7 m width) and remained there for 1 h during

2 consecutive days. During the 2 following days, pigs were habituated to the test pen individually, that is, each pig was moved from the housing pen to the test pen and remained there for 5 min. During the individual habituation sessions, a bucket with chopped apples was presented in front of one side (left or right) of the wall opposite to the pen door. The bucket was presented on the left side for half of the pigs and on the right side for the other half, following a balanced pattern for each housing pen. If the pig did not eat the chopped apples after the 5 min of habituation an experimenter entered the pen and put three apple chops half meter in front of the bucket. Later on, the experimenter left the pen and 3 more min were provided. After the habituation sessions, pigs performed 44 training sessions, 22 with access to chopped apples (rewarded session; R) and 22 without access to chopped apples (punishment session; P). A line was drawn on the floor 1 m around each bucket location. During the P sessions, when the front legs of a pig crossed the line around the bucket, an air puff was blown from the wall just over the bucket to the pig's face until it escaped (i.e. crossed back the line). Half of the pigs had the R location on the right and the other half had it on the left side according to the position of the bucket during the habituation sessions. Each pig performed two R and two P sessions per day. The training sessions finished 30 s after the pig crossed the line around the bucket with the front legs or 90 s after entering the test pen if the pig did not cross it. The latency to cross the line around the bucket, defined as the time taken by the pigs from the entrance into the test pen to cross the line around the bucket with the front legs, was recorded in all sessions. At the completion of the training sessions, the CBT1 was performed. Twelve training sessions (6 R and 6 P) were carried out before both the CBT2 and CBT3. Pigs that did not learn to discriminate between R and P after the end of each training sessions were excluded from the CBT. Following the criterion described by Carreras et al. (2015), pigs were excluded from the CBT if the mean time spent to cross the line around the bucket in the last two R sessions was equal or longer than in the last two P sessions.

2.2.2 Cognitive bias test (CBT)

The three CBT were performed following the same procedures. Two R, two P and one test session were performed each day during 5 days. Those R and P sessions were defined as reminder sessions. The order of these five sessions was random for each day. There were three test locations, one in the middle between R and P (M test) that was presented for 3 days, one between the R and the middle (R test) and the other between the P and the middle (P test) that were presented one day each. An M test was presented to all pigs during the first day, the other two M tests and the P test and R test were randomly distributed during the other 4 days. In all tests the bucket was empty. The sessions finished 30 s after the pig crossed the line around the bucket with the front legs or 90 s after entering the test pen if the pig did not cross it. As in the training sessions, the latency to cross the line around the bucket was recorded in all sessions.

2.3 Qualitative behaviour assessment (QBA)

Three QBA were carried out during the experiment, 3 days after each CBT (QBA1, QBA2 and QBA3). The QBA was performed following the Welfare Quality® protocol (Wefare Quality®, 2009) using descriptive terms with an expressive connotation to reflect animals' experience of a situation (Wemelsfelder, 2007). Each pen was observed for 10 min by a trained observer and a rating scale of 125 mm was used to score the pigs in each pen based on 20 behaviour terms, namely active, relaxed, fearful, agitated, calm, content, tense, enjoying, frustrated, sociable, bored, playful, positively occupied, listless, lively, indifferent, irritable, aimless, happy and distressed. A global score for each pen was obtained introducing all term scores in a formula described in the Welfare Quality® Assessment protocol for pigs (Welfare Quality®, 2009). Based on this formula, the higher the score obtained, the higher is the positive emotional state. More detailed information about the overall methodology of assessment and score obtained can be found in the Welfare Quality® Assessment protocol for pigs (2009).

2.4 Serum cortisol concentration analysis

Serum samples were collected on farm at three different times, 4 days after each CBT (S1, S2 and S3) and at the time of slaughter (S4) in all pigs. Blood samples were collected in 10 mL tubes without anticoagulant by cava venepuncture using a vacutainer needle at the farm and during exsanguination at the slaughterhouse. Blood was allowed to clot and serum was obtained by centrifugation at 2,000 x g for 10 min and it was frozen at -80°C until analysis. Cortisol concentrations were determined using commercial ELISA kits (DRG Diagnostics, Marburg, Germany).

2.5 Wounds on the carcass

The number of wounds on the carcass in each pig was assessed using the Welfare Quality® protocol for pigs (Dalmau et al., 2009) considering five anatomical regions (ears, front, middle, hind-quarters and legs) on one side of each carcass. Scores of 0 (<2 lesions in all regions), 1 (2-10 lesions in at least one region) and 2 (>10 lesions in at least one region) were given for each carcass by a trained observer on the slaughter line after scalding.

2.6 Statistical analyses

The Statistical Analyses System (SAS V9.2; software SAS Institute Inc., Cary, NC; 2002-2008) was used to analyse data and the significance threshold was ≤ 0.05. Descriptive data are presented with the mean and the standard error (mean ± SE).

Normality test of data and residuals was performed for each measure. If data were not parametric, they were log transformed to correct the distribution and, when possible, parametric statistics were used. The latency to cross the line around the bucket during the CBT showed a Poisson distribution and was analyzed using a GLIMMIX procedure, whereas serum cortisol concentration and the QBA score were normally distributed after a log transformation and was analyzed using a MIXED procedure. The score obtained from the number of wounds on the carcass showed a multinomial distribution and was analyzed using a GLIMMIX procedure. Each pig was considered as the experimental unit in all models, except for the QBA, whose data were analysed considering the housing pen as the experimental unit. When the latency to cross the line around the bucket during the training sessions was analyzed, the accessibility to the food (R and P sessions), the session order and their interaction were treated as fixed effects. In the analysis of the latency to cross the line around the bucket during each reminder (R and P) and test sessions (R test, P test and M test), the treatment (enriched vs. barren housing), the CBT (CBT2 and CBT3) and their interaction were included as fixed effects and the latency to contact the bucket during the CBT1 was considered as a co-variable. In the analysis of the serum cortisol concentrations, the treatment (enriched vs. barren housing), the sampling order (S2, S3 and S4) and their interaction were included as fixed effects and the serum cortisol concentrations during S1 were considered as a co-variable. When the QBA scores were analyzed, the treatment (enriched vs. barren housing), the QBA (QBA2 and QBA3) and their interaction were included as fixed effects and the QBA1 as a co-variable.

3. Results

During the CBT1, when compared with P training sessions, the latency to cross the line around the bucket during R training sessions was lower starting from training session 4 (36.26 ± 7.30 s vs. 58.90 ± 6.93 s; p = 0.014), meaning that the pigs learned to discriminate between the two reinforcers from this session onwards. However, after the 44 training sessions three pigs (6.82 %) were not able to discriminate between R and P, therefore the outputs of the CBT1 of those pigs were excluded from the analyses. Regarding the training sessions of the CBT2 and CBT3, pigs were able to discriminate between R and P from training session 1 (p <0.0001 in both trials) and in this case, two (4.55 %) and one (2.27 %) pigs were not able to discriminate between the two reinforcers, respectively, and therefore were excluded from the analyses.

The mean latencies to cross the line around the bucket for each session during the three CBT for each treatment group are presented in Table 1. When the CBT2 and CBT3 results are compared, the latency to cross the line around the bucket was only lower in the barren group for the R test in the CBT2 (6.14 ± 0.73 s vs. 19.10 ± 6.28 s; p = 0.03).

Table 1. Latency to cross the line around the bucket (mean ± S.E.; in s) in each cognitive bias test (CBT) for the two treatment groups (enriched vs. barren housing)[a]

		Housing conditions		
	Session	Enriched (n=22)	Barren (n = 22)	P - value
CBT1	R reminder	16.51 ± 1.95	9.24 ± 1.35	-
(co-variable)	R test	16.14 ± 5.68	11.95 ± 5.17	-
	M test	14.88 ± 3.32	20.50 ± 4.08	-
	P test	44.09 ± 8.44	37.18 ± 8.18	-
	P reminder	79.42 ± 1.61	82.76 ± 1.42	-
CBT2	R reminder	15.24 ± 1.82	6.37 ± 0.85	0.007
	R test	17.71 ± 6.45	6.14 ± 0.73	0.060
	M test	16.83 ± 3.13	20.38 ± 3.54	0.579
	P test	40.90 ± 7.29	49.27 ± 8.54	0.394
	P reminder	77.90 ± 1.79	85.90 ± 1.05	0.029
CBT3	R reminder	11.64 ± 1.41	7.90 ± 1.06	0.778
	R test	10.24 ± 2.53	19.10 ± 6.28	0.119
	M test	22.11 ± 3.60	24.58 ± 3.76	0.822
	P test	53.95 ± 8.26	57.95 ± 8.42	0.664
	P reminder	82.74 ± 1.41	86.51 ± 0.92	0.096

[a]R reminder: rewarded reminder; R test: rewarded test, between the rewarded reminder and the middle test positions; M test: middle test, between the rewarded and the punishment reminder positions; P test: punishment test, between the punishment reminder and the middle test positions; P reminder: punishment reminder

The means of the QBA scores of the two pens of each treatment and the means of the serum cortisol concentrations of all pigs of each treatment are presented in Table 2 and in Table 3, respectively. No difference in the serum cortisol concentration and the QBA score was found between S2 and S3 and between QBA2 and QBA3 for each treatment group (*p* > 0.05; data not shown).

Table 2. Qualitative behaviour assessment scores (QBA) (mean ± S.E.) in the two treatment groups (enriched vs. barren housing)[a]

	Housing conditions		
	Enriched (n = 2)	Barren (n = 2)	P - value
QBA1 (co-variable)	44.50 ± 7.50	47.50 ± 8.42	-
QBA2	67.60 ± 11.30	23.90 ± 2.45	0.022
QBA3	77.40 ± 4.10	31.80 ± 1.50	0.027

[a]QBA1: score obtained 3 days after CBT1; QBA2: score obtained after CBT2; QBA3: score obtained after CBT3

Table 3. Serum cortisol concentrations (mean ± S.E.; in ng/mL) in the two treatment groups (enriched vs. barren housing)[a]

	Housing conditions		
	Enriched (n = 2)	Barren (n = 2)	P - value
S1 (co-variable)	23.16 ± 2.88	24.69 ± 3.45	-
S2	18.32 ± 2.64	27.98 ± 3.07	0.008
S3	16.06 ± 2.06	25.25 ± 3.43	0.011
S4	18.06 ± 2.36	20.52 ± 2.29	0.527

[a]S1: sampling 4 days after CBT1; S2: sampling 4 days after CBT2; S3: sampling 4 days after CBT3; S4: sampling at slaughter

Pigs in the enriched group presented a lower score obtained from the number of wounds on the carcass than pigs in the barren group (0.82 ± 0.12 vs. 1.18 ± 0.12; $p = 0.05$).

4. Discussion

In this study, the latency to cross the line around the bucket did not differ between the enriched and the barren group for any test session (R test, M test and P test) in any CBT (CBT2 and CBT3). These results are in contrast with those reported by Douglas et al. (2012) who found that pigs housed in enriched conditions (1.9 m^2 of space per pig, solid floor, straw and several objects to manipulate) had a more positive cognitive bias compared with pigs housed in barren conditions (1.2 m^2 of space per pig, partially slatted floor and some wood to manipulate). The lack of differences between groups in the present study may suggest that the CBT is not valid or sensitive (as defined by Martin and Bateson, 1993) enough to assess variation of the affective state between pigs raised in different housing conditions. Indeed, Scollo et al. (2014) also failed to find differences in the latency to reach the bucket between groups of pigs raised at different densities.

In this study, when compared with the barren group, the enriched group took longer time during the R reminder sessions and shorter time during the P reminder sessions of the CBT2 to cross the line around the bucket. These results are surprising as pigs of both groups were trained to differentiate these stimuli in the same way and contrast with the common thinking about the beneficial effect of the environmental enrichment on animals' learning abilities (van Praag et al., 2000; Young, 2003). Thus, these results suggest that pigs raised in barren housing conditions had better abilities to learn about the reinforcement of both R and P stimuli compared with pigs raised in enriched conditions. An explanation may be the greater motivation of barren pigs to compete for resources they are not used to (Pedersen et al., 2002), resulting in a greater stimulation to discriminate between the two stimuli more accurately compared with pigs raised in an enriched environment.

Interestingly, the time taken by the pigs in the barren group to cross the line around the bucket in the R test was lower in the CBT2 compared with the CBT3. This result suggests that raising pigs in barren housing conditions may alter their cognitive bias after a certain period of time (i.e. 5 weeks). Those results are opposite to the ones reported by Löckener et al. (2016)

that suggested that cognitive bias differences between horses housed in different conditions were only found just after changing those conditions but disappear after a certain period of time. Indeed, during the CBT3 pigs were 20 weeks old and heavier than during the CBT2, making the reduced space allowance a resource to compete for. It is known that the competition for space in the pen results in stress in pigs (Turner et al., 2000) and may had a negative effect on the affective state of the pigs in the barren group.

In this study, pigs learned to discriminate between R and P stimuli after four training sessions, that is faster (10 training sessions) than in previous studies carried out at the same research facilities (Carreras et al., 2015, 2016). The introduction of the air puff in the P sessions aiming at increasing the severity of the negative reinforcer and balance the value between the negative and the positive reinforcers (Mendl et al., 2009) may have helped to speed up the learning process and discriminate between the positive and the negative stimuli, increasing the efficiency of the CBT (Murphy et al., 2014). Moreover, similarly to Carreras et al. (2015), during CBT2 and CBT3 pigs were able to remember the discrimination task from the first training session, while the percentage of pigs excluded from the CBT due to their incapacity to discriminate between R and P sessions was lower than that reported in previous studies (Carreras et al., 2015, 2016). A reason may be the larger number of training sessions (44) carried out before performing the CBT1 compared with previous studies (12-14; Carreras et al., 2015, 2016).

As expected, when compared with the barren group, pigs from the enriched group showed greater QBA scores in both QBA2 and QBA3. Temple et al. (2011) also found that Iberian pigs raised in extensive conditions had more positive mood than Iberian pigs raised in intensive conditions, suggesting that in both studies the QBA distinguished the type of housing conditions on the basis of expression of natural behaviour. Moreover, it has been suggested that housing conditions may have an effect on the observer's perception of welfare that would systematically associate pigs housed in enriched conditions to a more positive affective state. However, Wemelsfelder et al. (2009) have shown that QBA in pigs are sensitive to contextual bias, but that this sensitivity does not seriously distort observer characterizations of pig expression.

The enriched group had lower serum cortisol concentration than the barren group in both S2 and S3 according to our hypothesis. Pigs with more space are supposed to suffer less stress as they have to compete less for space (Anil et al., 2007) and therefore reduce the incidence of aggressive behaviour. Moreover the access to straw, allow them rooting, which is known to be a natural behaviour (Fraser et al., 1991), and also reduces the incidence of tail biting (EFSA, 2007). The results are in accordance with Janssens et al. (1994) that demonstrated that pigs in enriched housing conditions had lower serum cortisol concentration compared with the pigs in barren housing conditions.

The number of wounds on the body was also higher in pigs in the barren group compared with pigs in the enriched group. Other authors have reported more number of lesions in pigs confined in higher densities (e.g. Jensen, 1984; Turner et al., 2000). Anil et al. (2007) described two probable reasons for higher number of lesions when space is reduced: competition for resting space and competition to gain access to a feeder and maintain it even if feeding is *ad libitum*. The short distance between the experimental farm and the slaughterhouse and the maintenance of the housing groups during the lairage probably allowed that those factors did not mask the effect of housing conditions on the number of wound on the carcass of those pigs.

5. Conclusions

The enriched housing conditions applied in this study produced a more positive affective state in pigs than the barren ones as showed by better QBA scores, lower serum cortisol concentration and lower number of skin lesions on the carcass. However, the CBT failed to support these results suggesting that is not a valid or not a sufficiently sensitive measure to detect emotional variation in pigs raised in different housing conditions.

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General Discussion

1. Applicability and consistency of the cognitive bias test

1.1 Test design

Since Harding et al. (2004) first developed a test to assess cognitive bias in rats, a large number of studies have been carried out in different species. Despite this fact, there is a lack of standardised test design for studying the emotional influences on judgement (i.e. decision taken). Therefore, we developed a cognitive bias test (CBT) for pigs (chapter 1) which has been modified along the studies performed in order to improve the assessment of the judgment bias in those animals. In parallel other tests in pigs have been published (Brajon et al., 2015; Douglas et al., 2012; Düpjan et al., 2013; Murphy et al., 2014a, 2013; Scollo et al., 2014) using designs based on Harding et al. (2004) but offering several differences. The type of stimuli, the type of reinforcers and the number of the training and test sessions are the main features which differ between test designs of the different judgment bias studies in pigs.

Firstly, the learning and discriminative capabilities of the pig ought to be considered, since the assessment of the responses under ambiguity is contingent on the ability of the animal to first learn to associate a 'positive' and a 'negative' reinforcers with two different stimuli and then discriminate between these two stimuli. There is plenty of evidence about the well-developed spatial ability of pigs (Arts et al., 2009; Held et al., 2005), therefore the different spatial location of a bucket (left or right in the wall opposite the door in the test pen) was used as the negative and the positive stimuli in all our studies. Other alternatives such as auditory (e.g. tones of different frequency) (e.g. Douglas et al., 2012; Murphy et al., 2014a, 2013) and visual stimuli (e.g. different colours) (e.g. Brilot et al., 2010; Verbeek et al., 2014) could also be used but were discarded due to the difficulties of pigs to differentiate between some tone frequencies (Douglas et al., 2012) and the well known incapacity of pigs to discriminate between different colours (Eguchi et al., 1997; Tanida et al., 1991). According to our hypothesis the results show that pigs were able to differentiate between the two location stimuli faster than other studies using auditory or visual stimuli (around 15 vs. around 250) (Douglas et al., 2012; Murphy et al., 2013). These results are in agreement with other studies that used location stimuli (Düpjan et al., 2013; Scollo et al., 2014).

In most of the CBT carried out so far, including the studies presented in the present thesis, food was used as a positive reinforcer (Gygax, 2014). However, different alternatives have been proposed as a negative reinforcer. Three main possibilities are described: a reduction of the amount of food compared with the positive reinforcer (e.g. Murphy et al., 2013; Parker, 2008), absence of the positive reinforcer (e.g. Düpjan et al., 2013; Scollo et al., 2014; chapter 1, 2 and 3) and a “real” negative reinforcer, such as air puff, electric shock or waved bag in pigs face (e.g. Brajon et al., 2015; Douglas et al., 2012; chapter 4). The type of the negative reinforcer represents an important aspect due to two different reasons. The first one is related with the learning process and the second one is related with the ambiguity of the stimulus of the test. Our results suggest that a “real” negative reinforcer is the best option since it allows the pigs to learn faster to discriminate between the two stimuli during the training sessions. Moreover, a “real” negative reinforcer makes the stimulus of the test more ambiguous than using the others, since the intensity of the negative reinforcer is balanced with the intensity of the positive one (Mendl, 2009). Indeed, the mild severity of the negative reinforcer, i.e. a reduction or absence of the positive reinforcer, might predispose the animal to assume a certain risk when exposed to the ambiguous stimulus (Rolls, 2005). Comparing the results between the study of chapter 4 (using air puff as a negative reinforcer) and the previous studies (chapters 1, 2 and 3) (using the absence of the positive reinforcer as a negative reinforcer) it can be proved that pigs learnt faster (4 sessions vs. 10 sessions) to discriminate between the two stimuli during the training sessions. Moreover, regarding the test sessions, the latencies to respond in front the ambiguous stimuli increased (from 19.69 s, 19.08 s and 14.35 s for chapters 1, 2 and 3, respectively, to 23.5 s for chapter 4) during the study of chapter 4, probably due to the ambiguous stimuli were not judged as much positive (i.e. more ambiguous) as in the previous studies.

Two main strategies are described in the cognitive bias studies to ensure that animals learn to discriminate between the positive and the negative stimuli. The first one is to perform enough sessions to ensure that all animals have learnt (e.g. Bateson and Matheson, 2007; Burman et al., 2009) and the other option is to perform a number of sessions determined by pilot or previous studies and remove the animals that are not able to discriminate between the two stimuli after this number of sessions (e.g. Brajon et al., 2015; Düpjan et al., 2013; chapter 2 and 3). The first strategy has the advantage that does not remove animals that need more training sessions to discriminate between the two stimuli, however it is potentially a very time consuming option. The second strategy, proposed to remove animals that do not learn after a determined number of sessions, that is, in a not randomized way which can led to bias the sample, however it allows the technique to become more feasible. A good alternative could be the one carried out on the study of chapter 4, where the number of sessions was higher than the minimum recommended on previous studies (chapter 1, 2 and 3) but without pretending that all pigs learned the discrimination task, allowing the technique to become more feasible and decreasing the number of removed pigs. In chapters 1, 2 and 3 the number of pigs removed was higher (9.33%, 18.75% and 33.90%, respectively) than in chapter 4 (6.82%). According to our results Brajon et al. (2015) also removed a large percentage (59%) of pigs probably due to the lower number of training sessions performed. Contrary in other studies in which the number of sessions was higher no animals were removed (Douglas et al., 2012; Murphy et al., 2013).

Another issue related with the CBT design is its unsuitability for repeated testing (Murphy et al., 2014b) as animals quickly learn the reinforcer (in the case of the ambiguous stimulus the "reinforcer" will be refered as "outcome" since it is not used as a reinforcer) in the ambiguous tests, rendering them no longer ambiguous. When animals have to repeat the same or similar CBT, such as during the study of chapter 4, the ambiguous stimuli are commonly not rewarded to prevent outcome learning. Moreover, when the ambiguous test is performed once (chapter 2) or twice but with a period of time between them (chapters 1 and 3), the outcome should be between the positive and the negative reinforcer in order to be as much ambiguous as possible, e.g. the outcome can be rewarded but with lower amount of food compared with the positive reinforcer.

Most of the cognitive bias studies carried out so far, including the study of chapter 4, performed three types of ambiguous tests, one between the positive and the negative stimuli (middle stimuli) and the other two between the positive and the middle and between the negative and the middle stimuli. Those studies increase the opportunities to find differences between treatment groups but, as commented before, decrease the ambiguity of the tests as animals can easily learn the outcome of the ambiguous stimuli. Therefore the studies of chapters 1, 2 and 3 only carried out just one ambiguous stimulus, maintaining the ambiguity as much as possible but decreasing the opportunities to find differences between pigs or treatment groups compared with chapter 4.

1.2 The cognitive bias classification

A cognitive bias classification was proposed in chapter 1 to individually classify animals with a positive, neutral or negative cognitive bias. Although the CBT is performed individually, the results of most of the studies carried out so far are analyzed in groups. This classification is based on a formula that provides an adjusted score that expresses the result of the test session of a pig as a percentage of the difference between its own baseline results during the positive and negative training sessions. This individual classification allows comparing behavioural and physiological parameters variables between animals with different cognitive bias profiles (positive, negative and neutral cognitive bias). For example, in chapter 2, the results of the novel object test were compared among groups of pigs with different cognitive bias profiles. It also allows assessing if a pig first classified with negative cognitive bias can change to positive after a positive treatment, such as being positively handled or housed in enriched conditions and contrarily if a pig first classified with positive cognitive bias can change to negative after a negative treatment. However, in our studies most of the pigs were classified with a positive cognitive bias (84.85% and 79.55% in chapter 1 and 2, respectively), hindering the comparison between positive, neutral and negative cognitive bias profiles, as the number of pigs with negative and neutral cognitive bias was not representative. This higher percentage of animals classified with a positive cognitive bias might be explained due to the mild severity of the negative reinforcer (bucket without access to food) that might predispose the pig to assume a certain risk when exposed to the ambiguous stimulus (Ortony et al., 1988; Rolls, 2005; Mendl et al., 2009). Moreover, pigs might tend to be optimistic as an adaptive response to cope with difficult environments, such as the intensive farming systems (Metcalfe, 1998). Furthermore, the pigs used in the current studies were reared on a commercial farm during the first weeks of life and moved to the experimental facilities, probably with improved housing conditions. It is suggested that the previous environment, even if it was better or worse than the current one, can alter the perception of the animal and hence its cognitive bias (Bateson and Matheson, 2007; Douglas et al., 2012).

1.3 Consistency of the cognitive bias test

The term consistency refers to the propriety of a measure to produce the same result when measured several times under the same conditions (Martin and Bateson, 1993). Doyle et al. (2010) and Murphy et al. (2013) demonstrated that sheep and pigs, respectively, learn quickly the outcome of the ambiguous stimuli of the CBT, rendering the stimuli no longer ambiguous. Therefore, in order to assess the consistency of the CBT we decided to perform only one test each time to avoid the pigs learning the association between the ambiguous stimulus and its outcome. Chapter 1 showed no individual consistency between the same CBT carried out twice with the same animals and under the same housing conditions in a time lapse of 5 weeks. To our knowledge this was the first attempt to assess the consistency of the CBT in individual animals. Previous studies showed that the test was not consistent over time using the mean value of the latency to interact with the ambiguous stimuli of a group of animals (Doyle et al., 2010 and Murphy et al., 2013). We suggest that the inconsistency between the results of the two CBT carried out in chapter 1 can be due to the ability of the pigs to remember the outcome of the ambiguous stimulus during the second CBT. Moreover, other factors such as the age or the hunger state over time could also have an effect on the results, since older animals tend to be hungrier and therefore are more motivated to consume food (Pedersen et al., 2002).

2. Validity of the cognitive bias test

As no gold standard test of emotional-valence exists in animals, it is difficult to assess the validity of the CBT. However alternative ways to validate the CBT are possible, such as assessing the correlation with other behavioural, physiological and clinical measures of emotional state or assessing the effect of treatments (known to alter the emotional state) on the cognitive bias, such as drug administration (e.g. anxiolitics), using specific genotypes (e.g. rat models of depression) or housing and management conditions. In the present thesis, we correlate the novel object test (NOT), the defence cascade test (DCT) and the qualitative behaviour assessment (QBA) as behavioural measures, neurotransmitters and cortisol concentrations as physiological measures and the number of wound on pigs' carcass as a clinical measure with the results of the CBT. Furthermore, the effect of gender and halothane genotype and the effect of management and housing conditions were also assessed on cognitive bias.

2.1 Effect of the different treatments on cognitive bias

The main aim of most cognitive bias studies is to find differences of affective state using different treatments, such as housing conditions (Bateson and Matheson, 2007; Löckener et al., 2016) or drug administration (Anderson et al., 2013; Doyle et al., 2011a). We decided to use three treatments which are known to alter the welfare of the pigs and its affective state. Chapter 2 assessed the effect of the gender and the halothane genotype. Chapter 3 assessed the effect of positive and negative handling. And finally, Chapter 4 studied the effect of enriched and barren housing conditions. No differences in cognitive bias were found between treatment groups in any of the studies suggesting three possible causes: 1) the application of the treatment was too short and/or insufficient to alter the affective state, 2) the CBT was not valid or not sensitive enough to assess changes on affective state or 3) a combination of both factors. In chapter 2 and 3, no differences between treatment groups were neither found on the other behavioural test (NOT and DCT) and on the physiological measures (neurotransmitters concentration and cortisol concentration), supporting the hypothesis that the treatment was insufficient or unable to alter the affective state of the pigs. Neverteless, Chapter 4 found that pigs housed in enriched housing conditions had higher QBA scores, lower cortisol concentration and lower number of wounds on the carcass, compared with pigs in the barren group, suggesting that the treatment had an effect on the welfare and more likely on the affective state of the pigs. However, as described above, these differences were not found regarding the CBT, supporting the hypothesis that the CBT was not valid or not sensitive enough to assess the changes on affective state caused by the housing treatment. Therefore it can be suggested that even when the treatment has the potential to alter the welfare and the affective state of the pigs the CBT is not able to assess such differences.

Contrary to our results, Brajon et al. (2015) reported a more positive cognitive bias in positive handled piglets compared with negative and minimal handled ones. The discrepancy in the results may be explained by the different pigs' age of both studies, i.e. 25 days piglets in Brajon et al. (2015) study vs. 19 weeks old pigs in ours. Greater effects of management have been, in fact, reported on pigs during their first period of life than later (Hemsworth et al., 1986; Hemsworth and Barnett, 1992). Regarding housing conditions our results are in contrast with those reported by Douglas et al. (2012) who found that pigs housed in enriched conditions had more positive cognitive bias compared with pigs housed in barren conditions. Moreover, according to our results, Scollo et al. (2014) also failed to find differences in the latency to reach the bucket between groups of pigs raised at different densities. Different aspects could explain the differences between the results of these studies but the intensity of the treatment may be one of the main reasons.

The treatment carried out in some studies included a single test to assess the effect on the affective state (e.g. Doyle et al., 2010; Scollo et al., 2014), while the studies of chapters 3 and 4, as well as other studies such as Bethell et al. (2012) and Douglas et al. (2012), included several factors to induce such changes. From our point of view, the main aim of cognitive bias studies is to determine if the CBT is capable to assess differences in affective state but not if a single factor induces such differences. Therefore, it is suggested that, before using a test to assess if a single factor has an effect on the affective state it would be necessary to be certain that the test is capable to assess such effect.

2.2 Behavioural tests

Three different behavioural tests were carried out in the studies of this thesis: the NOT, the DCT and the QBA. Moreover, other authors have carried out other tests together with the CBT. For example, Wichman et al. (2012) carried out an anticipatory behaviour test and Doyle et al. (2011b) assessed the response of sheep to a novel object, to a novel environment and to a sudden object. In chapter 2 and 3 a NOT, a test to assess fear or anxiety to unfamiliarity, was carried out and in accordance with the results of the CBT no differences between treatments were found. Moreover, according with the results of Wichman et al. (2012) correlations between the results of both tests were found suggesting that both tests measure similar responses. Indeed, we suggested that the response of the CBT and the NOT was more influenced by other intrinsic factors of the animals, such as the level of fear, than by the treatments, in this case the gender and the halothane genotype (chapter 2) and the way pigs were handled (chapter 3).

In the study of chapter 3, a third behavioural test was carried out, the DCT. This test has been used to assess affective state in both humans and rodents (Lang et al., 1990; 2000). According to the results of the CBT and the NOT carried out in chapter 3, no differences were found between animals exposed to positive and negative handling. Moreover, the results obtained correlated positively with the results of the NOT and the CBT supporting the hypothesis that those tests are measuring similar responses, but these responses are more influenced by intrinsic factors rather than the treatments supposed to induce changes in the affective state.

Finally, a QBA was carried out in the study of chapter 4. Previous research had shown that this technique has high inter- and intra- observer reliability and is coherent with other behaviour and welfare measures (Wemelsfelder et al., 2000). Pigs in barren housing conditions had a lower QBA score, associated with negative affective states, compared with pigs in enriched housing conditions after 1 and 5 weeks housed in those conditions (chapter 4). Moreover, these differences were not found in CBT suggesting that the CBT was not sensitive enough or not valid to detect differences in affective state.

In summary, CBT, NOT and DCT were not sensitive or valid enough to detect the presumably effect induced by the treatments carried out. On the other hand differences between pigs in barren and enriched housing conditions affected the QBA as expected. It is relevant to emphasise that QBA is measured at group level on the housing pen, while the other three are measured individually on a test pen. Indeed, QBA measures the behaviour of the pigs during 20 min in their environment, while the CBT, the NOT and the DCT measure the response of pigs in front of an ambiguous, a novel and a sudden stimulus. Observing the pigs during longer time, in their housing environment and assessing its emotional state through general behaviour can probably lead to easily find differences between treatments compared with observing the pigs during less time, in a test pen and assessing its emotional state throw specific behaviour.

2.3 Physiological and clinical measures

The concentration of neurotransmitters (noradrenaline, dopamine, 3,4-dihydroxyphenylacetic acid, homovanillic acid, serotonin; 5-hydroxyindole-3-acetic acid) was analyzed in four brain areas during the study of chapter 2. Moreover, serum cortisol concentration was measured on chapters 3 and 4 and saliva and hair cortisol concentration was also measured on chapter 3. No correlation was found between the results of the CBT and both neurotransmitters and cortisol concentrations. The results of our study regarding neurotransmitters concentrations are opposite with the results of Doyle et al. (2011a) who administered a serotonin inhibitor (p-chlorophenylalanine) to sheep, which is known to induce negative affective states, and observed more negative cognitive bias compared with the control group. Moreover, Sharot et al. (2012) showed that the administration of a drug (dihydroxy-l-phenylalanine; l-DOPA) that enhances dopaminergic function and induces more positive affective states, increased the positive cognitive bias in humans, reducing negative expectations regarding the future. Moreover, no differences between treatments were found in the studies of chapters 2 and 3 regarding the neurotransmitters and cortisol concentration, respectively. However, expected differences between treatment groups were found on chapter 4, with higher serum cortisol concentration in the pigs housed in barren housing conditions, suggesting that those pigs were more stressed, i.e. with more negative affective state, according with the results of the QBA. The absence of differences between treatment groups regarding the neurotransmitters and cortisol concentrations in chapters 2 and 3, respectively, supports the hypothesis that the intensity of the treatments was not enough to alter the affective states. Moreover the results of the cortisol concentrations in chapter 4, suggest that the housing conditions altered the affective state of the animals but the CBT was not valid or not sensitive enough to assess such differences.

Finally, the carcass of the pigs in the barren housing conditions also showed a higher number of wounds compared with those in the enriched housing conditions, suggesting that those animals were in worse welfare conditions and therefore probably with a more negative affective state, supporting the results of the QBA and the serum cortisol concentration. This result is also supporting the hypothesis that housing conditions have an effect on the affective state of the pigs.

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Conclusions

1. Pigs are able to discriminate between the two location stimuli according to the associated reinforcers enabling the application of the cognitive bias test in these animals. In addition, the higher the intensity of the negative reinforce, the faster pigs learn and lower the number of animals that are not able to discriminate.

2. The cognitive bias classification allows differentiate groups according to the cognitive bias profiles (positive, neutral and negative cognitive bias). Moreover, it provides the possibility to assess if a pig with a cognitive bias profile change to another according to the treatment carried out.

3. Most of pigs are classified with a positive cognitive bias, suggesting that the mild severity of the negative reinforcer predispose pigs to judge more positively the ambiguous stimulus or pigs tend to be optimistic as an adaptive response.

4. The cognitive bias test performed is not consistent over time suggesting that pigs are able to remember the outcome of the ambiguous stimulus during the second cognitive bias test, thus losing the ambiguity required for the test. In addition, other factors such as the time of the day that pigs are tested, their age or their hunger may influence the results.

5. The gender, the halothane genotype and the management and the housing conditions do not affect the cognitive bias suggesting that there is a lack of effect of such treatments on the affective state and/or the cognitive bias test is not valid or not sensitive enough to assess the pigs' affective state.

6. Behavioural measures (novel object test and defence cascade test) and physiological measures (cortisol concentration and neurotransmitters concentration) are not affected by gender, halothane genotype and handling conditions. These results support the hypotheses that there is a lack of the effect of those treatments on the affective state of the pigs.

7. Pigs in the enriched housing conditions have better qualitative behaviour assessment scores, lower cortisol concentration and lower number of skin lesions on the carcass than pigs in the barren housing conditions. These results suggest that the housing conditions have an effect on the affective state of the pigs and therefore support the hypotheses that the cognitive bias test is not valid or not sensitive enough to assess such effect.

8. Positive correlations between cognitive bias test, novel object test and defence cascade test suggest that individual factors, other than the treatment performed, such as the fear level, the motivation or the coping style have an effect on the behavioural responses of pigs covering up the presumed effect of the treatments carried out.