Methods of inducing and assessing positive experiences in juvenile male Wistar rats

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Declaration

I, Tayla Jane Hammond, hereby declare that this thesis was composed by myself and that the work contained herein is my own except where explicitly stated otherwise in the text. This work has not been submitted for any other degree or professional qualification.

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Abstract

The importance of animals having positive experiences is essential for animal welfare as the balance of positive and negative affective states can be seen as critical to determining an animal's quality of life. However, a traditional focus on alleviating negative affective states has meant that the complete assessment of animal welfare is hindered by a relative lack of validated models of positive affective states. This disparity between the measurement of positive and negative affective states is particularly evident in rodents used for research. With over 1.9 million rats used for research purposes in the UK, USA and EU per annum as of 2018, contribution towards this gap in knowledge on positive affective states is vital to improving their welfare. As such, the aims of this doctoral research were twofold; first, to develop models of inducing a positive affective state in rats, and second, to investigate whether play is sensitive to the manipulation of affective state as an indicator of PAW.

Playful handling is a recently developed approach to positive interactions between humans and rats which aims to incorporate the diversity and unpredictability of juvenile rat social play. To validate the use of playful handling as a method of inducing a positive affective state in juvenile male rats, two independent cohorts were either playfully handled or control handled over successive days, as individuals or pairs. In response to playful handling, rats showed increased production of 50-kHz ultrasonic vocalisations (USVs) compared to control rats, a type of vocalisation associated with positive affect. Approach behaviour towards the experimenter was higher when rats were playfully handled as individuals compared to controls. I also measured rat preference for a high concentration (20%) of sucrose over water as a prospective behavioural test of positive affect. This was an adaption of the sucrose preference test which is a validated test of negative affect which measures preference for a low concentration (<1%) of sucrose over water. Rats which were playfully handled as individuals drank more sucrose solution than control rats. Thus, there is potential for using a high-concentration sucrose preference test as a measure of behavioural and neural changes induced by a positive experience. This increase in indicators of positive affect provides further evidence for the use of playful handling to induce a positive affective state in rats.

Presentation of positively valenced rat-produced USVs in playback paradigms is a novel method of altering affective state. This method is based on the premise that as vocalisations have a communicative function associated with affective state, the presentation of positive vocalisations will stimulate positive emotions in the recipient. Four independent cohorts were used to develop a novel paradigm in which acoustic stimuli could be presented in low-stress conditions. Across these experiments, four acoustic stimuli were used; positively valenced 50-kHz USVs, negatively valenced 22-kHz USVs, White Noise, and Background Noise. Across all four experiments using the novel playback paradigm, the presentation of 50-kHz stimuli increased subject-produced positively valenced 50-kHz USVs compared to Background Noise. A single presentation of White Noise also increased 50-kHz USVs to match those produced when presented with 50-kHz compared to Background Noise. This effect of White Noise on USV production was not seen after subsequent presentations, with USV production lowering to that of Background Noise on day five. When the speaker was placed on the side of the cage, there was also an increase in approach behaviour in response to 50-kHz stimuli compared to 22-kHz and Background Noise. The differences in measures of affective state in response to the auditory stimuli across cohorts provide evidence that playback is a promising method of stimulating a positive affective state in rats.

Play behaviour is proposed to be a promising indicator of positive emotions and welfare in domesticated animals. To investigate whether play changes with the manipulation of affective state, home cage behaviour was recorded before and after experiencing successive days of handling treatments or presentation of acoustic stimuli. After five days, there was an increase in play in the home cage prior to the experience in response to both playful handling and the playback of 50-kHz USVs. As these effects were specific to behaviour in the home cage occurring before the positive experience, this suggests that play may in part reflect a form of anticipatory behaviour. In the playback paradigm, the absence of a human-rat interaction also allowed the assessment of play during the presentation of acoustic stimuli. After five days of presentation with 50-kHz USVs, rats expressed more social play than those exposed to control stimuli. These results suggest there is a positive relationship between concurrent and pre-existing positive affective state and play.

Overall, this work provides support for the use of playful handling and presentation of 50-kHz USVs in a novel playback paradigm as methods to induce a positive affective state in rats. The successful manipulation of indicators of affective state allowed the investigation of play as an indicator of positive affect. As play was sensitive to increases in positive affect**,** there is evidence that play represents more than just the absence of a negative affective state and supports the use of play as an indicator of positive welfare.

Lay Summary

Scientists have accumulated a wealth of evidence that animals can feel a range of emotions, from pain to pleasure. However, there has been a traditional focus on investigating negative emotions like fear and anxiety. This focus has meant that there is a shortage of research investigating positive emotions in animals, such as enjoyment and happiness. This shortage of research is particularly evident in rats, despite being one of the most commonly used species for scientific research. With over 200 thousand rats used annually in the UK alone, investigating positive experiences is essential for rats to have a good life or a living worth living. As such, the aims of this research were; 1) to develop ways of stimulating a positive emotional state in rats and 2) to investigate whether rats in a positive emotional state express more play.

The first way of stimulating a positive emotional state I investigated was playful handling. Playful handling is a method of human-rat play that tries to mimic rat social play characteristics. During playful handling, the experimenter plays with a rat using a mixture of touching, tickling, chasing, and infrequent short pins. Across two experiments, I found that playfully handled rats were in a more positive emotional state than those who were not playfully handled. Playfully handled rats produced more 50-kHz ‘positive’ vocalisations, spent more time approaching the experimenter, and drank more of the rewarding sugary solution. The increase in my measurements of positive emotional state suggests that playful handling is a positive experience for rats.

The second way of stimulating a positive emotional state I investigated was the playback of positive vocalisations. This idea is based on the idea that as vocalisations allow animals to communicate how they are feeling, the presentation of positive vocalisations will stimulate positive emotions in the animals hearing them. Across four experiments, I found that rats presented with positive vocalisations were in a more positive emotional state than those presented with

neutral or negative vocalisations or noise. Rats presented with positive vocalisations produced more 50-kHz ‘positive’ vocalisations themselves and spent more time approaching the speaker from whom the vocalisations were presented. The increase in my measurements of positive emotional state provides evidence that playback of 50-kHz positive vocalisations is a positive experience for rats.

As playful handling and playback of 50-kHz positive vocalisations were a positive emotional experience, I could investigate whether rats in a more positive emotional state conducted more play. Play is a promising indicator of positive emotions and welfare; it is an enjoyable experience and disappears when animals experience poor living conditions. However, there is only a small body of evidence investigating the relationship between positive emotions and play. I investigated whether the rats which were in a more positive emotional state conducted more play during the experience and in the home cage before and after the handling or playback experience. After five days, I found that rats which were playfully handled or presented with 50-kHz vocalisations played more in the home cage before handling than those who experienced control handling or were presented with other neutral acoustic stimuli. The increase in play occurred before the experience, suggesting that rats positively anticipated the enjoyable experience. I also found that rats that were presented with 50-kHz vocalisations also played more during the experience than rats exposed to other neutral noises. These results suggest a relationship between a positive emotional state and play.

Overall, this research provides new ways to stimulate a positive emotional state in rats. Researchers can use playful handling and the presentation of 50-kHz to promote positive emotions and improve the welfare of rats. This research also provides evidence that a positive emotional state encourages rats to express more play. As such, play can sometimes indicate when rats are in a positive emotional state and are experiencing good welfare.

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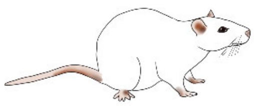

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Chapter 1: Positive animal welfare: how can we induce and assess positive affective states in rats?

Animal welfare science is a multidimensional field that encompasses all factors that may influence an animal's physical and emotional well-being. Relatively recently, from this broad remit came the development of positive animal welfare (PAW) (Yeates and Main, 2008), which is a facet of animal welfare science that emphasises the capacity for animals to have positive mental experiences. Despite the growing recognition of the importance of positive aspects of animal lives, the vast majority of animal welfare research has been and continues to be, concentrated on negative aspects (Lawrence *et al*., 2018). As such, the focus of this thesis is to contribute to the PAW literature by developing models of inducing positive affective states and indicators of PAW in juvenile male Wistar rats.

Positive animal welfare: what is it, and how can we assess it?

What is positive animal welfare?

The first formal use of PAW may have been in 2007 by Boissy *et al*. in their review of the evidence of positive emotions in animals. A year later, in 2008, Yeates and Main developed PAW into a distinct concept within animal welfare science by beginning discussions on methods to assess PAW. Since then, the concept of PAW has received increasing attention from animal welfare scientists, such that it is now regarded as an important facet of animal welfare science (Edgar *et al*., 2013; Jirkof *et al*., 2019; Lawrence *et al*., 2018, 2019; Makowska and Weary, 2020; Mellor, 2012, 2014a; Mellor and Beausoleil, 2015; Rault *et al*., 2020, 2022; Vigors, 2019).

The concept of animal emotions is related to terms such as 'feelings', 'mood' and 'affect' (Mendl and Paul, 2020). The definition and use of these terms in relation to animals are still a source of debate (Paul *et al*., 2019), with the distinctions between these terms proving difficult to separate with empirical testing (Lawrence *et al*., 2019; Vere and Kuczaj, 2016). The terms ‘affect’ and ‘affective state’ have been used widely across literature concerned with animal emotions (*e.g.,* Boissy *et al*., 2007; Jirkof *et al*., 2019; Mendl and Paul, 2020; Paul *et al*., 2005; Turner, 2019; Webb *et al*., 2019) as it encompasses both short-term, targeted emotions and longer-term, diffused moods (Paul *et al*., 2019). As such, I will use 'affective state' as the overarching term from this point.

While there is no universally agreed definition of PAW, the capacity of animals to experience positive affective states is central to the development of PAW as a concept within animal welfare (Lawrence *et al*., 2019). The recognition of positive behaviours and experiences dates back to the modern period of concern for animal welfare, for example, in the Brambell Report (Brambell, 1965). In the time since the Brambell Report, there has been mounting evidence and acceptance of the capacity of animals to experience positive affective states across different scientific fields, including neuroscience, psychology, and animal welfare science (Mellor, 2012; Phillips, 2008). With this evidence has come increased interest in approaches to conceptualise animal affective states (Lawrence *et al*., 2019).

There are two main approaches to conceptualising emotions which originate from human psychology; the discrete or categorical approach and the dimensional approach (Mendl and Paul, 2020). The discrete approach states that there are a small set of fundamental or ‘basic’ emotions, such as joy, sadness, fear, disgust, and anger, which arise in response to a specific event (Panksepp, 1998). While advocates of this model disagree on the number of discrete emotions, researchers agree that each state is mediated by specific neural circuitry and generates behavioural and physiological responses (Mendl and Paul, 2020). In contrast, the dimensional approach, such as the core affect model by Russell (2003), categorises affective states along two continuous, dimensional scales referred to as ‘valence’ and 'arousal' (Russell, 2003). Whilst valence refers to the extent to which an affective state is positive or negative, arousal refers to the level of activity associated with the affective state. Thus, according to this approach, each basic emotion emerges from a combination of the valence and arousal dimensions (Russell, 2003). For example, sadness would be defined as negative valence/low arousal, while joy would be defined as positive valence/high arousal (Mendl *et al*., 2010).

Mendl *et al*. (2010) have extended these models to the assessment of animal affective states by providing a framework that integrates discrete and dimensional approaches. They argue that there is an underlying bi-directional relationship between these approaches, such that discrete emotions influence affective states and vice versa (Mendl *et al*., 2010). As a result of this interdisciplinary approach, many of the terms used to describe positive affective states in animals are synonymous with those used in reference to humans (Lawrence *et al*., 2019; Proctor *et al*., 2013; Rault *et al*., 2020), such as pleasure (Berridge and Kringelbach, 2015), fun (Berridge and Kringelbach, 2015; Trezza *et al*., 2010) and enjoyment (Boissy *et al*., 2007).

In conjunction with the conceptualisation of animal affective states, there is also a need within the literature to understand the function of positive affective states and their relationship with PAW (Lawrence *et al*., 2019). Of primary focus has been the intrinsic functional link between positive affective states and behaviour (Lawrence *et al*., 2019). Whilst there are multiple proposed ideas to describe this relationship, such as the motivational affective states concept proposed by Fraser and Duncan (1998) and positive affective engagement by Mellor (2015), there is considerable overlap between these concepts. These concepts share the premise that positive affective states are involved in motivating goal-directed behaviour towards rewarding stimuli (Fraser and Duncan, 1998; Mellor, 2014b). In turn, the expression of these behaviours indicates the presence of positive affective states and PAW (Boissy *et al*., 2007; Yeates and Main, 2008). This opportunity to express and complete positively motivated behaviours is central to PAW (Lawrence *et al*., 2019) and the wider animal welfare concepts of ‘a good life’ and a ‘life worth living’ (Edgar *et al*., 2013)

How can we assess positive animal welfare?

The optimal approach for welfare assessment involves a combination of both resource-based and outcome-based approaches (Edgar *et al*., 2013). Relative to the assessment of negative affective states and associated implications for welfare, there is a lack of validated methods of assessing PAW (Keeling *et al*., 2013). This overview will present the current approaches used to assess positive affective states and their role in the practical assessment of PAW.

Resource-based approaches determine the welfare of an animal in terms of inputs into the animal’s environment, such as space, food, and environmental enrichment (Yeates and Main, 2008). Assessment of PAW is based on the premise that the provision of these resources allows the expression of motivated behaviours and expression of natural behaviours (Bracke and Hopster, 2006) which involves positive affect and, thus, indicates PAW (Rowe and Mullan, 2022). The use of a resource-based approach is a valuable tool for the practical assessment of PAW (Lawrence *et al*., 2018), with some authors arguing that this resource-based approach is currently the most promising method of assessing PAW (Rowe and Mullan, 2022). However, validation of the resource-based approach relies on using animal-based outcome measures to determine whether the resources provided are effective in eliciting positive affective states in animals (Yeates and Main, 2008).

Outcome-based approaches use the measurement of an animal’s physiology and behaviour (Fraser, 2008; Webb *et al*., 2019) and are thus regarded as a more direct measure of welfare (Yeates and Main, 2008). In this approach, welfare is assessed through the assumption that emotional states alter physiological markers and motivate behavioural responses (Boissy *et al*., 2007), *i.e.,* animals react differently when experiencing putatively positive affective states than negative states and in response to positive versus negative stimuli (Yeates and Main, 2008). As such, physiological markers and behavioural expressions are used as proxies for contrastingly valenced affective states (Makowska and Weary, 2013; Paul *et al*., 2005). Thus, through direct measurement of physiology and behaviour, we can extrapolate the valence of the underlying affective state and make inferences about the welfare status of the animal (Lawrence *et al*., 2018).

The use of physiological measures to determine affective state is based on changes in neuroendocrine, immunological, cardiovascular, and molecular parameters associated with affective states (Kremer *et al*., 2020). In terms of assessing PAW, the assessment of neuroendocrine function has been the most widely investigated physiological marker for PAW. This method typically relies on using peripheral hormones as a proxy for brain events and correlating the production of these hormones in the blood with the brain since the direct assessment of neuroendocrine factors is difficult or often impossible (*e.g.,* Huff and Davies, 2002, Vedhara *et al*., 2003). For example, dopamine has been widely implicated in determining the motivational value of rewarding behaviours, such as positive anticipation (Berridge and Robinson, 1998). However, dopamine release in the brain is difficult to assess directly (although see Hori *et al*., 2013). While physiological measures can be easily quantified, several potential confounds are introduced during sampling and interpretation (Kremer *et al*., 2020). These confounds place constraints on their use in the context of assessing positive affect in the assessment of PAW.

Confounds are first introduced when sampling physiological markers, as this often requires conducting aversive procedures: for example, in rodents, blood is collected through an incision in the tail (Broom and Johnson, 2019). In turn, the stress induced by sampling has potential effects on the physiological markers themselves, including changes in neuroendocrine levels (*e.g.,* Gärtner *et al*., 1980;

Haemisch *et al*., 1999; Riley, 1981), immunological parameters (Padgett and Glaser, 2003) and heart rate (Gärtner *et al*., 1980; Hubbard *et al*., 1986). As a result, the stress induced by the collection of physiological measures can confound data collection and mask the true impact of stimuli on affective state (Broom and Johnson, 2019). Further, physiological markers are sensitive to diurnal variation in baseline levels, such that the timing of collection in relation to the event of interest must take account of this variation (Axelrod and Reisine, 1984; Paul *et al*., 2005).

Interpretation of physiological measures is also challenging as both putatively positive and negative stimuli can induce the same response (Paul *et al*., 2005). For example, heart rate (Chabot *et al*., 1996; Marchant *et al*., 1995; Zupan *et al*., 2016) and glucocorticoid levels (Rushen, 1986; Starcevic *et al*., 2016) increase in response to both positive and negative situations. Further, while physiological markers may be able to distinguish changes in arousal, these markers may not be able to detect differences in valence (Kremer *et al*., 2020). This creates a further confound for the interpretation of the effect of stimuli on affective states. Overall, physiological measures are not yet reliable for assessing positive affective states (Webb *et al*., 2019) and, thus, cannot currently be used alone to determine the PAW status of an animal.

Behavioural measures are the most promising direction for the assessment of PAW, with behaviour regarded as the closest proxy to positive affective states (Lawrence *et al*., 2018). Accordingly, as the behavioural measures focus on the specific animal, it is important to be aware of species-typical nuances in behaviour, *i.e.,* knowledge of the ethological and ecological characteristics of the subject in question (Paul *et al*., 2005). The assessment of PAW can be conducted by observing and quantifying behaviour that occurs in the home environment or under staged test conditions (Kremer *et al*., 2020; Lawrence *et al*., 2019). In addition to these quantitative behavioural approaches, it is also important to briefly recognise another promising method to assess PAW, which uses a qualitative approach. This approach, known as qualitative behavioural assessment (QBA), focuses on the qualities associated which the expression of all behaviours instead of the physical characteristics (Wemelsfelder *et al*., 2000).

The expression of behaviours under home conditions is based on a traditional ethological approach: hypotheses are tested by analysing parts of the repertoire of behaviours which animals are free to express spontaneously within the constraints of their home pen or cage. Using this method, several promising parameters have been identified for the assessment of affective states. These parameters either focus on the whole animal, such as approach behaviour (Elliot and Thrash, 2002; Paul and Mendl, 2018), socio-affiliative behaviour (Boissy *et al*., 2007; Proctor and Carder, 2015) and play (Ahloy-Dallaire *et al*., 2017; Boissy *et al*., 2007; Held and Špinka, 2011; Paul *et al*., 2005), or on more specific expressions of the animal, such as facial expressions (Finlayson *et al*., 2016; Ross *et al*., 2008), ear postures (Raoult and Gygax, 2018; Reimert *et al*., 2013) and vocalisations (Hinchcliffe *et al*., 2020; Laurijs *et al*., 2021). Some of these specific expressions, such as the production of vocalisations, have also been used in the context of staged tests (Rygula *et al*., 2012).

The expression of behaviour under staged tests is stimulated by the test conditions: hypotheses are tested by asking questions about how animals express specific behaviours in a controlled artificial environment. As such, staged tests act can act as proxies for showing effects we would otherwise expect to find from longer-term ethological observation. Due to the relative efficiency of conducting staged tests compared to the observation of home cage behaviour, this method has been widely used to assess animal welfare (Paul *et al*., 2005). The most promising tests for the assessment of PAW are preference tests (animals show a preference for positively perceived resources) (Jensen *et al*., 2008), motivation tests (animals will work harder to achieve access to positively perceived resources) (Manser *et al*., 1996), and cognitive bias tests (animals in a positive affective state judge ambiguous stimuli as positive) (Saito *et al*., 2016).

While behavioural measures are the most promising direction for the assessment of PAW, as with physiological measures, they are not without limitations. One limitation is the reliance of behavioural measures on situational interference, *i.e.,* whether a stimulus or experience is classified as positive or negative is based on the human observer's evaluation (Mendl and Paul, 2020). As such, this poses the risk that the human observer perceives a situation differently from the animal subject. Further, interpretation of behavioural measures can be difficult since some behaviours are elicited in response to contrastingly valenced stimuli. For example, play occurs in response to both putatively stressful (Norscia and Palagi, 2011) and rewarding experiences (Hammond *et al*., 2019). However, these limitations can be addressed by using a range of behavioural measures.

Positive animal welfare for rats: why is it important, and how can we measure it?

The importance of promoting PAW for rats

The majority of the formal literature on PAW highlights the importance of positive experiences for farmed species, with a particular focus on birds and mammals such as poultry, pigs, cows, and sheep (Edgar *et al*., 2013; Lawrence *et al*., 2018; Proctor and Carder, 2015; Rowe and Mullan, 2022). However, the application of PAW and our responsibility to provide 'a good life' to laboratory species, particularly rodents, has been overlooked until relatively recently (Makowska and Weary, 2020). This discrepancy has meant that the current welfare standards for rodents have a basic and limited focus on mitigating unnecessary pain, harm, or distress (Makowska and Weary, 2020). Further, compared to studies using mammals, rodents are used in higher numbers for more invasive procedures and are less likely to receive pain relief (Makowska and Weary, 2013). While mice are currently the most widely used species in research, the number of rats used for scientific purposes is thought to bypass the number of both mice and fish (Abbott, 2009). This is partially due to advances in rat genomics which may reverse the decline in rat use relative to mice and the behavioural and neural complexity of rats compared to mice (Abbott, 2009). Considering that a conservative estimate reported that over 1.9 million rats were used for research purposes in the UK, USA and EU per annum as of 2018 (Carbone, 2021; UK Home Office, 2016), contributions toward understanding positive affective states and how to induce them can be seen as vital to improving rat welfare. Consequently, this project uses rats as the species of choice.

How can we measure the presence of positive affect in rats?

There are several proposed methods of assessing affective states in rats (for a review, see (Jirkof *et al*., 2019; Makowska and Weary, 2013). In line with the wider literature on PAW, as previously discussed, I believe that the most promising direction for the assessment of PAW in rats uses an outcome-based approach to assess behaviour under both home cage and staged test conditions. While some of the behavioural measures have been widely validated (*e.g.,* vocalisations, approach-avoidance behaviour, and preference tests), there is a need for further work to confirm the validity of others (*e.g.,* anticipatory behaviour) as measures of positive affect. Here, I will provide an overview of the three measures I will use in my thesis; ultrasonic vocalisations, approach and avoidance behaviour, and sucrose consumption.

Ultrasonic Vocalisations

The use of vocalisations as a potential indicator of animal welfare has gained momentum in the past twenty years across domestic species (*e.g.,* pigs: Briefer *et al*., 2022; cattle: Watts and Stookey, 2000; poultry: Marx *et al*., 2001; horses: Pond *et al*., 2010; dogs: Pongrácz *et al*., 2010 and rodents: Wöhr and Schwarting, 2013). As such, there is now considerable evidence across a variety of mammalian species that the underlying affective state influences the production of vocalisations (Brudzynski, 2013; Laurijs *et al*., 2021; Mendl and Paul, 2020). However, within animal welfare science, there is a significant bias towards the study of vocalisations under the context of negative affective states, such that the production of vocalisations in response to positive stimuli remains relatively unexplored (for a review, see Briefer, 2012). An exception to this is the study of vocalisations produced by rats.

In rats, the majority of vocalisations occur within the ultrasonic range, that is, of a high frequency, typically between 20 – 100-kHz, out with the human audible range of up to 20-kHz (Brudzynski and Zeskind, 2018). Since the initial discovery during the mid-20th century (Anderson, 1954; Noirot, 1968), these vocalisations, known as USVs, have been extensively studied with respect to their function as social signals, their underlying emotional context, and the role of fundamental brain mechanisms in their production (Schwarting *et al.,* 2018). Although initially hypothesised as merely a by-product of locomotion (Blumberg *et al.,* 1992), there is now considerable evidence for the role of USVs as a direct and non-invasive tool for measuring rat affective state.

Rats produce two distinct types of USVs, which differ in acoustic structure in terms of frequency, amplitude, and duration (Wöhr and Schwarting, 2009) and encompass several subtypes (Burke *et al*., 2017; Jennifer M. Wright *et al*., 2010). These broad types of USVs are evidenced to be associated with contrastingly valenced affective states through the different brain structures responsible for their initiation, the putative valence of the situations in which they are produced, and through pharmacological manipulation (*e.g.,* Barker, 2018; Brudzynski, 2013; Burgdorf *et al*., 2008; Burman *et al*., 2007; Knutson *et al*., 2002; Wöhr and Schwarting, 2013). USVs in the 30 – 90-kHz range, known as 50-kHz USVs, are associated with positive affect, and USVs in the 18 - 30-kHz range, known as 22-kHz USVs, are associated with negative affect.

The production of 50-kHz USVs is particularly relevant for the assessment of the presence of positive affective states in rats. These calls are modulated by the mesolimbic dopaminergic system (*i.e.,* the main reward pathway) (Brudzynski, 2013) and are produced in anticipation and receipt of hedonic stimuli, such as play (Knutson *et al*., 1998), sex (Barfield and Geyer, 1972) and opioid drugs (Burgdorf *et al*., 2011). As such, the presence of 50-kHz USVs is argued to reflect high arousal and positively valenced affective states, such as excitement and joy (Knutson *et al*., 1999). Some authors have even suggested that 50-kHz USVs are an ancestral homolog of laughter in humans (Panksepp, 2005; Panksepp and Burgdorf, 2003).

Over the past decade, there has been increasing evidence that the production of 50-kHz USVs can be used as a quantitative index of positive affect (Hinchcliffe *et al*., 2020). The majority of this evidence comes from neurobiological studies using the administration of psychostimulants, such as cocaine, or stimulation of brain areas associated with reward, which show that 50-kHz USV production is directly related to the amplitude of administration (Browning *et al*., 2011; Burgdorf *et al*., 2011, 2007; Meyer *et al*., 2012). However, there is more recent evidence of the graded nature of 50-kHz USVs, which does not rely on pharmacological manipulation or invasive brain procedures; Hinchcliffe *et al*. (2020) demonstrated that the number of 50-kHz USVs produced by individuals in response to short-term positive stimuli (*i.e.,* tickling by a human experimenter) was correlated with an independent test of affective state (an Affective Bias Test). As such, 50-kHz USV production is thought to act on a graded scale, such that individuals who produce the high rates of USVs in response to certain stimuli are in a more positive affective state and associate a higher value to the presented reward. As such, the production of 50-kHz USVs is a valuable method of measuring positive affect in rats.

Approach-avoidance behaviour

All species, from single-cell amoebae (Schneirla, 1965) to humans (Elliot and Thrash, 2002), show at least a basic form of approach and avoidance behaviour (Jones and Gosling, 2014). The ability to decide whether to approach or avoid certain stimuli is fundamental to survival by driving movement toward beneficial stimuli and away from potentially harmful stimuli (Kurt, 1936). As such, the perceived valence of specific stimuli (*i.e.,* positive or negative) underpins whether an animal will exhibit approach or avoidance movement (Kurt, 1936). As an evolutionarily conserved behaviour (Tooby and Cosmides, 1990), the balance between approach and avoidance is based on each species' innate strategy for survival and shows consistent species-specific responses (Elliot, 2013). Approach-avoidance is also sensitive to individual variation (Elliot, 2013), such that individual differences in approach and avoidance have been considered as measures of personality (Elliot, 2013) and individual motivation for specific stimuli (Greenberg, 2003).

In rodents, approach and avoidance behaviour have typically been assessed in standardised tests of negative affect, such as the Light-Dark Box and the Elevated Plus Maze (Bailey and Crawley, 2009). Rats have conflicting motivations to explore the environment to search for resources (*i.e.,* to approach positive stimuli) versus avoiding potentially harmful threats, such as predators (*i.e.,* to avoid negative stimuli) (Laricchiuta and Petrosini, 2014; La-Vu *et al*., 2020). Behavioural assays such as the Elevated Plus Maze measure the balance between these motivations and how induction of negative affect changes this balance (Paul *et al*., 2005). The potential of using approach and avoidance behaviour to evaluate positive stimuli has yet to be widely recognised. The balance between approach and avoidance can aid PAW measurement by providing insight into rat perception of specific stimuli (*i.e.,* rats will approach stimuli if perceived as positive) (Paul *et al*., 2005).

Sucrose preference test

In animal models of human psychiatric disorders, researchers have developed a range of tests for the assessment of depressive-like behaviours (Scheggi *et al*., 2018). One of the core symptoms of major depressive disorder and other psychiatric disorders, such as schizophrenia, is anhedonia (American Psychiatric Association, 2013). Anhedonia is defined as the reduced ability to feel pleasure from positive stimuli and is characterised by a lack of seeking such pleasurable situations (American Psychiatric Association, 2013). As anhedonia results from disruption within the reward-processing system, it can be assessed by testing the reaction to rewarding stimuli (Scheggi *et al*., 2018). The sucrose preference test is the most widely used method for assessing anhedonia (Scheggi *et al*., 2018), which uses the innate preference of rats for sweet substances (Corsini, 2002). Animals in putatively negative affect states consistently consume less sucrose than controls and show a decreased preference for sucrose over water (Papp *et al*., 1991; Willner, 1997; Willner *et al*., 1992).

While the sucrose preference test has traditionally been used as a behavioural assay for rodent models of psychiatric disorders, it has more recently been applied to assess animal welfare, focusing on the assessment of negative affective states. For example, Clarkson *et al.* (2018) found that sucrose preference was influenced by the handling method of mice, either the aversive method of tail handling or the refined method of tunnel handling. In response to tail handling, mice drank less sucrose, displaying more evidence of anhedonia and anxiety than when handling using a tunnel (Clarkson *et al*., 2018). While relatively unexplored, there is the potential for using this test to assess positive affective states and PAW, as the motivation to seek rewards is sensitive to both negative *and* positive affective states (Forbes and Dahl, 2005).

How can we induce positive affective states in rats?

Selected rat models of affective state

As a result of the wide use of rodents to study the behavioural and neurobiological implications of psychiatric disorders in humans (Ellenbroek and Youn, 2016), there are well-established models of inducing negative, but not positive, affective states in rats (Makowska and Weary, 2013; Scheggi *et al*., 2018). One such model is the Chronic Mild Stress Model, in which individuals are exposed to multiple mild stressors (*i.e.,* negatively perceived stimuli) over a prolonged period in an unpredictable and unrelenting sequence (Antoniuk *et al*., 2018; Willner, 2016). Logically, a model of inducing positive affect could be created by predictably presenting individuals with positively perceived stimuli. Two promising methods of inducing a positive affective state in rats are playful handling and playback of USVs.

Playful handling

Playful handling is a form of heterospecific play between rats and humans, developed from the Panksepp method of tickling (Panksepp and Burgdorf, 2000). Panksepp describes tickling as *'vigorous whole-body playful tickling (focusing on the ribs and ventral surface), with animals being repeatedly pinned four to six times, throughout the fifteen-second interval*' (Panksepp and Burgdorf, 2000) (**Figure 1.1ii**). Since the development of tickling over 20 years ago, more than 70 scientific articles on rat tickling have been published (Bombail *et al*., 2021). The majority of these published experiments report positive responses during tickling, *i.e.,* increased 50-kHz USV production, increased approach behaviour towards the human hand (LaFollette *et al*., 2017) and decreased indicators of anxiety and fear in standardised tests, such as the elevated plus-maze (Mällo *et al*., 2007) and the open-field test (Cloutier *et al*., 2013). Tickling also appears to mitigate the negative affective state induced by common procedures in rats (Cloutier *et al*., 2018). For example, when rats were exposed to restraint and intraperitoneal injection, those who had experienced tickling showed fewer markers of stress and less fear toward human handlers (Cloutier *et al*., 2012; Cloutier and Newberry, 2008).

However, there is growing evidence for individual variation in response to being tickled, primarily indicated by USV production (Bombail *et al*., 2021; Kõiv *et al*., 2016; Rygula *et al*., 2012). As previously discussed, USVs can act as a measure of affective state, such that the frequency of 50-kHz USVs can indicate the level of response during tickling. Using an affective bias test, Hinchcliffe *et al*. (2020) found that USVs correlated with responses to the affective bias test in a graded way, suggesting that high-calling rats found tickling a more positive experience than low-calling rats. However, some rats fail to produce 50-kHz USVs during tickling and exhibit behaviours indicative of a negative experience, such as freezing and production of 22-kHz USVs (Panksepp and Burgdorf, 2010; Schwarting *et al*., 2007). Thus, while tickling can induce a positive affective state in rats, there is evidence that the approach can potentially be improved (Bombail *et al*., 2021).

Rat social play is a fluid, complex interaction that involves many different behaviours, including chasing, pouncing, darting, wrestling, boxing, jumping, and pinning (*e.g.,* Whishaw *et al*., 2021) (**Figure 1.1i**). Each rat shows variation in the frequency and sequence of these behaviours, such that play does not follow a strict structure (Thor and Holloway, 1984). However, in the standard tickling protocol, the complexity seen during play is compromised by emphasising the need to deliver a set amount of pinning (Bombail *et al*., 2021).

Both the act of being involuntarily pinned and the frequency of its occurrence may explain why tickling is perceived by some rats less positively (Bombail *et al*., 2021). Pinning is a marker of social dominance between rats (Panksepp, 1981; Parent *et al*., 2013), with a high frequency of pinning marking the shift from positively valenced play to negatively valenced serious fighting (Boice and Adams, 1983). The standard tickling approach involves a high frequency of repeated pinning (four to six times during 15 seconds) (Panksepp and Burgdorf, 2010), which sharply contrasts with social play (less than ten pins per pair during five minutes) (Panksepp, 1981). Social play begins with one rat directing an attack toward the nape of the partner's neck from either the front or back (Pellis and Pellis, 2013). The defending rat will conduct a full rotation onto its back in response to this attack, *allowing* the attacking rat into the pinned position (Pellis and Pellis, 2013). Thus, during play, rats have a choice as to which behaviour they conduct, whereas, during tickling, the rat loses the ability to choose whether to be pinned, spending much of the 15-second interval being somewhat restrained on its back. This limits the capacity of the rat to express other playful behaviours and reduces the behaviours by which the experimenter can assess the response of the rat to the interaction (Bombail *et al*., 2021).

Schwarting *et al*. (2007) were one of the first to identify the opportunity to include a more expansive repertoire of behaviours into the tickling interaction. Their protocol involved using eight distinct types of manipulations that bear similarities to those seen in social play, such as the 'tail chase' and 'neck tickle'. However, this protocol did not adjust to the individual's response, as the sequence of manipulations was identical for each rat, with each manipulation applied for a defined number of times and duration (Schwarting *et al*., 2007). This strict procedure, similar to ticking, may explain why some individuals still produced 22-kHz USVs.

Playful handling is an alternative method of tickling that considers the complexity of play and takes into account individual variation. During playful handling, the experimenter responds to the individual rat by altering the interaction using a mixture of touching, tickling, and chasing and infrequent short pins (Bombail *et al*., 2019)(**Figure 1.1iii**). In this way, the rat chooses how they respond to a play solicitation by the experimenter and whether they want to solicit play themselves through gentle nips on the fingers. Thus, the interaction moves closer to heterospecific play and is likely to be a promising potential alternative to tickling (Bombail *et al*., 2021). So far, the evidence supports playful handling as an improved alternative to tickling. By incorporating more aspects of play and adjusting to the individual rat, Bombail *et al*. (2019) found that rats produced 233 USVs per minute on average during the fifth playful handling session. This is considerably more than the approximately 90 USVs per minute during the fourth session found by Burgdorf *et al*. (2005) using the Panksepp method. However, evidence for this method is in the early stages, and more validation is required for the use of playful handling as a model of positive affective state in rats.

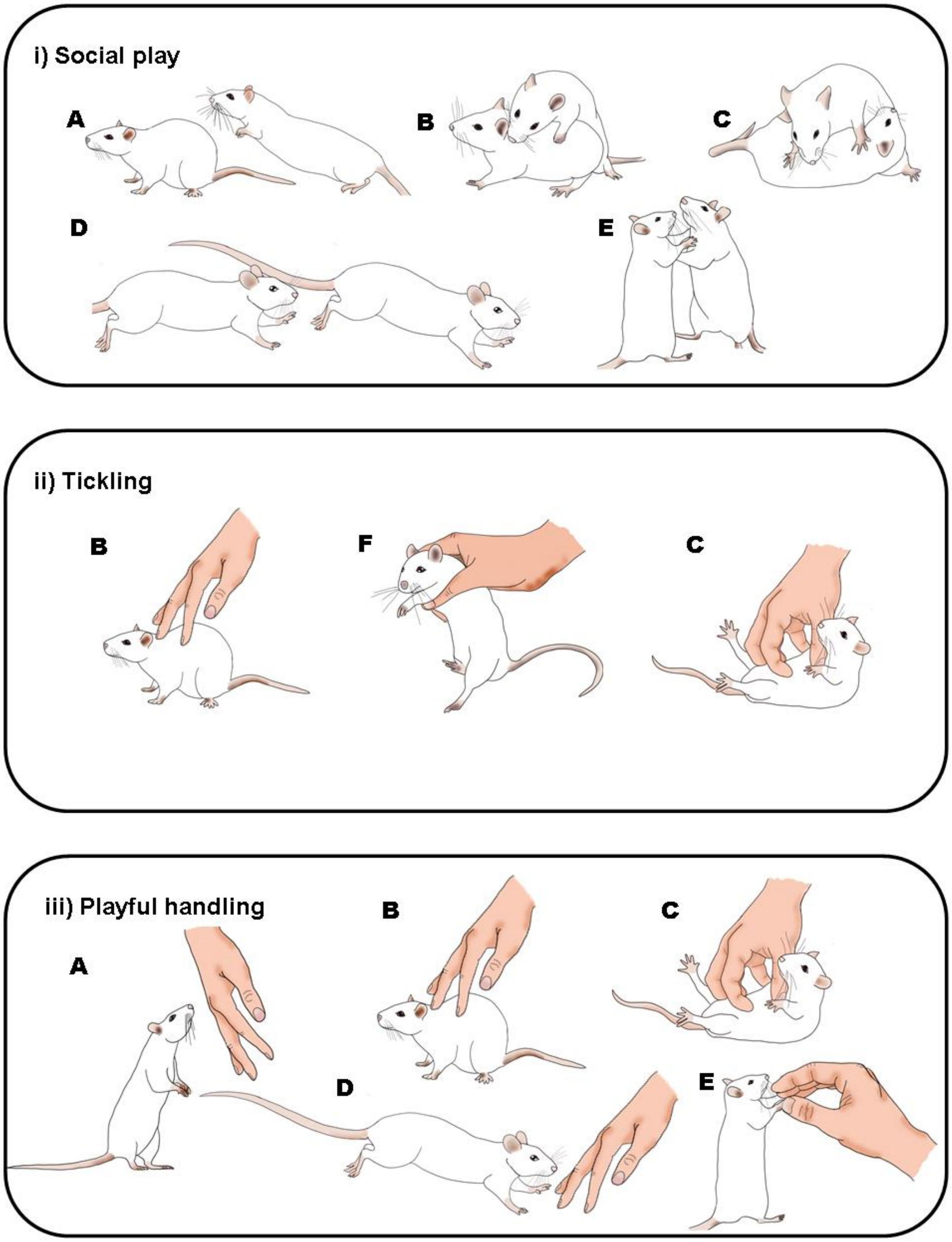


Figure 1.1 Illustration of the behaviours seen in rats during i) social play (Pellis and Pellis, 2013), ii) the Panksepp variation of tickling (Cloutier *et al.*, 2018) and iii) playful handling (Bombail *et al.*, 2021). Letters indicate behaviours that share similar physical characteristics; A) pouncing, B) nape contact, C) pinning, D) chasing, E) boxing, and F) flipping. During social play (i) or playful handling (iii), behaviours can occur in any order and do not always occur in each bout. During tickling, the sequence of behaviour is always B), F), and then C).

Playback of USVs

Emotional contagion is thought to be a basic form of empathy and is described as the process through which the affective state of one subject changes as a result of perceiving and matching the affective state of another (Hatfield *et al*., 1993; Nakahashi and Ohtsuki, 2015; Panksepp *et al*., 2001; Waal, 2008, 2007). It is this transfer of affective state which distinguishes emotional contagion from mimicry, which is defined as the involuntary copying of another's physical appearance or behavioural contagion (Chartrand and Bargh, 1999; Kennedy and Thorpe, 1964). Thus, a measure of affective state is required in order to distinguish emotional contagion from other empathy-related phenomena of behavioural contagion or mimicry (Adriaense *et al*., 2020; Pérez-Manrique and Gomila, 2021). Considering the adaptive value of emotional contagion through the promotion of behavioural and emotional synchronisation within social groups (Špinka, 2012; Spoor and Kelly, 2004) and the ability of the affective state from just a few individuals to influence entire social groups (Held and Špinka, 2011; Reimert *et al*., 2013; Špinka, 2012), this process is highly relevant for animal welfare.

Despite the potential of using emotional contagion to spread positive affective states and improve animal welfare, relatively few studies have explored this phenomenon across species. This is particularly evident in studies on emotional contagion in rats, where there is a considerable bias toward investigating negative emotional contagion (Pérez-Manrique and Gomila, 2021). For example, there are multiple studies evidencing negative emotional contagion through a 'fear observation' paradigm in which one demonstrator animal receives an electric shock resulting in increases in the freezing behaviour of a watching conspecific (Atsak *et al*., 2011; Carrillo *et al*., 2019, 2015; Han *et al*., 2020). One of the few available studies directly investigating emotional contagion of positive affective states in rats uses the playback of USVs.

In this study, Saito *et al*. (2016) investigated the presence of emotional contagion by testing the response of rats to playback of 50-kHz USVs or 22-kHz produced by a conspecific via a cognitive bias task. This experiment was based on the premise that as 50-kHz USVs are associated with a positive affective state, rats experiencing playback of these USVs prior to the cognitive bias task are likely to be in a positive affective state and show a positive ‘optimistic’ response towards an ambiguous cue (*i.e.,* pressing the reward lever). Whereas, as 22-kHz USVs are associated with a negative affective state, rats experiencing playback of these USVS prior to the cognitive bias task are likely to be in a negative affect state and show a negative ‘pessimistic’ response towards an ambiguous cue (*i.e.,* pressing the lever to avoid punishment). Saito *et al*. (2016) found that USV playback prior to the cognitive bias test had a stimulus-specific effect on performance, with 50-kHz USV playback leading to a more optimistic response compared with more pessimistic responses after playback of 22-kHz calls**.** As the performance of the rats in the judgement bias test matched the affective state of the playback stimuli, there is evidence that playback of vocalisations can facilitate emotional contagion.

A wealth of evidence of the effect of the playback of USVs on behaviour has been generated using the radial maze playback paradigm (Bigelow *et al*., 2022; Schwarting, 2018; Schwarting *et al*., 2018; Seffer *et al*., 2014; Wöhr *et al*., 2016; Wöhr and Schwarting, 2013)*.* Although the primary purpose of this paradigm was to develop a behavioural assay to assess rodent models of neurodevelopmental disorders in humans (Wöhr *et al*., 2016), results from these studies offer valuable insight into the potential of USVs to alter rat affective state. In this paradigm, an individual rat is placed in the centre of an elevated eight-arm maze and exposed to playback from a speaker at the end of one of the arms (Wöhr *et al*., 2016). Across studies, there are two main outcome measures: (1) the total distance travelled as an indicator of social exploratory behaviour and (2) the time spent on arms closest to the speaker versus distal arms as a measure of social approach (Schwarting *et al*., 2018). However, in a paper describing the protocol of the radial maze, Wöhr *et* *al*. (2016) also identify a third outcome measure: (3) the number of USVs produced by the subject individual (Wöhr *et al*., 2016). This third outcome measure is underexplored, despite the potential of USV production to offer an additional insight into affective state during the test.

In response to rat-produced 50-kHz positive calls, the radial maze paradigm has been successful in eliciting consistent behavioural responses, with both juvenile and adult rats showing increased approach behaviour and social exploration compared to background noise (Schwarting *et al.,* 2018). The strength of this effect is consistently dependent on the rat's developmental stage, being much stronger in juveniles than adults (Wöhr and Schwarting, 2009), which is in line with the critical prosocial role of 50-kHz USVs during social play, which has the same sensitive period (Knutson *et al*., 2002). In contrast, behavioural responses to 22-kHz calls in the radial maze have been less consistent. Rats display behaviours indicative of the fight or flight system in response to computer-generated sine-wave tones, yet rat-produced calls show no or weak effects on behaviour (Sadan *et al*., 2008; Schwarting *et al*., 2018; Wöhr and Schwarting, 2007). This may be due to a confound between acoustic stimuli, where sine-wave calls are both artificial and presented at a louder volume with a longer duration per call (Schwarting *et al*., 2018). In addition to the effects on behaviour, receivers of these USVs also show matched activation of the brain structures implicated in the production of such USVs, *i.e.,* playback of 50-kHz USVs activated the mesolimbic dopaminergic system, whereas playback of 22-kHz USVs activated the mesolimbic cholinergic system (Parsana *et al*., 2012; Wöhr and Schwarting, 2007).

The use of a playback paradigm offers several practical advantages as a method of inducing a positive affective state in rats. Studies investigating emotional contagion in rats typically involve a demonstrator animal (Hernandez-Lallement *et al*., 2020; Pérez-Manrique and Gomila, 2021). However, as previously discussed, the behavioural matching of one observer animal to the demonstrator animal indicates behavioural contagion rather than emotional contagion (Kennedy and

Thorpe, 1964; Zentall, 2001). Thus, in order to confer emotional convergence between the two animals, the affective state of the demonstrator animal must also be measured and known in addition to the subject animal. However, the literature rarely reports the measurement of the behaviour of the demonstrator animal and even less so the measurement of the affective state of such animals (Pérez-Manrique and Gomila, 2021). Investigation of emotional contagion using demonstrator animals also invariably requires the use of more individuals. This is contrary to the aims of reducing the number of animals in research, especially considering that the majority of demonstrator animals experience negative stimuli, such as foot shock or long-term fear conditioning (*e.g.,* Atsak *et al*., 2011; Knapska *et al*., 2010). As playback paradigms present pre-recorded tracks of known affective state from other studies without the presence of a demonstrator animal, these potential problems are mitigated. As such, playback of USVs appears to be a promising method to induce positive affective states in rats through the process of emotional contagion.

Play as a promising potential indicator of positive animal welfare

Why is play a promising indicator of PAW?

Happiness is never better exhibited than by young animals, such as puppies, kittens, lambs, etc., when playing together, like our own children (Darwin, 1872, p.47)

Despite the assistance of Charles Darwin in legitimising the study of animal behaviour, it was not until the early 1980s that non-human play received real scientific attention (Bekoff, 1984). Since then, ethologists, comparative psychologists, and evolutionary biologists alike have primarily focused on describing the forms and functions of play (Graham and Burghardt, 2010). While these questions are still the topic of current research (*e.g.,* Dawson *et al*., 2022), play undoubtedly represents a promising indicator of PAW (*e.g.,* Boissy *et al*., 2007; Held and Špinka, 2011). In addition, specific qualities of play are beneficial for the assessment of PAW under practical conditions; play is widely seen across species, rapidly identifiable, and occurs spontaneously (Burghardt, 2005; Fraser and Duncan, 1998; Held and Špinka, 2011).

Much of the initial investigation into play in domestic animals has focused on laboratory rodents (Pellis and Pellis, 2013). Rats are relatively cheap, being both small and short-lived, and as a widely used research animal, have the largest repository of detailed physiological, behavioural, neurological, and genetic information available for any mammal (Burghardt, 2009). Held and Špinka (2011) propose four aspects by which play is a promising direction to assess PAW. Here I provide the evidence for these aspects seen in rats.

First, play indicates when animals are under favourable conditions, as it is reduced and can even disappear when the animal's potential ability to survive is challenged (Held and Špinka, 2011; Boissy *et al*., 2007; Lawrence, 1987). For example, physical restraint for 30 minutes has been shown to completely suppress subsequent home cage play in rats (Klein *et al*., 2010). In contrast, enhanced conditions achieved through providing environmental enrichment promote play. For example, in juvenile rats that had experienced prenatal stress, increased space allowance and provision of different novel objects stimulated social play (Morley-Fletcher *et al*., 2003).

Second, play is a positively valenced affective experience, as indicated by the intrinsic motivation to play and its rewarding nature (Held and Špinka, 2011). Play can serve as a reward for juvenile rats during conditioned place preference testing and during operant conditioning (Rolls, 2005). Further, subcortical brain regions and neurotransmitter systems that mediate reward also play a central role in regulating social play (for full reviews, see Knutson *et al*., 1998; Siviy, 1998; Trezza *et al*., 2010). For example, the injection of opioid agonists, such as morphine, into the nucleus accumbens (an area associated with the mesolimbic reward pathway) increases hedonistic responses in rats to a sucrose solution and increases social play (Normansell and Panksepp, 1990).

Third, from an evolutionary perspective, as play has energetic costs, this must be balanced by some benefit to fitness (Held and Špinka, 2011). Many different hypotheses for the function of play have been generated, with the overarching theme that the functional benefits of play can have a positive impact in both the short and long term (Held and Špinka, 2011). In the shorter term, play may act as a coping mechanism to help individuals deal with immediate conditions which are causing a negative affective state (*e.g.,* Cloutier *et al*., 2014), and in the longer term, play may help individuals become more resilient to such environments (*e.g.,* Pellis and Pellis, 2013).

Finally, play can spread a positive affective state and promote PAW through its contagious nature (Held and Špinka, 2011). Play is considered an example of behavioural contagion in that the expression of play can stimulate play in others (Bekoff, 2001; Held and Špinka, 2011). For example, in rats, high-playing individuals induced more play in their partner than low-playing individuals (Pellis and McKenna, 1992). As play is associated with a positive affective experience and the stimulation of play in another animal is also likely to result in a positive affective state, play can also be considered a form of emotional contagion (Hammond *et al*., 2019; Held and Špinka, 2011; Špinka *et al*., 2016). Considering that play contagion would result in the spread of positive affective state, stimulating the expression of play behaviour is a promising method of promoting PAW (Held and Špinka, 2011; Špinka, 2012).

The current state of play as an indicator of PAW

The majority of research has focused on the impact of negative affect and negative welfare conditions on play (Ahloy-Dallaire *et al*., 2017). Across animal species, there is evidence that play is suppressed by poor welfare conditions, such that the absence of play is now widely considered an indicator of a negative affective state. However, in a review of the evidence on the impact of positive affective states on play, Ahloy-Dallaire *et al*. (2017) argue that there is insufficient evidence to conclude whether play is sensitive to increases in positive affect.

To fill this gap in understanding the relationship between play behaviour and PAW, we must study animals that are unambiguously not experiencing situations associated with negative affect and develop methods of inducing a positive affective state (Ahloy-Dallaire *et al*., 2017). We can then assess the impact of this experimentally induced positive affect on play behaviour and make conclusions about the potential use of play as an indicator of PAW. This research is particularly important for my model species, as rodents are widely used under experimental conditions yet typically receive a lower standard of care than other mammals (Makowska and Weary, 2013; Makowska and Weary, 2020). Thus, a key direction for achieving a good life for rats under our care is to develop methods of inducing and assessing positive affective states in rats.

Thesis Aims and Outline

The aims of this doctoral research were twofold:

1. To develop methods of inducing a positive affective state in rats.
2. To investigate whether play is sensitive to the manipulation of affective state as an indicator of PAW.

In all experiments, the main measures of affective state were USV production and approach-avoidance behaviour, with the addition of a sucrose preference test in Chapters 2 and 5.

In **Chapter 2**, I investigate the response of rats to a recently developed method of heterospecific play named playful handling. All rats were playfully handled, or control handled for five days as individuals in an arena. The successful induction of a positive affective state in playfully handled rats allowed the investigation of play in the home cage before and after handling as an indicator of PAW.

In **Chapter 3,** I investigate rats' responses to playful handling under a different environmental context. All rats were playfully handled, or control handled for three days as pairs in the home cage. This protocol reduced the time investment required per rat, which has been cited as the main barrier to the uptake of heterospecific play in laboratory staff.

In **Chapter 4**, I investigate the response of rats to the playback of acoustic stimuli in the home cage as a method of altering affective state. Acoustic stimuli were 50-kHz and 22-kHz USVs, as well as man-made White Noise and Background Noise. Using two cohorts, all rats were presented with all acoustic stimuli in a Latin Square design across three days. Assessment of play as an indicator of PAW was conducted during playback in the home cage.

Following the initial support for the use of playback to induce a positive affective state in rats in Chapter 4, in **Chapter 5**, I investigate the response of rats to the repeated playback of 50-kHz USVs, White Noise, and Background Noise over five days. The assessment of play as an indicator of PAW was conducted both during playback and in the home cage before and after playback.

Finally, in **Chapter 6**, I critically examine how my research has addressed these two aims and highlight potential areas for future research to expand our understanding of positive affective states to ultimately promote PAW.

Chapter 2: Application of playful handling as individuals in an arena to induce a positive affective state and measure changes in play in juvenile male Wistar rats

Abstract

A promising method for inducing positive affective states in laboratory rats is heterospecific play. The Panksepp method of tickling is the most popular method of heterospecific play, with some rats demonstrating an increase in markers of positive affective state; *i.e.,* increased production of positively valenced 50-kHz ultrasonic vocalisations (USVs) and increased approach behaviour towards the experimenter. However, there is vast individual variation in response to tickling, which poses the risk that tickling is aversive for some individuals. Playful handling is an alternative to tickling, which incorporates more aspects of social play and takes account of individual variation. As a relatively novel method, the aims of this study were to validate the use of playful handling to induce a positive affective state and investigate variation in responses to playful handling. On the evidence that playful handling induced a positive affective state, another aim of this study was to investigate whether play can act as a quantifiable indicator of positive affect. Play is a promising indicator of affective state in animals. Yet, there remains uncertainty on how play changes in response to positive experiences. Juvenile male Wistar rats (N = 24; 6 playfully handled cages and 6 control cages; 45 days old) were housed in pairs and assigned by cage to receive playful handling or control handling for five days. All handling was conducted individually in an arena separate from the home cage. Playful handling involved the experimenter touching, tickling, chasing, and gently pinning the rat. The interaction lasted for two minutes, with 15 seconds of active play alternated with 15-second pauses. During the

pauses and for the entire interaction for control rats, the hand remained stationary on the side of the arena. On the first and last days of handling, rats were also exposed to a 30-second free roam test before and after handling as measures of anticipation and lasting positive affective state. Affective state in response to handling was measured through USV production, approach behaviour, and a high concentration (20%) sucrose preference test on the evening of day five as a novel measurement of the rewarding properties of heterospecific play. In support of previous evidence that playful handling is a positive experience for rats, playfully handled rats showed increased markers of positive affect compared to control rats; playfully handled rats produced three times more USVs (96.8 vs 29.5 ± 14.1 USVs/minute on day five; $F_{1, 383} = 61.9$, $p < 0.001$), conducted more approach behaviour (6.7 vs 5.3 ± 0.4 seconds/minute on day five; $F_{1, 191} = 5.1$, $p = 0.05$) and drank more sucrose than control rats (31.3 vs 19.3 ± 1.7 ml/16 hours; $F_{1,23} = 8.76$, $p = 0.008$). Playfully handled rats showed increased variation in response to handling than control rats in USV production (CV on day five = 10.4% vs 52.6%) but not approach behaviour (CV on day five = 65.6% vs 73.8%) or sucrose consumption (CV on day five = 11.5% vs 18.3%). In order to investigate changes in play, home cage behaviour was recorded for 15 minutes before and after handling on days one and five. Playfully handled rats performed more solitary (4.1 vs 0.8 ± 0.9 events per 15 minutes on day five; $F_{1,47} = 3.9$, $p = 0.05$) but not social play ($F_{1,47} = 0.1$, $p = 0.6$) in the home cage before handling. This was not seen in control rats. In general, these results confirm that playful handling is a positive experience for rats and suggests that play is sensitive to increases in positive affect.

Introduction

Across animal welfare science, it is becoming more widely recognised that it is the balance of positive and negative affective states which determines an animal's welfare status (Fraser and Duncan, 1998; Green and Mellor, 2011). To achieve a state of positive animal welfare, positive affective states should predominate over negative states across an animal's lifetime (Lawrence *et al*., 2017). However, the traditional focus on the study of alleviating negative states has meant this balance between affective states cannot be measured. This traditional focus is particularly evident in laboratory species, including rats. As a result, while there are well-established examples of experiences that can induce negative affective states, such as the Chronic Mild Stress Model (Willner *et al*., 1992), there is less knowledge of experiences that induce positive affective states (Makowska and Weary, 2013).

One promising method for inducing positive affective states is heterospecific play between humans and rats. The predominant form of heterospecific play is the Panksepp method of tickling, with over 70 scientific articles published on rat tickling as of 2021 (LaFollette *et al*., 2017). Tickling aims to mimic aspects of social play in rats by using the human hand to stimulate areas of the rat's body, which are also contacted during social play (Panksepp and Burgdorf, 2000). This method mainly focuses on the rats' nape of the neck and ventral areas where access to the latter is gained through the experimenter repeatedly flipping and pinning the rat onto its back (Panksepp and Burgdorf, 2000).

Rats often emit positively-valenced ultrasonic vocalisations (USVs) in response to tickling by a human experimenter (*e.g.,* Burgdorf and Panksepp, 2001; Cloutier *et al*., 2013; Hammond *et al*., 2019; Panksepp and Burgdorf, 2000). USVs are recognised as a direct and non-invasive tool for measuring a rat's subjective affective state (Barker, 2018; Wöhr and Schwarting, 2009), with evidence that USV response to tickling acts as a graded measure of positive affect (Hinchcliffe *et al*., 2020). As discussed in Chapter 1, rats produce two types of USVs which are thought to reflect contrasting affective states; 50-kHz USVs are produced in response to a wide range of putatively positive stimuli, such as during mating, receipt or anticipation of a food reward, and social play (Burgdorf *et al*., 2019), while 22-kHz USVs are produced in response to negative stimuli, such as in response to predator cues and during electric footshock (Wöhr and Schwarting, 2013). In addition to the production of 50-kHz USVs, tickled rats also show shorter latencies to approach the experimenter's hand (LaFollette *et al*., 2017) and increased time in contact with the experimenter's hand (Hammond *et al*., 2019).

However, there is increasing evidence of individual variation in response to tickling, with some rats failing to produce indicators of positive affect (Bombail *et al*., 2021, 2019; LaFollette *et al*., 2017). The graded scale of 50-kHz USVs suggests that individuals who produce fewer USVs enjoy tickling less than conspecifics who produce more USVs (Hinchcliffe *et al*., 2020). The number of 50-kHz USVs produced in response to tickling varies considerably between individuals (LaFollette *et al*., 2017), such that it is possible to selectively breed for high and low callers in response to tickling (Burgdorf *et al*., 2005). 50-kHz USV responses to tickling also tend to be consistent across sessions within individuals, suggesting that USV production is a stable individual trait (Bombail *et al*., 2019; Mällo *et al*., 2007). This variation in response to tickling poses a risk that some individuals perceive the procedure as neutral or possibly aversive and raises the question of whether tickling can act as a universal method of improving rat welfare.

In Bombail *et al*. (2021), we suggest that this individual variation in response to tickling may be due to the emphasis on pinning and a lack of flexibility in response to the behaviour of individual rats. Identifying these potential limitations of tickling as a method of heterospecific play led to the development of playful handling (Bombail *et al*., 2021). Playful handling aims to incorporate the diversity and unpredictability of social play. The experimenter reacts to the individual rat through variation in the type and intensity of behaviours seen in social play, such as touching, tickling, chasing, and being chased. In the first research paper that uses playful handling, Bombail *et al*. (2019) reported a significant increase in 50-kHz USV production during playful handling compared to days when they were not handled and compared with control rats. It is worth noting that although the authors use the term 'tickling' throughout, the more flexible method used is what our group now terms as playful handling (Bombail *et al*., 2021). Further evidence of the ability of playful handling to induce a positive affective state in rats is needed to validate playful handling as an alternative protocol to tickling. There is also the opportunity to investigate other methods of measuring rat perception towards handling in addition to USV production and approach behaviour as the primary indicators of affective state. For example, the measurement of preference for sucrose over water is a widely-used test of anhedonia in rodent models of depression, which could be adapted to measure behavioural and neural changes induced by a positive experience.

In addition to the limited methods of inducing positive affective states, there are insufficient quantifiable indicators of positive affect in animals (Lawrence *et al*., 2017). Play is proposed as a promising potential indicator of affective state and welfare in animals (Boissy *et al*., 2007). There is considerable evidence within animals and children that play is sensitive to adverse psychological and environmental conditions, such as social structure, food availability, and experiencing pain or fear (reviewed in Held and Špinka, 2011). While this evidence provides support for lack of play to be used as an indicator of negative affective state and poor welfare (Bateson and Martin, 2014; Boissy *et al*., 2007; Burghardt, 2005; Held and Špinka, 2011; Lawrence, 1987), there is a relative lack of evidence to support the use of play as an indicator of positive affective state and welfare.

If play were a marker of positive affective state, putatively positive experiences would increase the frequency of play (Ahloy-Dallaire *et al*., 2017). However, in a review of the current evidence, Ahloy-Dallaire *et al*. (2019) state there is insufficient evidence to support this argument due to the small body of research. The use of playful handling to induce a positive affective state in rats and measure changes in play would potentially help fill this gap in the literature. While there is currently no investigation into the impact of playful handling on spontaneous play, previous work has suggested a relationship between tickling and play (Hammond *et al*., 2019; Knutson *et al*., 1998; LaFollette *et al*., 2018a, 2018b). For example, tickling was shown to increase solitary play in the home cage prior to a predicted handling session (Hammond *et al*., 2019). Logically, playful handling would be expected to have a similar or more prominent effect on play as tickling, considering the relationship between the two methods of heterospecific play.

The aims and hypotheses of this study were;

i) To investigate whether playful handling of individuals in an arena induces a positive affective state in rats as indicated by USV production, approach behaviour, and sucrose preference. I hypothesised that playfully handled rats would produce more 50-kHz USVs, show increased approach behaviour towards the experimenter and consume more sucrose than control rats as a reflection of their increased positive affective state.

ii) To investigate inter-individual and intra-individual variation in response to playful handling as individuals in terms of USV production and approach behaviour. I hypothesised that playfully handled rats would show high inter-individual variation and low intra-individual variation in USV production and approach behaviour.

iii) To investigate whether the induction of a positive affective state through playful handling as individuals in an arena results in a change in play in the home cage before and after handling. I hypothesised that the positive affective state induced by playful handling, but not control handling, would be reflected by an increase in play in the home cage.

Methods

Ethics

This study was conducted in accordance with the European Union directive of 22nd September 2010 (2010/63/EU), and the UK Animals (Scientific procedures)

Act 1986. There was further approval by the local animal experimentation ethics committees (Comité d'Ethique en Expérimentation Animale INRA IdF-Jouy-en-Josas/AgroParisTech (Comethea); permission #16–17), Roslin Institute Animal Welfare and Ethical Review Body (AWERB), the Royal (Dick) School of Veterinary Studies Veterinary Ethical Review Committee (VERC) and SRUC's Animal Ethics Committee.

Animals, housing, and husbandry

All work was conducted at the National Research Institute for Agriculture, Food and Environment (INRAE) at the Île-de-France-Jouy-en-Josas Research Centre in France between 1st April and 10th April 2019. Rats were bred and raised in-house from primiparous mothers of Wistar strain. Wistar rats are the third most commonly used strain in tickling experiments (23%, N = 13) (LaFollette *et al*., 2017). From weaning (21 post-natal days), male Wistar pups (N = 24) were kept firstly in groups of four for five days (to reduce stress) then in pairs for the rest of the study (N = 12 cages). Within pairs, rats were randomly selected across litters and were unrelated to prevent the possibility of any affinity or animosity due to shared early life experiences. Rats were also matched by weight within 2g to account for the relationship between body condition and play behaviour (Hammond *et al.*, 2019).

Data collection began 37 post-natal days as play shows the highest absolute levels between 32 and 40 days of age depending on the rat strain, with the peak of play being on the latter end of this range for Albino rats (Panksepp, 1981; Thor and Holloway, 1984). Adolescent males show higher frequencies of play fighting (Pellis and Pellis, 2013) and more robust responses to tickling than adults (Burgdorf and Panksepp, 2001). The sample size was calculated using Mead's resource equation (Arifin and Zahiruddin, 2017; Mead, 1988), with 17 total degrees of freedom of the error component.

Rats were housed in clear plastic cages with a wire lid (Techniplast 1291H, 42.5 × 26.6 × 18.5cm). Each cage was supplemented with wood chip bedding, ad libitum access to food (M25 Extralabo, Dietex France), and water. Cage cleaning was carried out by a familiar caretaker once a week at least two hours before any experimental procedure taking account of the experimental timeline. Rats were in a reverse light cycle, so handling could occur during the dark period when the rats are naturally active (lights off at 0900 with handling between 1000 and 1500). Low-intensity red lighting (4.5 lx measured using an Isotech digital light meter Lux-1337) was used during the dark phase to keep the rats within the active dark photoperiod and encourage USV production (Knutson *et al*., 1998). The room was maintained at a constant temperature (23 ± 0.3 °C) and humidity (34.5 ± 8.9%). Cages were situated on two tiers of a standard cage rack with four cages per rack (N = 12 cages). Both rats within a cage received marks on the tail using a non-toxic black marker for individual identification.

Experimental design

Twelve cages were assigned to receive either playful or control handling (N = 6 playfully handled cages and N = 6 control handled cages). Cages were assigned to treatment according to bodyweight by taking an average cage weight after the first habituation session and assigning cages of the closest weight to playful or control handling. There was a negligible 0.26g difference in average cage weight between cages assigned to playful handling and control handling. Overall mean weight and standard deviation on the day before handling was 138.3 ± 4.5g*.* As there is evidence that light intensity, noise level, and human activity can impact rat behaviour (Cloutier and Newberry, 2010), the distribution of cages on the rack was balanced between treatment and control. Within each rack of four cages, two were assigned to receive playful handling treatment, and two were assigned to control handling.

After four days of habituation, rats received five sessions of either playful handling or control handling in the arena as individuals across five days. Within these five days, days one and five were assigned as recording days, with home cage behaviour recorded for 15 minutes before and after handling. These home cage recordings allowed for investigation of the effect of playful handling on play and other spontaneous behaviour. On non-recording days (days two to four), rats were still handled according to the assigned treatment without home cage recording. On the evening of day five, rats were given a sucrose preference test.

Experimental set-up

All testing was conducted in a different room to the holding room as there is evidence that the emotional state of one animal can be transferred (emotional contagion) to another through USV production, odours, or another mechanism (Hammond *et al.*, 2019). Similar to the holding room, this room was lit with red lighting, which delivered a low-intensity illumination (4.5 lx) to keep the rats within the active dark photoperiod and encourage USV production (Knutson *et al*., 1998).

Rats were handled as individuals in an arena. The experimental arena was made of Plexiglass® with an open-top (66 × 41 × 40 cm), lined with a base of woodchip shavings to provide a non-slip surface. Any faeces and urine patches were removed between rats before the shavings were mixed to evenly spread olfactory cues. Across all habituation and handling sessions, the handling order was randomised for each session using a random number generator. This randomisation was to prevent systematic transmission of cues, *e.g.,* olfactory cues, related to treatment.

Experimental procedures

Habituation to handling: Habituation was conducted over four days using the same equipment and under the same environmental conditions as the handling phase, aiming to expose the rats gradually to test conditions, first as pairs, then individually. Rats were habituated to travel to and from the holding room, the experimental arena, and general handling to prevent neophobia. When travelling between rooms, the home cage was wrapped in a black plastic bag to prevent exposure to bright light, considering rats were in the dark photoperiod. Rats were not individually handled during transport. This protocol was refined during a previous study (Hammond *et al*., 2019).

Daily procedure during testing: On recording days (days one and five), before handling, each cage was placed on a designated recording rack within the homeroom and filmed for 15 minutes. Cages were then transported to the procedure room and placed on a bench. After handling, the cage was returned to the recording rack and filmed for another 15 minutes. On return, the wire cage lid was topped with cardboard to create a barrier for USVs, odours, and other signals, which may stimulate emotional contagion, affecting other rats in the homeroom. Only one female experimenter (TH) handled the rats for all handling stimulations, always wearing the same knitted glove on top of a nitrile glove.

Free roam test: On days one and five, rats were given a 30-second opportunity to approach the experimenter's hand or conduct play before (FR1) and after (FR2) handling. The behavioural responses expressed during FR1 on day one would act as a baseline measurement as rats had not experienced any handling treatment. On day five, the responses during FR1 would act as an indicator of anticipation for the respective handling treatments. Responses during FR2 would act as an indicator of lasting changes to affective state. The experimenter conducted the 'free roam test' by placing her hand on the long side of the arena with her fingertips on the bedding against the right-side wall (**Figure 2.1**). This test was only conducted on recording days (days one and five) to preserve novelty.

Handling treatment: Handling techniques remained the same regardless of the day (**Figure 2.1**). Playful handling involved the experimenter using one hand, covered by a soft knitted glove, to touch, tickle, chase, and gently attempt to pin the rat to mimic rough and tumble play (Bombail *et al*., 2019). Rats were playfully handled in this way for repeated bouts of 15 seconds alternated with 15-second "pauses" lasting two minutes (adapted from Panksepp and Burgdorf, 2010). During pauses, the hand remained stationary on the side of the arena nearest the experimenter with the palm flat to measure rat interest in the experimenter's hand. The gloved hand moved between resting with the palm flat on two adjacent sides of the arena wall nearest the experimenter for control animals. The resting location alternated every 15 seconds, resulting in a match with the pause phases in playful handling. This allowed for direct behavioural comparison between treatment and control animals.

Recording and analysis of behaviour

During handling, behaviour in the home cage and arena was recorded using a Sony HD camcorder (HDR- PJ810E) and subsequently analysed using Observer XT 14 software (Noldus Information Technology, Wageningen, the Netherlands). In the home cage, behaviour was recorded for 15 minutes before and after handling. During handling, behaviour was coded in sections according to the free-roam and pause phases of the experimental procedure; as playfully handled and control rats experienced different interactions during the handling phase, the behaviour was only compared during the pause phases. Using continuous focal sampling, the frequency and duration of each behaviour were coded according to the below ethogram (**Table 2.1**). During the collection of data from videos of home cage behaviour, the coder (TH) was blinded to treatment to prevent bias. This blinding was not possible during the videos of handling as the coder could see the treatment.

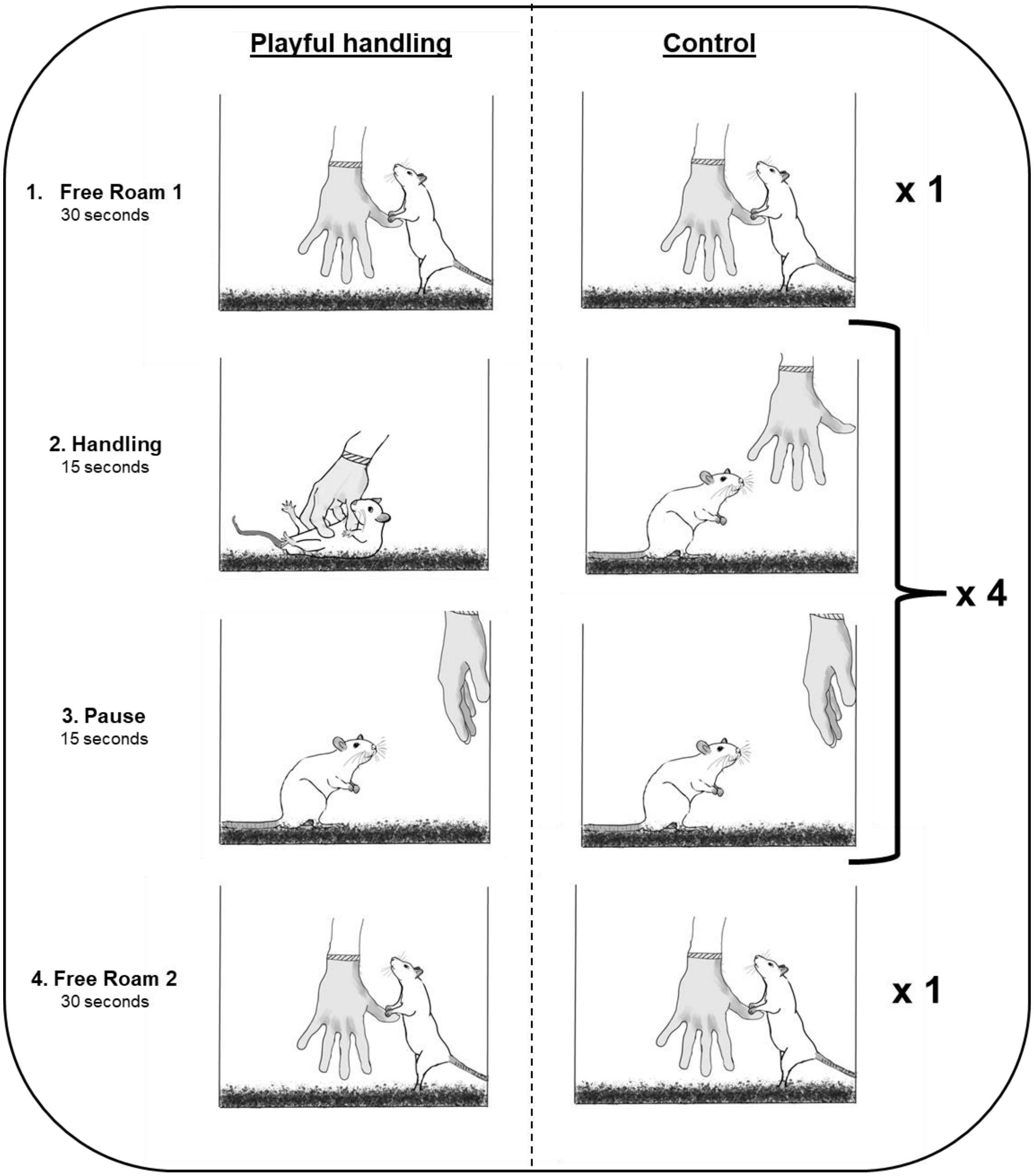


Figure 2.1. Illustration of handling techniques used on individual juvenile male Wistar rats in an arena (N = 24 rats housed in pairs; 6 playfully handled cages and 6 control handled cages). The playful handling treatment is in the left pane, and the control is in the right. Steps one and four indicate the 30-second free roam tests conducted once before handling and once after handling. Steps two to three indicate the 30-second handling phase, repeated 4 times. This figure is not to scale.

Table 2.1. Ethogram developed for recording behaviour of juvenile male Wistar rats (aged between 39 – 50 days old) in the home cage housed as pairs before and after handling and during handling (N = 24 rats housed in pairs; 6 playfully handled cages and 6 control handled cages). The table indicates which behaviours were recorded during each phase (the home cage and/or during handling).

Behaviour	**Description**	**When scored**
Solitary play	Rat conducts fast locomotor movement involving at least one hop by an individual, where hops involve all four paws leaving the ground at the same time, not in the direction of a play partner or during a play bout. The behaviour starts with fast running or a hop from stationary or during locomotor movement and ceases when this movement stops. (Hammond *et al*., 2019)	Home cage and during handling
Social play	One rat jumps or lunges towards the partner's nape, resulting in the partner either chasing the soliciting rat, rearing (in which pairs make rapid pawing movements at each other), or rotating so that one rat is on its back with the other standing over it in a pin. The frequency of pinning and dorsal contacts within social play was also coded. Multiple pins and dorsal contacts can occur during a single social play bout. The behaviour starts with fast running, a jump or lunge towards a play partner and ceases when there is no chasing, rearing, pins or dorsal contacts between the play partners. (Kerkhof *et al*., 2013; Webber *et al*., 2012)	Home cage
Rearing	Rat raises both front paws off the ground (can be in contact with the wall or not), standing up on hind legs. Does not include rears directed toward the experiment's hand or body. (Lever *et al*., 2006)	Home cage and during handling
Approach towards experimenter	Rat orients its body and head so that the nose is directed towards the experimenter's hand and within 1cm or touching the hand. (Hammond *et al*., 2019)	Home cage and during handling
Inactive	Any behaviour where the rat's body is unmoving, such as freezing, resting, sitting, or lying still. (LaFollette *et al*., 2018)	Home cage

Recording and analysis of subject produced USVs

USVs were recorded using a high-quality USB microphone (Pettersson M500-384 USB Ultrasound microphone, Pettersson Electronik; Sweden) connected via a USB interface to a PC laptop. This microphone was placed over the centre of the arena (height 51cm), pointing downwards from the arena floor. Recorded sound was digitised at the sampling rate of 384 kHz and a bit depth of 24 bit using Audacity (https://www.audacityteam.org; Audacity, Version 2.1.3, Pennsylvania, United States of America).

USVs were manually counted from spectrograms produced by Audacity and labelled as 50-kHz USVs (peak frequency between 30 and 80-kHz and duration between 10–150ms) or 22-kHz USVs (bandwidth of >4kHz, peak frequency between 20-29 kHz and duration of 300ms or more) (Brudzynski, 2009; LaFollette *et al.*, 2018; Wright *et al.,* 2010*)*. Overlapping calls were counted as one call (Wright *et al*., 2010). Vocalisations were counted in sections according to the experimental procedure's free-roam, handling, and pause phases. USV rate was expressed as the number of calls emitted per minute (calls/minute). During data collection from spectrograms, the coder (TH) was blinded to treatment to prevent bias.

Sucrose preference test (SPT)

Rats were presented with a novel adaptation of the sucrose preference test as an additional method of assessing the impact of playful handling on affective state. The sucrose preference test is a reward-based test initially developed to measure preference for low concentrations of sucrose (0.5 to 1%) over water (Willner *et al*., 1987) in rodent models of depression. Following exposure to chronic stress protocols, rats will show no preference for the sweet-tasting sucrose over water, such that the resulting anhedonic state changes the reward value of the sucrose (Forbes *et al*., 1996; Fraser and Duncan, 1998; Willner *et al*., 1992, 1987). As I was interested in measuring the impact of positive handling and a putatively enhanced positive affect on motivation for positive stimuli, I raised the sucrose concentration to 20% to enhance its rewarding properties. A higher sucrose concentration is typically used in studies examining reward sensitivity (Brennan *et al*., 2001; Clarkson *et al*., 2018; Flaherty *et al*., 1973; Sclafani and Ackroff, 2003). For example, Wilmouth and Spear (2009) used a range of sucrose concentrations from 3.4% to 34% to investigate the presence of evolutionary conserved facial reactions to favourable tastes in rats. Rodents will also work harder for more concentrated sucrose solutions than dilute solutions (Bailey *et al*., 1986). The concentration of 20% sucrose used in this study was based on research examining the naturally occurring variation of sucrose consumption according to different concentrations (Brennan *et al*., 2001). The most significant difference between high and low consumers was found at the 20% concentration, suggesting that 20% is the ideal concentration to measure individual differences in reward sensitivity.

For the first test, rats were presented with two pre-weighed bottles (one containing tap water and one containing 20% sucrose solution) in the home cage for two hours (1530 to 1730) as a short-term preference test. For the second test, the bottles were then weighed and left overnight (16 hours). Each bottle was weighed before and after both tests to measure cage-level sucrose and water consumption. Throughout testing, rats had *ad libitum* access to food, and the location of the bottles was balanced to prevent any effects of side bias (Strekalova *et al*., 2004). During the weighing of the bottles, the experimenter was blinded to treatment to prevent bias.

Statistical analysis

Tests of intra-observer reliability were conducted prior to statistical analysis. As only one observer scored all behaviours, inter-observer reliability tests were unnecessary. I conducted a repeat scoring of a minimum of 12.5% of the data seven days later. For USV data, I conducted Spearman's rank correlations between the first count and second count for each spectrogram (Martin and Bateson, 1993). To meet the reliability criteria, correlations were above the acceptable cut-off defined by Martin and Bateson of 0.7. For behaviour, these correlations were conducted using Observer XT 11 software and met a minimum Cohen's kappa of 0.8 with a percentage agreement of 64% (McHugh, 2012).

All data were analysed in Genstat 19.1 using General Linear Models (GLM), linear regression models, and correlations. Figures were generated using GraphPad Prism (ver 9.2). Assumptions of these models were tested on the residuals using Bartlett's test for homogeneity of variances and the Anderson-Darling test for normality. Tukey's honest significance difference (HSD) procedure was conducted for multiple comparisons on significant differences ($a = 0.05$). Means (M) and standard errors of the mean (SEM) reported were back-transformed to the original scale for biological significance. For all tests, the level of statistical significance was set at $p < 0.05$.

Data on home cage behaviour were taken from the first and fifth consecutive days of handling. As rats were tested together in the home cage, the frequency and duration of each rat's behaviour were summed to give a cage level of expression. To investigate the effect of handling treatment on behaviour in the home cage, handling treatment (playful handling or control handling), the timing of the recording in relation to handling (before or after), and day (one or five) were modelled as the main effects with additional interactions between the three main effects. As behaviour in the home cage was recorded in pairs, the blocking factor was the cage. For all measurements of solitary play, pinning, and dorsal contacts, I only recorded the frequency due to the short duration of these events. This was to reduce the likelihood of false positives, as coding would depend on the coder's reaction time.

USV data were taken from the first and fifth consecutive days of handling. USV counts were converted into a rate per minute to compare USV production between the free roam tests and handling phases of different durations. In all periods, the production of 22-kHz USVs was too infrequent to fit to a GLM. To investigate USV production during handling, handling treatment (playful handling or control handling), handling phase (during handling or pauses), and day (one or five) were modelled as the main effects with additional interactions between the three main effects. To investigate USV production and behaviour during the free roam tests, handling treatment (playful handling or control), the timing of the free roam test (before or after handling), and day (one or five) were modelled as the main effects with additional interactions between the three main effects. To investigate behaviour during the pauses in handling, handling treatment (playful handling or control) and day (one or five) were modelled as the main effects with additional interactions between the two main effects. As responses to handling were as individuals in the arena, the blocking factor was (cage number/rat) for all of the models above. This blocking structure reflected that each rat was tested alone and was nested within a cage, with this identification remaining unchanged across all test days. Pearson's correlation was used to investigate the relationship in USV production during handling and pauses within each treatment on day five.

As previously discussed in Chapter 1 and the introduction of this chapter, there is considerable individual variation in measured responses to heterospecific play (LaFollette *et al*., 2017). To investigate this, I present the coefficient of variation as a statistical measure of the difference in variation between the two treatments for USV production, approach behaviour, sucrose preference, and play.

Linear regression was used to determine whether there was evidence of anticipation for handling in the free roam test prior to handling on day five on an individual level. The number of USVs or the duration of approach towards the experimenter during the free roam test before handling on day five were fitted as the explanatory variable, and the number of USVs or the duration of approach towards the experimenter during handling day five were fitted as the response variable.

To investigate the relationship within measures of affect (USV production and approach behaviour) across handling treatments, these variables were summed across pause phases on an individual level. Linear regression was used to determine whether responses to handling on day one predicted responses on day five within each treatment. The number of USVs or the duration of approach towards the experimenter on day one was fitted as the explanatory variable. The number of USVs or the duration of approach towards the experimenter on day five was fitted as the response variable.

For the SPT, the main effects of the GLM were the volume of substance consumed (20% concentration of sucrose or water) and handling treatment with additional interactions between the two main effects. The weight of rats three days before the test was also fitted as a covariate. As rats were tested as pairs in the home cage and were likely to influence each other's behaviour, the cage was fitted as the blocking factor.

I created a Pearson's correlation matrix within each treatment to investigate the relationship between measures of affect during handling (USV production and approach behaviour), sucrose consumption, and home cage play. All responses were taken on a cage level from day five, using the average USV production and approach behaviour during handling, sucrose consumption, and the frequency of solitary play or duration of social play in the home cage play before handling. Data were taken at a cage level as rats were housed in pairs.

Results

Playfully handled rats produced more 50-kHz USVS than controls during handling on day five

There was a tendency towards a significant main effect of handling treatment on USV production during handling, with playfully handled rats producing more USVs than controls across both days ($F_{1, 383} = 4.7$, $p = 0.06$; **Figure 2.2**). There was a significant main effect of day, with rats across both treatments producing more USVs on day five than on day one ($F_{1, 383} = 139.5$, $p < 0.001$). There was also a significant interaction between treatment and day ($F_{1, 383} = 61.9$, $p < 0.001$), with playfully handled rats showing a higher magnitude of increase in USV production from day one to five than control rats. Tukey's HSD test indicated that playfully handled rats produced more USVs than control rats on day one ($T = 2.74$, $p = 0.03$) and day five ($T = 10.51$, $p < 0.001$). USV production increased from day one to five in playfully handled ($T = 9.71$, $p < 0.001$) but not control rats ($T = 1.95$, $p = 0.21$).

The number of USVs emitted by playfully handled rats on day one predicted the number of USVs produced on day five ($T = 3.07$, $p = 0.01$, $r^2 = 48.5\%$). The number of USVs emitted by control rats on day one did not predict the number of USVs produced on day five ($T = 2.07$, $p = 0.07$, $r^2 = 29.9\%$). The coefficient of variation for USVs differed between the two handling treatments, with higher variation in playfully handled rats compared to control rats on both days. On day one, the coefficient of variation for playfully handled rats was 54.4% and 16.5% for control rats. On day five, the coefficient of variation for playfully handled rats was 52.6% and 10.4% for control rats.

Rats produced USVs during both the handling and pause phases when the hand was resting on the inside of the cage. There was no significant main effect of handling phase (during handling or pauses) on the number of USVs produced ($F_{1,\,383} = 1.4$, $p = 0.19$). USV production was influenced by a significant interaction between treatment, handling phase and day ($F_{1,\,383} = 6.9$, $p = 0.009$; **Figure 2.3**). Tukey's HSD test indicated that playfully handled rats produced more USVs during pauses than handling on day one ($T = 6.38$, $p < 0.001$), which was reversed on day five ($T = 6.86$, $p < 0.001$). There was no difference in USV production between handling and pause phases for control rats across both testing days ($T = 0.01$, $p = 1.0$). The number of USVs between the handling and pause phases on day five were strongly correlated for both playfully handled rats ($R_p = 0.96$; $p < 0.001$) and control rats ($R_p = 0.90$; $p < 0.001$).

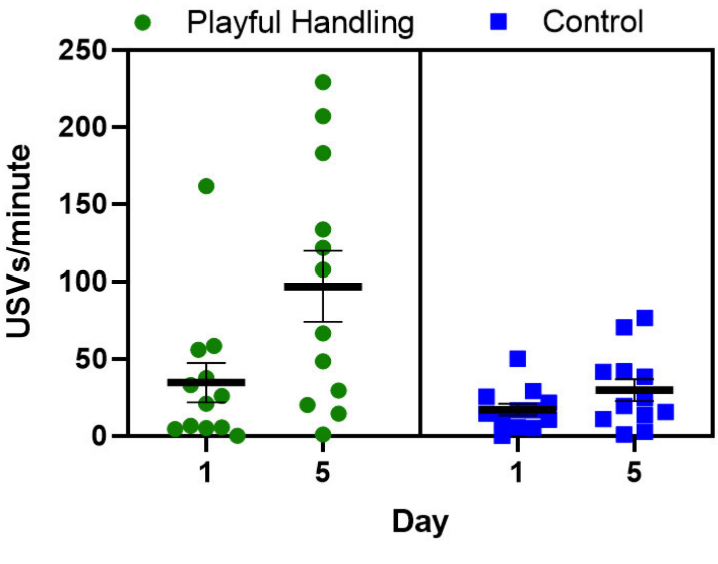


Figure 2.2. Graph showing the mean number of 50-kHz ultrasonic vocalisations (USVs) per minute produced by individual juvenile male rats (N = 24 rats housed in pairs; 6 playfully handled cages and 6 control handled cages) during the handling sessions on the first and fifth handling days. Rats received playful handling (left pane; green circles) or control handling (right pane; blue squares). Values reflect means for each rat, with the black bar indicating the mean ± SEM for each treatment by day.

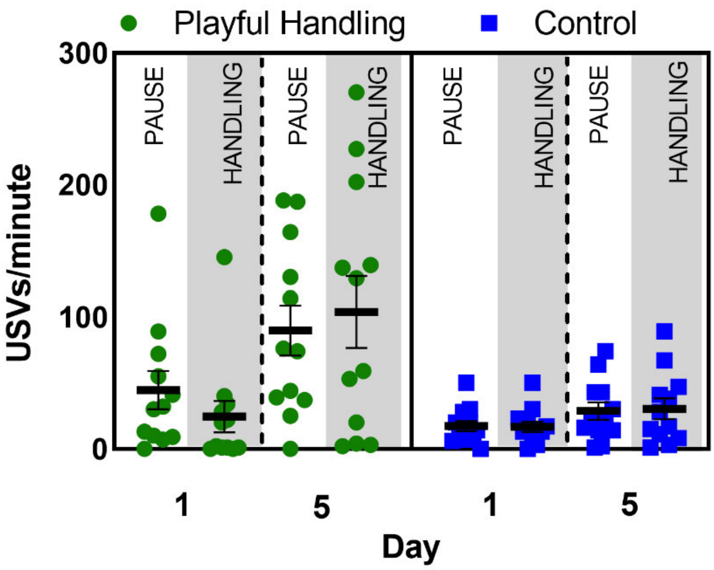


Figure 2.3. Graph showing the mean number of 50-kHz ultrasonic vocalisations (USVs) per minute produced by individual juvenile male rats (N = 24 rats housed in pairs; 6 playfully handled cages and 6 control handled cages) during the first and fifth handling days during handling and pauses in handling. White background bars indicate the number of USVs produced during pause phases, and grey bars indicate when rats were handled. Rats received playful handling (left pane; green circles) or control handling (right pane; blue squares). Values reflect means for each rat, with the black bar indicating the mean ± SEM for each treatment by day.

Playfully handled rats expressed more approach behaviour towards the experimenter than control rats

Results of the GLM modelling of rat behaviour during the pause phases of playful handling are shown in **Table 2.2**. Rearing and solitary play were not influenced by the main effects of treatment of day. The only behaviour that was influenced by the main effect of treatment was approach behaviour towards the experimenter's hand, with playfully handled rats conducting more approach behaviour than control rats. There was also a significant effect of day on approach behaviour, with a decrease on day five compared to day one. The duration of approach behaviour by playfully handled rats on day one did not predict approach behaviour on day five (T = 0.25, p = 0.81, r^2 = 0.6%). The duration of approach behaviour by control rats on day one also did not predict approach behaviour on day five (T = 1.52, p = 0.16, r^2 = 18.7%). The coefficient of variation for the duration of approach behaviour did not differ between treatments. On day one, the coefficient of variation for playfully handled rats was 47.1% and 45.1% for control rats. On day five, the coefficient of variation for playfully handled rats was 65.6% and 73.8% for control rats.

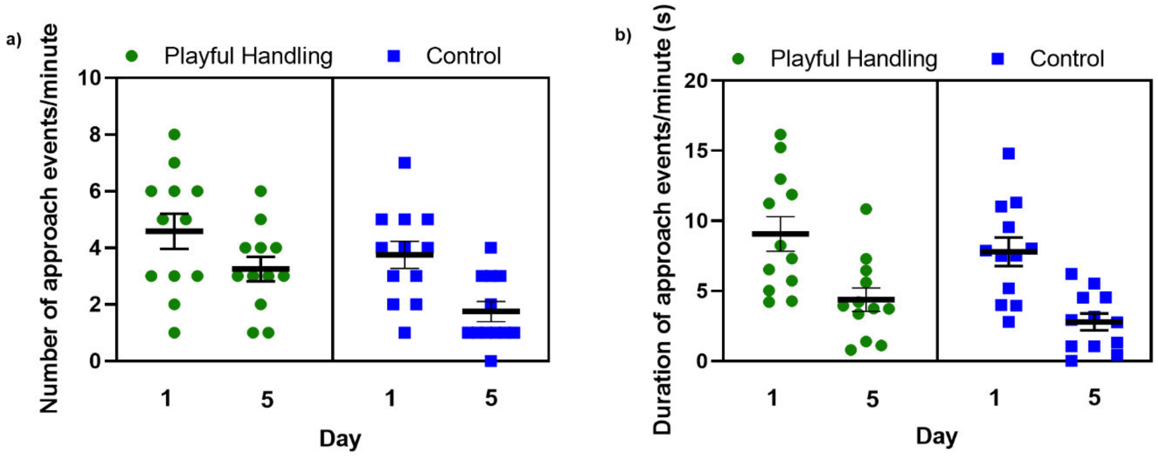


Figure 2.4. Graph showing the mean a) frequency and b) duration (s) of approach behaviour towards the experimenter's hand (s) by individual juvenile male rats (N = 24 rats housed in pairs; 6 playfully handled cages and 6 control handled cages) during pauses on the first and fifth days of handling. The treatments comprised either playful handling (left pane; green circles) or control handling (right pane; blue squares). Values reflect means for each rat, with the black bar indicating the mean ± SEM for each treatment by day.

Table 2.2. Table presenting results of an ANOVA ran on the frequency and duration of behaviours expressed by juvenile male rats (N = 24 rats housed in pairs; 6 playfully handled cages and 6 control handled cages) during pauses in handling. Significant P values are indicated in bold script.

	Variable	**Treatment**		**Day (1 or 5)**	
Behaviour		$F_{1,191}$	*p*-value	$F_{1,191}$	*p*-value
Approach towards experimenter	frequency	7.61	**0.02**	12.82	**0.002**
	duration	5.12	**0.05**	22.13	**<0.001**
Rearing	frequency	3.43	0.54	0.11	0.96
	duration	5.67	0.32	0.54	0.32
Solitary play	frequency	2.91	0.13	0.34	0.68

There was no relationship between USV production and approach behaviour during the pauses in handling

To determine whether there was a relationship between the two measures of affective state, I conducted correlations between USV production and the duration of approach behaviour during pauses in handling on day five; day five was chosen as the responses to handling treatments would be expected to be established by that point. There was no significant relationship between USV production and the duration of approach behaviour towards the experimenter for playfully handled (R_p = - 0.07, p = 0.84) or control rats (R_p = - 0.06, p = 0.86).

Playfully handled rats maintained the number of USVs produced across the free roam tests

There was a significant main effect of treatment on USV production during the free roam tests ($F_{1, 95}$ = 5.2, p = 0.04), with playfully handled rats producing more USVs compared to control rats. There were also significant main effects of timing of the free roam test (*i.e.,* whether it was applied before or after handling) ($F_{1, 95}$ = 76.8, $p <$ 0.001; **Figure 2.5**) and day ($F_{1, 95}$ = 6.9, p < 0.001), with rats producing more USVs during FR1 than FR2 and more USVs on day five compared to day one.

The coefficient of variation of USV production differed between the two handling treatments, with greater variation in playfully handled rats compared to control rats in both free roam tests. During FR1, the coefficient of variation for playfully handled rats was 61.1% and 46.3% for control rats. During FR2, the coefficient of variation for playfully handled rats was 73.9% and 49.9% for control rats.

The number of USVs emitted by playfully handled rats during FR1 on day five predicted the number of USVs emitted during handling (T = 3.55, p = 0.005, r^2 = 55.7%). Likewise, the number of USVs emitted by control rats during FR1 on day five also predicted the number of USVs emitted during handling (T = 4.49, p = 0.001, r^2 = 66.8%).

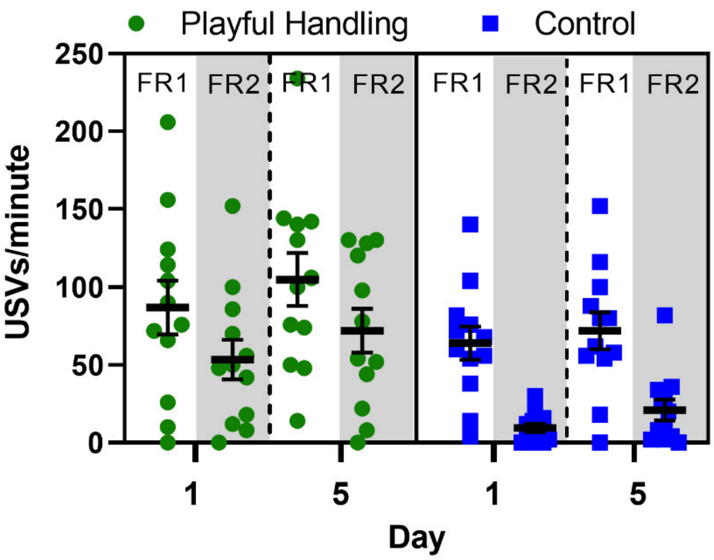


Figure 2.5. Graph showing the mean number of 50-kHz ultrasonic vocalisations (USVs) per minute produced by individual juvenile male rats (N = 24 rats housed in pairs; 6 playfully handled cages and 6 control handled cages) during free roam(FR) tests on the first and fifth days of handling. These tests provided a 30-second opportunity for the rat to express anticipation, approach the hand or conduct other behaviours before and after receiving handling treatments. White background bars indicate the number of USVs produced during FR1 (before handling), and grey background bars indicate the number of USVs produced during FR2 (after handling). The treatments consisted of either playful handling (left pane; green circles) or control handling (right pane; blue squares). Values reflect means for each rat, with the black bar indicating the mean ± SEM for each treatment by day.

Rearing and solitary play were influenced by the timing of the free roam test

Results of the GLM modelling rat behaviour during free roam tests are shown in **Table 2.3**. There was no significant effect of treatment or day on approach behaviour towards the experimenter, rearing, or solitary play. There was a main effect of timing of the free roam test (before or after handling) on rearing, with rats conducting more rearing during FR2 than FR1 (42.3 vs 32.0 ± 1.85 seconds per minute). Solitary play was also influenced by the timing of the free roam test, with solitary play occurring more frequently during FR1 than FR2 on both days (0.4 vs 0.0 ± 0.2 events per minute). There was no main effect of the timing of the free roam test on approach behaviour. The duration of approach behaviour by playfully handled rats during FR1 on day five did not predict the duration of approach behaviour during handling on day five (T = 1.02, p = 0.33, r^2 = 9.4%). The duration of approach behaviour by control rats during FR1 on day five also did not predict the duration of approach behaviour during handling on day five (T = 1.21, p = 0.25, r^2 = 12.8).

The coefficient of variation for the duration of approach behaviour did not differ between the two handling treatments. During FR1, the coefficient of variation for playfully handled rats was 45.6% and 42.8% for control rats. During FR2, the coefficient of variation for playfully handled rats was 44.5% and 42.6% for control rats.

Table 2.3. Table presenting results of an ANOVA ran on the frequency and duration of behaviours expressed by juvenile male rats (N = 24 rats housed in pairs; 6 playfully handled cages and 6 control handled cages) during free roam (FR) tests on the first and fifth handling days. These tests provided a 30-second opportunity for the rat to express anticipation, approach the hand or conduct other behaviours before (FR1) and after (FR2), receiving either playful handling or control handling. Significant P values are indicated in bold script.

	Variable	**Treatment**		**Day (1 or 5)**		**Timing of test (before or after)**	
Behaviour		$F_{1, 95}$	p-value	$F_{1, 95}$	p-value	$F_{1, 95}$	p-value
Approach towards experimenter	frequency	0.21	0.73	0.27	0.63	3.22	0.19
	duration	0.52	0.58	2.65	0.17	0.89	0.37
Rearing	frequency	0.05	0.87	6.78	0.78	28.78	**0.03**
	duration	0.26	0.56	0.32	0.36	7.69	**0.04**
Solitary play	frequency	0.32	0.62	0.68	0.47	6.14	**0.02**

There was no relationship between USV production and approach behaviour during the free roam tests

To determine whether there was a relationship between the two measures of affective state during the free roam tests, I conducted correlations between USV production and duration of approach behaviour during free roam tests on day five. Day five was chosen as the responses to handling treatments would be expected to be established by that point. During FR1, there was no relationship between USV production and approach towards the experimenter for playfully handled ($R_p = 0.13$, $p = 0.70$) or control rats ($R_p = 0.13$, $p = 0.70$). During FR2, there was also no relationship between USV production and approach towards the experimenter for playfully handled ($R_p = 0.26$, $p = 0.42$) or control rats ($R_p = 0.10$, $p = 0.75$).

Playfully handled rats conducted more solitary play in the home cage before handling than control rats

Solitary play was measured by frequency alone due to the short duration of events. There was no main effect of handling treatment on solitary play ($F_{1,47} = 2.3$, $p = 0.14$). There was a main effect of the timing of recording (before or after handling), with solitary play occurring more frequently before handling than after ($F_{1,47} = 24.4$, $p < 0.001$). There was a main effect of day on solitary play, with rats across both treatments conducting more play on day five compared to day one ($F_{1,47} = 4.1$, $p = 0.05$). Solitary play was influenced by an interaction between treatment and the timing of recording ($F_{1,47} = 3.9$, $p = 0.05$; **Figure 2.6**). Tukey's HSD test indicated that playfully handled rats conducted more solitary play events than control rats before handling ($T = 3.66$, $p < 0.001$) but not after handling ($T = 0.01$, $p = 1.00$).

The coefficient of variation for the frequency of solitary play was higher in playfully handled rats than control rats on both days. On day one, the coefficient of variation of solitary play for playfully handled rats was 76.5% and 55.6% for control rats. On day five, the coefficient of variation for playfully handled rats was 62.3% and 47.9% for control rats.

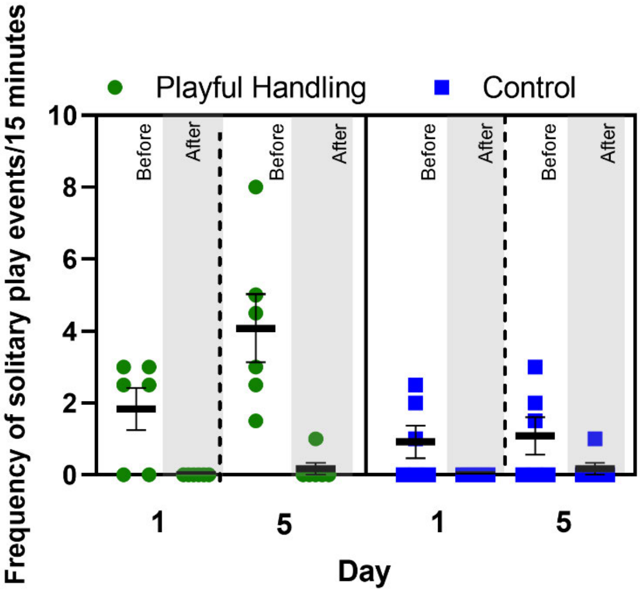


Figure 2.6. Graph showing the mean frequency of solitary play events by pairs of juvenile male rats (N = 24 rats housed in pairs; 6 playfully handled cages and 6 control handled cages) in the home cage measured for 15 minutes before and after handling on the first and fifth days. The treatments consisted of either playful handling (left pane; green circles) or control handling (right pane; blue squares). Values reflect means for each cage with the black bar indicating the mean ± SEM for each treatment by day.

There was no difference between playfully handled and control rats in the frequency ($F_{1,47}$ = 1.3, p = 0.3) or duration of social play ($F_{1,47}$ = 0.1, p = 0.61). All rats conducted more social play before than after handling in both frequency (0.8 vs. 0.1 ± 0.04 2 events per 15 minutes; $F_{1,47}$ = 116.1, p < 0.001) and duration of events (40.2 vs. 12.9 ± 6.6 seconds per 15 minutes; $F_{1,47}$ = 459.8, p < 0.001). The frequency of social play increased from day one to five (1.3 vs. 2.0 ± 0.1 events per 15 minutes: $F_{1,47}$ = 4.11, p = 0.05). This increase was not seen in the duration of social play (7.6 vs. 10.6 ± 1.7 seconds per 15 minutes; $F_{1,47}$ = 1.9, p = 0.2).

As the majority of social play occurred before handling, I investigated the frequency of pinning and dorsal contacts during this period on the final day of handling as additional proxies of social play. I found no difference in pinning frequency between playfully handled and control rats during social play before handling (4.3 vs 3.6 ± 1.0 events per 15 minutes; $F_{1,23}$ = 1.7, p = 0.2). Likewise, there was no difference in the frequency of dorsal contacts between playfully handled and control rats during social play before handling (7.8 vs 7.3 ± 2.5 events per 15 minutes for playfully handled and control rats; $F_{1,23}$ = 0.2, p = 0.9).

The coefficient of variation for the duration of social play differed between the two treatments. On day one, the coefficient of variation of the duration of social play for playfully handled rats was 74.8% and 56.7% for control rats. On day five, the coefficient of variation of the duration for playfully handled rats was 55.1% and 97.3% for control rats.

Treatment had no effect on inactivity in the home cage

There was no effect of treatment on the frequency of inactive events ($F_{1,47} = 3.6$, $p = 0.08$) or duration of inactivity in the home cage ($F_{1,47} = 0.1$, $p = 0.9$). There was a significant main effect of timing of recording (before or after handling) on the frequency and duration of inactivity, with rats being less active after handling than before (frequency: 0.4 vs. 0.0 ± 0.04 events per 15 minutes; $F_{1,47} = 34.1$, $p < 0.001$ and duration: 138.1 vs. 3.5 ± 19.7 seconds per 15 minutes; $F_{1,47} = 23.2$, $p < 0.001$). There was also a significant main effect of day on the frequency and duration of inactivity, with rats spending more time inactive on day one compared to day five (frequency: 0.5 vs. 0.1 ± 0.03 events per 15 minutes; $F_{1,47} = 10.3$, $p = 0.002$ and duration: 132.7 vs. 9.6 ± 19.7 seconds per 15 minutes; $F_{1,47} = 19.4$, $p < 0.001$).

Playfully handled rats showed a higher preference for sucrose than control rats in the SPT

There was a main effect of handling treatment on sucrose consumption in the two-hour test ($F_{1,23} = 13.93$, $p = 0.001$; **Figure 2.7**) and the sixteen hour test ($F_{1,23} = 8.76$, $p = 0.008$), with playfully handled rats consuming more sucrose than control rats. The treatment effect was specific to sucrose consumption as there was a main effect of substance, with all rats consuming more sucrose than water across the 2-hour ($F_{1,23} = 12.21$, $p < 0.001$) and 16-hour time points ($F_{1,23} = 7.74$, $p = 0.01$), as well as no difference in water consumption ($F_{1,23} = 0.26$, $p = 0.34$). Consumption was independent of body weight, with average cage weight having no effect on the volume of sucrose or water drank ($F_{1,23} = 0.13$, $p = 0.72$).

The coefficient of variation was broadly similar between the two handling treatments, with reduced variation in sucrose consumption in playfully handled rats compared to control rats in both tests. During the two-hour test, the coefficient of variation for playfully handled rats was 17.2% and 27.9% for control rats. During the sixteen-hour test, the coefficient of variation for playfully handled rats was 11.5% and 18.3% for control rats.

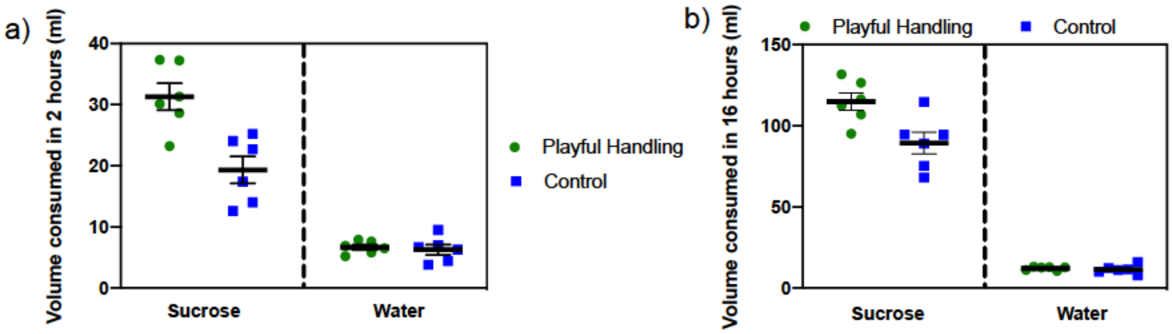


Figure 2.7. Graph showing consumption of 20% sucrose (left pane) and water (right pane) by pairs of juvenile male Wistar rats in a sucrose preference test (N = 24 rats housed in pairs; 6 playfully handled cages and 6 control handled cages) over a) two hours and b) 16 hours. Rats were playfully handled (left pane; green circles) or control handled (right pane; blue squares) for five days, then presented with sucrose (20% concentration) and water as pairs in the home cage. Values reflect means for each cage with the black bar indicating the mean ± SEM for each treatment by substance.

Relationships between measures of affective state and play were dependant on treatment

Results of a Pearson's correlation matrix ran between measures of affective state are shown in **Table 2.4**. There was a significant positive relationship between solitary play before handling and approach behaviour during handling on day five for playfully handled rats. There was a significant positive relationship between social play before handling and USV production during handling on day five for control rats.

Table 2.4. Results of a Pearson's correlation matrix generated from the responses of pairs of juvenile male Wistar rats to five days of handling (N = 24 rats housed in pairs; 6 playfully handled cages and 6 control handled cages). Pearson's correlations were run within each treatment to investigate the relationship between measures of affect during handling (frequency of USV production and duration of approach behaviour), sucrose consumption over 2 hours, and home cage play (frequency of solitary play and duration of social play before handling) on day five. The table reports the mean (M), standard deviation, Pearson correlation on the left, and p-value on the right Significant P values are indicated in bold script.

	Behaviour	M ± SEM	USVs	Approach	Solitary play	Social play	Sucrose
Playful handling	USVs	98.6 ± 25.6	-				
	Approach	4.4 ± 0.6	0.02, 0.97	-			
	Solitary play	10.8 ± 1.7	0.09, 0.85	0.95, **0.004**	-		
	Social play	142.5 ± 30.3	0.70, 0.12	0.15, 0.78	0.25, 0.64	-	
	Sucrose	31.3 ± 2.2	0.48, 0.34	0.17, 0.75	0.01, 0.99	0.56, 0.25	-

	Behaviour	M ± SEM	USVs	Approach	Solitary play	Social play	Sucrose
Control	USVs	29.5 ± 6.5	-				
	Approach	2.8 ± 0.6	0.41, 0.42	-			
	Solitary play	3.8 ± 1.1	0.03, 0.96	0.16, 0.76	-		
	Social play	132.7 ± 31.8	0.81, **0.05**	0.01, 0.98	0.36, 0.49	-	
	Sucrose	19.3 ± 2.2	0.03, 0.95	0.06, 0.91	0.66, 0.15	0.26, 0.15	-

Discussion

Playful handling is a novel approach to heterospecific play with rats that aims to mimic the flexibility and unpredictable nature of social play (Bombail *et al*., 2021). This method was introduced by Bombail *et al*. (2019) and is currently the only published paper using playful handling. Here, I provide further validation for the use of playful handling to induce a positive affective state in juvenile male rats. First, playfully handled rats produced more 50-kHz USVs than control rats, with 50-kHz USV production regarded as a valid measure of positive affective state (Hinchcliffe *et al*., 2020; Seffer *et al*., 2014). Secondly, playfully handled rats actively solicited interaction with the experimenter, as shown by increased approach behaviour. Finally, playfully handled rats consumed more high concentration sucrose than controls in a novel test of the preference for rewarding stimuli in response to handling. The successful induction of a positive affective state in playfully handled rats allowed for investigation of the impact of a positive experience on play behaviour. After five days of handling, solitary play in the home cage increased in playfully handled rats compared with control rats. This effect was not seen in social play and only occurred in the home cage before and not after handling. As the positive affective state induced by playful handling increased solitary play, this provides evidence for play to act as an indicator of improved welfare.

Playful handling induced a positive affective state in rats

As discussed in Chapter 1 and the introduction of this chapter, playful handling was developed as an alternative method of heterospecific play, which aims to imitate aspects of social play that are lacking when using tickling. Rats produce the highest rates of 50-kHz USVs during social play compared to other putatively pleasurable experiences, such as mating and feeding (Schwarting *et al*., 2007). Thus, plentiful production of USVs during playful handling would suggest that rats perceive this form of handling as a positive and putatively playful experience. In the first paper to use playful handling, Bombail *et al*. (2019) reported a significant increase in 50-kHz USV production during playful handling compared to days when they were not handled and compared with control rats. Consistent with Bombail *et al*. (2019), USV production in playfully handled rats was over threefold higher than control rats on day five of handling, providing further support that playful handling is a positive experience for juvenile male rats. The lack of treatment effect on USVs on day one can be explained by evidence that the response of USVs to heterospecific play appears to develop over time. The increase in USV production over subsequent sessions in response to tickling is reported in earlier (*e.g.,* Burgdorf and Panksepp, 2001; Cloutier and Newberry, 2008; Panksepp and Burgdorf, 2000) and more recent research (Hammond *et al*., 2019; LaFollette *et al*., 2018a). The increase in USV production in response to playful handling was also found in Bombail *et al*. (2019), with the number of USVs produced by playfully handled rats increasing threefold from the first to the fifth session. Considering that control rats also produced more USVs on day five compared to day one, this suggests that the protocol for individually handling rats in the arena was not aversive and may be positive in itself.

Comparison of USV production during handling and pause phases may also aid the interpretation of the development of USV responses. While there was no difference in USV production between phases for control rats, USV production in playfully handled rats was affected by an interaction between phase and day. During the first day, playfully handled rats produced the majority of USVs during pauses in handling, whereas, by the fifth day, the majority of USVs were produced during handling. Bombail *et al*. (2019) report similar results, with the number of USVs being greater during the handling than the pause phases during the fifth session. Amongst other functions, 50-kHz USVs are produced to establish social contact (Wöhr *et al*., 2008) and maintain social play (Himmler *et al*., 2014). For example, rats elicit 50-kHz USVs when exposed to odours of a conspecific (Brudzynski and Pniak, 2002) and show reduced social play when a partner is deafened (Siviy and Panksepp, 1987). In reference to these qualities of 50-kHz USVs, this suggests that on the first day, the increase in USV production during pauses may represent the rats' motivation to communicate with the experimenters' hand. By the fifth day, the increase in USV production during handling may reflect the rat wanting to maintain the play interaction with the experimenter. This development of a positive perception of playful handling over subsequent sessions is also supported by the overall increase in USV production from day one to five and the strong positive correlation in USV production between handling and pauses on day five.

Approach behaviour indicated that rats have a positive perception of the experimenter

Measurement of approach behaviour is often used to measure rat behaviour in studies of rat tickling (LaFollette *et al*., 2017). The propensity of a rat to approach the experimenter's hand has been interpreted as a reflection of the positive reward value of tickling, which motivates the rat to move towards the hand to receive more tickling (Burgdorf and Panksepp, 2001; LaFollette *et al*., 2017). Approach behaviour has been quantified using a variety of protocols by either measuring the latency to approach the hand (*e.g.,* Burgdorf and Panksepp, 2001) or time spent in contact with the experimenter's hand (*e.g.,* Hammond *et al*., 2019), which can either be moving (hand following) (Hammond *et al*., 2019; Ishiyama and Brecht, 2016; Lampe *et al*., 2017) or stationary (hand seeking) (Burgdorf and Panksepp, 2001; LaFollette *et al*., 2018a). Across these different protocols, tickled rats consistently show an increase in approach behaviour towards the experimenter's hand compared to controls (*e.g.,* Burgdorf *et al*., 2009; Burgdorf and Panksepp, 2001; Hammond *et al*., 2019; Hori *et al*., 2013, 2014; Yamamuro *et al*., 2010, 2013).

Both Bombail *et al*. (2019) and this study found that playfully handled rats spent more time in contact with the experimenter than control rats in the pauses between handling when the hand was placed on the side of the arena. This suggests that, as with tickling, playfully handled rats were more motivated than control rats to interact with the handler and perceived the human-rat interaction as positive. In addition to an overall treatment effect, there was a decline in approach behaviour across both treatments on day five compared to day one. However, a comparison with Bombail *et al*. (2019) cannot be made as in this experiment, behaviour was only measured during the fifth day and not on both days one and five. Despite this, a similar decline in time spent in contact with a moving hand in response to tickling was seen in Hammond *et al*. (2019). Similarly, a comparison with tickling studies cannot be made as the protocol of using a stationary hand and measuring time spent in contact with the hand has not been used in previous research, with latency to approach as the more common measure. This raises the discussion of which protocol for assessing interaction with the human hand is more applicable to the measurement of affective state in response to handling.

There was no evidence of anticipatory USVs in playfully handled rats

Anticipatory behaviour is thought to reflect the appetitive or ‘wanting’ phase of positive emotions (Mendl *et al*., 2010; Spruijt *et al*., 2001) and, thus, can reflect the value an animal places on rewarding stimuli (van der Harst *et al*., 2003). The presence of anticipatory behaviour before the stimulus is presented infers that the stimulus is rewarding, with the magnitude of the response indicating the reward value of the particular stimulus (Makowska and Weary, 2013). Anticipation is most commonly measured by calculating the number of transitions between different behaviours a rat conducts during a set time, with a high number of behavioural transitions indicating anticipatory behaviour. However, anticipation prior to a rewarding stimulus may also be expressed by an increase in specific behaviours, which are also produced in receipt of that reward and are associated with positive affect (Anderson *et al*., 2020). Previous work has shown that 50-kHz USVs production increases in anticipation of putatively positive experiences such as copulation (Bialy *et al*., 2000), contact with a conspecific (Burke *et al*., 2017) and access to a running wheel (Heyse *et al*., 2015). As such, the presence and magnitude of USV production may indicate the reward value associated with playful handling and control handling.

To test this, rats were presented with a free roam test on the first and final day of handling for 30 seconds before and after handling. While the free roam test before handling (FR1) allowed rats to express anticipation for handling, the test after handling (FR2) allowed the expression of lasting changes to affective state. FR1 on day one acted as a baseline measurement since rats had no knowledge of the handling treatment they were about to receive. Both treatments showed an increase in USV production from day one to five and produced more USVs in FR1 than FR2, suggesting that being placed into the arena as an individual was an actively anticipated positive experience. From the baseline measurement during FR1 on day one, playfully handled rats produced more USVs than control rats. The number of USVs produced during this test was maintained on day five, suggesting that there is no evidence of an increased reward value associated with playful handling over control handling. However, during FR2, playfully handled rats also produced more USVs than control rats, suggesting that the positive affective state induced by playful handling lasted after the handling treatment was completed.

There was no effect of treatment, day, or timing of the test on approach behaviour during the free roam tests, suggesting that anticipation of playful handling is not expressed through approach behaviour. As there was no difference between playfully handled and control rats in approach behaviour after handling, this also suggests that approach behaviour may not be sensitive to lasting changes in affective state as seen when measuring USV production.

The change in positive affective state was expressed by changes in sucrose preference

To further validate the effects of playful handling on affective state, I adapted a behavioural test of the reward value of handling by measuring preference of 20% sucrose against water. This test is based on evidence that both tickling and sucrose consumption increase dopamine release in the mesolimbic reward pathway, specifically in the nucleus accumbens (Hori *et al*., 2013; Ishiyama and Brecht, 2016; Oliveira-Maia *et al*., 2011). This pathway is essential in regulating the motivation for rewarding experiences, such as play, food, sex, and social interactions (Wager and Cox, 2009). The capabilities of neuroplasticity in the mesolimbic system are evidenced by the neuronal alterations resulting from psychostimulants and opiates, which are considered common drugs of abuse (Chiara and Imperato, 1988; Robinson and Kolb, 1999). Similar functional and morphological changes to those seen in response to psychostimulants are also seen in response to the natural reward of mating (Pitchers *et al*., 2010). As such, repeated exposure to a positive experience, such as playful handling, could be expected to change this pathway in a similar manner to that seen with psychostimulants.

As hypothesised, playfully handled rats drank more 20% sucrose solution than control rats. Thus, there is potential for using a high-concentration sucrose preference test as a measure of behavioural and neural changes induced by a positive experience. However, further work is necessary to consider other factors that influence sucrose consumption to validate this test as a measure of affective state. Sucrose consumption is also sensitive to motivational factors, such as hunger and satiety (Brennan *et al*., 2001). As playfully handled rats were induced into play with the handler and conducted more free-choice play in the home cage, they likely had higher energy needs and were consequently hungrier than control rats. Thus, the increase in sucrose consumption in playfully handled rats could be explained by the need to balance energy consumption with energy intake (Strouthes, 1971) and may explain why playfully handled rats conducted more ingestive behaviour overall. Future tests could use saccharine, an artificial sweetener with zero or low caloric content to counter the potential confound related to differences in energy requirements between playfully handled and control rats (Hoffman, 2016; Strouthes, 1971). Thus, the motivation of rats to consume sucrose would be based on palatability and not nutritional value (Strouthes, 1971).

Rats show consistent responses to playful handling as individuals in the arena in terms of USV production but not approach behaviour

There is growing evidence of individual variation in responses to tickling (Bombail *et al*., 2021, 2019; LaFollette *et al*., 2017) and playful handling (Bombail *et al*., 2019). In order to investigate variation in responses to playful handling between individuals, I reported the coefficient of variation of USV production and approach behaviour during the free roam tests and handling. Similar to tickling, rats showed considerable individual variation in response to playful handling. In playful handled rats, the coefficient of variation in USV production during handling was higher by threefold on day one and fivefold on day five compared to control rats. This effect was specific to USV production, as there was no difference in the coefficient of variation of the duration of approach behaviour or sucrose consumption during the free roam tests or handling. In order to promote the uptake of positive welfare measures for lab animals, this variation in USV production should be further investigated by evaluating different protocols of heterospecific play.

Previous research on tickling reports that USV production in response to tickling is stable within individuals and correlates to standardised tests of anhedonia and anxiety in rats (Mällo *et al*., 2007; Schwarting *et al*., 2007). In both Bombail *et al*. (2019) and this study, rats showed consistency in USV production within individuals in response to playful handling. The number of USVs emitted during handling on day one predicted the number of USVs produced on day five for playfully handled but not control rats. Approach behaviour was not individually consistent as the duration of approach behaviour on day one did not predict the response on day five. Further, on day five, USV production during FR1 predicted USV production during handling for both playfully handling and control rats. This effect was not seen in approach behaviour, with no relationship between approach during FR1 and handling. Compared with approach behaviour, USV production seems to be the more robust measure of individual variation in response to playful handling.

The increase in play in response to playful handling provides support for the use of play as an indicator of a positive affective state

Investigation into the effect of positive affective states on play is lacking in humans and animals (Ahloy-Dallaire *et al*., 2017). The results of this study help to fill this gap and provide supporting evidence that play can respond to enhanced positive affect, as the positive affective state induced by playful handling increased play behaviour in the home cage. The effect of playful handling on play was specific to solitary play before handling, which is analogous to my previous work examining the effect of tickling on play in the home cage (Hammond *et al*., 2019).

In Hammond *et al*., 2019, we suggest that as the treatment differences in play occur before the expected experience, with play overall being more commonly before handling, play may represent a form of positively-valenced anticipation. By day five, rats could have reacted to the stimulus of the experimenter moving the cage and actively anticipated access to the arena for handling, represented by an increase in play. This link between play and anticipation of a positive experience has also been seen in response to tickling, where social play increased in the home cage before handling after three days (LaFollette *et al*., 2018). However, the analysis on play by

LaFollette *et al*. (2018) was limited in comparison to this study, when the author only measured one type of play, social play, by using the frequency of pins as a marker of overall play and having no control treatment. As such, these results tentatively suggest that anticipatory solitary play is sensitive to the presence of a positive affective state and PAW.

Relationships between measures of affective state and play were dependent on treatment

As playful handling had a significant effect on USV production, approach behaviour, sucrose consumption, and solitary play, it would be logical to expect a positive relationship between these measures in playfully handled but not control rats. In order to investigate this relationship between the measures of affective state and play, I performed a Pearson's correlation matrix within each treatment for responses on day five. Between USV production and social play, there was a strong positive relationship in control rats and a strong tendency in playfully handled rats. This tentatively suggests that rats that conduct more social play in the home cage before handling also produce more USVs during handling. In playfully handled but not control rats, there was a strong positive relationship between approach behaviour and solitary play and a strong tendency between USV production and sucrose consumption. As there were relationships between some indicators of affective state and not others, this suggests that each measurement may be reflecting a different component of rat perception towards the positive experience, which may not necessarily be related.

The results described above are tentative and require further investigation due to some limitations in the analysis. First, this analysis was carried out on a cage level due to the inability to distinguish individual sucrose consumption and play, resulting in low degrees of freedom and a loss of information at the individual level. Further, as approach behaviour was only measured during the pauses in handling, measurement of USV production was also limited to production during pauses. As previously discussed, rats show high levels of inter-individual variation in USV production. Providing a cage-level measure during only the pauses in handling may have limited the ability to find relationships between measures and play.

Conclusion

This work further validates playful handling as an alternative method of inducing a positive affective state in rats through a heterospecific interaction. In comparison with control handling, playful handling increased USV production, approach behaviour, and sucrose consumption which can be considered as indicators of positive affect. The positive affective state induced by playful handling allowed for investigation of the response of play to a positive experience. As play increased in response to playful handling, this provides evidence that play represents more than just the absence of a negative affective state and can indicate the presence of positive affect and PAW in rats.

Chapter 3: Application of playful handling as pairs in the home cage to induce a positive affective state and measure changes in play in juvenile male Wistar rats

Abstract

Playful handling is a method of inducing a positive affective state in rats which can potentially improve rat welfare in laboratory settings. Current evidence suggests that playful handling is a positive experience for rats, yet this is limited to handling as individuals in an arena separate from the home cage. As this protocol requires a lengthy time investment per rat, it is likely to act as a barrier that prevents the uptake of playful handling in laboratory personnel. The aims of this study were to validate the use of playful handling as pairs in the home cage to induce a positive affective state and investigate variation in responses to playful handling. Juvenile male Wistar rats (N = 14 cages; 7 playfully handled cages and 7 control cages; 42 days old) were housed in pairs and assigned by cage to receive playful handling or control handling for three days. All handling was conducted as pairs in the home cage. Playful handling involved the experimenter touching, tickling, chasing, and gently pinning the rat, with the experimenter reacting to the behaviour of the individual rat. The interaction lasted for three minutes, with 15 seconds of active play with each individual alternated with 15-second pauses. During the pauses, the hand was removed from the home cage. For control rats, during the periods when playfully handled rats received active play, the hand was placed inside the side of the home cage with the hand removed during pauses. On the first and last days of handling, rats were also exposed to a 30-second free roam test before and after handling as measures of anticipation and lasting positive affective state. Affective state in response to handling was measured through USV production and

approach behaviour. Playfully handled rats produced more 50-kHz USVs than control rats across all three days of handling (170.2 vs 108.5 ± 17.1 USVs/minute across all three days of recording; $F_{1, 335} = 6.5$, $p = 0.03$). There was also more anticipation for playful handling than control handling, as evidenced by USV production during the free roam test prior to handling (122.6 vs 91.4 ± 17.3 USVs/minute on day three; $T = 3.83$, $p = 0.002$). There was no significant difference in approach behaviour between treatments ($F_{1, 41} = 0.3$, $p = 0.57$). However, there is the potential that playfully handled rats were attempting to locate the experimenter as evidenced by an increase in rearing in playfully handled rats compared to control rats (16.4 vs 12.6 ± 1.2 events in one minute; $F_{1, 41} = 4.7$, $p = 0.03$). Further, playfully handling rats as pairs in the home cage resulted in reduced variation in USV production between cages compared to control rats (CV on day three = 50.6% vs 11.1%), suggesting that this protocol was an enjoyable experience for more individuals. In general, these results support playfully handling as a method of inducing a positive affective state under conditions that are practical for laboratory personnel.

Introduction

Over 200k rats per annum are used for research in the UK alone (UK Home Office, 2016). Current welfare standards for rats used in research primarily aim to mitigate unnecessary pain, harm, or distress (Makowska and Weary, 2020). However, the absence of negative affective states does not indicate that animals are in a positive welfare state or have ‘a good life’ (Boissy *et al*., 2007). Instead, it is the balance of negative and positive states that determines an animal's overall welfare status (Lawrence, 2016; Webb *et al*., 2019). As such, momentum is gaining for the argument that this basic standard of care is not enough and must be enhanced by promoting positive experiences in rats and other laboratory species (Makowska and Weary, 2020). In order to achieve this higher standard of care, there is a need to address the lack of well-validated methods of inducing a positive affective state in rats.

A promising method of promoting positive experiences in rats is heterospecific play with a human caretaker. The most widely used protocol is the Panksepp method of rat tickling, which involves vigorous, rapid finger movements or tickling on the nape of the neck, followed by repeated pins, in which the rat is flipped onto its back and tickled ventrally (Panksepp and Burgdorf, 2000). Generally, tickling is perceived as a positive experience by rats, primarily evidenced by an increase in positively valenced ultrasonic vocalisations, also known as 50-kHz USVs (LaFollette *et al*., 2017). The production of 50-kHz USVs is the most promising measurement of positive affect in rats since USV production in response to tickling occurs on a graded scale which directly reflects the strength of the associated affective state (Hinchcliffe *et al*., 2020). Thus, individuals who produce fewer USVs can be assumed to receive less enjoyment from the experience than conspecifics who produce more USVs. The highest rates of 50-kHz USVs are seen during social play (Schwarting *et al*., 2007), such that rats would be expected to produce plentiful USVs in response to a positive and playful interaction. However, as discussed in Chapter 2, it is well-established that USV responses to tickling vary considerably between individuals (Bombail *et al*., 2021, 2019; LaFollette *et al*., 2017), such that there is the risk that some individuals perceive the interaction as aversive (Bombail *et al*., 2021). Observation of this variation in our own (Hammond *et al*., 2019) and others' work (*e.g.,* LaFollette *et al*., 2017, Hinchcliffe *et al*., 2020) led to the development of playful handling as an alternative method to tickling (Bombail *et al*., 2021).

In comparison to tickling, playful handling aims to mimic rat social play more closely. Social play in rats is a fluid, complex interaction that involves many different behaviours, including chasing, pouncing, darting, wrestling, boxing, jumping, and pinning (Whishaw *et al*., 2021). Rats display individual variation in the frequency and sequence of these behaviours (Thor and Holloway, 1984). During playful handling, the experimenter reacts to the individual and varies the type and intensity of behaviours seen in social play, such as touching, tickling, chasing, and being chased (Bombail *et al*., 2021). As such, the individual has more choice within the interaction, and it may be a positive experience for a wider number of rats than tickling (Bombail *et al*., 2021). So far, the evidence provided by Bombail *et al*. (2019) and Chapter 2 supports playfully handling as a positive experience for rats. In Chapter 2, I found that rats that were playfully handled as individuals showed increased production of 50-kHz USVs, increased approach behaviour towards the experimenter's hand, and consumed more sucrose than control rats as an independent test of affect. Playful handling also increased home cage play, with play regarded as a promising potential indicator of positive welfare (Boissy *et al*., 2007).

The majority of research, including the two studies on playful handling (*i.e.,* Bombail *et al*., 2019 and Chapter 2), conducts heterospecific play as individuals in an arena that is separate from the home cage (LaFollette *et al*., 2017). This protocol has successfully been used for a variety of experimental purposes. For example, the investigation of potential biomarkers of affective state (Hori *et al*., 2009; Yamamuro *et al*., 2013, 2010) or examining the relationship between aspects of play and tickling (Hammond *et al*., 2019). However, while this protocol may be adapted for experimental investigations, the significant time investment required per rat is unlikely to be conducive to the other prospective use of playful handling; to improve the welfare of rats in laboratory settings. In a review examining the uptake of tickling in almost 800 research personnel, LaFollette *et al*. (2018) found that only 11% of participants implemented tickling, with a lack of time stated as the main barrier.

Considering the link between tickling and playful handling, it is likely that promoting the widespread implementation of playful handling will face a similar problem. A potential method of increasing the efficiency of playful handling is to provide heterospecific play within the home cage environment. This maximises the use of time by handling multiple individuals at once without the need to transfer the animals into a separate arena. Further, handling rats in their home cage with a familiar conspecific is also likely to positively affect rat perception of playful handling. For example, when rats were exposed to novel stimuli, those exposed with a conspecific showed fewer indicators of negative affect than those exposed alone in a phenomenon known as social buffering (Davitz and Mason, 1955; Kikusui *et al*., 2006; Taylor, 1981).

Considering the points raised above, the aims and hypotheses of this study were;

i) To investigate whether playful handling of rats as pairs in the home cage induces a positive affective state in rats as indicated by USV production and approach behaviour. I hypothesised that playfully handled rats would produce more 50-kHz USVs and show increased approach behaviour towards the experimenter than control rats as a reflection of their increased positive affective state.

ii) To investigate inter-individual and intra-individual variation in response to playful handling as pairs in terms of USV production and approach behaviour. I hypothesised that playfully handled rats would show high inter-individual variation and low intra-individual variation in USV production and approach behaviour.

Methods

Ethics

This study was conducted in accordance with the European Union directive of 22nd September 2010 (2010/63/EU), and the UK Animals (Scientific procedures) act 1986. There was further approval by the Roslin Institute Animal Welfare and Ethical Review Body (AWERB), the Royal (Dick) School of Veterinary Studies

Veterinary Ethical Review Committee (VERC), and SRUC's Animal Ethics Committee.

Animals, housing, and husbandry

All work was conducted at the Roslin Institute, Edinburgh, UK, between 7th and 10th September 2021. To reduce the use of experimental animals, the rats in this experiment were used after the completion of Chapter 4 for Experiment 3, in which they had all undergone the same experimental procedure and received the same acoustic stimuli. In brief, in a Latin Square design, all rats were exposed to playback of three different acoustic stimuli (50-kHz, White Noise, and Background Noise) in the home cage as pairs over three days. As such, the previous experiment did not expose rats to the playful handling used in this experiment. For Chapter 4, juvenile male rats from females of unknown parity were imported from Charles River Laboratories, Margate, UK. On arrival, rats were assigned to pairs (N = 14 cages), randomly selected across litters and matched by weight within 2g to account for the relationship between body condition and play behaviour (Hammond *et al*., 2019). For this experiment, rats were kept in the same pairs as in Chapter 4, considering housing rats in pairs is the most common method of socially housing rats after weaning (Cloutier *et al*., 2013). Data collection began when the rats were 42 days old, which is in line with the wider literature as the average age of rats receiving tickling is 42 days old (N = 32 experiments; taken from data in (LaFollette *et al*., 2017). All 14 cages were used from Chapter 4 with the reduction principle of the 3Rs in mind. A retrospective power calculation indicated power of 83% (Kane, 2018).

Rats were housed in clear plastic cages with a wire lid (48 x 26.3 x 20.5 cm; Techniplast, Italy). Each cage was supplemented with wood chip bedding, ad libitum access to food (Teklad Global Rodent Maintenance Diet (14% protein) from Envigo, England), and water. Cage cleaning was carried out by the same caretaker once a week, ensuring no disturbance occurred at least two hours before any experimental procedure. Rats were in a reverse light cycle so that handling could occur during the dark period when the rats are naturally active (lights off at 0900 with handling between 1000 and 1500). Low-intensity red lighting (4.5 lx measured using an Isotech digital light meter Lux-1337) was used during the dark phase to keep the rats within the active dark photoperiod and encourage USV production (Knutson *et al*., 1998). The holding room was maintained at a constant temperature (23 ± 0.2 °C) and humidity (37.5 ± 6.7%). Cages were situated on a single rack across four tiers. Both rats within a cage received marks on the tail using a non-toxic black marker for individual identification.

Experimental design

All cages from Experiment 3 in Chapter 4 were used in this experiment. Fourteen cages were assigned to receive either playful handling or control handling (N = 7 playfully handled cages and N = 7 control handled cages). Cages were assigned to treatment according to body weight by taking an average cage weight after the second habituation session and assigning cages of the closest weight to either playful handling or control handling. There was a negligible 0.35g difference in average cage weight between cages assigned to playful handling and control handling. Overall mean weight and standard deviation on the day before handling was 119.4 ± 7.9g. Following two habituation sessions on one day, rats received three sessions of either playful handling or control handling in the home cage across three days.

Experimental set-up

All testing was conducted within the holding room at least three metres away from other rats as there is evidence that the emotional state of one animal can be transferred (emotional contagion) to another through USV production, odours, or another mechanism (Hatfield *et al*., 1993). Rats were handled in their home cage. Across all habituation and handling sessions, the handling order was randomised for each session using a random number generator. This randomisation was to prevent systematic transmission of cues, *e.g.,* olfactory cues, related to treatment.

Experimental procedures

Habituation to handling: Habituation was conducted on one day across two sessions under the same environmental conditions as the handling phase, aiming to expose the rats gradually to the movement and touch of the experimenter's hand. Rats were not individually handled during transport. This protocol was reduced from Chapter 2 as the rats were previously habituated to the movement of the cage onto the desk in Chapter 4.

Daily procedure during testing: Each cage was moved from the rack across the homeroom to a bench at least 3 metres away from other rats. Rats were then handled according to treatment in the home cage. On return to the rack, the wire cage lid was topped with cardboard to create a barrier for USVs and odours. Only one female experimenter (TH) handled the rats for all handling stimulations, always wearing the same knitted glove on top of a nitrile glove.

Free roam test: On days one and five, rats were provided with a 30-second opportunity to approach the experimenter's hand or conduct play before (FR1) and after (FR2) handling (adapted from LaFollette *et al*., 2018). The behavioural responses expressed during FR1 on day one would act as a baseline measurement as rats had not experienced any handling treatment. On day three, these responses would act as an indicator of anticipation for the respective handling treatments. The experimenter conducted the 'free roam test' by placing her hand on the long side of the home cage with her fingertips on the bedding against the right-side wall (**Figure 3.1**).

Handling treatment: Handling techniques remained the same regardless of the day (**Figure 3.1**). As in Chapter 2, playful handling involved the experimenter using one hand, covered by a soft knitted glove, to touch, tickle, chase, and gently attempt to pin the rat in a manner that mimics rough and tumble play (Bombail *et al*., 2019). Each individual rat was playfully handled in this way for a bout of 15 seconds each, followed by a 15-second "pause" lasting for a total of three minutes (adapted from Panksepp and Burgdorf, 2010). In this way, rats received the same duration of tactile stimulation phases and pause phases as in Chapter 2. For control rats, instead of handling, the gloved hand rested with the palm flat on the wall of the home cage nearest the experimenter. There was one procedural change in handling from Chapter 2. Instead of placing the hand on the side of the arena during pauses, the experimenter's hand was removed from the home cage so rats could not interact with the hand. This was to take account of the reduced dimensions of the home cage compared to the arena, which could make the assessment of approach behaviour less accurate. This also allowed the hand to remain active throughout handling. When the hand was placed on the side of the arena during pauses in Chapter 2, rats would interact with the hand through sniffing and biting and receive no response. In this way, the hand could be seen as inactive and create an inconsistency in the expectation of the rat. The removal of the hand during pauses also allowed for direct behavioural comparison between treatment and control animals.

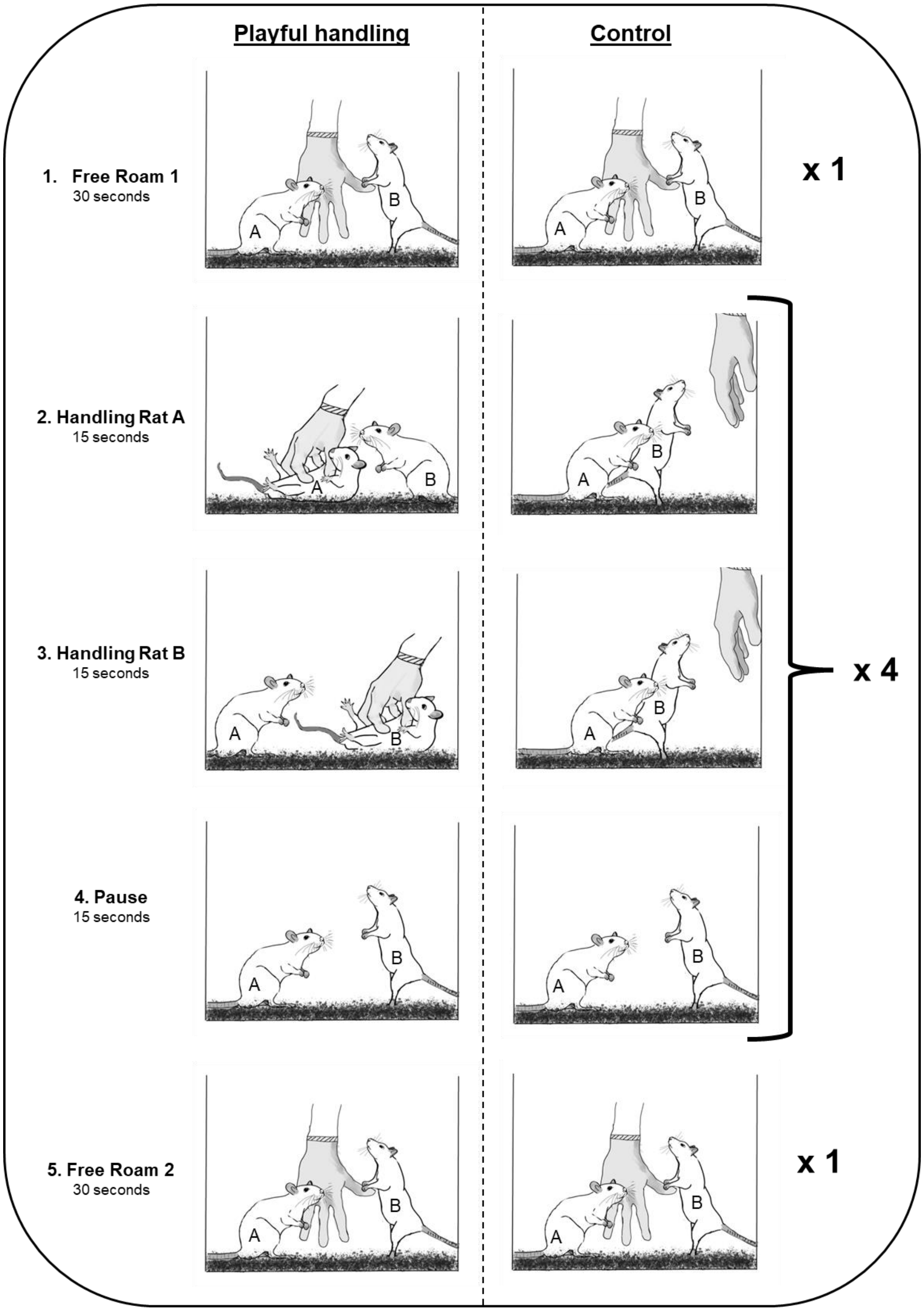


Figure 3.1. Illustration of handling techniques used on juvenile male Wistar rats as pairs in the home cage (N = 28 rats housed as pairs; 7 playfully handled cages and 7 control handled cages). The playful handling treatment is in the left pane, and the control is in the right pane. A and B identify the individual. Steps one and five show the 30-second free roam tests, which were conducted once before handling and once after handling. Steps two to four indicate the 45-second handling phase, which was repeated 4 times. This figure is not to scale.

Recording and analysis of behaviour

Behaviour during handling was recorded using a Sony HD camcorder (HDR-PJ810E) and subsequently analysed using Observer XT 14 software (Noldus Information Technology, Wageningen, the Netherlands). Behaviour was coded in sections according to the free roam and pause phases of the experimental procedure; as playfully handled and control rats experienced different interactions during the handling phase, the behaviour was only compared during the pause phases. Using continuous focal sampling, frequency and duration of each behaviour were coded according to the ethogram below (**Table 3.1**). During the collection of data from spectrograms and videos of home cage behaviour, the coder (TH) was blinded to treatment to prevent bias. This blinding was not possible during the videos of handling as the coder could see the treatment. During the collection of data from videos of home cage behaviour, the coder (TH) was blinded to treatment to prevent bias.

Table 3.1 Ethogram developed for recording behaviour of pairs of juvenile male Wistar rats (N = 28 rats housed as pairs; 7 playfully handled cages and 7 control handled cages; aged between 42-45 days old) during free roam tests and in the pauses between handling (either playful handling or control handling).

Behaviour	**Description**
Solitary play	Rat conducts fast locomotor movement involving at least one hop by an individual, where hops involve all four paws leaving the ground at the same time, not in the direction of a play partner or during a play bout. The behaviour starts with fast running or a hop from stationary or during locomotor movement and ceases when this movement stops. (Hammond *et al*., 2019)
Social play	One rat jumps or lunges towards the partner's nape, resulting in the partner either chasing the soliciting rat, rearing (in which pairs make rapid pawing movements at each other), or rotating so that one rat is on its back with the other standing over it in a pin. The frequency of pinning and dorsal contacts within social play was also coded. Multiple pins and dorsal contacts can occur during a single social play bout. The behaviour starts with fast running, a jump or lunge towards a play partner and ceases when there is no chasing, rearing, pins or dorsal contacts between the play partners. (Kerkhof *et al*., 2013; Webber *et al*., 2012)
Rearing	Rat raises both front paws off the ground (can be in contact with the wall or not), standing up on hind legs. Does not include rears directed toward the experiment's hand or body. (Lever, *et al*., 2006)
Approach towards experimenter	Rat orients its body and head so that the nose is directed towards the experimenter's hand and within 1cm or touching the hand.(Hammond *et al*., 2019)

Statistical analysis

Tests of intra-observer reliability were conducted prior to statistical analysis. As only one observer scored all behaviours, inter-observer reliability tests were unnecessary. I conducted a repeat scoring of a minimum of 12.5% of the data seven days later. For USV data, I conducted Spearman's rank correlations between the first count and second count for each spectrogram (Martin and Bateson, 1993). To meet the reliability criteria, correlations met the acceptable cut-off defined by Martin and Bateson of 0.7. For behaviour, these correlations were conducted using Observer XT 11 software and met a minimum Cohen’s kappa of 0.8 with a percentage agreement of 64% (McHugh, 2012).

All data were analysed in Genstat 19.1 using General Linear Models (GLM), linear regression models, and correlations. Figures were generated using GraphPad Prism (ver 9.2). Assumptions of these models were tested on the residuals using Bartlett's test for homogeneity of variances and the Anderson-Darling test for normality. Tukey's honest significance difference (HSD) procedure was conducted for multiple comparisons on significant differences ($a = 0.05$). Means (M) and standard errors of the mean (SEM) reported were back-transformed to the original scale for biological significance. For all tests, the level of statistical significance was set at $p < 0.05$.

USV and behaviour data were taken from all three days of handling. As rats were tested together in the home cage, the frequency and duration of each rat's USV production and behaviour were summed to give a cage level of expression. To account for this, the blocking factor was cage in all models. USV counts were converted into a rate per minute to compare USV production between the free roam tests and handling phases which were of different durations. In all periods, the production of 22-kHz USVs, solitary or social play, was too infrequent to fit into a GLM. To investigate USV production during handling, handling treatment (playful handling or control handling), handling phase (during handling or pauses), and day (one, two, or three) were modelled as the main effects with additional interactions between the three main effects.

To investigate USV production and behaviour during the free roam tests, handling treatment (playful handling or control), the timing of the free roam test (before or after handling), and day (one, two, or three) were modelled as the main effects with additional interactions between the three main effects. To investigate behaviour during the pauses in handling, handling treatment (playful handling or control) and day (one, two, or three) were modelled as the main effects with additional interactions between the two main effects. Pearson's correlation was used to investigate the relationship in USV production during handling and pauses within each treatment on day three.

As previously discussed in Chapters 1 and 2, there is considerable individual variation in measured responses to heterospecific play (LaFollette *et al*., 2017). To investigate this, I present the coefficient of variation as a statistical measure of the difference in variation between the two treatments for USV production and approach behaviour.

Linear regression was used to determine whether there was evidence of anticipation for handling in the free roam test prior to handling on day three on a cage level. The number of USVs or the duration of approach toward the experimenter during the free roam test before handling on day three was fitted as the explanatory variable and the number of USVs or the duration of approach toward the experimenter during handling day three was fitted as the response variable.

To investigate the relationship within measures of affect (USV production and approach behaviour) across handling treatments, these variables were summed across handling and pause phases on a cage level. Linear regression was used to determine whether responses to handling on day one predicted responses on day three within each treatment. The number of USVs or the duration of approach toward the experimenter on day one was fitted as the explanatory variable, and the number of USVs or the duration of approach toward the experimenter on day three was fitted as the response variable.

To investigate the relationship between measures of affect (USV production and approach behaviour) during pauses in handling, I performed Pearson's correlations on the average USV production and duration of approach behaviour on day three within each treatment on a cage level.

Results

Playfully handled rat pairs produced more 50-kHz USVs than controls during handling

There was a significant main effect of handling treatment on USV production during handling, with playfully handled rats emitting significantly more 50-kHz USVs than those who experienced control handling ($F_{1, 335} = 6.5$, $p = 0.03$; **Figure 3.2**). There was also a significant main effect of day, with rats across both treatments producing more USVs on day three than on day one ($F_{2, 335} = 60.7$, $p < 0.001$).

The number of USVs emitted by playfully handled rats on day one did not predict the number of USVs produced on day 3 ($T = 1.16$, $p = 0.30$, $r^2 = 5.5\%$). The number of USVs emitted by control rats on day one did predict the number of USVs produced on day three ($T = 6.35$, $p < 0.001$, $r^2 = 86.8\%$). The coefficient of variation for USVs differed between the two handling treatments, with reduced variation in playfully handled rats compared to control rats on both days. On day one, the coefficient of variation for playfully handled rats was 19.5% and 64.4% for control rats. On day three, the coefficient of variation for playfully handled rats was 11.1% and 50.6% for control rats.

Rats produced USVs during both the handling and pause phases when the hand was out of the home cage. There was no significant effect of handling phase (during handling or pauses) on the number of USVs produced ($F_{1, 335} = 1.8$, $p = 0.23$). While the number of USVs between the handling and pause phases on day three were strongly correlated for control rats ($R_p = 0.94$; $p = 0.002$), there was no significant correlation between the two phases for playfully handled rats ($R_p = 0.57$; $p = 0.27$).

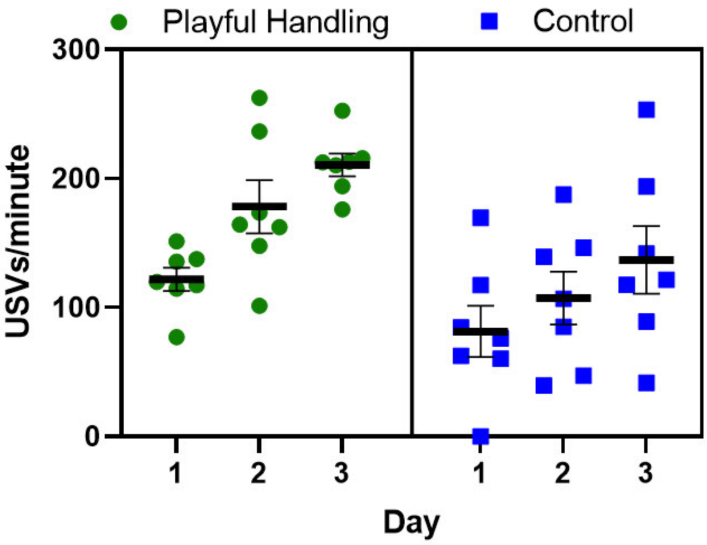


Figure 3.2. Graph showing the mean number of 50-kHz ultrasonic vocalisations (USVs) per minute produced by pairs of juvenile male rats (N = 28 rats housed as pairs; 7 playfully handled cages and 7 control handled cages) across three consecutive sessions of handling. Rats received playful handling (left pane; green circles) or control handling (right pane; blue squares). Values reflect means for each cage with the black bar indicating the mean ± SEM for each treatment by day.

Playfully handled rats conducted more rearing than control rats during handling

Results of the GLM modelling rat behaviour during the pause phases of playful handling are shown in **Table 3.2**. The only behaviour that was influenced by the main effect of treatment was the frequency of rearing, with playfully handled rats conducting more rears than control rats (16.4 vs 12.6 ± 1.2 events in one minute). Frequency of rearing and frequency of approach behaviour towards the experimenter was influenced by the main effect of day, with an increase on day three compared to day one (rearing: 8.5 vs 5.1± 0.9 events per minute and approach behaviour: 11.9 vs 15.7 ± 0.7 events per minute).

The duration of approach behaviour by playfully handled rats on day one did not predict approach behaviour on day three (T = 0.63, p = 0.56, r^2 = 0.0%). The duration of approach behaviour by control rats on day one also did not predict approach behaviour on day 3 (T = 0.73, p = 0.50, r^2 = 0.0%). The coefficient of variation for the duration of approach behaviour differed between the two handling treatments on day one but not day three, with reduced variation in playfully handled rats compared to control rats. On day one, the coefficient of variation for playfully handled rats was 39.8% and 98.2% for control rats. On day three, the coefficient of variation for playfully handled rats was 44.9% and 39.8% for control rats.

Table 3.2. Table presenting results of an ANOVA ran on the frequency and duration of behaviours expressed by juvenile male rats (N = 28 rats housed as pairs; 7 playfully handled cages and 7 control handled cages) during pauses in playful handling or control handling across three days. Significant P values are indicated in bold script.

	Variable	**Treatment**		**Day**	
Behaviour		$F_{1, 41}$	*p*-value	$F_{2,41}$	*p*-value
Approach towards experimenter	frequency	0.8	0.34	6.1	**0.007**
	duration	0.3	0.57	0.7	0.49
Rearing	frequency	4.7	**0.05**	5.7	**0.009**
	duration	4.2	0.06	0.1	0.87
Digging	frequency	0.7	0.4	2.7	0.09
	duration	0.6	0.46	1.4	0.26

There was a relationship between USV production and approach behaviour during the pauses in handling for playfully handled rats

To determine whether there was a relationship between the two measures of affective state, I conducted correlations between USV production and duration of approach behaviour during pauses in handling on day 3; day 3 was chosen as the responses to handling treatments would be expected to be established by that point. There was a significant relationship between USV production and approach towards the experimenter for playfully handled (R_p = 0.77, p = 0.04) but not control rats (R_p = 0.21, p = 0.66).

Playfully handled rats produced more USVs than control rats in the free roam tests

There was a significant main effect of treatment on USV production during the free roam tests, with playfully handled rats producing more USVs than control rats ($F_{1, 55}$ = 7.6, p = 0.008). There was a significant main effect of the timing of the free roam test (*i.e.,* whether it was applied before or after handling), with rats producing more USVs during FR2 than FR1 ($F_{1, 55}$ = 14.5, p < 0.001) and on day three compared with day one ($F_{1, 55}$ = 39.4, p < 0.001). There was also a significant interaction effect between treatment, phase and day ($F_{1, 55}$ = 6.2, p = 0.02; **Figure 3.3**). Tukey's HSD indicated that playfully handled rats produced more USVs than control rats in during all free roam tests (T = 3.83, p = 0.002) bar FR1 on day one (T = 0.38, p = 0.98). There was an increase in USV production from FR1 day one to three during all other FR tests for playfully handled (T = 3.40, p = 0.004) and control rats (T = 4.67, p = 0.03).

The coefficient of variation differed between the two handling treatments, with reduced variation in playfully handled rats compared to control rats in both free roam tests. During FR1, the coefficient of variation for playfully handled rats was 28.0% and 49.5% for control rats. During FR2, the coefficient of variation for playfully handled rats was 11.1% and 50.6% for control rats.

The number of USVs emitted by playfully handled rats during FR1 on day three did predict the number of USVs emitted during handling on day 3 (T = 2.90, p = 0.03, r^2 = 55.3%). The number of USVs emitted by control rats during FR1 on day three did not predict the number of USVs emitted during handling on day 3 (T = 1.35, p = 0.23, r^2 = 0.0%).

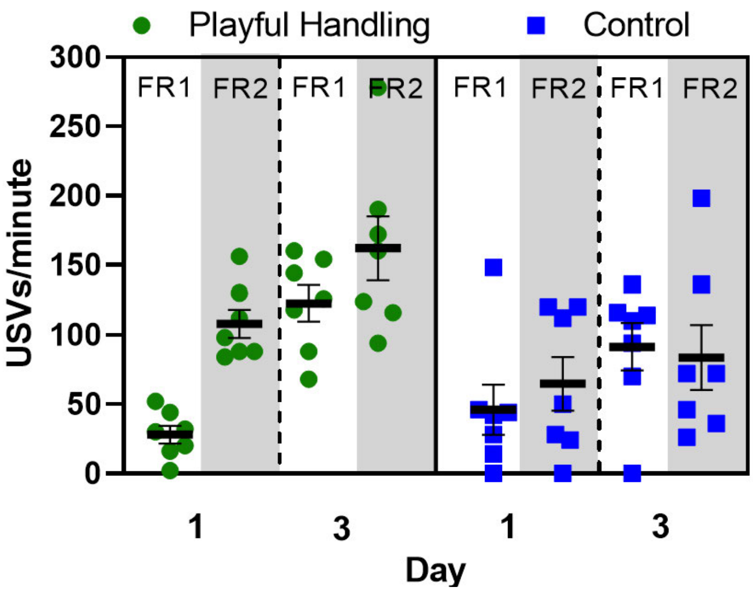


Figure 3.3. Graph showing the mean number of 50-kHz ultrasonic vocalisations (USVs) per minute produced by pairs of juvenile male rats (N = 28 rats housed as pairs; 7 playfully handled cages and 7 control handled cages) during free roam (FR) tests on the first and third consecutive sessions of handling. These tests provided a 30-second opportunity for the rat to express anticipation, approach the hand or conduct other behaviours before and after receiving handling treatments. White background bars indicate the number of USVs produced in the free roam test before handling (FR1), and grey background bars indicate the number of USVs produced after (FR2). The handling treatments comprised either playful handling (left pane; green circles) or control handling (right pane; blue squares). Values reflect means for each cage with the black bar indicating the mean ± SEM for each treatment by day.

Approach behaviour and rearing were influenced by the timing of the free roam test

Results of the GLM modelling of rat behaviour during free roam tests are shown in **Table 3.3**. There was no significant effect of treatment or day on approach behaviour towards the experimenter or rearing. There was a main effect of timing of the free roam test (before or after handling) on the duration of approach behaviour, with rats conducting more approach behaviour during FR1 than FR2 on both days (42.3 vs 32.0 ± 1.85 seconds per minute). Rearing was also influenced by the timing of the free roam test, with the frequency and duration of rearing increasing in FR2 on both days (frequency: 2.9 vs 5.7 ± 1.9 events per minute, duration: 20.1 vs 27.6 ± 1.6 seconds per minute).

The coefficient of variation of the duration of approach behaviour differed between the two handling treatments, with reduced variation in playfully handled rats compared to control rats in both free roam tests. During FR1, the coefficient of variation for playfully handled rats was 16.8% and 52.9% for control rats. During FR2, the coefficient of variation for playfully handled rats was 44.9% and 67.5% for control rats.

The duration of approach behaviour by playfully handled rats during FR1 on day three did not predict the duration of approach behaviour during handling on day three (T = 0.01, p = 1.00, r^2 = 0.0%). The duration of approach behaviour by control rats during FR1 on day three also did not predict the duration of approach behaviour during handling on day three (T = 0.20, p = 0.85, r^2 = 0.0).

Table 3.3. Table presenting results of an ANOVA ran on the frequency and duration of behaviours expressed by juvenile male rats (N = 28 rats housed as pairs; 7 playfully handled cages and 7 control handled cages) during free roam tests (FR) within playful handling or control handling across three days. These tests provided a 30-second opportunity for the rat to express anticipation, approach the hand or conduct other behaviours before (FR1) and after (FR2), receiving either playful handling or control handling. Significant P values are indicated in bold script.

	Variable	**Treatment**		**Day**		**Timing of test (before or after)**	
Behaviour		$F_{1, 55}$	*p*-value	$F_{2,55}$	*p*-value	$F_{1,55}$	*p*-value
Approach towards experimenter	frequency	0.71	0.38	1.55	0.16	0.71	0.38
	duration	0.01	0.89	1.78	0.18	15.61	**<0.001**
Rearing	frequency	0.01	0.94	5.42	0.60	16.14	**0.03**
	duration	0.13	0.83	0.46	0.50	9.32	**0.01**

There was no relationship between USV production and approach behaviour during the free roam tests

To determine whether there was a relationship between the two measures of affective state during the free roam tests, I conducted correlations between USV production and duration of approach behaviour during free roam tests by that point. Day 3 was chosen as the responses to handling treatments would be expected to be established by day three. During FR1, there was no relationship between USV production and approach towards the experimenter for playfully handled ($R_p = 0.73$, $p = 0.06$). or control rats ($R_p = 0.08$, $p = 0.86$). During FR2, there was also no relationship between USV production and approach towards the experimenter for playfully handled ($R_p = 0.04$, $p = 0.69$) or control rats ($R_p = 0.04$, $p = 0.92$).

Discussion

Playful handling is a promising method of inducing a positive affective state in rats through heterospecific play. Current evidence that playful handling is a positive experience is limited to individually handled rats in an arena separate from the home cage (Bombail *et al*., 2019; Chapter 2). This protocol requires a lengthy time investment per rat and is likely to act as a barrier that prevents the uptake of playful handling in laboratory personnel. To reduce the time investment required to conduct playful handling, rats were playfully handled, or control handled as pairs in their home cage. Here, I provide validation for the use of playful handling to induce a positive affective state in pairs of juvenile male rats in the home cage, as primarily evidenced by 50-kHz USV production. Further, playfully handling rats in the home cage with the conspecific resulted in reduced variation in USV production between cages, suggesting that this protocol results in a consistent increase across individuals in positive affective state.

Playful handling induced a positive affective state in rats

As a novel method of heterospecific play, the first two studies on playful handling have determined the responses of individual rats to playful handling in an arena. So far, the evidence suggests that playful handling is a positive experience for rats, with increased 50-kHz USV production (Bombail *et al*., 2019; Chapter 2), approach behaviour (Bombail *et al*., 2019; Chapter 2), and sucrose consumption (Chapter 2). However, the wider uptake of playful handling in laboratory settings is likely to face barriers, with time cited as the main barrier to the uptake of tickling (LaFollette *et al*., 2018b). As such, I investigated whether playful handling is a positive experience for rats under conditions that are more likely to promote heterospecific play in laboratory facilities. To reduce the time investment required per rat, rats were playfully handled as pairs in the home cage, considering that rats are most commonly housed in pairs after weaning (Cloutier *et al*., 2013). This method is relatively time-efficient compared to the standard protocol in that the number of steps required to handle rats is significantly reduced, yet rats receive the same duration of stimulation as when handled individually.

Consistent with previous results using the individual protocol (Bombail *et al*., 2019; Chapter 2), the production of 50-kHz USVs indicated that playfully handled rats were in a more positive affective state than control rats. Playfully handled rats produced more 50-kHz USVs than control rats across all three days, with an increase in USV production from day one to three. As discussed in Chapter 2, USV responses to heterospecific play appear to develop over time, with USV production increasing across subsequent sessions in response to both playful handling and tickling (Bombail *et al*., 2019; Burgdorf and Panksepp, 2001; Cloutier *et al*., 2013; Hammond *et al*., 2019; LaFollette *et al*., 2018a). However, the increase in USV production from day one to three was not specific to playfully handled rats, with USV production also increasing across handling days in control rats. Taken with evidence that USV production acts on a graded scale (Hinchcliffe *et al*., 2020), this suggests that the development of USV responses is not specific to heterospecific play and could potentially be used to measure how rats perceive a certain experience. For example, experiences could be ranked according to the strength of positive affect by comparing the gradient of the change in USV production over subsequent days. The increase in USV production in control rats resulting from moving the home cage to the workbench also highlights the potential ease by which even a small increase in positive affective state can be promoted in laboratory rats. There was no difference in USV production between the handling and pause phases in playfully handled or control rats. This may be attributed to the reduced variation in USV production between cages in playfully handled rats compared to control rats, which I will return to later.

In contrast to when rats were handled as individuals in an arena, there was no difference in approach behaviour between playfully handled or control rats. This lack of treatment effect may be explained by the difference in the location of the experimenter's hand during pauses between individually handled, and pair-handled rats. When rats were individually handled, the experimenter's hand was placed on the inside of the wall of the arena, within touching distance of the rat: when the rats were handled as pairs, the experimenter's hand was removed from the home cage. As discussed in the Methods section (see Experimental procedures), this change aimed to reduce the likelihood of false-positive results and maintain the experimenter's hand as an active play partner. However, it is possible that the rats could not locate the experimenter when the hand was removed from the home cage. When the hand was located within the arena, the rat could use olfactory cues to locate the experimenter, considering that rats rely on odours to discriminate between different humans (Driel and Talling, 2005; McCall *et al*., 1969). When the hand was out of the cage, olfactory cues would have been blocked by the cage wall, and thus, rats would have to rely on weaker sensory modalities such as vision, which has poor acuity in albino rats (Burn, 2008). However, there is the potential that rats were able to learn the location of the experimenter over subsequent sessions, as suggested by the increase in the frequency of approach behaviour from day one to three in both treatments. Playfully handled rats also conducted more rears than control rats, with rats using rearing as a method of information gathering (Lever, *et al*., 2006). This is further supported by the strong positive relationship between USV production and approach behaviour during handling on day three, which was found for playfully handled but not control rats. As such, while there was a lack of treatment effect to approach behaviour, this suggests that playfully handled rats were more motivated to be handled than control rats. Ideally, rats would have experienced the same sucrose preference test per Chapter 2 as a third measure of affective state. However, due to Covid-19 delaying experiments, this test could not be conducted.

As discussed in Chapter 2, further insight into how rats perceive playful handling could be gained from examining rat behaviour prior to handling during the anticipatory period. Anticipatory behaviour is thought to reflect the value an animal places on a reward (van der Harst and Spruijt, 2007). For example, 50-kHz USVs have been seen to increase prior to putatively positive experiences such as copulation (Bialy *et al*., 2000) and contact with a conspecific (Burke *et al*., 2017). As such, behaviour prior to handling may indicate the reward value associated with the specific handling treatment. As per Chapter 2, USV production and approach behaviour were measured during a free roam test on days one and three of handling. In this test, the experimenter placed her fingertips on the floor of the home cage for 30 seconds, both before and after handling. The free roam test before handling (FR1) provided rats with an opportunity to express anticipation for handling, whilst the test after handling (FR2) allowed the expression of lasting changes to affective state. FR1 on day one acted as a baseline measurement since rats had no knowledge of the handling treatment they were about to receive. Whilst there was no difference between treatments on day one, playfully handled rats produced more USVs than control rats during FR1 on day three. Both playfully handled and control rats showed an increase in USV production during FR1 from day one to three. In playfully handled rats, this was a fourfold increase in USV production, whereas, in control rats, USV production was doubled. This suggests that while rats actively anticipated both handling treatments, the higher magnitude of USV production suggests that playfully handling is a significantly more rewarding experience than control handling. Further, on day three, the number of USVs produced during FR1 predicted the number of USVs produced during handling in playfully handled but not control rats. This suggests that anticipatory USVs were directly related to the reward value of the stimulus. Overall, the presence of anticipatory USVs in control rats provides further support for the idea raised earlier in this discussion that the act of moving the home cage to the workbench is a positive experience. On both days, playfully handled rats produced more USVs than control rats during FR2, suggesting that the positive affective state induced by playful handling lasted even after the handling experience had concluded.

In contrast, there was no significant effect of treatment or day on approach behaviour towards the experimenter. Rats of both treatments and across both days conducted an increased duration of approach behaviour during FR1 than FR2. This cautiously suggests that both treatments expressed anticipation for handling through approach behaviour since it more commonly occurred prior to the expected experience. However, approach behaviour during FR1 did not predict the duration of approach behaviour during handling on day three for playfully handled or control rats. As such, approach behaviour may not be a sensitive enough measure of treatment differences in anticipation.

Playful handling as pairs in the home cage reduced variation between individuals in USV production compared to control handled rats

It is increasingly evident that rats show variation in responses to heterospecific play both between and within individuals (Bombail *et al*., 2021, 2019; LaFollette *et al*., 2017). Production of 50-kHz USVs, in particular, appears to be sensitive to individual variation in response to heterospecific play (Hinchcliffe *et al*., 2020). Typically, a wide range of USV rates is produced between individuals, with this rate remaining stable over time within individuals (Bombail *et al*., 2021, 2019; LaFollette *et al*., 2017). In this study, playfully handled rats showed reduced variation between cages in USV production compared to control rats across the handling and the free roam tests from the first day. This effect was particularly apparent during handling, where the between-cage variation in USV production in playfully handled rats was fivefold lower than in control rats on day five. Playful handling also reduced between-cage variation in approach behaviour compared to control rats, although this was specific to day one of handling. These findings are distinct from previous work, suggesting that the novel properties of the protocol used in this experiment resulted in the experience being positive for a higher number of rats.

Playful handling was developed to incorporate two key aspects; first, to incorporate the diversity and unpredictability of social play, and second, to react to the behaviour of the individual rat (Bombail *et al*., 2021). Most importantly, this interaction allows rats to have control over the stimulation in that the experimenter tailors the experience according to each rat (Bombail *et al*., 2021). Pairing this interaction with the presence of the cage-mate, who was playfully handled at the same time, and conducting all handling in the home cage could explain the reduced variation in USV production. Although habituation steps were taken to reduce the likelihood of inducing stress, the first experience of playful handling is an unknown and novel experience. The presence of a partner rat is known to act as a buffer for stressful situations in the form of social support (Taylor, 1981). For example, rats exposed to an anxiogenic experience, such as a novel environment, show fewer behavioural and physiological indicators of fear if with a conspecific than when alone (Davitz and Mason, 1955; Kikusui *et al*., 2006; Taylor, 1981).

The presence of a partner rat also provides the opportunity for emotional contagion to occur, whereby the emotional state of one animal is transferred to another as indicated by the change in the behaviour of one rat to match the behaviour of another (Held and Špinka, 2011). Play is well-documented to have contagious properties; for example, rats' play level increases in response to a more playful partner (Pellis and McKenna, 1992). There is also evidence that tickling has contagious properties, as non-handled cage mates of tickled rats have shown increased hand-following behaviour toward the experimenter than control rats (Hammond *et al*., 2019). Thus, the perception of one rat to playful handling may influence the partner and spread a positive emotional state.

Further, handling rats in the home cage mitigated the need for rats to be habituated to the arena, which could potentially be another source of stress. As a novel study, this work was conducted using a small number of cages. Further work on a larger cohort is necessary to support the potential of playful handling under these conditions to reduce variation in USV production.

The reduced variation in USV production between cages in playfully handled rats could explain the lack of consistency in USV production in response to playful handling, *i.e.,* if the variation in USV production between cages is small, then this reduces the ability of extreme individuals to influence the correlation. While USV production on day one predicted USV responses on day three in control rats, this was not seen in playfully handled rats. Similarly, the number of USVs between the handling and pause phases on day three were strongly correlated for control rats but not playfully handled rats. Further, the experiments which found consistency in USV production in playfully handled rats for five rather than three days (Bombail *et al*., 2019; Chapter 2). Considering the evidence that USV production in response to heterospecific play develops over time (Bombail *et al*., 2019; Cloutier and Newberry, 2008; Hammond *et al*., 2019; LaFollette *et al*., 2018a; Panksepp and Burgdorf, 2010), three days may not have been sufficient to develop each individual's perception of playful handling. Thus, given a longer period, there may be consistency in USV production in response to playful handling. This treatment-specific effect was not found in approach behaviour, with no relationship between approach behaviour within cages on days one and three in playfully handled and control rats. As discussed earlier and in Chapter 2, approach behaviour may not be a sensitive measure of rat affect.

Conclusion

This work validates the use of playful handling as a method of inducing positive affective states in rats under conditions that are practical for laboratory personnel. Playfully handled rats produced more indicators of a positive affective state and increased anticipation to be handled than control rats. In addition to being efficient for laboratory personnel, the reduced variation in USV responses to playfully handling in the home cage as pairs suggests that a wider number of rats perceived the experience as positive. This has important implications for rat welfare by reducing the likelihood that playful handling is aversive.

Chapter 4: Application of a novel acoustic playback paradigm to induce positive affective state and measure changes in play in juvenile male Wistar rats

Abstract

Playback of positively-valenced vocalisations is a potential method of inducing a positive affective state in rats. Previous work has shown that presentation of contrastingly valenced ultrasonic vocalisations (USVs) to individual rats in a radial maze changes approach behaviour towards the sound source; positively valenced 50-kHz USVs induce approach behaviour, while negatively valenced 22-kHz USVs increase behavioural indicators of the fight or flight response. The home cage playback paradigm used here for the first time was developed as a low-stress environment that allows the measurement of behaviours indicative of a change in affective state. The primary aim of this study was to investigate whether the playback of acoustic stimuli to juvenile male Wistar rats (37 days old) in this paradigm alters affective state in rats as indicated by USV production and approach behaviour. On the evidence that playback of acoustic stimuli induced a positive affective state, a secondary aim of this study was to investigate whether play was sensitive to this manipulation of affective state. Across all experiments, rats received acoustic stimuli treatments in the home cage as pairs. In Experiment 1 (N = 14; 7 cages), rats were exposed to a single presentation of 50-kHz USVs and Background Noise to establish whether rats could perceive the playback stimulus. In Experiments 2 and 3, two independent cohorts of rats were presented with four acoustic stimuli in a pseudo-randomised Latin square design across three days. In Experiment 2 (N = 30; 15 cages), rats were presented with 50-kHz USVs, 22-kHz USVs, and Background Noise with the speaker located at the top of the cage. In Experiment 3 (N = 28; 14 cages), 50-kHz USVs, White Noise (within the 30 – 100kHz range), and Background Noise were presented with the speaker at the side of the cage. A change of study site for Experiment 3 allowed for a change in speaker location to better match previous work (Wöhr and Schwarting, 2012a). In Experiment 1, rats showed increased USV production during the playback phases of the acoustic track compared to the pause phases. In Experiment 2, the playback of 50-kHz USVs increased subject-produced 50-kHz USVs compared with the playback of 22-kHz USVs and Background Noise (11.9 vs. 7.2 vs. 5.7 ± 1.6 USVs per minute; $F_{2,251} = 5.81$, $p = 0.05$). There were no effects of playback on play or other behaviours. In Experiment 3, USV production increased in response to both 50-kHz USVs and White Noise, compared with Background Noise (107.5 vs. 103.6 vs. 85.7 ± 8.9 USVs per minute; $F_{2,239} = 6.05$, $p = 0.05$). While the playback of both 50-kHz and White Noise increased the production of subject-produced USVs, a matched increase in social play was only seen in response to White Noise compared to Background Noise (duration of social play: 21.8 vs 17.7 vs 13.4 ± 2.2 seconds per minute; $F_{2,239} = 3.55$, $p = 0.03$). The effect of White Noise on social play could be due to its arousing properties and therefore has potential for use as an acoustic enrichment for laboratory rats. The increase in subject-produced USVs in response to playback of 50-kHz USVs suggests an enhancement of affective state, but this was not associated with an increase in play behaviour. In general, these results suggest that the playback of 50kHz USVs and filtered White Noise is a positive experience for rats.

Introduction

In the past five centuries, rats have made, and continue to make, significant contributions to science (Kaliste and Mering, 2007; Sengupta, 2012). Although it is difficult to provide an accurate estimate of the number of individuals used for scientific purposes across institutes each year, this is a considerable sum. For example, over 200 thousand rats are used per annum for research in the UK alone

(UK Home Office, 2016). Constant revision and improvement to the welfare standards for these animals are vital from both ethical and scientific viewpoints; we have a moral responsibility to give animals a ‘life worth living’ at a minimum (Hubrecht, 2014) and there is evidence that scientific validity is compromised when subject animals are experiencing poor welfare (Bayne and Würbel, 2014; Garner, 2005; Neville *et al*., 2022; Olsson *et al*., 2003; Richter *et al*., 2009). However, the current welfare standards for rats are limited by focusing solely on mitigating unnecessary harm, pain, or distress (Cait *et al*., 2022; Makowska and Weary, 2020). In recognition of this discrepancy, there has been a much-needed drive to promote positive experiences for these animals, such that providing laboratory rats with a ‘good life’ should be a precondition for their use (Makowska and Weary, 2020). In order to do this, it is necessary to develop practical and validated methods of inducing and assessing positive affective states in rats.

A promising method to induce positive affective states in animals is the playback of vocalisations. The prospect of using the playback of vocalisations to modulate receiver affective state was noted in the early 2000s (Burman *et al*., 2007; Manteuffel *et al*., 2004), yet has remained widely unexplored within animal welfare science (Briefer, 2018). The use of acoustic playback is based on the evidence that the production of vocalisations, and their acoustic structure, is sensitive to the affective state of the producer (Briefer, 2012). Considering that the primary role of vocalisations is communication, playback of these calls will likely result in valence-matched effects on conspecifics (*e.g.,* Manteuffel *et al*., 2004). This convergence of affective state occurs through emotional contagion, which is widely regarded as a simple form of empathy (Hatfield *et al*., 1993; Nakahashi and Ohtsuki, 2015; Panksepp *et al*., 2001; Waal, 2008, 2007) and involves measurable changes across neurological, physiological, and behavioural levels (Waal, 2007). This change in affective state in the receiver animals distinguishes emotional contagion from other empathy-related phenomena of behavioural contagion or mimicry (Adriaense *et al*., 2020; Pérez-Manrique and Gomila, 2021).

Playback of vocalisations to manipulate affective state is particularly promising for rats, considering that the ultrasonic vocalisations (USVs) produced by juvenile and adult rats are widely accepted as affective signals (Wöhr and Schwarting, 2009). Broadly, rats produce two types of USVs which perform different communicative functions and are associated with contrasting affective states; calls in the 30 – 90-kHz range, known as 50-kHz USVs, and calls in the 18 - 30-kHz range, known as 22-kHz USVs. As the role of 50-kHz USVs is to facilitate social interaction, perception of these calls is likely to induce a positive affective state in the recipient (Burgdorf *et al*., 2008). In contrast, as 22-kHz USVs serve as alarm calls for conspecifics in aversive or threatening situations, such as in response to a predator, perception of these calls is likely to induce a negative affective state in the recipient (Litvin *et al*., 2007). As these types of USVs do not overlap in their acoustic structure (*i.e.,* frequency, amplitude, or duration), the recipient of the USVs receives an unambiguous signal to the affective state of the producer without the need to visually perceive the producer or the USV-inducing situation (Brudzynski, 2013, 2007). The differential perception of these USVs by rats is further evidenced when tested using cognitive bias: Saito *et al*. (2016) found that USV playback prior to a judgement bias test had a stimulus-specific effect on performance, with 50-kHz USV playback leading to a more "optimistic" response compared with more "pessimistic" responses after playback of 22-kHz calls.

Most research examining the effect of the playback of USVs on rat behaviour uses the radial maze paradigm. This paradigm measures the locomotory behaviour of an individual rat after being placed in the centre of an elevated eight-arm maze with playback emitting from a speaker at the end of one of the arms (Wöhr *et al*., 2016). Rats show consistent responses to the playback of contrastingly valenced USVs from the speaker; while 50kHz ‘positive communicative’ USVs induce approach behaviour towards the speaker and increase exploration (Schwarting *et al*., 2018), 22kHz ‘negative' alarm USVs induce behaviours indicative of the fight/flight system, *e.g.,* reduced locomotor activity and freezing (Wöhr and Schwarting, 2007).

While the radial maze playback paradigm has successfully been used within neuroscience as a behavioural assay for animal models of human neurodevelopmental disorders, it has some limitations for studying playback in relation to animal welfare. Due to the design of the maze, this paradigm relies on using two outcome measures which are based solely on locomotory behaviour, *i.e.,* total distance travelled as an indicator of social exploratory behaviour and time spent in the arms closest the speaker versus time in the arms furthest from the speaker as an indicator of social approach (Wohr and Schwarting, 2007). As a result, the potential for using multiple behavioural measures to assess affective state and welfare is restricted (Broom, 1993; Vere and Kuczaj, 2016). The assessment of welfare with the radial maze approach is also hindered by USVs often not being used to assess the animal's affective state. Although listed as a third measure in a paper describing the protocol of the radial maze playback paradigm (Wöhr 2016), the measurement of subject-produced USVs during playback is not widely used across studies. Measurement of USV production during playback would offer an indicator of affective state and provide the necessary information to distinguish between emotional and simpler forms of behavioural contagion or mimicry, as previously discussed. Further, behavioural expression may be confounded by a number of stressors introduced when using the radial maze as rats are exposed to handling, isolation, and bright light, all of which are known to increase behavioural and physiological markers of stress in rats (Begni *et al*., 2020; Gärtner *et al*., 1980; Korte and Boer, 2003; Nathiya and Vanisree, 2010). Thus, validation of a novel playback paradigm is necessary to use playback as an approach for inducing positive affective states and PAW in rats.

In addition to developing a novel approach to use playback for inducing positive affective state in rats, the second aim of this thesis was to investigate whether play is sensitive to this manipulation of affective state as an indicator of PAW. Play has been proposed as a promising indicator of positive emotions and welfare in domesticated animals (*e.g.,* Boissy *et al*., 2007; Held and Špinka, 2011). In brief, play tends to occur when animals are under favourable conditions, as it is reduced and can even disappear when the animal's potential ability to survive is challenged (Held and Špinka, 2011; Boissy *et al*., 2007; Lawrence, 1987). In a recent review of play as a potential indicator of welfare, Ahloy-Dallaire *et al*. (2018) conclude that although there is sufficient evidence to support that play is suppressed by negative affect, there is a lack of evidence to support that play is sensitive to increases in positive affect. Since 50-kHz USVs are positively valenced and important during social play (Pellis and Pellis, 2013), I hypothesised that the positive affective state induced by the playback of these calls would be accompanied by an increase in play behaviour.

The aims and hypotheses of this study were;

i) To investigate whether playback of acoustic stimuli in the novel home cage playback paradigm alters affective state in rats as indicated by USV production and a wide ethogram of behaviours. I hypothesised that when rats are exposed to playback of 50-kHz USVs, I predicted that they would produce more 50-kHz USVs and show increased approach behaviour towards the speaker than when exposed to Background Noise or White Noise. In turn, when rats are exposed to 22-kHz USVs, they will produce fewer 50-kHz USVs and reduced approach behaviour than when exposed to Background Noise and 50-kHz USVs. In turn,
ii) To investigate whether the induction of a positive affective state through playback results in a change in play behaviour during playback of acoustic stimuli. I hypothesised that the change in play would be analogous to the valence of each state. Explicitly, the positively valenced state induced by playback of 50-kHz USVs would increase play, and the negatively valenced state induced by playback of 22-kHz USVs would decrease play. These

changes in play would be relative to both control stimuli of Background Noise and White Noise.

To meet these aims, I conducted three experiments using three independent cohorts of rats; Experiments 1 and 2 were conducted at the National Research Institute for Agriculture, Food and Environment (INRAE), Île-de-France-Jouy-en-Josas Research Centre, France, while Experiment 3 was conducted at the Roslin Institute, Edinburgh, UK. The change in the study site in Experiment 3 allowed for a change in speaker location to match previous work (Wöhr and Schwarting, 2012a). Experiment 1 acted as a proof-of-concept test to establish whether rats could perceive the playback stimulus. To confirm this, I measured changes in USV production and behaviour during a single presentation of 50-kHz USVs compared to Background Noise. Following support from the proof-of-concept test, in Experiment 2, I presented rats with playback of contrastingly valenced USVs (50-kHz and 22-kHz) using Background Noise as a control. The use of Background Noise as a control indicated whether changes in behaviour were specific to the properties of the presented USVs rather than the presentation of a general noise stimulus. Experiment 3 was conducted to test the effects of 50-kHz USVs against Background Noise and White Noise as an alternative control stimulus. White Noise contains the same acoustic pressure as rat-produced USVs but presents across all frequencies in the chosen range (30-100kHz), resulting in no distinguishable single-frequency audible signal and a lack of communicative function.

Methods

Ethics

This study was conducted in accordance with the European Union directive of 22nd September 2010 (2010/63/EU) and the UK Animals (Scientific procedures) Act 1986. There was further approval by the local animal experimentation ethics committees (Comité d'Ethique en Expérimentation Animale INRA IdF-Jouy-en-Josas/AgroParisTech (Comethea); permission #16–17), Roslin Institute Animal Welfare and Ethical Review Body (AWERB), the Royal (Dick) School of Veterinary Studies Veterinary Ethical Review Committee (VERC) and SRUC's Animal Ethics Committee.

Animals, housing, and husbandry

Experiments 1 and 2 were conducted at the National Research Institute for Agriculture, Food and Environment (INRAE) at the Île-de-France-Jouy-en-Josas Research Centre in France between 28th March and 5th April 2019. Experiment 3 was conducted at the Roslin Institute, Edinburgh, UK, between the 4th and 12th of August 2020. The change in location of these experiments was partially due to the Covid-19 outbreak and the closure of the French facility. Although not optimal for consistency across experiments, this did offer an advantage of testing the home cage playback paradigm in rats of a different population pool and in a different laboratory environment.

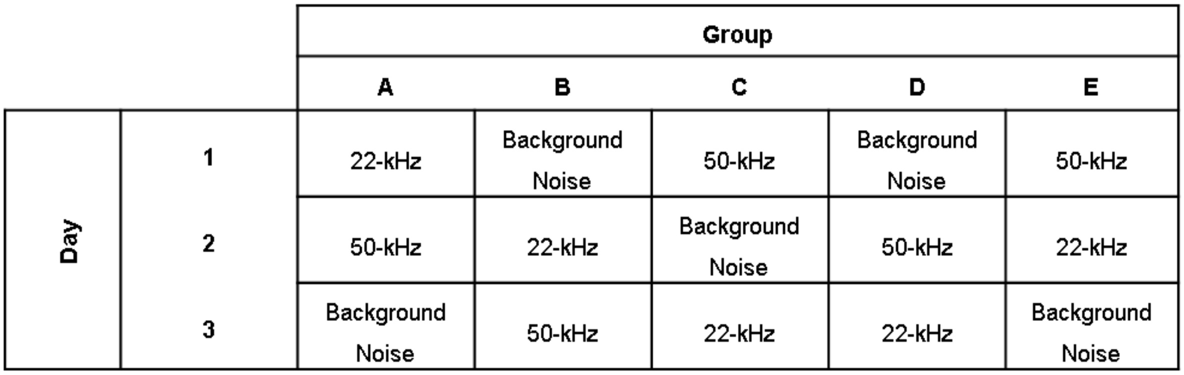
For Experiments 1 and 2, male rats were bred and raised in-house from primiparous females of Wistar strain. For Experiment 3, male rats were imported from females of unknown parity from Charles River Laboratories, Margate, UK. In previous experiments, male rats have been shown to emit more USVs, with Wistars as a commonly used strain for experiments examining USV production (*e.g.,* Wöhr and Schwarting, 2009). Juveniles were used as they show more pronounced changes in behaviour in response to acoustic playback than adult rats (Wöhr and Schwarting, 2007). For Experiment 1 as a proof-of-concept test, I used a cohort of 12 juvenile male Wistar rats from the same breeding pool as Experiment 2, housed in pairs and 39 days old at testing. These animals were then used in an unrelated electrophysiology study to reduce the use of experimental animals in research. For Experiments 2 and 3, I used juvenile males (N = 30 in Experiment 2; N= 28 in Experiment 3 due to an accident resulting in the loss of one pair). Within pairs, rats were randomly selected across litters and were unrelated to reduce any confounding effects of a shared life experience. Rats were also matched by weight within 2g as body condition has been shown to impact play behaviour in rats (Hammond *et al.*, 2019). Data collection began at 37 post-natal days as play shows the highest absolute levels between 32 and 40 days of age depending on strain, the peak of play being on the latter end of this range for Albino rats (Panksepp, 1981; Thor and Holloway, 1984).

Rats were housed in clear plastic cages with a wire lid (France: 42.5 × 26.6 × 18.5cm and UK: 48 x 26.3 x 20.5 cm; both from Techniplast, Italy). Each cage was supplemented with wood chip bedding and *ad libitum* access to food and water. Rats in Experiment I and II were fed M25 Extralabo from Dietex France, and rats in Experiment III were fed Teklad Global Rodent Maintenance Diet (14% protein) from Envigo, England. Cage cleaning was carried out by the same caretaker once a week, ensuring no disturbance occurred at least two hours prior to any experimental procedure. The home room was maintained on an inverted 12 h light/dark cycle (lights on at 2100) and at a constant temperature (23 ± 0.2 °C) and humidity (37.5 ± 6.7%). During the dark interval, red lighting was used to allow behavioural recording (4.5 lx measured using an Isotech digital light meter Lux-1337). For Experiments 1 and 2, cages were situated on two tiers across four standard cage racks, with four cages per rack. For Experiment 3, cages were situated on a single rack across four tiers. Both rats within a cage received marks on the tail using a non-toxic black marker for individual identification.

Experimental design

Experiment 1: Each pair of rats received the single 50-kHz playback stimuli in a randomised order on the same day, with all pairs tested within three hours. This short period of testing was to minimise the effects of circadian rhythm on natural activity patterns. For Experiment 1, no record of body weight was taken.

Experiments 2 and 3: Each pair of rats received all acoustic stimuli treatments in the home cage in a pseudo-randomised Latin square design across three days (**Figures 4.1 and 4.2**). To achieve this, cages were assigned to one of five groups (3 cages per group), with groups balanced according to total cage weight on the day before playback began so that average group weight was within 1.63g of each other. Overall mean weight and standard deviation on the day before playback was 189.8 ± 10.1g for Experiment 2 and 103.2 ± 6.3g for Experiment 3. The order in which each group received each treatment was pseudo-randomised in that all cages would receive each treatment once across three days. Within each day, the order of each cage was randomised, making sure the same treatments did not follow each other to prevent the potential build-up of response to one treatment (*e.g.,* olfactory cues).



		Group				
		A	B	C	D	E
Day	1	22-kHz	Background Noise	50-kHz	Background Noise	50-kHz
	2	50-kHz	22-kHz	Background Noise	50-kHz	22-kHz
	3	Background Noise	50-kHz	22-kHz	22-kHz	Background Noise

Figure 4.1. Figure showing the pseudo-randomised Latin Square design generated for presenting different acoustic stimuli to paired juvenile male Wistar rats (N = 30 rats housed in pairs) in Experiment 2. All rats were presented with all three different acoustic stimuli across three days, with the order of presentation determined by the Latin Square design. Treatments consisted of playback of 50-kHz USVs, 22-kHz USVs, and Background Noise. Presentation occurred as pairs in the home cage.

		Group				
		A	B	C	D	E
Day	1	White Noise	Background Noise	50-kHz	Background Noise	50-kHz
	2	50-kHz	White Noise	Background Noise	50-kHz	White Noise
	3	Background Noise	50-kHz	White Noise	White Noise	Background Noise

Figure 4.2. Figure showing the pseudo-randomised Latin Square design generated for presenting different acoustic stimuli to paired juvenile male Wistar rats (N = 28 rats housed in pairs) in Experiment 3. All rats were presented with all three different acoustic stimuli across three days, with the order of presentation determined by the Latin Square design. Treatments consisted of playback of 50-kHz USVs, White Noise, and Background Noise. Presentation occurred as pairs in the home cage.

Experimental setup

Rats were accustomed to an inverse light cycle, so that experimental testing could occur during the dark period when the rats are naturally active (lights off at 0900 with testing between 1000 and 1500). Testing was conducted in another room or at least three metres away from other rats to prevent the potential for emotional contagion (Hammond *et al.*, 2019). This room was lit with red lighting, which delivered a low-intensity illumination (4.5 lx) to keep the rats within the active dark photoperiod and encourage USV production (Knutson *et al*., 1998).

Experiments 1 and 2: All testing was performed in the home cage. The standard wire rack lid was swapped for another standard lid fitted with an ultrasonic speaker and a high-quality ultrasonic microphone (**Figure 4.3**).

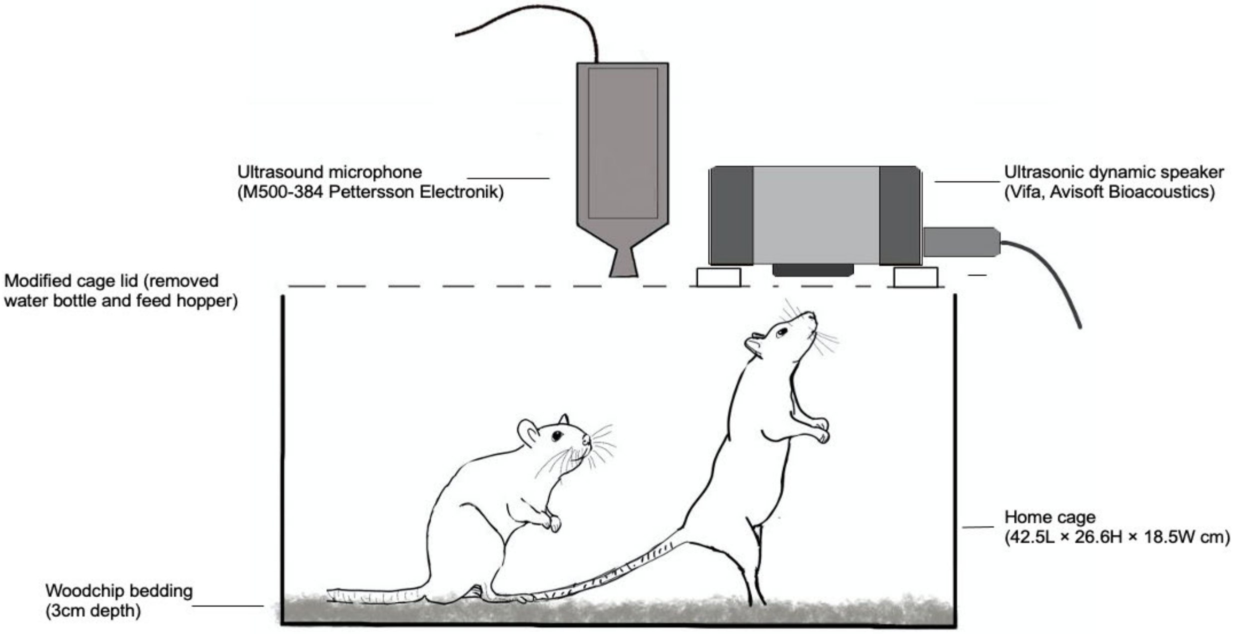


Figure 4.3. Equipment setup used in Experiments 1 (N = 6 cages) and 2 (N = 15 cages) for presenting different acoustic stimuli in the home cage to paired juvenile male Wistar rats in two independent playback experiments. This figure is not to scale.

Experiment 3: The home cage configuration of the Edinburgh facility allowed for refinement of the equipment set up for Experiment 3 (**Figure 4.4**). Wöhr *et al*. (2016) state that to encourage USV production and approach behaviour the speaker should be on the same vertical plane as the rats, mimicking natural circumstances. Removing the food hopper on the side of the cages in the Edinburgh facility created a space for a grill and the speaker to be fitted.

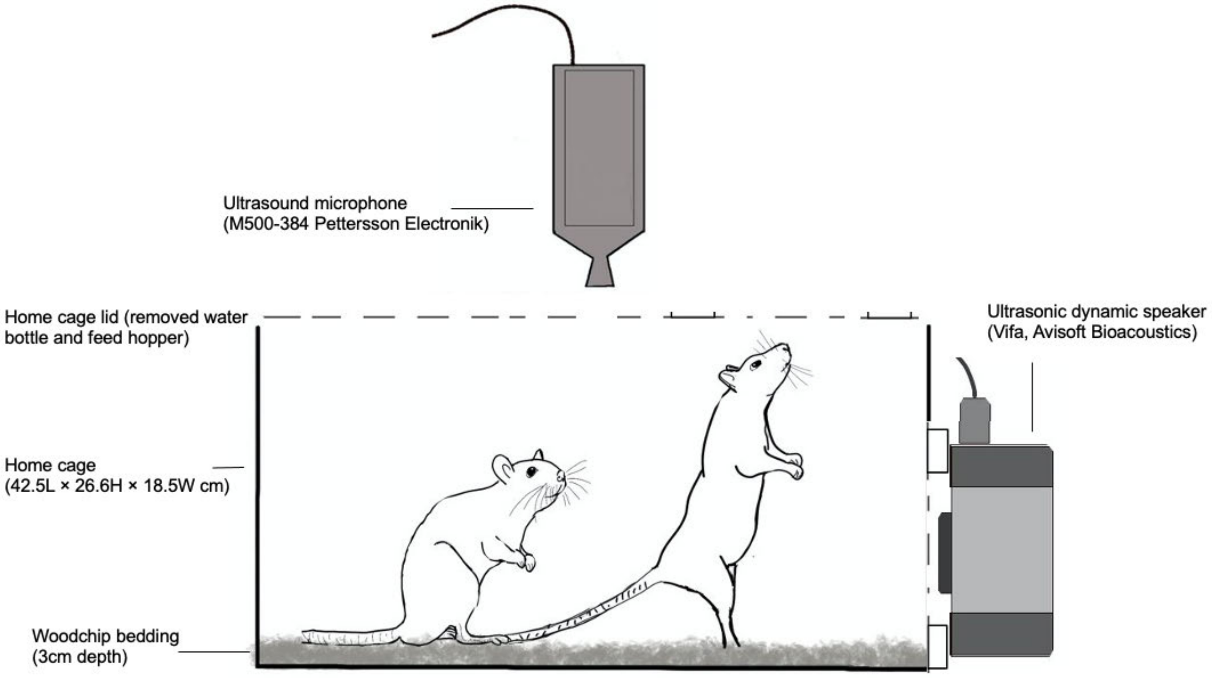


Figure 4.4. Equipment setup used in Experiment 3 (N = 14 cages) for presenting different acoustic stimuli in the home cage to paired juvenile male Wistar rats in one playback experiment. This figure is not to scale.

Acoustic stimuli

The acoustic stimuli presented below are adapted from those used by Wöhr and Schwarting in their radial maze playback paradigm (for a review, see Seffer *et al*., 2014). Acoustic stimuli were presented through the Avisoft-RECORDER USGH software (Avisoft Bioacoustics, Berlin, Germany) using an ultrasonic dynamic speaker (Vifa, Avisoft Bioacoustics) with a frequency range of 1 – 120 kHz (at 12dB) and an ultrasonic power amplifier with a frequency range of 1–180 kHz (UltraSoundGate Player 216H, Avisoft Bioacoustics).

For the generation of playback stimuli, USVs were selected from rats used in other studies. All recordings were produced using the same equipment from rats of the same developmental stage, sex, and strain as the rats used in this Experiment (juvenile male Wistar rats between 37 and 49 days old). Recordings had a sampling rate of 384 kHz in 16-bit format. To generate the presentation of a high number of USVs within the one-minute playback intervals, sequences of USVs were manually selected from recordings and compiled together. Sequences of USVs produced from the same rat were kept together for consistency in recordings, as individuals may sound different from each other and to reduce inter-individual variation in vocalisation properties. As rats rapidly habituate to playback stimuli, no sequence of USVs was repeated (Schwarting and Wöhr, 2012). A high pass filter (15kHz) was applied to each playback track to remove environmental noise. All playback pauses consisted of recordings of baseline noise from the experimental room under experimental conditions.

Experiment 1: Experiment 1 acted as a proof-of-concept test to establish whether rats perceive the playback stimulus. I presented rats with a playback track that consisted of 1) 150 seconds of unrecorded habituation to the equipment set up with no playback of USVs, 2) 30 seconds of Background Noise, 3) 30 seconds of 50-kHz USVs, and 4) 30 seconds of Background Noise. The total 30-second 50-kHz stimuli consisted of 152 USVs, consisting of four different sequences between five and ten seconds long. The 50-kHz USVs were recorded from individual rats during playful handling (for settings and recording parameters, see Chapter 2). The USVs had a mean call duration of 69ms with a mean dominant frequency of 63.2kHz. All playback tracks were produced in Audacity (https://www.audacityteam.org; a free and open-source digital audio editor and recording application software; Audacity, Version 2.1.3, Pennsylvania, United States of America). Playback also contained Background Noise produced by the rats at the same time as recording. Outcome measures were changes in USV production and behaviour during the 30 seconds of 50-kHz USVs compared with Background Noise (**Table 4.1**).

Experiment 2: Three acoustic stimuli were used: 50-kHz USVs, 22-kHz USVs, and Background Noise (**Figure 4.5**). 50-kHz USVs and Background Noise were presented at approximately 60 dB, with 22-kHz USVs presented at approximately

35 dB (measured from a distance of 30cm) due to their lower frequency and, thus, a higher power output during playback. Noise levels were approximated empirically from visual comparison with spectrograms of previously recorded rat-produced USVs at the same microphone distance. The plot spectrum tool in Audacity indicated decibel range. The speaker power settings were confirmed by recording the playback signal and ensuring it had a similar acoustic intensity to rat USVs. As the context of the source USVs used for playback is likely to be important, I presented acoustic stimuli from multiple contexts:

50-kHz: The 50-kHz stimuli presented in the radial maze paradigm (Wöhr *et al*., 2016) were produced from a single adult male rat exploring a cage containing the smell of a recently removed cage mate. While this was sufficient to induce approach behaviour, I thought that this context might not match the more positive affective state that is assumed to accompany play. Therefore, I generated acoustic stimuli from USVs emitted during playful handling in a handling arena (Chapter 2) and spontaneous solitary and social play in the home cage (generated for these experiments). The 50-kHz call playback track consisted of two contexts; the 1st and 3rd playback were recorded from individual rats during playful handling (for settings, recording, and ethical approval, see Chapter 2). The 2nd interval was recorded from pairs of rats during spontaneous social play in the home cage. The total three-minute 50-kHz stimuli consisted of 837 USVs made up of 26 different sequences between two and 24 seconds long. The USVs had a mean call duration of 85ms with a mean dominant frequency of 63.2kHz. Playback also contained Background Noise produced by the rats while recording (Wöhr and Schwarting, 2012).

22-kHz: The 22-kHz call playback track consisted of two contexts; the 1st and 3rd playback were recorded from isolated rats during a 16hr food deprivation (Champeil-Potokar *et al*., 2021). The 2nd interval was recorded from isolated rats during the presentation of a fox odour (for setting, recording, and ethical approval, see Bombail *et al*., 2019 and Champeil-Potokar *et al*., 2021). The total three-minute 22-kHz stimuli consisted of 116 USVs made up of 19 different sequences between three and ten seconds long. The USVs had a mean duration of 644.5ms and a mean dominant frequency of 22.1kHz. As with the 50-kHz acoustic stimuli, playback also contained Background Noise produced by the rats while recording.

Background Noise: Playback tracks could potentially contain Background Noise in the ultrasonic range other than USVs, including those that occur from rat movement or equipment noise. To account for this, Background Noise from a rat that did not produce USVs, conduct play, or approach behaviour during playful handling was presented as a control. This track contained no visible rat-produced USVs and was recorded under the same experimental conditions as this study.

Experiment 3: For Experiment 3, I presented three acoustic stimuli; the same 50-kHz USVs and Background Noise as Experiment 2 and White Noise (**Figure 4.5**). Each track consisted of a habituation interval and three sets of alternate presentations of the acoustic stimuli and pauses for a total of seven minutes. 50-kHz USVs, Background Noise, and White Noise were presented at approximately 60 dB. Noise levels were approximated using the same method as in Experiment 2.

White Noise: White Noise is an acoustic signal of equal intensity across all frequencies, allowing the experimenter to present the same level of an auditory signal without the modulated properties of USVs. The White Noise acoustic stimuli track was generated by matching the time and amplitude of the natural USVs in the 50-kHz track and replacing them with artificial White Noise generated within Audacity (Wöhr and Schwarting, 2012). Duration of White Noise and when it occurred was matched to the original track to the nearest millisecond. Instead of individually matching the amplitude of each 50-kHz USV (Wöhr and Schwarting, 2012), I took an average dB reading from all 837 USVs and matched the White Noise amplitude to this (-77dB). To ensure that the intensity of the White Noise and 50-kHz corresponded, I used Audacity's 'plot spectrum' feature, which exports the data used to create the spectrum of a call and allows comparison of dB levels at set frequencies (for details, see Gailey, 2015). I set a criterion that the intensity of USVs and White Noise at each level must be no more than 5dB different. Optimisation of the track was completed when 40 randomly selected USVs, and associated White Noise (selected via random.org number generator) met this criterion. Previous work has suggested that White Noise can induce behavioural inhibition and avoidance behaviour, which has been suggested to be due to the White Noise occurring in the same frequency range of aversive 22-kHz USVs (Wöhr and Schwarting, 2012). Further I also wanted to ensure that the White Noise presented did not include any low frequency background noise which is a by-product of recording. Thus, I removed all audible sounds, including White Noise, below 30kHz using a high pass filter in Audacity (Gailey, 2015). As with the other acoustic stimuli presented, Background Noise was added between the White Noise 'USVs' (Wöhr and Schwarting, 2012).

Validation measures: I undertook several testing steps to confirm that the acoustic tracks were what was played through the speaker. First, I played the USVs through the ultrasonic speaker and recorded the output using an ultrasound microphone in two situations: 1) in a soundproof box with the speaker and microphone facing each other 20 cm apart and 2) in the home cage with the speaker and microphone placed as used in the study. I then compared the playback tracks and the recordings of the playback tracks when played through the speaker. As well as comparing the presence of USVs, I also optimised the settings of the speaker volume so that the sound intensity produced by the speaker was the same as the original call recordings. To do this, I used the plot spectrum feature of Audacity and conducted a visual analysis of spectrograms and the width of waveforms (Gailey, 2015).

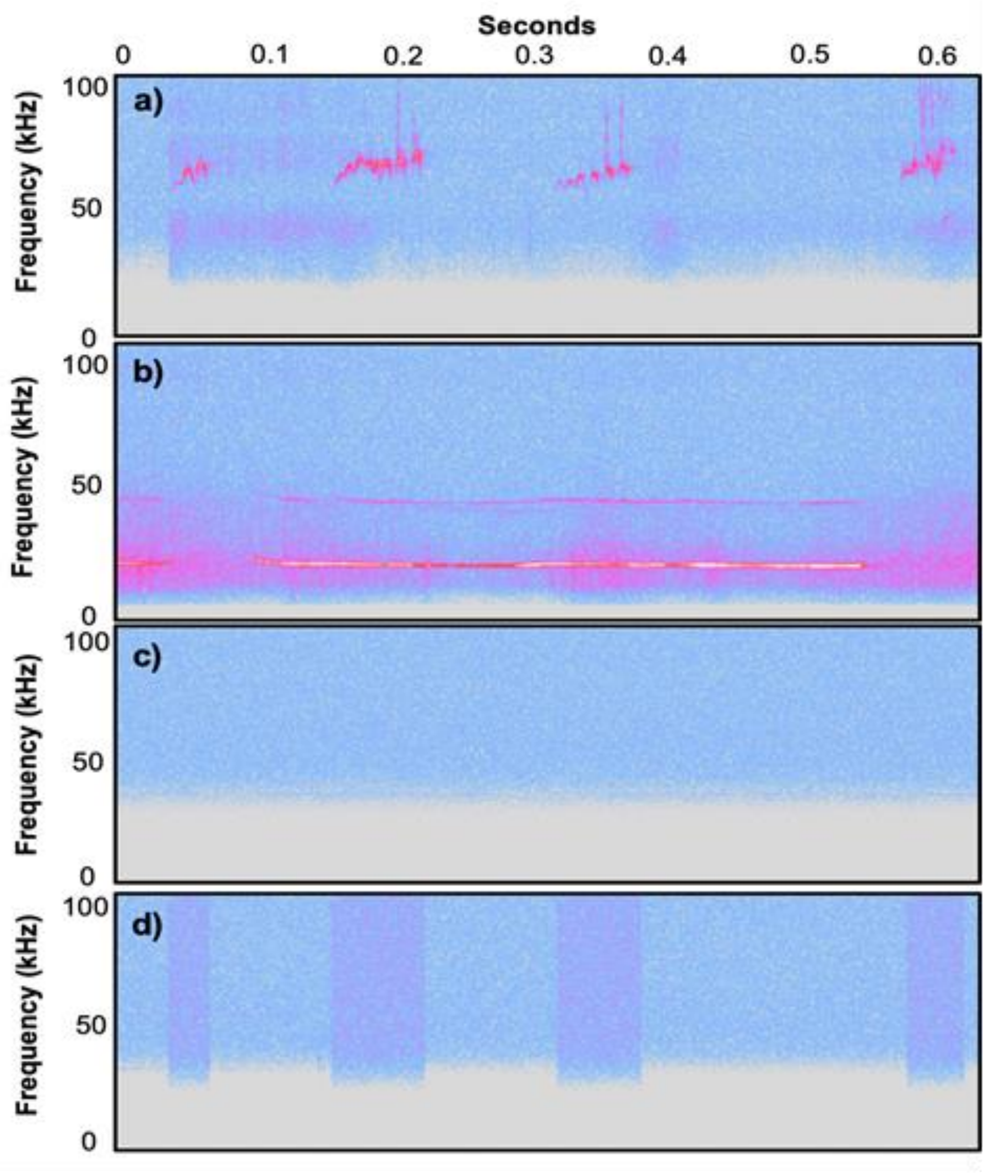


Figure 4.5. Example spectrograms of the four types of acoustic stimuli presented to juvenile male Wistar rats (N = 70; split into the three experiments) in playback trials; a) 50-kHz USVs, b) 22-kHz USVs, c) Background Noise, and d) White Noise. All USVs were recorded from rats used in other studies from the same developmental stage, sex, and strain. The 50-kHz USVs were recorded from individual rats during playful handling (Chapter 2) and from pairs of rats during spontaneous social play in the home cage (generated for these experiments). The 22-kHz USVs were recorded from isolated rats following a 16hr food deprivation or a fox faeces odour presentation (Bombail *et al*., 2019; Champeil-Potokar *et al*., 2021). Background Noise was recorded under the same experimental conditions with no rats present in the room. The White Noise acoustic stimuli track was generated by matching the time and amplitude of the natural USVs in the 50-kHz track and replacing them with artificial White Noise generated within Audacity (Wöhr and Schwarting, 2012). A high-pass filter was applied to the White Noise stimuli to remove all sound below 30kHz.

Experimental procedures

Habituation to equipment: For all experiments, prior to experimental testing, all rats were habituated to the experimental setup to prevent neophobia. Rats were not individually handled during transport or during experimentation bar when weighed and marked at the start of each Experiment. For Experiment 1, habituation consisted of a single session. For Experiments 2 and 3, habituation was conducted over three days during three separate sessions, with increased exposure to the equipment and under environmental conditions. Rats were left in their home cage and moved slowly onto the testing desk. For Experiments 1 and 2, the standard lid with the food hopper was swapped to one fitted with the speaker and microphone used for testing, and the water bottle was removed (**Figure 4.3**). For Experiment 3, the food hopper and water bottle were removed then a grille was fitted in front of the speaker (**Figure 4.4**). During habituation, both setups were left for an increasing time each day up to a total of seven minutes to match the time of each playback trial. Following a session, the wire cage lid was topped with cardboard to create a barrier for USVs and odours, which may have stimulated emotional contagion in the other rats in the homeroom.

Daily procedure during testing: The same procedure was followed during testing. Once the equipment was set up, the video and USV recording was started, and then the playback track was selected. I then left the room during playback testing and returned on completion. This aimed to prevent any human disturbance which could alter rat behaviour.

Recording and analysis of subject-produced USVs during playback

USVs were recorded using a high-quality USB microphone (Pettersson M500-384 USB Ultrasound microphone, Pettersson Electronik; Sweden) connected via a USB interface to a PC laptop. This microphone was placed over the centre of the home cage (height of approx. 51cm), pointing downwards towards the home cage floor.

Recorded sound was digitised at a sampling rate of 384 kHz and a bit depth of 24 bit using Audacity.

USVs were manually counted from spectrograms produced by Audacity and labelled as 50-kHz USVs (peak frequency between 30 and 80 kHz and a duration between 10–150ms) or 22-kHz USVs (bandwidth of >4kHz, peak frequency between 20-29 kHz and duration of 300ms or more) (Brudzynski, 2009; LaFollette *et al.*, 2018; Wright *et al.,* 2010*)*. Overlapping USVs were counted individually, as there were two rats present during the recording. Vocalisations were counted according to habituation, playback, and pause intervals of the acoustic playback track. USV rate was expressed as the number of calls emitted per minute (calls/min).

After counting the number of USVs produced by rats, I compared the recorded subject-produced USV track directly in time with the relevant playback track for all experiments. This was to prevent the double-counting of USVs, which may have been recorded from the inputted playback track rather than produced by the rats themselves. If a vocalisation was deemed as a duplicate of the playback track, it was not counted. During the initial collection of data from spectrograms, the coder (TH) was blinded to treatment to prevent bias.

Recording and analysis of behaviour during playback

During playback, behaviour in the home cage was recorded using a Sony HD camcorder (HDR- PJ810E) and subsequently analysed using Observer XT 14 software (Noldus Information Technology, Wageningen, the Netherlands). Frequency and duration were coded according to the following ethograms for Experiment 1 (**Table 4.1**) and Experiments 2 and 3 (**Table 4.2**). During the collection of data, the coder (TH) was blinded to treatment to prevent bias.

Table 4.1. Ethogram used in Experiment 1 developed for recording behaviour of pair-housed male Wistar rats (N = 12) in the home cage during playback of 50-kHz USVs.

Behaviour	Description
Approach towards the speaker	Rat orients its body and head so that the nose is directed towards the speaker and within 1cm of the speaker. (Lever *et al*., 2006)
Rearing (undirected)	Rat raises both front paws off the ground (can be in contact with the wall or not), standing up on hind legs. Includes all rears, bar those with the rat's nose directed towards the speaker. (Lever *et al*., 2006)
Locomotor activity	Rat crosses the centre line of the cage with its' whole body. The centre line was defined by a marker on the side length of home cage.
Inactive	Any behaviour where the rat's body is still and unmoving, such as freezing, resting, sitting, or lying still. The rat can make facial movements with eyes open or closed. (LaFollette *et al*., 2018)

Table 4.2. Ethogram used in Experiments 2 (N = 15 cages) and 3 (N = 14 cages) developed for recording behaviour of pair-housed male Wistar rats in the home cage during playback of four different acoustic stimuli (50-kHz, 22-kHz, White Noise and Background Noise).

Behaviour	**Description**
Solitary play	Rat conducts fast locomotor movement involving at least one hop by an individual, where hops involve all four paws leaving the ground at the same time, not in the direction of a play partner or during a play bout. The behaviour starts with fast running or a hop from stationary or during locomotor movement and ceases when this movement stops. (Hammond et al., 2019)
Social play	One rat jumps or lunges towards the partner's nape, resulting in the partner either chasing the soliciting rat, rearing (in which pairs make rapid pawing movements at each other), or rotating so that one rat is on its back with the other standing over it in a pin. The frequency of pinning and dorsal contacts within social play was also coded. Multiple pins and dorsal contacts can occur during a single social play bout. The behaviour starts with fast running, a jump or lunge towards a play partner and ceases when there is no chasing, rearing, pins or dorsal contacts between the play partners. (Kerkhof *et al*., 2013; Webber *et al*., 2012)
Approach towards the speaker	Rat orients its body and head so that the nose is directed towards the speaker and within 1cm of the speaker. (Lever *et al*., 2006)
Rearing (undirected)	Rat raises both front paws off the ground (can be in contact with the wall or not) standing up on hind legs. Includes all rears, bar those with the rat's nose directed towards the speaker. (Lever *et al*., 2006)

Digging	Clear movement of bedding with the front or hind paws or face. The rats front paws and/or face are not visible because it is beneath the bedding. (Makowska and Weary, 2016)
Self-grooming	All self-directed grooming behaviour including licking the fur, grooming with forepaws and scratching with any limb. (Gaskill and Pritchett-Coming, 2015)
Locomotor activity	Rat crosses the centre line of the cage with its whole body. The centre line was defined by a marker on the side length of home cage.
Inactive	Any behaviour where the rat's body is still and unmoving, such as freezing, resting, sitting or lying still. The rat can make facial movements with eyes open or closed.
Freezing	The rat's body is motionless in a crouching position. Rat makes vibrissae movements of the whiskers, gentle bobbing head movements and has bulging eyes. (Brudzynski, 2019)
Resting	The rat's body is still and unmoving, either sitting or lying. The rat can make facial movements with eyes open or closed. (LaFollette *et al*., 2018)
Other non-social/ social	Includes urinating, defecating, chewing of any object and any other unclassified behaviour not directed towards the cage mate/includes any behaviour towards the cage mate such as social grooming.

Statistical analysis

Tests of intra-observer reliability were conducted prior to statistical analysis. As only one observer scored all behaviours, inter-observer reliability tests were unnecessary. I conducted a repeat scoring of a minimum of 12.5% of the data seven days later. For USV data, I conducted Spearman's rank correlations between the first count and second count for each spectrogram (Martin and Bateson, 1993). To meet the reliability criteria, correlations were above the acceptable cut-off defined by Martin and Bateson of 0.7. For behaviour, these correlations were conducted using Observer XT 11 software and met a minimum Cohen's kappa of 0.8 with a percentage agreement of 64% (McHugh, 2012).

All data were analysed in Genstat 19.1 using General Linear Models (GLM). Figures were generated using GraphPad Prism (ver 9.2). Assumptions of the GLMs were tested using Bartlett's test for homogeneity of variances and the Anderson-Darling test for normality. Tukey's honest significance difference (HSD) procedure was conducted for multiple comparisons on significant differences ($a = 0.05$). Means (M) and standard errors of the mean (SEM) reported were back-transformed to the original scale for biological significance. For all tests, the level of statistical significance was set at $p < 0.05$.

Across all three experiments, USV and other behavioural data were collected during each session. USV counts were converted into a rate per minute to compare USV production between different experiments. In all experiments, the production of 22-kHz USVs was too infrequent to fit to a GLM. The frequency and duration (seconds) were calculated for most behaviours bar two exceptions; general locomotor activity was measured by the frequency of crossing over from one side to the other, and solitary play was only measured using the frequency due to the short duration of these events. This was to reduce the likelihood of false positives, as coding would depend on the coder's reaction time. Of the eleven behaviours measured in Experiments 2 and 3 (**Tables 4.1**

and 4.2), four (resting, freezing, other social and non-social behaviours) were not fitted to a General Linear Model as they were deemed too rare for statistical analysis (only eight total occurrences displayed by 3% of rats). As rats were tested together in the home cage and were likely to influence each other's behaviour, the frequency and duration of each rat's USV production and behaviour were summed to give a cage level of expression. To account for this, cage was included as a blocking factor in all models.

For Experiment 1, to investigate the effect of a single presentation of acoustic stimuli on USV production and behaviour, interval type (playback of acoustic stimuli or pause intervals) was modelled as the main effect, with cage as the blocking factor. For Experiments 2 and 3, to investigate the effect of acoustic stimuli on rat USV production and behaviour, acoustic treatment (50-kHz, 22-kHz, Background Noise or White Noise) and interval type (playback of acoustic stimuli or pause intervals) were modelled as the main effects with an additional interaction between the two main effects. The blocking factor was (Group/Cage_number)*Day to reflect that each cage was nested within a group, with this identification remaining unchanged across all test days. To investigate the effect of acoustic stimuli on rat location within the cage, treatment, interval type, and location (left or right) were modelled as the main effects with additional interactions between the three main effects. The blocking factor was also (Group/Cage_number)*Day. For all models, the appropriate frequency or duration of the behaviour of interest including USVs during the one minute of habituation for each day was fitted as a covariate. This was to account for any baseline differences in cages that were not dependent on treatment. Effects of treatment order were accounted for within the Latin square design. To investigate individual variation in measured responses to playback of acoustic stimuli, I present the coefficient of variation as a statistical measure of the difference in variation between the three treatments for USV production, approach behaviour, and play. Due to camera failure, one video recording of a pair of rats during one playback session was missing in Experiment 2. As this represented only 2.2% of all behaviour data within this Experiment (two rats out of 90 measurements), no further action was made to correct this.

Results

Experiment 1: Proof-of-concept test to establish whether rats hear the playback stimulus

Rats increased USV production in response to playback of 50-kHz acoustic stimuli

There was a significant main effect of interval type on subject-produced USVs, with rats producing approximately four-fold the number of 50-kHz USVs during the 30 seconds of 50-kHz acoustic stimuli compared with the Background Noise before and after playback ($F_{2,17} = 74.81$, $p < 0.001$; **Figure 4.6**). Tukey's HSD revealed that the number of USVs produced in the pauses before and after playback were not significantly different from each other (T = 1.97, $p = 0.32$).

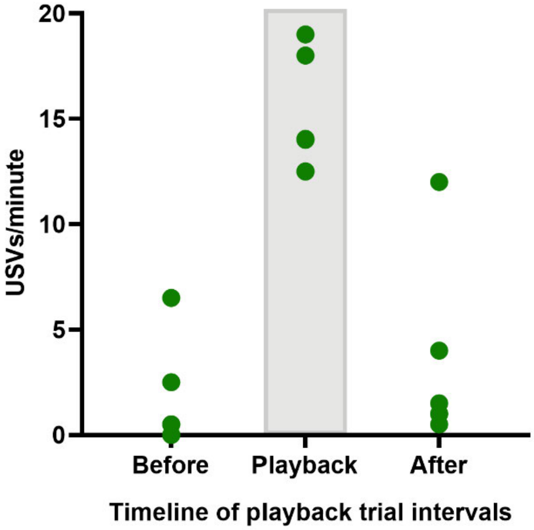


Figure 4.6. Graph showing the total number of 50-kHz ultrasonic vocalisations (USVs) per minute produced by pairs of juvenile male rats. Each green circle represents one cage (N = 12 rats housed in pairs; six cages) during presentation and pauses of 50-kHz USV recordings in Experiment 1. All rats were presented with the acoustic stimuli once as pairs in the home cage on the same day. Each interval (before, during, and after playback) was 30 seconds long, and intervals are shown linearly from left to right along the x-axis, with data scaled to the number of USVs per minute.

Other behaviours were not influenced by a 30-second playback of 50-kHz acoustic stimuli

Results of the GLM run on the frequency and duration of behaviours during Experiment 1 are shown in **Table 4.3**. When exposed to the playback track, there was no significant main effect of interval type on the frequency or duration of approach behaviour towards the speaker or any other behaviour during the 30-second period of acoustic stimuli compared with the before and after Background Noise intervals (**Table 4.3**).

Table 4.3. Table presenting results of an ANOVA ran on the frequency and duration of behaviours expressed by juvenile male rats (N = 12 rats housed in pairs; six cages) during presentation and pauses of 50-kHz ultrasonic vocalisation recordings in Experiment 1. All rats were presented with the acoustic stimuli once as pairs in the home cage on the same day.

	Variable	**Interval type (playback or pause)**	
Behaviour		$F_{2,35}$	*p*-value
Approach towards speaker	frequency, duration	0.11, 0.19	0.92, 0.78
Undirected rearing	frequency, duration	0.29, 0.52	0.77, 0.56
Inactive	frequency, duration	0.14, 0.12	0.10, 0.93

Experiment 2: Playback of 50-kHz, 22-kHz, and Background Noise

Subject-produced 50-kHz USVs increased in response to the playback of 50-kHz acoustic stimuli

There was a significant main effect of acoustic stimuli treatment on subject-produced USVs, with rats producing more USVs in response to 50-kHz acoustic stimuli compared to 22-kHz USVs and Background Noise ($F_{2,251}$ = 5.81, p = 0.05, **Figure 4.7**). Tukey's

HSD test indicated that the mean number of USVs produced during the 50-kHz stimuli presentation was significantly different from both the 22-kHz stimuli (T = 11.31, $p < 0.001$) and Background Noise (T = 10.29, $p < 0.001$), which were not different from each other (T = 11.57, $p = 0.23$). There was also a significant main effect of interval type, with rats producing more USVs during the playback intervals of the acoustic stimuli than the pauses (9.6 vs 7.0 ± 1.0 USVs per minute, $F_{1, 251} = 3.87$, $p = 0.05$). The coefficient of variation for USVs was similar between the three acoustic stimuli; for rats exposed to 50-kHz stimuli, the coefficient of variation was 86.7%, 22-kHz was 87.1%, and Background Noise was 87.7%.

Rats conducted more locomotor activity and digging during the playback intervals of the trials

Results of the GLM run on the frequency and duration of behaviours during Experiment 2 are shown in **Table 4.4**. There was no significant main effect of treatment on approach behaviour, locomotor activity, undirected rearing, self-grooming, digging, or solitary play. The coefficient of variation for the duration of approach behaviour was highest in response to 50-kHz stimuli and White Noise at 64.1% and 62.9%, respectively. The coefficient of variation in response to Background Noise was 47.2%.

Play occurred infrequently, with solitary play only being expressed for a total of 72.8 seconds and social play for a total of 205 seconds across all trials. There was a significant main effect of interval type on digging and locomotor activity, with increases in the expression of these behaviours during the playback intervals compared with pause intervals (digging frequency: 1.8 vs 1.1 ± 0.1 events per minute and duration: 7.6 vs 4.8 ± 0.8 seconds per minute, locomotor activity: 4.0 vs 3.3 ± 0.1 events per minute).

The coefficient of variation of solitary play frequency between rats were similar; 50-kHz stimuli at 57.7%, 22-kHz stimuli at 57.1% and Background Noise at 56.7%. There was also no difference in the coefficient of variation of social play duration between rats exposed to 50-kHz stimuli at 51.8%, 22-kHz stimuli at 51.3% and Background Noise at 54.2%.

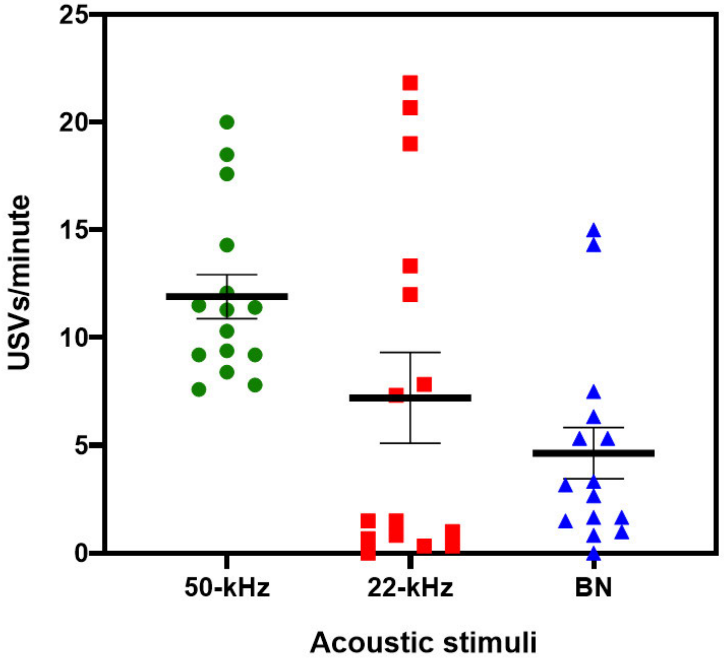


Figure 4.7. Graph showing the mean number of 50-kHz ultrasonic vocalisations (USVs) per minute produced by pairs of juvenile male rats (N = 30 rats housed in pairs; 15 cages) during Experiment 2. All rats were presented with all three treatments (50-kHz acoustic stimuli (green circles), 22kHz acoustic stimuli (red squares), or Background Noise (BN) (blue triangles)) in a Latin square design as pairs in the home cage. Values reflect means for each cage with the black bar indicating the mean ± SEM for each treatment by day.

Table 4.4. Table presenting results of an ANOVA ran on the frequency and duration of behaviours expressed by juvenile male rats (N = 30 rats housed in pairs; 15 cages) during the presentation of three different acoustic stimuli (50-kHz, 22-kHz, or Background Noise). All rats were presented with all three treatments in a Latin square design as pairs in the home cage. Significant P values are indicated in bold script.

	Variable	**Treatment**		**Interval type (playback or pause)**	
Behaviour		$F_{2, 251}$	*p*-value	$F_{1,251}$	*p*-value
Approach towards speaker	frequency, duration	1.82, 1.08	0.26, 0.41	0.16, 0.96	0.69, 0.33
Locomotor activity	frequency	1.38	0.33	13.28	**<0.001**
Undirected rearing	frequency, duration	0.67, 1.43	0.55, 0.43	11.70, 0.08	0.59, 0.77
Self-grooming	frequency, duration	0.11, 0.14	0.90, 0.87	0.19, 0.01	0.66, 0.94
Digging	frequency, duration	0.95, 1.15	0.45, 0.39	9.28, 6.66	**0.003, 0.01**
Inactive	frequency, duration	2.22, 1.83	0.20, 0.25	1.40, 1.96	0.24, 0.16
Solitary play	frequency	1.32	0.35	0.03	0.87
Social play	frequency, duration	0.89, 0.46	0.46, 0.47	0.48, 0.01	0.49, 0.98

Experiment 3: Playback of 50-kHz, White Noise, and Background Noise.

Subject-produced 50-kHz USVs increased in response to playback of 50-kHz acoustic stimuli and White Noise

There was a significant main effect of acoustic stimuli treatment on subject-produced USVs, with rats producing more USVs in response to 50-kHz and White Noise compared to Background Noise ($F_{2,239} = 6.05$, $p = 0.05$; **Figure 4.8**). Tukey's HSD test indicated that the mean number of USVs produced during the 50-kHz stimuli (T = 11.45, $p = 0.02$) and White Noise (T = 13.47, $p = 0.02$) differed significantly from Background Noise but not each other (T = 3.79, $p = 0.23$). There was no main effect of interval type on USV production ($F_{1,239} = 3.63$, $p = 0.06$). The coefficient of variation for USVs was highest in response to White Noise at 89.9%, Background Noise at 63.2%, and then 50-kHz stimuli at 52.9%, respectively.

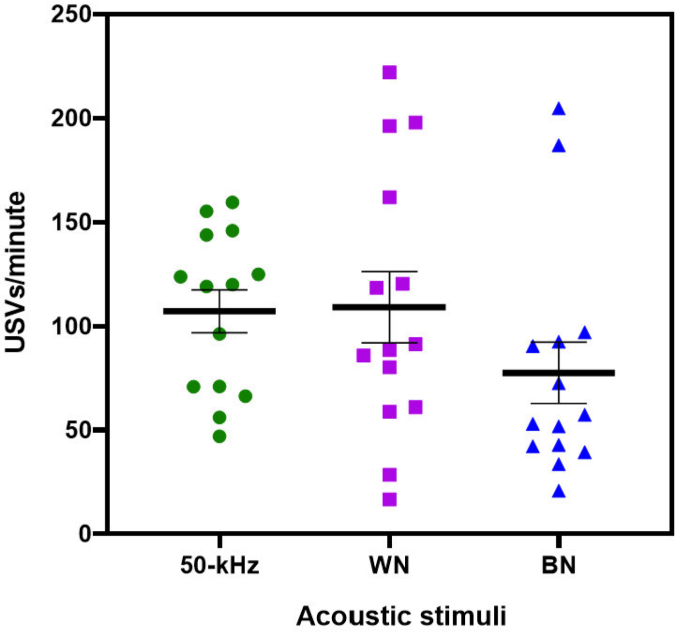


Figure 4.8. Graph showing the mean number of 50-kHz ultrasonic vocalisations (USVs) per minute produced by pairs of juvenile male rats (N = 28 rats housed in pairs; 14 cages) during Experiment 3. All rats were presented with all three treatments (50-kHz acoustic stimuli (green circles), White Noise (WN) (purple squares), or Background Noise (BN) (blue triangles)) in a Latin square design as pairs in the home cage. Values reflect means for each cage with the black bar indicating the mean ± SEM for each treatment by day.

There was a tendency towards an effect of treatment on approach behaviour

Results of the GLM run on the frequency and duration of behaviours during Experiment 3 are shown in **Table 4.5**. There was no significant main effect of treatment on locomotor activity, undirected rearing, self-grooming, digging, and solitary play.

There was a tendency towards a main effect of treatment on the duration of approach behaviour towards speaker, with rats exposed to 50-kHz and White Noise conducting the longest duration of approach behaviour, followed by rats exposed to Background Noise (26.4 vs 25.7 vs 21.4 ± 1.7 seconds per minute). Tukey's HSD test indicated that the duration of approach behaviour produced during the 50-kHz stimuli (T = 10.91, $p = 0.04$) and White Noise (T = 9.89, $p = 0.05$) differed significantly from Background Noise but not each other (T = 2.65, $p = 0.98$). The coefficient of variation for the duration of approach behaviour was highest in response to 50-kHz stimuli and White Noise at 64.1% and 62.9%, respectively. The coefficient of variation in response to Background Noise was 47.2%.

There was a significant main effect of interval type on solitary play, with rats conducting more solitary play during the pause than playback intervals (1.4 vs 0.9 ± 0.1 events per minute). There was no difference in the coefficient of variation of solitary play frequency between rats exposed to 50-kHz stimuli at 77.6%, White Noise at 76.1%, and Background Noise at 69.7%.

Table 4.5. Table presenting results of an ANOVA ran on the frequency and duration of behaviours expressed by juvenile male rats (N = 28 rats housed in pairs; 14 cages) during the presentation of three different acoustic stimuli (50-kHz, White Noise, and Background Noise). All rats were presented with all three treatments in a Latin square design as pairs in the home cage. Significant P values are indicated in bold script.

	Variable	**Treatment**		**Interval type (pause or playback)**	
Behaviour		$F_{2, 239}$	*p*-value	$F_{5,239}$	*p*-value
Approach towards speaker	frequency, duration	0.20, 2.51	0.82, **0.08**	0.18, 0.38	0.67, 0.54
Locomotor activity	frequency	1.87	0.16	1.29	0.26
Undirected rearing	frequency, duration	0.17, 6.68	0.84, 0.12	0.58, 0.80	0.45, 0.37
Self-grooming	frequency, duration	0.17, 6.67	0.84, 0.30	0.58, 0.80	0.45, 0.37
Digging	frequency, duration	1.39, 2.51	0.25, 0.08	0.57, 0.26	0.45, 0.61
Solitary play	frequency	0.76	0.47	7.75	**0.006**

Rats exposed to White Noise conducted more social play than rats exposed to 50-kHz or Background Noise

In contrast with the low levels of play in the cohort of rats in Experiment 2, rats in Experiment 3 spent 4440 seconds conducting social play. There was a main effect of treatment on the frequency and duration of social play, with rats conducting more social play in response to White Noise, followed by Background Noise and then 50-kHz (frequency: $F_{2,239} = 2.98$, $p = 0.05$ and duration: $F_{2,239} = 3.55$, $p = 0.03$; **Figure 4.9**). Tukey's HSD test indicated that the frequency of social play during each treatment were significantly different from each other (50-kHz vs WN: T = 11.10, $p = 0.03$, 50-kHz vs BN: T = 12.07, $p = 0.03$, WN vs BN: T = 9.88, $p = 0.05$). Similarly, Tukey's HSD test indicated that the duration of social play during each treatment were significantly different from each other (50-kHz vs WN: T = 12.63, $p = 0.02$, 50-kHz vs BN: T = 11.71, $p = 0.03$, WN vs BN: T = 8.06, $p = 0.05$). There was no significant effect of interval type on social play (frequency: $F_{1,239} = 0.40$, $p = 0.53$ and duration: $F_{1,239} = 0.06$, $p = 0.80$).

The coefficient of variation of social play duration between rats were similar between different acoustic stimuli; 50-kHz stimuli at 60.4%, White Noise stimuli at 60.9%, and Background Noise at 60.5%.

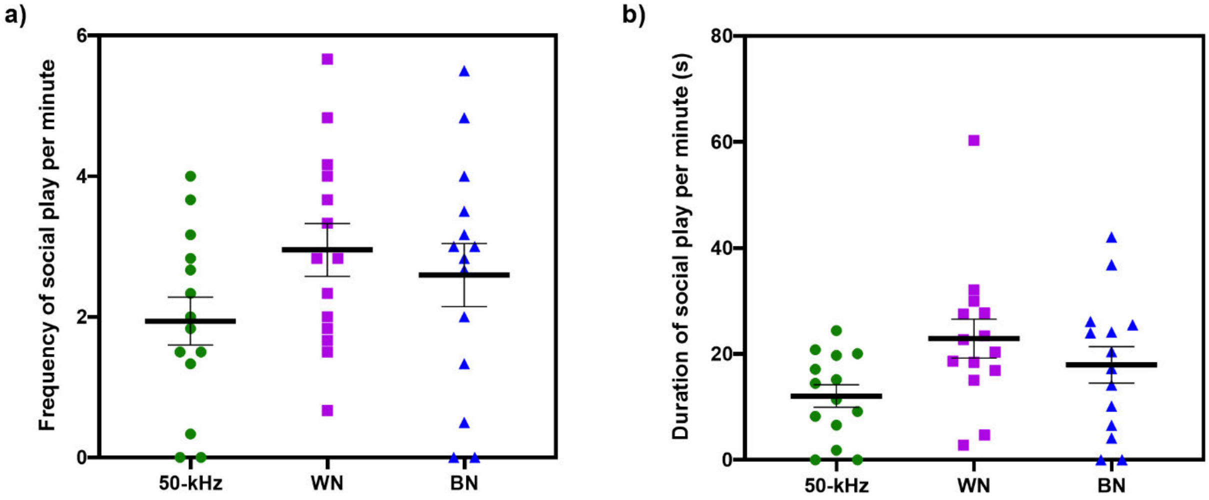


Figure 4.9. Panels representing the a) frequency and b) duration of social play events (s) of juvenile male rats (N = 28 rats housed in pairs; 14 cages) during Experiment 3. All rats were presented with all three treatments (50-kHz acoustic stimuli (green circles), White Noise (WN) (purple squares), or Background Noise (BN) (blue triangles)) in a Latin square design as pairs in the home cage. Values reflect means for each cage with the black bar indicating the mean ± SEM for each treatment by day.

Discussion

Previous work has shown that playback of contrastingly valenced USVs is a promising approach to study the effects of playback on rat behaviour and underlying affective states (Schwarting *et al*., 2018; Wöhr *et al*., 2016; Wohr *et al*., 2008). However, the potential of using playback of positively valenced USVs to induce a positive affective state and promote PAW has yet to be widely explored. The home cage playback paradigm developed in this chapter was designed to be conducive to measuring more of the full behavioural repertoire of rats for the purpose of welfare assessment. Explicitly, this paradigm was designed to meet four criteria (in no specific order of importance): the provision of sufficient space, a conspecific with which to enact social behaviours, the removal of potential stressors (*i.e.,* bright lighting, handling, and novelty) and the ability to measure USV production as an indicator of affective state. Here, I found support for the use of the home cage playback paradigm to manipulate affective state; in response to 50-kHz acoustic stimuli and, unexpectedly, White Noise, rats produced more 50-kHz USVs than when exposed to 22-kHz acoustic stimuli or Background Noise. The successful induction of a positive affective state when rats were exposed to 50-kHz acoustic stimuli and White Noise allowed the investigation of the impact of a positive experience on play behaviour. While the positive affective state induced by White Noise was accompanied by a corresponding increase in social play during playback, this was not seen when rats were exposed to 50-kHz acoustic stimuli. These results illustrate the potential of playback to lead to positive affective states in rats but also demonstrate that play may not always be a sound indicator of PAW.

Do rats perceive the acoustic stimuli? Confirmation through USV production

Experiment 1 was a proof-of-concept test, using a single 30-second presentation of 50-kHz USVs to cages of paired rats in between two intervals of Background Noise. A rapid increase in subject-produced USVs during this 30-second playback interval confirmed that rats could perceive the acoustic stimulus presented by the speaker. The production of 50-kHz USVs and the absence of 22-kHz USVs also provided evidence that the rats did not find the equipment set up or protocol aversive, even with minimal habituation. However, the effect of playback was restricted to USV production, as rats showed no change in other behavioural expressions. This could be because this experiment used a short exposure to the 50-kHz stimuli, which did not allow sufficient time for rats to process or locate the source. For example, in the radial maze paradigm, rats are exposed to a minimum of 60 seconds in total, with rats consistently exhibiting increased approach behaviour and social exploration compared to background noise in response to 50-kHz USVs (Wöhr *et al*., 2016; Schwarting *et al.,* 2018). Another possibility relates to how rats perceive the playback of USVs when isolated from other social signals, as discussed later.

The playback of 50-kHz and White Noise stimuli induced a positive affective state in rats

50-kHz USVs are associated with appetitive or hedonic situations, such as feeding, mating, and play, and are thought to indicate a positive affective experience (*e.g.,* Knutson *et al*., 1998; Schwarting *et al*., 2007). Thus, in response to the playback of 50-kHz USVs, I expected rats to respond with more 50-kHz USVs themselves as an expression of an enhanced positive affective state. In contrast, 22-kHz USVs are associated with aversive situations, such as in response to a predator or foot shock, and are thought to indicate a negative affective experience (Schwarting and Wöhr, 2012). Thus, in response to exposure to an apparent alarm via playback of 22-kHz USV, I expected a dampening of subject-produced 50-kHz USVs and reduced locomotion or an increase in freezing.

In Experiment 2, when rats were exposed to 50-kHz USVs, I found that they produced twice the overall number of USVs than when exposed to 22-kHz USVs or Background Noise, which did not differ from each other. In Experiment 3, rats produced significantly more USVs in response to the playback of 50 kHz USVs relative to Background Noise. Considering the evidence that the production of 50-kHz USVs can be used as a quantitative index of positive affect (Burgdorf *et al*., 2005; Hinchcliffe *et al*., 2020; Knutson *et al*., 1999), this increase in 50-kHz USVs suggests that playback of 50kHz acoustic stimuli induced a positive affective state in both experiments. This convergence of affective state between the acoustic stimuli and subject animals suggests that emotional contagion has occurred (Hatfield *et al*., 1993; Waal, 2007). Unexpectedly in Experiment 3, rats also produced a similar number of USVs during White Noise to those produced during exposure to 50-kHz. This increase in 50-kHz USV production compared to Background Noise suggests that playback of White Noise induced a positive affective state in rats, to a similar level of that in response to the playback of 50-kHz USVs. As White Noise has no communicative meaning or associated affective state, it would seem logical to propose that the positive affective state induced by this stimulus occurred without emotional contagion.

Previous use of White Noise in playback experiments has resulted in a profound and long-lasting reduction in locomotion and avoidance of the sound source (Endres *et al*., 2007; Wöhr and Schwarting, 2012a). In these experiments, White Noise was presented from 0 to 27-kHz (Endres *et al*., 2007) or from 0 to 100-kHz (Wöhr and Schwarting, 2012a). Wöhr and Schwarting (2012) suggested that as this sound energy also occurs within the same range as negatively valenced 22-kHz USVs, it may be perceived as aversive. Also, I have observed that matching the short duration of 50-kHz USVs with White Noise across this frequency range produces audible bursts of 'hissing'. Thus, although White Noise contains all frequencies and has no communicative value, presenting it across this range may trigger aversion. As such, the White Noise used in this study was filtered to remove all sound below 30-kHz, including White Noise and human-audible Background Noise. The frequency range at which White Noise was presented is the only difference between previous work and this study, as the duration of each White

Noise burst was matched to the duration of each USV within the 50-kHz playback track (Wöhr and Schwarting, 2012a).

The positive affective state induced by this filtered White Noise method could be a result of arousal. Compared with the Background Noise of the animal facility, the pattern and frequency range in the White Noise stimuli may have been physiologically arousing and potentially enriching to some animals. Although speculative, previous work supports the idea of the enriching properties of acoustic stimuli in rodents. For example, playback of rainforest sounds to mice resulted in increased movement and a prolonged lifespan by almost 17% (Yamashita *et al*., 2018), while playback 'pure tone pips' promoted recovery from damage to cortical systems associated with auditory function in rats (Zhu *et al*., 2014). Wöhr and Schwarting (2007) have also shown that frequency modulation (a defining factor of some 50-kHz USVs) is not required for acoustic stimuli to have appetitive value in the short-term. For example, rats will still conduct approach behaviour in response to artificially generated 50-kHz sine wave tones, which lack the amplitude and frequency modulation characteristics of natural rat-produced USVs (Wöhr and Schwarting, 2007). Overall, the effect of filtered White Noise on affective state raises essential questions about rat perception of auditory stimuli used in playback experiments.

The few studies that measure the effect of acoustic stimuli on subject-produced USVs report an overall lack of effect of *all* acoustic stimuli on USV production (Sadananda *et al*., 2008), which may be an artefact of the low overall calling rate of subjects (Sadananda *et al*., 2008; Wöhr and Schwarting, 2007, 2009). These experiments were conducted in the radial maze as lone rats in a novel environment, and thus, could potentially be stressful. In response to this, Olszyński *et al*. (2020) investigated whether conducting playback under comparatively low-stress experimental conditions could induce USV emission in single and paired rats. The authors made several fundamental changes to reduce experimental stress: using cages similar to the home cage for playback, shorter exposure to playback stimuli, and an increased number of handling and habituation sessions (Olszyński *et al*., 2020). Olszyński *et al*. (2020) attribute these changes to the overall increase in USV emission in their study compared to previous work. They also found that rats presented as individuals or pairs increased production of USVs in response to playback of 50-kHz stimuli compared with 22-kHz stimuli across both single and paired rats. Along the same lines, I took several steps to induce a low-stress environment: the use of the home cage itself facilitating no need for handling, low-intensity red lighting, prolonged habituation to the playback equipment, and exposure in pairs with no isolation. Thus, using a low-stress environment might be essential to elicit USV production in response to USV playback and allow their measurement as an indicator of affective state.

The change in positive affective state was expressed by changes in approach behaviour towards the source of the sound when placed at the side of the cage

The efficacy of the radial maze playback paradigm is primarily based on the contrasting effects of 50-kHz and 22-kHz acoustic stimuli on approach behaviour toward the sound source (Schwarting *et al*., 2018; Wöhr *et al*., 2016). While both juvenile and adult rats show increased approach behaviour towards the speaker in response to 50-kHz USVS, 22kHz USVs induce freezing (Schwarting *et al*., 2018; Wöhr and Schwarting, 2007). In this study, there was no effect of acoustic stimuli on approach behaviour in Experiment 2. However, in Experiment 3, there was a tendency towards an increase in approach behaviour in response to 50-kHz and White Noise acoustic stimuli.

The lack of main effect in Experiment 2 may be explained by the difference between the home cage paradigm and the radial maze paradigm in the measurement of approach behaviour. In the radial maze paradigm, approach behaviour is measured by comparing the time spent on arms closest to the speaker versus distal arms as a measure of social approach (Schwarting *et al*., 2018). In the home cage playback paradigm, approach behaviour is measured by the frequency and duration of time rats spent with their nose within 1cm of the speaker. As such, the measure used in this study is more specific than that used in the radial maze paradigm and thus requires a higher level of behaviour change to result in a statistically significant difference. However, taken with the change in subject-produced USVs, the tendency towards an increase in approach behaviour in response to 50-kHz and White Noise suggests that these stimuli were perceived as positive (Paul *et al*., 2005b).

The change in speaker location from the top to the side of the cage may be responsible for the tendency of an effect of acoustic stimuli on approach behaviour in Experiment 3 but not Experiment 2. When designing the initial setup of the playback equipment, one of the critical features I aimed to include was to present acoustic stimuli on the same horizontal plane as the rats. Previous work states that this is more likely to induce approach behaviour as it mimics the conditions in which they are most likely to receive conspecific vocalisation (Wöhr *et al*., 2016). However, in practice, this was not achievable using the home cages provided at the animal facility in France for Experiments 1 and 2. As such, the speaker was placed on top of the cage at one side in these experiments. As a result, the sound radius may have been limited to one side, preventing the acoustic stimuli from diffusing widely throughout the cage and likely hindering rat ability to localise the sound source (Seffer *et al*., 2014). Therefore, when conducting Experiment 3 at another facility in Scotland, the home cages' layout allowed the speaker's location to change. As such, the tendency towards an effect of acoustic stimuli on approach behaviour tentatively suggests that the relocation of the speaker from the top to the side of the cage was an improvement.

In order to systematically test the reliability of the home cage playback paradigm, I recognise there should have been no changes to the equipment between cohorts. However, considering the changes already made in terms of study location, the source of the subjects, and the aim of developing a novel protocol, I decided to alter the location. Thus, the difference in approach behaviour towards the different speaker locations is more likely due to the diffusion of sound through the cage rather than a change in the communicative value of the playback of 50-kHz USVs. Testing this would require presenting the same 50-kHz stimuli in a range of locations and assessing whether approach behaviour is consistent toward these locations.

Contrary to the hypothesis that 22-kHz acoustic stimuli would induce the expression of behaviours indicated by the fight or flight response, there was no effect of 22kHz acoustic stimuli on activity or freezing. The lack of effect of 22-kHz acoustic stimuli on affective state is further evidenced by subject-produced USVs, with rats producing a similar calling rate of 50-kHz USVs to Background Noise and a lack of 22-kHz ‘alarm’ USVs. Previous work has reported a lack of effect of 22-kHz USVs on rat behaviour (Sadan *et al*., 2008; Schwarting *et al*., 2018; Wöhr and Schwarting, 2007), with Schwarting and Wöhr (2012) suggesting two main explanations. First, the combination of testing conditions and acoustic stimuli used in this paradigm was not perceived as critically aversive. Indeed, this paradigm was designed to minimise stress and anxiety, as previously discussed. Second, although all rats have the genetic predisposition to respond to alarm USVs, the lack of previous aversive experiences in these rats likely meant that they had not acquired their affective valence (Schwarting and Wöhr, 2012). Schwarting and Wöhr (2012) suggest that behavioural responses to 22-kHz USVs are dependent on an interaction between an innate evolutionary mechanism and learned experience. For example, when acoustic stimuli are paired with the aversive experience of foot shocks, rats conducted more freezing when presented with 22-kHz USVs than other paired stimuli, including 50-kHz USVs (Parsana *et al*., 2012). As such, the lack of effect of 22-kHz USVs found in this study could be explained by one or a combination of these explanations and does not diminish the potential of the home cage playback paradigm to a induce positive affective state.

An increase in play behaviour as a result of the induction of a positive affective state was only seen in response to White Noise

Play is a promising indicator of affective state in animals, yet there are few examples of how play changes in response to a positive experience (Ahloy-Dallaire *et al*., 2017). As such, the second aim of this study was to investigate whether play is sensitive to the presence of positive affective states and can thus act as an indicator of PAW. Given that the playback of 50-kHz and White Noise increased USV production, matched increases in play would be expected in response to both acoustic stimuli as an expression of positive affective state. In contrast to this hypothesis, social play was reduced when rats were exposed to 50-kHz acoustic stimuli compared to Background Noise.

A potential explanation for this effect of 50-kHz acoustic stimuli on play relates to the generation of USVs used in the playback tracks. During the generation of the acoustic stimuli tracks, decisions were made carefully considering rat perception. However, considering that this was one of the first explorations of the impact of the playback of USVs on affective state with PAW in mind, some of these decisions were based on logic based on the available evidence. For example, evidence suggests that individual rats have a vocal signature that is recognised by conspecifics (Vielle *et al*., 2021). As such, sequences of USVs produced from the same rat, or pairs of rats were kept together to prevent the potential aversive effect of presenting the ‘voices’ of multiple different rats in a mixed manner. However, there is the potential that the 50-kHz USVs used in this study are mismatched in both timing and meaning to the USVs produced by the subject rat, dampening the communicative function of these calls and play. As a result, while the playback of the 50-kHz acoustic used in this study was sufficient to induce a positive affective state, the specific calls and track used in this study did not induce play behaviour. In contrast, the positive affective state induced by playback of White Noise was expressed by an increase in social play. As previously discussed, the White Noise stimuli could have been arousing, with the combination of arousal and positive affective state being expressed by rats through an increase in social play. This effect of White Noise on play suggests that the expression of play behaviour can be sensitive to affective states generated from positive experiences. However, there is further complexity within this relationship, such that play cannot be used as a sole indicator of positive affect in response to playback of acoustic stimuli.

There were differences in average USV production and play behaviour between cohorts

Significant differences existed between the cohorts in the overall USV production and play. Rats in Experiment 2 appeared to have a low calling rate and reduced play compared to previous studies (*i.e.,* Bombail *et al*., 2019; Hammond *et al*., 2019). While Experiments 1 and 2 were conducted using rats bred in-house at INRAE, France, rats used for Experiment 3 were imported from Charles River, England. Thus, although these cohorts were of the same age, sex and strain, there may have been baseline genetic and environmental differences in development due to heterogeneous source breeding populations. Both USV production and play are notorious for having high levels of individual variation (Pellis and Pellis, 2013; Pellis and McKenna, 1992; Schwarting *et al*., 2018). Further, in a study investigating USV production and play in response to playful handling, we also found evidence of cohort effects *within* breeding populations (Hammond *et al*., 2019). Both USV production and play were reduced in one cohort, despite all rats being sourced from the same rodent breeding facility (Hammond *et al*., 2019). In this paper, we suggested this could be related to differences in start bodyweight, as a proxy for physical condition, between the two cohorts (Hammond *et al*., 2019). Unsurprisingly, we also found that start bodyweight differed between the two breeding pools, further indicating a divergence between rats used in Experiment 2 and 3. Despite this discrepancy, it does provide evidence that this playback protocol could induce changes in USV production across two heterogeneous cohorts.

Conclusion

Overall, this work provides the first support for the novel home cage playback paradigm to induce a positive affect in rats. In response to the playback of 50-kHz USVs and filtered White Noise, rats were in a more positive affective state than when exposed to 22-kHz USVs or Background Noise, as indicated by an increase in subject-produced 50-kHz USVs. The playback of acoustic stimuli also influenced play behaviour, which has been proposed as a promising potential indicator of PAW. Unexpectedly, social play increased during the playback of White Noise stimuli but not during the playback of 50-kHz USVs. Further work is necessary to investigate whether this effect remains consistent over repeated presentations of these acoustic stimuli. In general, these results suggest that while the 50-kHz and White Noise stimuli were enriching to rats, there is a complex relationship between positive affective state and play as an indicator of PAW.

Chapter 5: Repeated application of a novel acoustic playback paradigm to induce positive affective state and measure changes in play in juvenile male Wistar rats

Abstract

The home cage playback paradigm was developed to investigate the potential of using contrastingly valenced ultrasonic vocalisations (USVs) to manipulate affective state. The results from the first experiments using the home cage playback paradigm reported in Chapter 4 were promising; in response to a single presentation of positively valenced 50-kHz USVs and filtered White Noise, rats showed an increase in subject-produced 50-kHz USVs as an indicator of a positive affective state. While both of these stimuli appeared to induce a positive affective state, only the playback of White Noise led to an increase in play. As such, the aims of this study were to further investigate the ability of the home cage playback paradigm to induce a positive affective state in rats and whether this results in a matched change in play. Juvenile male Wistar rats (37 days old) were presented with either 50-kHz USVs (N = 14; 7 cages), White Noise (N = 14; 7 cages) or Background Noise acoustic stimuli (N = 12; 6 cages) as pairs in the home cage for five consecutive days. Affective state in response to handling was measured through USV production, approach behaviour and a sucrose preference test on the evening of day five. There was a significant interaction between treatment and day on USV production during playback ($F_{2,479} = 3.03$, $p = 0.05$). On day one, USV production increased in response to both 50-kHz USVs and White Noise, compared with Background Noise (56.7 vs 62.0 vs 42.5 ± 6.7 USVs per minute). On day five, USV production further increased in rats exposed to 50-kHz acoustic stimuli and decreased in rats exposed to White Noise, with no change in response to Background Noise (63.2 vs 44.8 vs 42.5 ± 6.7 USVs per minute). On both days, rats exposed to 50-kHz USVs conducted the highest frequency and duration of

approach behaviour, followed by those exposed to White Noise and then Background Noise (duration of approach behaviour: 12.9 vs 8.7 vs 7.2 ± 2.1 seconds per minute; $F_{2,479} = 10.55$, $p < 0.001$). These results suggest that rats became habituated to the White Noise stimuli, but not the 50-kHz stimuli after five days of presentation. There was no significant main effect of treatment on sucrose consumption ($F_{1,39} = 1.12$, $p = 0.35$) in the sucrose preference test. There was a significant interaction between treatment and day on social play during playback (frequency: $F_{2,479} = 4.22$, $p = 0.02$ and duration: $F_{2,479} = 4.57$, $p = 0.01$). On day one, social play was lower in response to 50-kHz stimuli, whilst social play during White Noise was similar to that during Background Noise (duration of social play: 2.4 vs 4.9 vs 4.7 ± 4.2 seconds per minute). On day five, there was an increase in the expression of social play in rats exposed to 50-kHz stimuli, with a decrease in rats exposed to White Noise and Background Noise (duration of social play: 4.8 vs 3.8 vs 2.8 vs ± 3.4 seconds per minute). There was no effect of acoustic stimuli treatment on play in the home cage in the ten minutes before and after playback on days one and five (frequency: $F_{2,159} = 0.09$, $p = 0.91$ and duration: $F_{2,159} = 1.41$, $p = 0.27$). These results suggest complexity concerning rat perception of acoustic stimuli and the impact of positive affect state on play behaviour. However, there is a promise for the use of the home cage playback paradigm to investigate these questions.

Introduction

Animal welfare science now recognises the importance of positive affective states and experiences, as evidenced by the increasing interest in positive animal welfare (PAW) (Lawrence *et al*., 2017, 2019; Rault *et al*., 2022; Vigors, 2019). However, much PAW research has focused on farmed species (Edgar *et al*., 2013; Lawrence *et al*., 2018; Proctor and Carder, 2015; Rowe and Mullan, 2022), whilst overlooking laboratory species, such as rats. Considering the number of individuals involved (UK Home Office, 2020) and the evidenced ability of rats to experience a range of both positive and negative affective states (Burgdorf *et al*., 2005; Makowska and Weary, 2013; Panksepp and Burgdorf, 2000; Rygula *et al*., 2012), some authors have argued that providing rats with 'a good life' should be a fundamental condition for their use in research (Makowska and Weary, 2020). In light of this, there have been efforts to promote PAW for rats, primarily by providing enrichment, *i.e.,* the provision of resources that promote natural behaviour (van der Harst *et al*., 2003; Mellor, 2014b; Ratuski and Weary, 2022) or a rewarding human-rat interaction such as tickling or playful handling (Bombail *et al*., 2021; Hammond *et al*., 2019; LaFollette *et al*., 2017).

Another potential method of inducing positive affective states in rats is through the playback of vocalisations. Using playback of vocalisations to manipulate affective state is based on the premise that as the primary function of vocalisations is to communicate information, there will likely be valence-matched effects on conspecifics when hearing these calls (*e.g.,* Manteuffel *et al*., 2004). This method is particularly promising in rats considering that two broad types of ultrasonic vocalisations (USVs) are widely accepted as serving as signals of contrasting affective state (Wöhr and Schwarting, 2009). USVs which occur in the 30 – 90-kHz range, collectively termed 50-kHz USVs, are associated with positive affect and facilitate social interaction (Burgdorf *et al.,* 2008). In contrast, USVs which occur in the 18 - 30-kHz range, collectively termed 22-kHz USVs, are associated with negative affect and serve as alarm calls for conspecifics (Litvin *et al*., 2007). Thus, the playback of 50-kHz USVs is likely to induce a positive affective state, whereas hearing the playback of 22-kHz USVs is likely to induce a negative affective state in the recipient (Litvin *et al.,* 2007).

Previous work using the radial maze playback paradigm provides evidence for the contrasting effect of USVs on rat behaviour (Wöhr *et al*., 2016). This paradigm assesses the locomotory behaviour of an individual rat in an eight-armed maze in relation to the speaker which presents the acoustic stimuli. Experiments using this paradigm consistently report that 50kHz ‘positive communicative' USVs induce approach behaviour towards the speaker and increase exploration (Wöhr, 2017; Wöhr and Schwarting, 2012, 2009), whereas 22kHz ‘negative' alarm USVs induce behaviours indicative of the fight/flight system, *e.g.,* reduced locomotor activity and freezing (Schwarting *et al*., 2007; Schwarting and Wöhr, 2012). However, the radial maze paradigm is limited for the study of playback in relation to animal behaviour and welfare. As such, the aim of Chapter 4 was to develop a suitable paradigm to assess the impact of playback of acoustic stimuli on affective state.

In the home cage playback paradigm, a pair of rats are presented with acoustic stimuli in the home cage under red light after a period of habituation to the equipment. By presenting acoustic stimuli to pairs of rats in the home cage, the potential stress encountered from handling, isolation and bright light in the radial maze paradigm is mitigated (Begni *et al*., 2020; Gärtner *et al*., 1980; Korte and Boer, 2003; Nathiya and Vanisree, 2010). Use of the home cage allows the measurement of a wider range of behaviours offers insight into affective state of the rats during playback of acoustic stimuli. The results from Chapter 4 are promising; in response to playback of 50-kHz USVs and filtered White Noise to pairs of rats in their home cage, there was a significant increase in subject-produced 50-kHz calls compared to 22-kHz USVs and Background Noise. There was also a tendency for rats exposed to 50-kHz USVs to conduct more approach behaviour towards the speaker. However, as this study only measured the responses of rats to a single exposure of each acoustic stimulus, further work is needed to investigate whether repeated playback of acoustic stimuli will result in a sustained increase in positive affective state in rats.

Given the evidence that playback of 50-kHz USVs and White Noise increased indicators of positive affect, a secondary aim of this research was to investigate the sensitivity of play to the manipulation of affective state using the home cage playback paradigm. Play has long been proposed as a promising potential indicator of PAW, as it tends to occur when animals are under favourable environmental conditions and is associated with a positively valenced affective experience (Held and Špinka, 2011; Boissy *et al*., 2007; Lawrence, 1987). Play can also be considered a form of emotional contagion in that the expression of play can stimulate play in others and spread the associated positive affective state (Hammond *et al*., 2019; Held and Špinka, 2011; Špinka *et al*., 2016). As such, stimulating play in animals is a promising method of promoting PAW (Held and Špinka, 2011; Špinka, 2012). However, in a recent review of play as an indicator of welfare, Ahloy-Dallaire *et al.* (2019) conclude that there is currently insufficient evidence that play levels are enhanced with increases in positive affect.

The aims and hypotheses of this study were;

i) To investigate whether repeated playback of specific acoustic stimuli alters affective state, as indicated by USV production, approach behaviour and sucrose preference. Based on the results from Chapter 4, I hypothesised that when rats are exposed to repeated playback of 50-kHz USVs and White Noise, they would produce more 50-kHz USVs, show increased approach behaviour towards the speaker and show increased sucrose preference relative to rats exposed to Background Noise.
ii) To investigate whether the induction of a positive affective state through repeated playback results in a matched change in play, both during playback and in the home cage before and after playback. Based on the results from Chapter 4, I hypothesised that the positively valenced state induced by White Noise would be expressed by an increase in social play during playback, whereas social play would be lower during the playback of 50-kHz USVs, both compared to Background Noise.

Methods

Ethics

This study was conducted in accordance with the European Union directive of 22nd September 2010 (2010/63/EU) and the UK Animals (Scientific procedures) act 1986. There was further approval by the Roslin Institute Animal Welfare and Ethical Review Body (AWERB), the Royal (Dick) School of Veterinary Studies Veterinary Ethical Review Committee (VERC) and SRUC's Animal Ethics Committee.

Animals, housing and husbandry

All work was conducted at the Roslin Institute, Edinburgh, UK, between 25th August and 7th September 2021. Subjects were juvenile male Wistar rats (N = 40) from females of unknown parity imported from Charles River Laboratories, Margate, UK. Across different litters, rats were matched into pairs by weight, so individuals were within 2g of each other. This was to take account of any effects of a shared life experience and the potential impact of body condition on play behaviour in rats (Hammond *et al.*, 2019). Data collection began at 37 days from birth as play shows the highest absolute levels between 32 and 40 days of age depending on the rat strain, with the peak of play being towards the latter end of this range for Albino rats (Panksepp, 1981; Thor and Holloway, 1984).

Rats were housed in clear plastic cages with a wire lid (UK: 48 x 26.3 x 20.5 cm; Techniplast, Italy). Each cage was supplemented with wood chip bedding and *ad libitum* access to food and water. Rats were fed Teklad Global Rodent Maintenance Diet (14% protein) from Envigo, England. Cage cleaning was carried out by the same caretaker once a week, ensuring no disturbance occurred at least two hours prior to any experimental procedure. The homeroom was maintained on an inverted 12 h light/dark cycle (lights on at 2100) and at a constant temperature (23 ± 0.2 °C) and humidity (37.5 ± 6.7%). Low-intensity red lighting (4.5 lx measured using an Isotech digital light meter Lux-1337) was used during the dark phase to keep the rats within the active dark photoperiod (Knutson *et al*., 1998). Cages were situated on a single rack across four tiers. Both rats within a cage received marks on the tail using a non-toxic black marker for individual identification.

Experimental design

Across the 20 cages of paired rats, seven cages were assigned to receive 50-kHz acoustic stimuli, seven cages received White Noise acoustic stimuli and six received Background Noise. This division of cage numbers per treatment was randomly assigned. Treatment groups were balanced according to total cage weight three days before playback began, so the groups' weights were within 0.8g of each other. The overall mean weight and standard deviation three days before playback was 193.8 ± 16.7g. Within each day, the order of each cage was randomised, making sure the same treatments did not follow each other to prevent the potential build-up of response to one treatment (*e.g.,* olfactory cues).

Rats received playback of acoustic stimuli in the home cage for five days according to the assigned treatment (50-kHz, White Noise and Background Noise). Within these five days, days one and five were assigned as recording days, with home cage behaviour recorded for ten minutes before and after handling. These recordings allowed for investigation of the effect of playback on play and other spontaneous behaviour in the home cage. On days two to four, rats were exposed to playback according to the assigned treatment but were not recorded in the home cage before and after playback. Data were analysed from days one and five only. On the evening of day five, all rats were given a sucrose preference test.

Experimental setup

Rats were accustomed to an inverse light cycle, so experimental testing could occur during the dark period when the rats are naturally active (lights off at 0900 with testing between 1000 and 1500). All testing was conducted within the holding room at least three metres away from other rats to minimise the potential for emotional contagion (Hammond *et al*., 2019; Hatfield *et al*., 1993). This room was lit with red lighting, which delivered a low-intensity illumination (4.5 lx) to keep the rats within the active dark photoperiod and encourage USV production (Knutson *et al*., 1998).

Playback was conducted using the same experimental setup and acoustic stimuli as Experiment 3 in Chapter 4 (see for detailed methodology). All testing was performed in the home cage, with an ultrasonic microphone placed above the cage (see the recording of USVs section below) and an ultrasonic speaker replacing the food hopper (**Figure 5.1**).

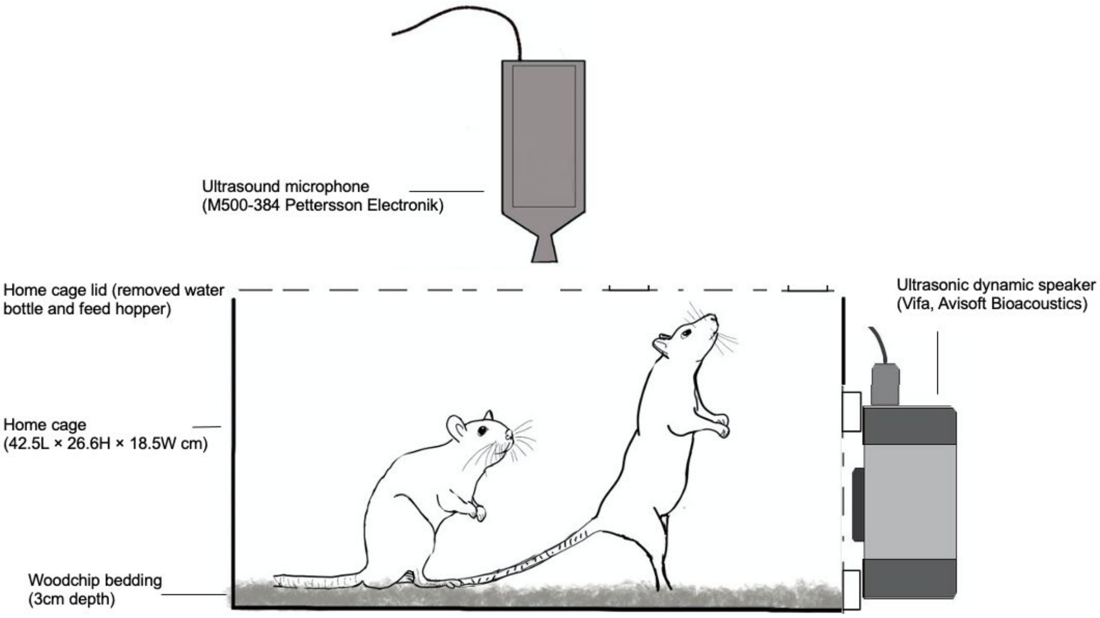


Figure 5.1. Equipment setup used for presenting different acoustic stimuli in the home cage to paired juvenile male Wistar rats (N = 20 cages). This figure is not to scale.

Experimental procedures

Habituation to equipment: All rats were habituated to the experimental setup before experimental testing over three days. The playback speaker was fitted to the home cage by removing the food hopper and water bottle and then placing the speaker behind a grille (**Figure 5.1**). The equipment was left for an increasing time each day, up to a total of seven minutes, to match the maximum time of each playback trial. Following a session, the wire cage lid was topped with cardboard for ten minutes to create a barrier for USVs and odours affecting other rats in the room.

Daily procedure during testing: On recording days (days one and five), each cage was placed on a designated recording rack within the homeroom and filmed for ten minutes before playback. Cages were then transported to the bench for the presentation of acoustic stimuli. The cage was then returned to the recording rack and filmed for another ten minutes after playback. On non-recording days, rats were not recorded in the home cage but were placed immediately on the bench for presentation of acoustic stimuli. The experimenter left the room during recording of home cage behaviour and during playback to prevent any human disturbance which could alter rat behaviour.

Acoustic stimuli

Acoustic stimuli were presented through the Avisoft-RECORDER USGH software (Avisoft Bioacoustics, Berlin, Germany) using an ultrasonic dynamic speaker (Vifa, Avisoft Bioacoustics) with a frequency range of 1 – 120-kHz (at 12dB) and an ultrasonic power amplifier with a frequency range of 1–180-kHz (UltraSoundGate Player 216H, Avisoft Bioacoustics).

As detailed in Chapter 4, playback stimuli were generated by collecting USVs from rats used in other studies. The 50-kHz USVs were recorded from individual rats during playful handling (Chapter 2) and from pairs of rats during spontaneous social play in the home cage (generated for Chapter 4). The White Noise acoustic stimuli track was generated by matching the time and intensity of the natural USVs in the 50-kHz track and replacing them with artificial White Noise generated within Audacity (Wöhr and Schwarting, 2012). A high-pass filter was applied to all stimuli to remove all sounds below 30-kHz. Background Noise was recorded under the same experimental conditions with no rats in the room. All recordings were produced using the same equipment from rats of the same developmental stage, sex, and strain as the rats in this experiment (juvenile male Wistar rats between 37 and 49 days old). Recordings had a sampling rate of 384-kHz in 16-bit format. Examples of acoustic stimuli can be seen in **Figure 5.2**.

Each track consisted of a one minute habituation phase and three sets of alternate presentations of the acoustic stimuli and pauses for one minute each. The total playback track was seven minutes long. 50-kHz USVs, White Noise and Background Noise were presented at approximately 60 dB (measured from a distance of 30cm). To prevent habituation over the five days of testing (Wöhr and Schwarting, 2012), the playback track within each treatment was different for each day. Each one-minute playback section was split into 15-second phrases and reordered to achieve this.

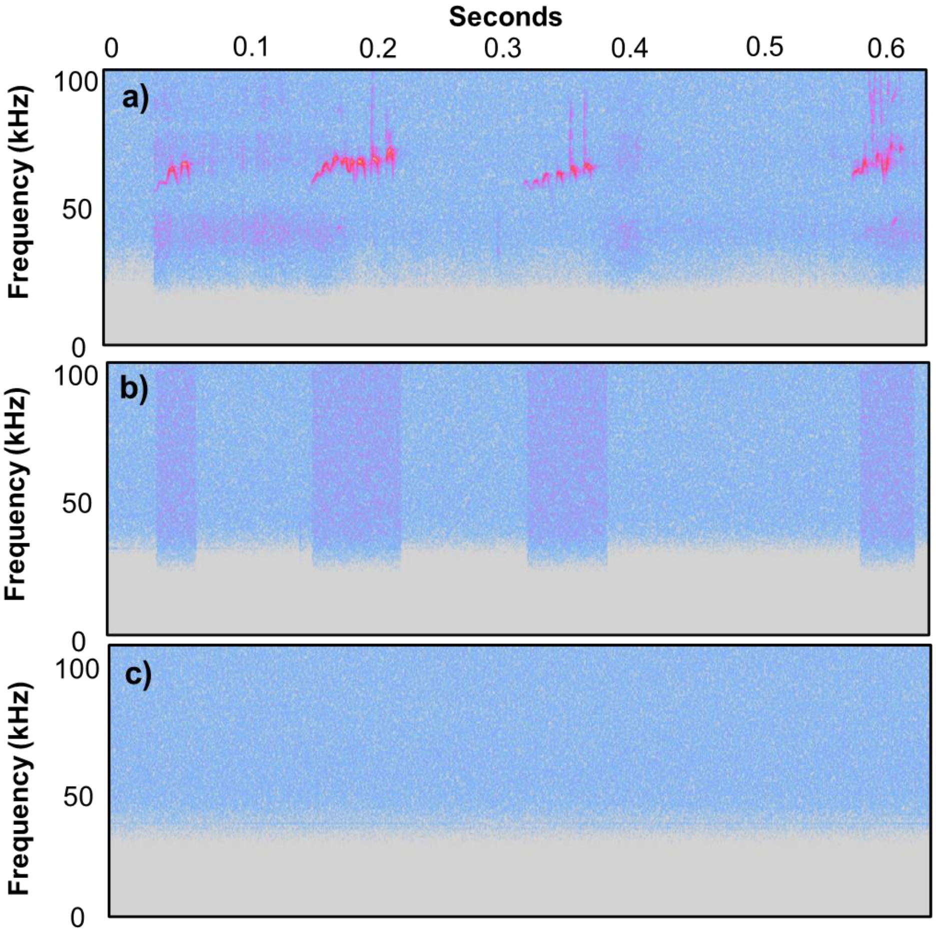


Figure 5.2. Example spectrograms of the three types of acoustic stimuli presented to juvenile male Wistar rats (N = 20 cages); a) 50-kHz USVs, b) White Noise, and c) Background Noise. All USVs were recorded from rats used in other studies from the same developmental stage, sex and strain.

Recording and analysis of behaviour

Behaviour in the home cage before and after playback and during playback were recorded using a Sony HD camcorder (HDR- PJ810E) and subsequently analysed using Observer XT 14 software (Noldus Information Technology, Wageningen, the Netherlands). Using the same ethogram as Chapter 2, behaviour was coded using continuous focal sampling (**Table 5.1**). During the data collection, the coder (TH) was blinded to treatment to prevent bias.

Recording and analysis of subject-produced USVs during playback

USVs were recorded using a high-quality USB microphone (Pettersson M500-384 USB Ultrasound microphone, Pettersson Electronik; Sweden) connected via a USB interface to a PC laptop. This microphone was placed over the centre of the home cage (height of approx. 51cm), pointing downwards towards the home cage floor. Recorded sound was digitised at a sampling rate of 384 kHz and a bit depth of 24 bit using Audacity (https://www.audacityteam.org; a free and open-source digital audio editor and recording application software; Audacity, Version 2.1.3, Pennsylvania, United States of America).

USVs were manually counted from spectrograms produced by Audacity and labelled as 50-kHz USVs (peak frequency between 30 and 80 kHz and duration between 10–150ms) or 22-kHz USVs (bandwidth of >4kHz, peak frequency between 20-29 kHz and duration of 300ms or more) (Brudzynski, 2009; LaFollette *et al.*, 2018b; Wright *et al.,* 2010*)*. Overlapping USVs were counted individually, as two rats were present during the recording. Vocalisations were counted according to the habituation, playback and pause intervals of the acoustic playback track. USV rate was expressed as the number of calls emitted per minute (calls/min).

After counting the number of USVs produced by rats, I compared the recorded subject-produced USV track directly in time with the relevant acoustic stimuli track for all experiments. This was to prevent the double-counting of USVs, which may have been recorded from the inputted playback track rather than produced by the rats themselves. If a vocalisation was deemed as a duplicate of the playback track, it was not counted. During the initial collection of data from spectrograms, the coder (TH) was blinded to treatment to prevent bias.

Sucrose preference test

As in Chapter 2, rats were presented with an adaptation of the sucrose preference test as an additional method of assessing the impact of playback of acoustic stimuli on affective state. In this test, rats were given a choice of two pre-weighed bottles (one containing tap water and one containing 20% sucrose solution) given in the home cage for two hours after the fifth day of playback (1530 to 1730) as a short-term preference test. The concentration of 20% sucrose used in this study was based on research examining the naturally occurring variation of sucrose consumption according to different concentrations (Brennan *et al*., 2001). Each bottle was weighed before and after both tests to measure cage-level sucrose and water consumption. Throughout testing, rats had *ad libitum* access to food, and the location of the bottles was balanced to prevent any effects of side bias (Strekalova *et al*., 2004). While weighing the bottles, the experimenter was blinded to treatment to prevent bias.

Table 5.1. Ethogram used for recording behaviour of pair housed male Wistar rats aged between 37 – 44 days old) in the home cage before and after playback of acoustic stimuli and during playback of three different acoustic stimuli (N = 20 rats housed in pairs; 7 cages exposed to 50-kHz, 7 cages exposed to White Noise and 6 cages exposed to Background Noise). The table indicates which behaviours were recorded during each phase (the home cage and/or during handling).

Behaviour	**Description**	**When scored**
Solitary play	Rat conducts fast locomotor movement involving at least one hop by an individual, where hops involve all four paws leaving the ground at the same time, not in the direction of a play partner or during a play bout. The behaviour starts with fast running or a hop from stationary or during locomotor movement and ceases when this movement stops. (Hammond et al., 2019)	Before, after and during playback
Social play	One rat jumps or lunges towards the partner's nape, resulting in the partner either chasing the soliciting rat, rearing (in which pairs make rapid pawing movements at each other), or rotating so that one rat is on its back with the other standing over it in a pin. The frequency of pinning and dorsal contacts within social play was also coded. Multiple pins and dorsal contacts can occur during a single social play bout. The behaviour starts with fast running, a jump or lunge towards a play partner and ceases when there is no chasing, rearing, pins or dorsal contacts between the play partners. (Kerkhof et al., 2013; Webber et al., 2012)	Before, after and during playback
Approach towards the speaker	Rat orients its body and head so that the nose is directed towards the speaker and within 1cm of the speaker. (Lever *et al*., 2006)	During playback

Rearing (undirected)	Rat raises both front paws off the ground (can be in contact with the wall or not) standing up on hind legs. Includes all rears, bar those with the rat's nose directed towards the speaker. (Lever *et al*., 2006)	During playback
Digging	Clear movement of bedding with the front or hind paws or face. The rats front paws and/or face are not visible because it is beneath the bedding. (Makowska and Weary, 2016)	During playback
Self-grooming	All self-directed grooming behaviour including licking the fur, grooming with forepaws and scratching with any limb. (Gaskill and Pritchett-Coming, 2015)	During playback
Locomotor activity	Rat crosses the centre line of the cage with its whole body. The centre line was defined by a marker on the side length of home cage.	During playback
Inactive	Any behaviour where the rat's body is still and unmoving, such as freezing, resting, sitting or lying still. The rat can make facial movements with eyes open or closed.	Before, after and during playback
Freezing	The rat's body is motionless in a crouching position. Rat makes vibrissae movements of the whiskers, gentle bobbing head movements and has bulging eyes. (Brudzynski, 2019)	During playback
Resting	The rat's body is still and unmoving, either sitting or lying. The rat can make facial movements with eyes open or closed. (LaFollette *et al*., 2018b)	During playback

Other non-social/ social	Includes urinating, defecating, chewing of any object and any other unclassified behaviour not directed towards the cage mate/includes any behaviour towards the cage mate such as social grooming.	During playback

Statistics

Tests of intra-observer reliability were conducted prior to statistical analysis. As only one observer scored all behaviours, inter-observer reliability tests were unnecessary. I conducted a repeat scoring of a minimum of 12.5% of the data seven days later. For USV data, I conducted Spearman's rank correlations between the first count and second count for each spectrogram (Martin and Bateson, 1993). To meet the reliability criteria, correlations exceeded the acceptable cut-off defined by Martin and Bateson of 0.7. For behaviour, these correlations were conducted using Observer XT 11 software and met a minimum Cohen's kappa of 0.8 with a percentage agreement of 64% (McHugh, 2012).

All data were analysed in Genstat 19.1 using General Linear Models (GLM). Figures were generated using GraphPad Prism (ver 9.2). Assumptions of the GLMs were tested using Bartlett's test for homogeneity of variances and the Anderson-Darling test for normality. For all tests, the level of statistical significance was set at $p < 0.05$. Tukey's honest significance difference (HSD) procedure was conducted for multiple comparisons on significant differences ($a = 0.05$). Means (M) and standard errors of the mean (SEM) reported were back-transformed to the original scale for biological significance.

All USV and behavioural data were taken from the first and fifth consecutive days of playback. In all sessions, the production of 22-kHz USVs was too infrequent to fit a GLM. To investigate USV production and behaviour during playback of different acoustic stimuli, interval type (playback of acoustic stimuli or pause intervals) and day (one or five) were modelled as the main effects with additional interactions between the three main effects. Cage was the blocking factor. As rats were tested together in the home cage, the frequency and duration of each rat's behaviour were summed to give a cage level of expression. The frequency and duration (seconds) were calculated for most behaviours bar two exceptions; general locomotor activity was measured by the frequency of crossing over from one side to the other, and solitary play was only measured using the frequency due to the short duration of these events. This was to reduce the likelihood of false positives, as coding would depend on the coder's reaction time. Of the eleven behaviours measured during playback (**Table 5.2**), four (resting, freezing, other social and non-social behaviours) were not fitted to a General Linear Model as they were deemed too rare for statistical analysis. To investigate individual variation in measured responses to playback of acoustic stimuli, I present the coefficient of variation as a statistical measure of the difference in variation between the two treatments for USV production, approach behaviour, sucrose preference and play.

All data on home cage behaviour were taken from the first and fifth consecutive days of playback. To investigate the effect of handling treatment on behaviour in the home cage, acoustic treatment (50-kHz, Background Noise or White Noise), the timing of the recording in relation to playback (before or after) and day (one or five) were modelled as the main effects with additional interactions between the three main effects. As behaviour in the home cage was recorded in pairs, the blocking factor was the cage.

Results

USV production was dependent on an interaction between acoustic stimuli and day

There was significant main effect of acoustic stimuli treatment ($F_{2,479} = 0.75$, $p = 0.05$) and day ($F_{1,479} = 4.25$, $p = 0.04$) on subject-produced USVs (**Figure 5.3**). Rats produced the most USVs in response to 50-kHz USVs, followed by White Noise and Background Noise and on day one compared to day five. There was no significant main effect of interval type on subject-produced USVs ($F_{1,479} = 1.69$, $p = 0.19$). There was also significant interaction between treatment and day ($F_{2,479} = 3.03$, $p = 0.05$). On day one, rats produced more USVs in response to 50-kHz and White Noise compared to Background Noise. Tukey's HSD test indicated that on day one, the mean number of USVs produced during the 50-kHz stimuli ($T = 10.67$, $p = 0.02$) and White Noise ($T = 12.58$, $p = 0.03$) differed significantly from Background Noise but not each other ($T = 2.03$, $p = 0.97$). On day 5, USV production decreased in rats exposed to White Noise and Background Noise and increased in rats exposed to 50-kHz acoustic stimuli. Tukey's HSD test indicated that on day five, the mean number of USVs produced during the 50-kHz stimuli (T = 10.67, *p* = 0.02) differed significantly from White Noise (T = 12.98, *p* = 0.003) and Background Noise (T = 14.37, *p* = 0.002) which were not different from each other (T = 2.03, *p* = 0.84).

The coefficient of variation for USVs did not differ between the three acoustic stimuli. On day one, the coefficient of variation for rats exposed to 50-kHz stimuli was 90.4%, White Noise was 92.3%, and Background Noise was 88.4%. On day five, the coefficient of variation for rats exposed to 50-kHz stimuli was 76.6%, White Noise was 81.2%, and Background Noise was 93.4%.

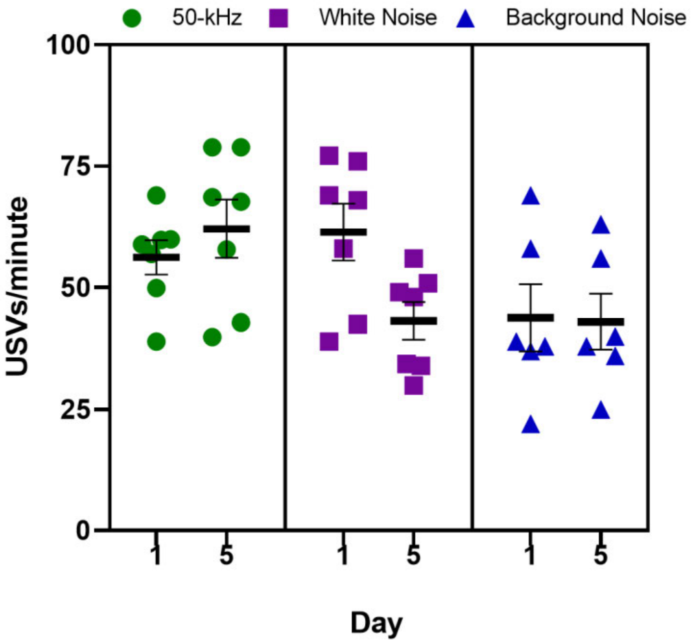


Figure 5.3. Graph showing the mean number of 50-kHz ultrasonic vocalisations (USVs) per minute produced by pairs of juvenile male rats (N = 20 rats housed in pairs; 7 cages exposed to 50-kHz, 7 cages exposed to White Noise and 6 cages exposed to Background Noise) on days one and five of acoustic playback. Rats were presented with either 50-kHz acoustic stimuli (green circles), White Noise (WN) (purple squares) or Background Noise (BN) (blue triangles). Values reflect means for each cage with the black bar indicating the mean ± SEM for each treatment by day.

Playback of 50-kHz acoustic stimuli increased approach behaviour towards the speaker

Results of the GLM modelling rat behaviour during the pause phases of playful handling are shown in **Table 5.3**. The only behaviour that was influenced by the main effect of treatment was approach behaviour towards the speaker, with rats exposed to 50-kHz acoustic stimuli conducting the highest frequency and duration of approach behaviour, followed by those exposed to White Noise and then Background Noise (**Figure 5.4**). Tukey's HSD test indicated that the mean duration of approach behaviour significantly differed between all three treatments across both days (50-kHz vs WN: T = 10.12, p = 0.04, 50-kHz vs BN: T = 13.56, p = 0.01, WN vs BN: T = 9.80, p = 0.04). There was a significant effect of day on the frequency and duration of approach behaviour, with rats conducting more approach behaviour on day one compared to day five (frequency: 2.8 vs 1.9 ± 0.1 events per minute, $F_{1,479}$ = 4.08, p = 0.02 and duration: 11.2 vs 8.9 ± 0.6 seconds per minute, $F_{1,479}$ = 7.57, p = 0.008). There was also a main effect of interval type on the frequency and duration of approach behaviour, with rats conducting more approach behaviour during the playback phases compared with the pauses (frequency: 2.1 vs 1.9 ± 0.1 events per minute and duration: 11.1 vs 8.9 ± 0.6 seconds per minute).

The coefficient of variation for the duration of approach behaviour differed between the three acoustic stimuli on day one but not day five. On day one, the coefficient of variation for rats exposed to 50-kHz stimuli was 77.5%, White Noise was 91.1%, and Background Noise was 82.0%. On day five, the coefficient of variation for rats exposed to 50-kHz stimuli was 98.0%, White Noise was 92.6%, and Background Noise was 95.6%.

There was no significant main effect of treatment on locomotor activity, undirected rearing, self-grooming, digging and solitary play. There was a significant main effect of interval type on locomotor activity and self-grooming, with rats becoming more active during the playback phases than the pause phases (2.0 vs 1.8 ± 0.04 events per minute) and conducting more self-grooming during the pause phases than the playback phases (frequency: 0.4 vs 0.2 ± 0.04 events per minute and duration: 3.0 vs 1.4 ± 0.5 seconds per minute). There was a significant main effect of day on locomotor activity, with rats becoming more active on day five compared to day one (1.8 vs 2.9 ± 0.04 events per minute).

There was no relationship between USV production and approach behaviour during playback of acoustic stimuli

To determine whether there was a relationship between the two measures of affective state, I conducted correlations between USV production and the duration of approach behaviour during playback of acoustic stimuli on day five; day five was chosen as the responses to acoustic stimuli treatments would be expected to be established by that point. There was no significant relationship between USV production and the duration of approach behaviour towards the speaker in response to 50-kHz ($R_p = 0.09$, $p = 0.58$), White Noise ($R_p = 0.14$, $p = 0.38$) or Background Noise ($R_p = 0.17$, $p = 0.33$).

Table 5.3. Table presenting results of an ANOVA ran on the frequency and duration of behaviours expressed by juvenile male rats (N = 20 rats housed in pairs; 7 cages exposed to 50-kHz, 7 cages exposed to White Noise and 6 cages exposed to Background Noise) during the presentation of one of three different acoustic stimuli (50-kHz, White Noise and Background Noise) on days one and five during pauses and playback intervals. Significant P values are indicated in bold script.

		Treatment		**Day**		**Interval type (pause or playback)**	
Behaviour		$F_{2, 479}$	*p*-value	$F_{1, 479}$	*p*-value	*p*-value	*p*-value
Approach towards speaker	frequency, duration	4.07, 10.55	**0.04**, **<0.001**	4.08, 7.57	**0.02**, **0.008**	3.16, 7.03	**0.05**, **0005**
Locomotor activity	frequency	0.89	0.43	8.40	**0.004**	10.55	**0.001**
Undirected rearing	frequency, duration	0.14, 0.30	0.87, 0.75	0.95, 2.16	0.33, 0.14	1.75, 0.17	0.19, 0.68
Self-grooming	frequency, duration	1.32, 3.27	0.31, 0.07	0.02, 0.45	0.89, 0.50	10.73, 9.63	**0.001**, **0.002**
Digging	frequency, duration	0.13, 0.33	0.87, 0.76	3.31, 0.72	0.56, 0.48	0.80, 0.92	0.07, 0.37
Inactive	frequency, duration	0.81, 1.64	0.46, 0.18	4.90, 2.44	0.31, 0.16	1.45, 0.54	0.18, 0.48
Solitary play	frequency	0.21	0.61	0.04	0.84	0.67	0.41

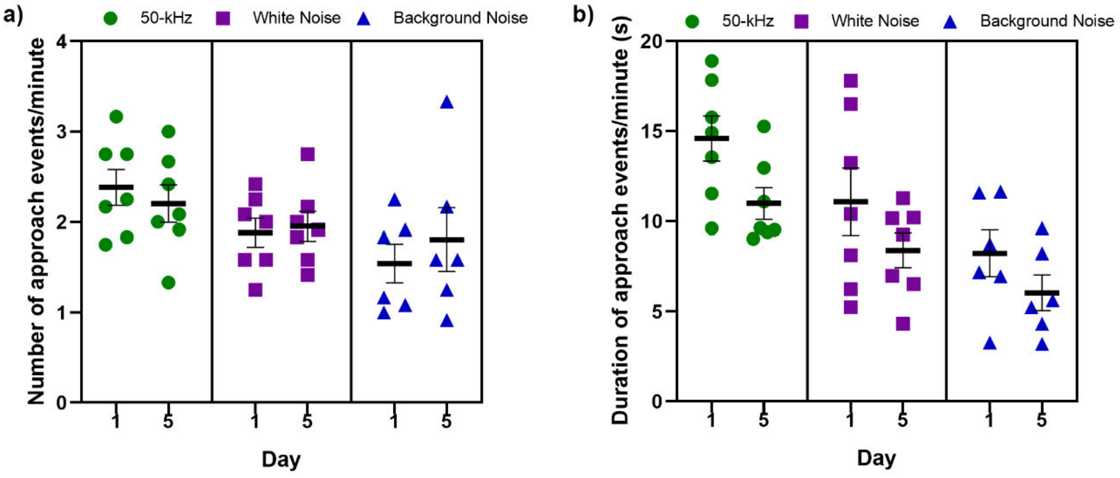


Figure 5.4. Panels showing a) the mean frequency and b) duration (s) of approach behaviour in pairs of juvenile male rats (N = 20 rats housed in pairs; 7 cages exposed to 50-kHz, 7 cages exposed to White Noise and 6 cages exposed to Background Noise) during the presentation of one of three acoustic stimuli on the first and fifth day of playback. Rats were presented with either 50-kHz acoustic stimuli (green circles), White Noise (purple squares) or Background Noise (blue triangles) for five days. Values reflect means for each cage with the black bar indicating the mean ± SEM for each treatment by day.

Social play during playback was dependent on an interaction between acoustic stimuli and day

There was no significant main effect of treatment on the frequency and duration of social play during playback of acoustic stimuli (frequency: $F_{2,479} = 0.07$, $p = 0.93$ and duration: $F_{2,479} = 0.01$, $p = 0.99$). There was also no main effect of interval type on the frequency or duration of social play during playback (frequency: $F_{1,479} = 0.08$, $p = 0.78$ and duration: $F_{1,479} = 0.17$, $p = 0.68$). There was a significant interaction effect on the frequency and duration of social play between treatment and day (frequency: $F_{2,479} = 4.22$, $p = 0.02$ and duration: $F_{2,479} = 4.57$, $p = 0.01$) (**Figure 5.5**). On day one, rats exposed to White Noise and Background Noise conducted the most social play, then 50-kHz. Tukey's HSD test indicated that on day one, the mean duration of social play during playback of White Noise ($T = 12.76$, $p = 0.05$) and Background Noise ($T = 13.54$, $p = 0.05$) differed significantly from 50-kHz but not each other ($T = 2.30$, $p = 0.76$). On day five, this effect was reversed, with rats exposed to 50-kHz conducting the most social play, followed by White Noise and Background Noise. Tukey's HSD test indicated that on day five, the mean duration of social play during playback of White Noise ($T = 13.50$, $p = 0.03$) and Background

Noise (T = 11.93, *p* = 0.02) differed significantly from 50-kHz but not each other (T = 3.01, *p* = 0.81).

The coefficient of variation for the duration of social play did not differ between the three acoustic stimuli. On day one, the coefficient of variation for rats exposed to 50-kHz stimuli was 76.7%, White Noise was 74.7%, and Background Noise was 77.4%. On day five, the coefficient of variation for rats exposed to 50-kHz stimuli was 76.3%, White Noise was 70.3%, and Background Noise was 79.3%.

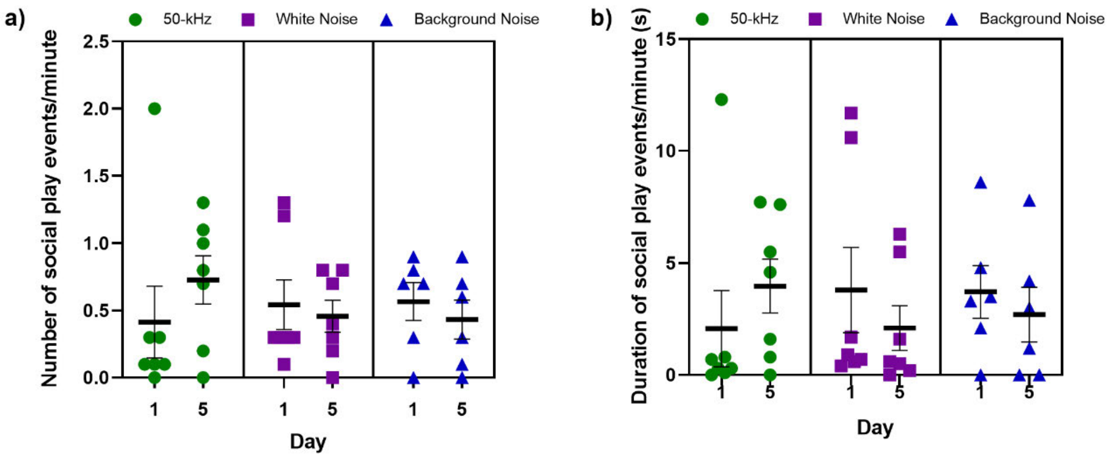


Figure 5.5. Panels showing a) the mean frequency and b) duration (s) of social play in pairs of juvenile male rats (N = 20 rats housed in pairs; 7 cages exposed to 50-kHz, 7 cages exposed to White Noise and 6 cages exposed to Background Noise) during presentation of one of three acoustic stimuli on the first and fifth day of playback. Rats were presented with either 50-kHz acoustic stimuli (green circles), White Noise (WN) (purple squares) or Background Noise (BN) (blue triangles) for five days. Values reflect means for each cage with the black bar indicating the mean ± SEM for each treatment by day.

Playback of acoustic stimuli influenced play in the home cage

There was no significant main effect of treatment on the frequency of solitary play in the home cage 10 minutes before and after playback ($F_{2,159}$ = 0.09, *p* = 0.91). There was a significant main effect of timing of recording (before or after playback) on the frequency of solitary play, with rats conducting more solitary play before than after playback (2.3 vs 0.5 ± 0.4 events; $F_{1,159}$ = 19.88, *p* < 0.001). There was also a significant main effect of day on the frequency of solitary play in the home cage, with the frequency of solitary play increasing from day one to five (0.8 vs 2.1 ± 0.4 events per ten minutes $F_{1,159} = 11.45$, $p < 0.001$).

Similarly, there was no significant main effect of treatment on the frequency or duration of social play in the home cage 10 minutes before and after playback (frequency: $F_{2,159} = 0.09$, $p = 0.91$ and duration: $F_{2,159} = 1.41$, $p = 0.27$). There was a significant main effect of timing of recording (before or after playback) on the frequency and duration of social play, with rats conducting more social play before than after playback (frequency: 7.5 vs 1.6 ± 0.6 events per ten minutes; $F_{2,159} = 83.22$, $p < 0.001$ and duration: 79.8 vs 18.1 ± 8.8 seconds per ten minutes; $F_{2,159} = 46.98$, $p < 0.001$).

However, there was a significant interaction effect on the frequency of social play between treatment, day and timing of recording ($F_{2,159} = 4.61$, $p = 0.01$; **Figure 5.6**). During the baseline recording before playback on day one, rats exposed to Background Noise conducted the highest frequency of social play events, followed by 50-kHz and then White Noise. Tukey's HSD test indicated that before playback on day one, the mean frequency of social play significantly differed between all three treatments (50-kHz vs WN: T = 13.74, $p = 0.03$, 50-kHz vs BN: T = 14.55, $p = 0.02$, WN vs BN: T = 15.49, $p = 0.01$). Tukey's HSD test indicated that before playback on day five, there was no difference in the mean frequency of social play in response to the 50-kHz stimuli (T = 1.73, $p = 0.15$), White Noise (T = 3.60, $p = 0.64$) or Background Noise (T = 1.52, $p = 0.88$). There was no significant main effect of day on the duration of social play ($F_{2,159} = 0.50$, $p = 0.48$).

The coefficient of variation for the duration of social play differed between the three acoustic stimuli on day one but not on day five. On day one, the coefficient of variation for rats exposed to 50-kHz stimuli was 57.4%, White Noise was 83.0%, and Background Noise was 77.4%. On day five, the coefficient of variation for rats exposed to 50-kHz stimuli was 69.2%, White Noise was 59.6%, and Background Noise was 57.8%.

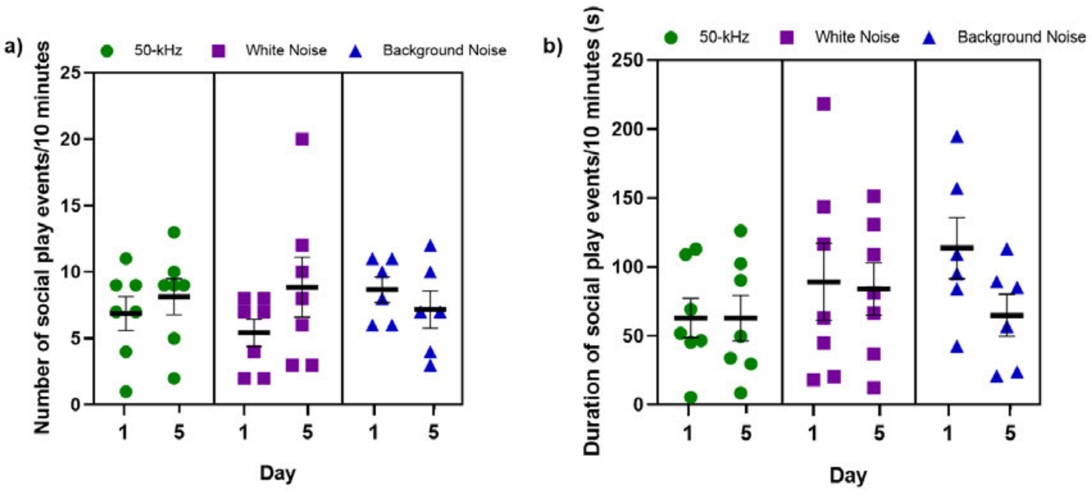


Figure 5.6. Panels showing a) the mean frequency and b) duration (s) of social play in pairs of juvenile male rats (N = 20 rats housed in pairs; 7 cages exposed to 50-kHz, 7 cages exposed to White Noise and 6 cages exposed to Background Noise) measured in the home cage 10 minutes before playback on the first and fifth day of playback. Rats were presented with either 50-kHz acoustic stimuli (green circles), White Noise (WN) (purple squares) or Background Noise (BN) (blue triangles) for five days. Values reflect means for each cage with the black bar indicating the mean ± SEM for each treatment by day.

Periods of inactivity in the home cage decreased on day five

There was no effect of treatment on the frequency of inactive events ($F_{2,159}$ = 0.79, p = 0.47) or duration of inactivity in the home cage ($F_{2,159}$ = 0.50, p = 0.62). There was a significant main effect of timing of recording (before or after handling) on the frequency and duration of inactivity, with rats being more active after playback than before (frequency: 0.0 vs 0.29 ± 0.09 events per ten minutes; $F_{1,159}$ = 25.06, p < 0.001 and duration: 75.5 vs 0.0 ± 32.3 seconds per ten minutes, $F_{1,159}$ = 10.85, p = 0.001). There was also a significant main effect of day on the frequency and duration of inactivity, with rats spending more time inactive on day one compared to day five (frequency: 0.25 vs 0.04 ± 0.04 events per ten minutes; $F_{1,159}$ = 13.69, p < 0.001 and duration: 64.6 vs 10.9 ± 11.5 seconds events per ten minutes, $F_{1,159}$ = 21.41, p < 0.001).

Acoustic stimuli treatment does not influence sucrose preference

There was no significant main effect of treatment on sucrose consumption ($F_{1,39}$ = 1.12, p = 0.35) or water consumption $F_{1,39}$ = 0.41, p = 0.67) in the sucrose preference test. There was a significant main effect of the substance, with rats consuming more sucrose than water across the 2 hours (20.3 vs 8.6 ± 0.2 ml; $F_{1,39} = 193.8$, $p < 0.001$). Consumption was independent of body weight, with average cage weight having no significant effect on the volume of sucrose or water drank ($F_{1,39} = 0.16$, $p = 0.64$).

The coefficient of variation for the volume of sucrose consumed did not differ between the three acoustic stimuli. The coefficient of variation for rats exposed to 50-kHz stimuli was 27.5%, White Noise was 21.2%, and Background Noise was 29.7%.

Discussion

The home cage playback paradigm was designed to investigate the affective responses of rats to different acoustic stimuli, with the practical aim of using this method to promote PAW (Chapter 4). In order to measure changes in affective state, the aim was to facilitate the expression and measurement of more of the behavioural repertoire of rats than previously used approaches. The results in Chapter 4 provided the first evidence of the ability of this paradigm to induce a positive affective state in response to a single exposure to 50-kHz USVs and White Noise. Here, I provide further validation for the use of the home cage playback paradigm to induce a positive affective state in rats after repeated exposure to the same acoustic stimuli. The production of 50-kHz USVs by subjects and approach behaviour towards the speaker on day one matched previous work (Chapter 4); rats produced more USVs in response to 50-kHz and White Noise acoustic stimuli than Background Noise. After five days of repeated playback of these stimuli, USV production in response to White Noise dropped to that of Background Noise and increased in response to 50-kHz stimuli. Rats also conducted the most approach behaviour towards the playback speaker in response to 50-kHz acoustic stimuli on both days. This suggests that while rats became habituated to the White Noise stimuli, the positive response to 50-kHz stimuli remained consistent after the five days of presentation. The increase in social play during playback on day five in response to 50-kHz stimuli suggests that play can be sensitive to the experience of positive affect.

The playback of 50-kHz and White Noise stimuli induced a positive affective state in rats, as indicated by USV production

Playback paradigms are based on the premise that as vocalisations are associated with a particular affective state, the playback of these vocalisations will have valence-matched effects on receiving conspecifics (Briefer, 2018; Manteuffel *et al*., 2004). The process through which the affective state of the producer and receiver converges is termed emotional contagion (Hatfield *et al*., 1993; Waal, 2007). In rats, 50-kHz USVs are the primary vocalisation type associated with positive affect (Barker, 2018; Browning *et al*., 2011; Burgdorf *et al*., 2007) and, thus, are most likely to facilitate emotional contagion and induce a positive affective state in receivers. In Chapter 4, there was evidence that the presentation of 50-kHz USVs in the home cage playback paradigm successfully facilitated the transfer of positive emotional contagion; in response to a single exposure of positively-valenced 50-kHz USVs, both independent cohorts of rats produced significantly more 50-kHz USVs than those exposed to Background Noise. Similarly, on day one of this study, using the same equipment, stimuli tracks, and procedures as in Experiment 3 in Chapter 4, there was a significant increase in subject-produced 50-kHz calls in response to 50-kHz USVs compared to Background Noise. Overall, the positive effect of 50-kHz acoustic stimuli on USV production during a single exposure is consistent with previous work, in which rats presented both as individuals or as pairs increased production of USVs in response to playback of 50-kHz stimuli (Olszyński *et al*., 2020). While there was replication of the results found in Chapter 4, the rate of USV production in this study was approximately half that of the rate of USV production in Chapter 4. This difference in overall calling rate is likely due to inherent variation in USV production between genetically distinct cohorts (Burgdorf *et al*., 2005; Hammond *et al*., 2019; Harmon *et al*., 2008; Webber *et al*., 2012).

There are no other studies, as far as I am aware, that monitor the change in USV production or other behaviours after repeated presentations of acoustic stimuli in rats. In this study, rats were presented with the same acoustic stimulus treatment for five consecutive days. After five days of exposure, there was the same overall effect of 50-kHz stimuli on USV production in that rats exposed to 50-kHz acoustic stimuli produced significantly more 50-kHz USVs than Background Noise. There was also a slight increase in USV production on day five compared to day one in those exposed to 50-kHz stimuli but not Background Noise. This consistent effect of 50-kHz acoustic stimuli on USV production suggests that 50-kHz USVs retain their appetitive properties after five days of repeated exposure (Wöhr, 2017). However, it is likely that the process used to generate different acoustic tracks for each day also contributed to reducing habituation to the acoustic stimuli (Wöhr and Schwarting, 2012). Overall, the increase in subject-produced USVs in comparison to Background Noise suggests that exposure to 50-kHz acoustic stimuli successfully induces a positive affective state in juvenile male Wistar rats during both single and repeated presentations. The convergence of positive affective state between acoustic stimuli and subject rats could be interpreted as emotional contagion having occurred (Hatfield *et al*., 1993; Waal, 2007).

In both Chapter 4 and this study, there was an unexpected effect of White Noise on subject-produced 50-kHz USVs during a single exposure; subject produced USVs increased in response to White Noise to a similar level to those produced on exposure to 50-kHz acoustic stimuli. The White Noise stimulus used in these studies was generated with the initial aim of providing an additional control stimulus that provides the same level of an auditory signal without the modulated properties of USVs. As previous work has suggested that white noise in the range of 0 to 27-kHz produces behavioural indicators of aversion (Endres *et al*., 2007; Wöhr and Schwarting, 2012), the White Noise presented here was filtered to remain above 30-kHz. As a result of this process, the White Noise acoustic stimuli could potentially be arousing and of positively-valenced novelty compared with the general noise of the facility and the Background Noise acoustic stimuli generated from recordings of this general noise.

Considering that rats show rapid habituation to complex acoustic stimuli, *i.e.,* 50-kHz USVs (Wöhr and Schwarting, 2012), it is likely that exposure to a less complex stimulus such as White Noise would result in decreased interest and arousal over repeated presentations. Although the White Noise acoustic stimulus underwent the same process as the 50-kHz USVs, the lack of complexity or relevance for social communication is also likely to contribute to rapid habituation to White Noise. Consistent with this, the effect of White Noise on USV production by subjects did not remain consistent after five days of repeated exposure; on day five, USV production in response to White Noise dropped to a similar level to that produced in response to Background Noise. Overall, the increase in subject-produced USVs on day one suggests that exposure to White Noise acoustic stimuli successfully induces a positive affective state in juvenile male Wistar rats during a single but not repeated presentation. The lack of communicative information in the White Noise acoustic stimulus suggests that the increase in positive affective state is likely to result from positively-valenced arousal rather than emotional contagion.

Approach behaviour indicated that rats consistently perceived the 50-kHz stimuli as positive

The use of approach behaviour to measure an animal's perception of certain stimuli is based on the theory that the perceived valence of specific stimuli underpins whether an animal will exhibit approach or avoidance movement (Kurt, 1936). In this study, acoustic stimuli had a consistent effect on the frequency and duration of approach behaviour towards the speaker across days one and five; rats conducted the most approach behaviour in response to 50-kHz acoustic stimuli, followed by those exposed to White Noise and then Background Noise. While there was a decrease in time spent conducting approach behaviour towards the speaker on day five compared to day one, this was constant across all acoustic stimuli, suggesting a universal habituation effect. This result is analogous to that found in the radial maze paradigm, where playback of 50-kHz USVs consistently increases approach behaviour towards the sound source (Schwarting *et al*., 2018; Wöhr and Schwarting, 2007).

Similarly, in the first use of the home cage playback in Chapter 4 (Experiment 3, Chapter 4), there was higher levels of approach behaviour in response to 50-kHz acoustic stimuli. Taken together, these results provide evidence that rats are the most motivated to approach the 50-kHz acoustic stimuli.

The radial maze paradigm relies on approach toward the sound source as one of two main outcome measures (Schwarting *et al*., 2018; Wöhr *et al*., 2016). However, tests of approach behaviour have been criticised for their use to infer affective state (Waiblinger *et al*., 2006). While approach behaviour can indicate whether a presented stimulus is perceived as positive or negative (Kurt, 1936), it does not directly indicate the valence of the emotion which initially drove the motivation to approach or avoid the stimulus (Berridge, 1996; Fraser and Duncan, 1998). For example, the motivation to eat a certain substance can be driven as a result of pleasure, associated with a positive affective state, or hunger, associated with a negative affective state (Kirkden and Pajor, 2006). Further, the inclination for an individual to approach a stimulus may be a result of simpler curiosity rather than enjoyment (Waiblinger *et al*., 2006). As such, whilst approach behaviour can indicate whether an animal is motivated to approach a stimulus, an additional indicator of valence is required to determine the underlying affective state, such as USVs (Chapter 1).

The production of 50-kHz USVs increased in response to 50-kHz acoustic stimuli, suggesting that the underlying affective state driving motivation towards the 50-kHz acoustic stimulus across both days is positive. Following this logic, rats also appeared to perceive the White Noise acoustic stimuli as positive during the first presentation on day one, considering the number of subject-produced 50-kHz USVs and the intermediate level of approach behaviour compared to 50-kHz acoustic stimuli and Background Noise. However, as USV production in White Noise decreased to a similar level to that produced in response to Background Noise on day five, the positive perception of White Noise can be argued to reduce over time. This effect appears to be somewhat independent of approach behaviour, as rats were still motivated to interact with the speaker more than rats exposed to Background Noise. These results further support the need to use an additional indicator of valence to allow interpretation of underlying affective state.

The change in positive affective state was not expressed by changes in sucrose preference

The sucrose preference test is widely employed for assessing anhedonia in rodent models of depression (Scheggi *et al*., 2018). Anhedonia is a negatively valenced affective state described as the inability to feel pleasure (Gorwood, 2008) and is generated in rats through chronic exposure to stressors (Deussing, 2007). In the sucrose preference test, anhedonia is expressed through reduced consumption of a low-concentration sucrose solution than controls and a decreased preference for sucrose over water (Papp *et al*., 1991; Willner, 1997; Willner *et al*., 1992). The reduced value placed on sucrose when animals are in a negative affective state results from functional and morphological changes to the mesolimbic reward pathway (Scheggi *et al*., 2018). Changes to this pathway are also seen in response to positively valenced experiences, such as mating and consumption of psychostimulants (Pitchers *et al*., 2010; Robinson and Kolb, 1999). Considering this, it could be expected that the reward value of sucrose also changes in response to positive stimuli, such that the sucrose preference test could also be considered a test of hedonia, as well as anhedonia.

As far as I am aware, the first use of the sucrose preference test for this purpose was in Chapter 2. After five days of the positive experience of playful handling, rats showed increased preference and consumption of a high concentration sucrose solution (20%) over water compared with control rats. As such, the sucrose preference test was applied in this study after five days as an additional method of assessing the impact of playback of acoustic stimuli on affective state. Specifically, I hypothesised that rats exposed to 50-kHz and White Noise acoustic stimuli would consume more sucrose than rats exposed to Background Noise as an expression of the positive affective state induced by these stimuli as evidenced in Chapter 4. In contrast to this hypothesis, there was no effect of acoustic stimuli treatment on sucrose consumption, with all rats showing a preference for sucrose over water. This suggests that the sucrose preference test is not sensitive to changes in positive affective state through the playback of acoustic stimuli and requires further validation to be used as a method of assessing positive affect.

The positive affective state induced by 50-kHz acoustic stimuli was expressed by an increase in social play during playback on day five

Whilst play has long been proposed as a promising indicator of positive affective state in animals, investigations into the change in play in response to a positive experience are limited in number (Ahloy-Dallaire *et al*., 2017). As such, a secondary aim of this research was to investigate the sensitivity of play to the manipulation of affective state using the home cage playback paradigm. The production of 50-kHz USVs and approach behaviour indicates that rats exposed to 50-kHz acoustic stimuli were in a positive affective state on both days one and five. These measures also indicate that rats were in positive affective state in response to White Noise on day one. Accordingly, if play were sensitive to this induction of positive affective state, an increase in play during playback of these acoustic stimuli would be expected.

In contrast with this hypothesis, on day one, the positive affective state induced by 50-kHz and White Noise stimuli was not matched with increased play; social play was reduced in response to 50-kHz stimuli, whilst social play during White Noise was similar to that during Background Noise. These results are similar to those found in Chapter 4, where social play was reduced when rats were exposed to 50-kHz acoustic stimuli compared to Background Noise yet induced when exposed to White Noise. In Chapter 4, I suggest that this lack of effect of 50-kHz acoustic stimuli on play may be due to the absence of other sensory input associated with play behaviour other than USV production, *i.e.,* olfactory, visual, and tactile stimulation (Pellis and Pellis, 2013). However, the increased expression of social play during playback on day five in response to the 50-kHz acoustic stimulus compared to White Noise and Background Noise observed in this chapter does not support this theory. Instead, these results suggest that presentation of 50-kHz USV alone is sufficient to stimulate play after repeated presentations.

The need for repeated presentations to induce social play, despite the presence of a consistent positive affective state, may be explained by a change in the motivational priorities of the rats from day one to five. Rats are highly motivated to explore their environment, forgoing rewards and increasing risk to explore (Franks *et al*., 2013). Exploration behaviour is one of the most basic but critical adaptive behaviours, as it allows animals to evaluate and adapt to their environment, increasing the chance of survival (Alvarez and Alvarez, 2008). In comparison, play is regarded as a 'luxury' behaviour in that is thought to only occur in the absence of threats to survival (Fraser and Duncan, 1998; Lawrence, 1987). Thus, on day one, when rats are first exposed to the playback stimuli for the first time, they are likely to place a high motivational priority on exploring this new stimulus. By day five, rats have had time to evaluate the risk-value of the stimulus to survival and subsequently place less priority on exploration behaviour. This allows rats the low-risk opportunity to conduct social play as an expression of positive affective state. The decrease in approach behaviour towards the speaker and general locomotor behaviour on day one to day five supports this theory. Overall, this suggests that play can indicate the presence of positive affective state but given the sensitivity of play to the animals' priorities it may be inhibited by higher priority motivations and behaviours (see Fraser and Duncan, 1998).

The effect of a positive affective state induced by the 50-kHz and White Noise stimuli on play may also be expressed outwith the direct experience. All rats conducted more solitary and social play in the home cage before than after the playback experience. Previous work suggests that behaviour that occurs before an expected experience could be taken as a reflection of the value an animal places on a reward and the anticipation associated with receiving that reward (van der Harst and Spruijt, 2007). Thus, the amount of play expressed before the expected presentation of acoustic stimulus could potentially indicate the level of positive affect associated with that stimulus. However, the effects of acoustic stimuli on home cage play were limited to the number of social play events; the highest increase in the number of social play events from baseline was seen in response to White Noise, with little change in social play before playback from day one to five in response to 50-kHz or Background Noise. This finding suggests that the positive affective state induced by acoustic stimuli is not reflected in a change in play in the home cage either before or after the experience.

Conclusion

Overall, this work provides further support for the application of playback in the home cage playback to induce a positive affective state in rats. In response to five consecutive days of presentation with 50-kHz USVs, rats were in a more positive affective state than when exposed to White Noise or Background Noise, as indicated by an increase in subject-produced 50-kHz USVs and approach behaviour towards the sound source. While the effect of 50-kHz acoustic stimuli on indicators of positive affective state remained consistent over repeated presentations, the effect of White Noise on these measures was short-lived. The effect of positively perceived acoustic stimuli on play suggests that social play was sensitive to the playback of acoustic stimuli. However, further work is necessary to untangle the complex relationship between positive affective state and play as an indicator of PAW.

Chapter 6: General discussion

The aims of this doctoral research were twofold; first, to develop methods of inducing a positive affective state in rats, and second, to investigate whether play is sensitive to the manipulation of affective state as an indicator of PAW. In this chapter, I will critically examine how my research has addressed these two aims and highlight potential areas for future research to expand our understanding of positive affective states to promote PAW.

Thesis aims

Aim 1: To develop methods of inducing a positive affective state in rats

While there are well-established methods for inducing negative affective states in rats, there are fewer methods for inducing positive affective states (Makowska and Weary, 2013; Scheggi *et al*., 2018). As such, my primary aim was to further develop methods of inducing a positive affective state in rats under laboratory conditions. There are several stimuli that are perceived positively by rats, such as rewarding foodstuffs (Berridge, 2000), access to conspecifics for social and sexual interaction (Brudzynski and Pniak, 2002; van der Harst *et al*., 2003; Pfaus *et al*., 2001), electrical stimulation of rewarding brain areas (Burgdorf *et al*., 2000) and addictive drugs, such as amphetamines and morphine (Burgdorf *et al*., 2001; Knutson *et al*., 1999). However, these methods typically require aversive steps, such as food and water deprivation, isolation, and surgical procedures to induce a positive affective state. Thus, the positive affective state induced by these methods may be *relatively* positive, *i.e.,* more positive than a negative experience (Ahloy-Dallaire *et al*., 2017). Given this, I selected two methods that did not require aversive procedures. Further, I also wanted to use methods that could later be developed to be used outside of applied research settings, by laboratory personnel, to improve rat welfare on a broader scale. As such, I also considered the complexity, expense of equipment, and level of human involvement required when selecting my potential methods for inducing a positive affective state. Explicitly, the ideal methods for inducing PAW under practical conditions would be simple, inexpensive, and require a small investment once developed.

Since heterospecific play or ‘rat tickling’ was first introduced in the early 2000s, over 70 scientific articles on rat tickling have been published (Bombail *et al*., 2018). As the majority of published experiments report positive responses during tickling, *i.e.,* increased 50kHz USV production and increased approach behaviour towards the human hand (LaFollette *et al*., 2017), I identified tickling as a promising method to induce a positive affective state. Prior to the beginning of this doctoral research, I was involved in discussions on whether tickling is the best way to play with a rat based on our previous research (Bombail *et al*., 2019; Hammond *et al*., 2019). As a result, we determined that an alternative method of heterospecific play, termed ‘playful handling’, might be enjoyable to a wider number of rats (Bombail *et al*., 2021).

The novelty of this method meant that there is no direct evidence that playful handling is an improved method of heterospecific play, with limited evidence to suggest that playful handling is a positive experience for rats. Thus, in order to provide further validation for playful handling as a positive experience, in Chapters 2 and 3, I investigated whether playful handling induced a positive affective state in rats across two different environments; as individuals in an arena and as pairs in the home cage. Across both environments, rats showed an increase in positive affective state, suggesting that playful handling is a successful method of inducing a positive affective state, meeting the primary aim of this research.

The second method I chose to induce a positive affective state was also relatively novel; the playback of ultrasonic vocalisations. In rats, the playback of vocalisations has primarily been used as a behavioural assay for animal methods of human neurodevelopmental disorders with socio-communicative deficits, such as schizophrenia (*e.g.,* Schwarting *et al*., 2018). Considering that the playback of contrastingly valenced USVs reliably induced behaviours indicative of a matched affective state in receivers in these studies (*e.g.,* Wöhr and Schwarting, 2009), playback of specific acoustic stimuli seemed a promising method of inducing a positive affective state. As this work is the first time in which the playback of USVs has been used to promote PAW, I had to design a paradigm for this purpose. In Chapters 4 and 5, I report evidence that playback of 50-kHz USVs induced a positive affective state after a single exposure, having a more robust effect than White Noise after five days of consecutive presentation. This suggests that playback of 50 kHz USVs in the home cage is a successful method of inducing a positive affective state, meeting the primary aim of this research.

Despite meeting the primary aim of inducing a positive affective state in rats there was evidence for individual variation in the level of positive affective state achieved through playful handling and playback of 50-kHz USVs. Individual variation in 50-kHz USV production has been widely reported under different environmental conditions, such as in response to tickling (LaFollette *et al*., 2018a) and receipt of social (Seidisarouei *et al*., 2021) and non-social rewards (Mulvihill and Brudzynski, 2018; Seidisarouei *et al*., 2021). Similarly, there is individual variation in approach behaviour (Elliot and Thrash, 2002; LaFollette *et al*., 2018b). To investigate individual variation in my experiments, I calculated the coefficient of variation, which is a descriptive measure of the relative dispersal of data points around the mean, with a higher percentage indicating a higher level of dispersal and, thus, higher individual variation. In response to both methods, the coefficient of variation in USV production was over 50% suggesting that while both methods induced a more positive affective state than controls, there was a high degree of individual variation. The coefficient of variation tentatively suggests that playful handling was a more successful method of inducing a positive affective state based on individual variation. This effect could be explained by the richer sensory experience of playful handling, in which rats receive tactile, olfactory, and visual stimulation, compared to playback, in which rats only receive auditory stimulation. Overall, these results suggest that any form of treatment that aims to induce positive affective states must consider individual variation in the enjoyment of the experience (Bombail *et al*., 2021).

The main limitation across both methods was the use of male rats of a particular strain, sex and age range. The choice to focus on one sex and strain was based on a trade-off between time available and the number of individuals required for statistical power, keeping the reduction of animals used in research in mind. The decision to use males was based on previous work on tickling and playback experiments, which are biased toward males (LaFollette *et al*., 2017; Olszyński *et al*., 2020; Seffer *et al*., 2014). For example, LaFollette *et al*. (2017) report that only 28% of studies on tickling used female rats. Considering that my studies were somewhat novel, the consistency in using males would assist in providing comparison to previous work. I also chose to use Wistar rats rather than other strains for a similar reason; Wistar rats are a commonly used strain for tickling and are the predominant strain used in playback studies (LaFollette *et al*., 2017; Seffer *et al*., 2014). The experiments in this thesis also only used juveniles between 37 and 43 days old, with rats reaching adulthood at around 60 days. This age range was chosen in light of previous work, which indicates that juveniles produce more robust responses to tickling and playback of acoustic stimuli than adults (Burgdorf and Panksepp, 2001; Wöhr and Schwarting, 2009, 2007, 2012). These age-dependent effects can be attributed to higher levels of social motivation in juveniles compared to adults (Seffer *et al*., 2014). Further, this age range also aligns with the sensitive period of play in which juvenile males show higher frequencies of play fighting (Pellis and Pellis, 2013), allowing the investigation of my second aim. Thus, before playful handling and playback of 50-kHz USVs can be widely advocated as promoting PAW for all rats, further work would be necessary to establish the effects on females, adults, and rats of other strains.

Aim 2: To investigate whether play is sensitive to the manipulation of affective state as an indicator of PAW

A secondary aim of this research was to investigate whether play is sensitive to the manipulation of affective state and can act as an indicator of PAW. While play has long been proposed as a promising indicator of positive emotions and welfare in domesticated animals (*e.g.,* Boissy *et al*., 2007; Held and Špinka, 2011), there is insufficient evidence to support the use of play as a universal indicator of an animal's welfare, particularly PAW (Ahloy-Dallaire *et al*., 2017). As such, this aim was included to address the lack of research into the expression of play when animals are experiencing positive affect. The investigation of this aim was reliant on the success of achieving the primary aim of playful handling and playback of acoustic stimuli inducing a positive affective state. As both methods effectively induced a positive affective state, I investigated the relationship between affective state and play during the experience and out with the experience in the home cage.

The results in this thesis suggest there is complexity in whether play reflects the concurrent positive affective state of rats. While there was a relationship between positive affective state and play expressed *during* the presentation of acoustic stimuli, this was not seen in response to playful handling. There was no difference in play behaviour between rats who were playfully handled or control rats as individuals in an arena or as pairs in the home cage during handling. During the first day of playback, the positive affective state induced by the presentation of White Noise stimuli, but not 50-kHz USVs, was expressed by increased social play. On the fifth day of repeated playback, only 50-kHz USVs induced a positive affective state, with rats exposed to 50-kHz USVs conducting more social play. This effect of White Noise on positive affective state and play did not last until day five of repeated presentation. These results suggest that the increase in play as a reflection of a concurrent positive affective state is context-specific.

The discrepancy in the expression of play in response to playful handling and playback of acoustic stimuli can speculatively be explained by two factors. First, the positive affective state induced by playful handling may not induce rats to play as the experience involves a playful interaction with the experimenter. Playful handling was developed to include as many characteristics of rat-rat social play as can be achieved in a human-rat interaction. While we cannot currently state that rats perceive playful handling as a direct alternative to social play, there is the potential that the motivation to play during the experience is at least partly fulfilled through playful handling. Second, the assessment of changes in play in response to playful handling is hindered by the time available for these changes to be expressed. During playful handling, the rat only has a total of 1 minute to express play behaviour in the pauses between handling bouts. In comparison, during the playback of acoustic stimuli, rats have a total of 6 interrupted minutes to express changes in play behaviour. Overall, these results suggest that while play can reflect a concurrent positive affective state in some contexts, it is not universally expressed when rats are experiencing positive affect. As such, the simple presence or absence of play cannot comprehensively be used as an indicator of PAW.

There were more consistent links between positive affective state and the expression of play in the home cage across my methods of inducing a positive affective state; after five days, there was an increase in play in the home cage prior to the experience in response to both playful handling (Chapter 2) and the playback of 50-kHz USVs (Chapter 5). While these results suggest an overall relationship between pre-existing positive affective state and play, there were nuanced differences in the type of play expressed in response to each method. In response to playful handling, the relationship between positive affect and play was specific to solitary play. In response to the playback of 50-kHz USVs, the effect was specific to social play.

It has long been theorised that that different types of play may have evolved independently, based on the observation that some species conduct only one type of play while others conduct multiple types (Burghardt, 2005). For example, while rats engage in solitary and social play, mice only conduct solitary play (Pellis and Pellis, 1991). As such, solitary and social play may represent distinct motivational systems (Melotti *et al*., 2014) controlled by different brain mechanisms (Papini, 2020). The investigation into this question is further obstructed by the standard practice within animal research to focus on one type of play or aggregate subtypes of play together (Winder *et al*., 2017). This practice is particularly evident in studies on rat play, with the seminal works on play in rats having a narrow focus on social play (Panksepp, 1981; Pellis and Pellis, 2013; Vanderschuren and Trezza, 2013). In contrast, categorising play into sub-types is a basic standard in research on human play, with researchers often taking further steps to examine the nature of this play, *e.g.,* by measuring different elements of play and their temporal expression (Ahloy-Dallaire *et al*., 2017). These detailed measurements have allowed for a comprehensive investigation into qualitative and quantitative aspects of play in children, revealing previously unfound links with well-being (Ahloy-Dallaire *et al*., 2017). Considering this and the results in this thesis, further research should, at a minimum, include measurement of both types of play, with a preference to look at more qualitative aspects, such as the preference for different elements or using Qualitative Behavioural Assessment (as discussed later).

As with my primary aim, the main limitation of this study in relation to the investigation of play as an indicator of PAW was using only males of one particular strain and age. Considering the known differences between males and females in the quantity and characteristics of play (Schwarting, 2018), the results found in this study on males play may not apply to females. However, the literature suggests that sexual dimorphism in play is limited to social play, with rats of both sexes expressing similar trends in solitary play (Pellis and Pellis, 1983). As such, if these experiments were repeated in females, I would expect the effects of positive affective state on solitary play on females to be similar to males, with any differences seen in social play. I would hypothesise that females would show reduced effects of the positive affective state on playful handling and playback of 50-kHz USV stimuli expressed by social play, given that they naturally play less than males (Pellis, 2002). Differences in social play may also be seen in more specific aspects of play than the overall duration. For example, as there is sexual dimorphism in pinning frequency, there may be differences in dorsal contacts and pinning during play in female playfully handled rats compared to controls, which were not seen here in males (Auger and Olesen, 2009).

Overall, the results in this thesis contribute to filling the gap in the literature examining the relationship between positive affective state and play as identified by Ahloy-Dallaire *et al*. (2018). While there is a positive relationship between concurrent and pre-existing positive affective state and play, this relationship is not straightforward and is subject to certain complexities. First, the absence of play does not indicate the absence of positive affective state and PAW. Secondly, play is not a single behaviour: it includes sub-types with distinct qualitative characteristics that differ in their underlying motivation for expression.

Future work

There are many potential directions for the continuation of this research, for example, the investigation of the effectiveness of playful handling compared to the standard tickling protocol as outlined by Bombail *et al*. (2022). Here, I focus on promising areas for future work to assess the presence of positive affective state and the associated implication for PAW assessment.

A deeper investigation into the meaning of USVs in rats

In this study and within the wider literature, USVs have proved to be a promising tool for the measurement of affective state in rats (Burgdorf *et al*., 2005; Hinchcliffe *et al*., 2020; LaFollette *et al*., 2018a; Panksepp and Burgdorf, 2000; Wöhr and Schwarting, 2013). In this thesis, I chose to use the total number of 50-kHz USVs as an indicator of positive affective state. However, the ‘50-kHz USVs’ category encompasses calls with significant variations in frequency, shape, and duration with as many as 14 subtypes of 50-kHz USVs (Wright *et al*., 2010). Thus, there is the potential that only certain types of 50-kHz USVs are associated with positive affect (Wöhr *et al*., 2008). Investigation into the distinction between subtypes of 50-kHz USVs has been the subject of studies for over the past ten years (Wright *et al*., 2010) yet there is still no clear consensus on the number of subtypes of 50-kHz USVs and the associated functions of these different subtypes.

A detailed examination into the meaning of the subtypes of USV will aid the interpretation of rats’ affective responses. However, this investigation is currently limited by human capability in terms of capacity and level of detail. Manual coding of USVs is laborious and time-consuming, even when classifying USVs into the broad group of 50-kHz calls rather than according to subtype. As with all behavioural assessment using manual coding, the complexity of USVs means that there is always the risk of human error when categorising sub-types and a low degree of inter and intra-observer reliability (Martin and Bateson, 1993). Manual coding by a human also limits the complexity at which analysis of USV production can be conducted, *e.g.,* we cannot look for patterns or relationships in the structure of USVs (Lawson *et al*., 2021). In light of these limitations, I strongly believe that the development of USVs as an indicator of not only positive affective states but rat subjective state overall relies on the optimisation of USV detection and analysis software solutions.

In the last few years, there has been a rise in the development of different software packages for this purpose, such that there is demand across research fields, including pre-clinical neuroscience and animal welfare (Binder *et al*., 2021; Coffey *et al*., 2019; Goussha *et al*., 2022; Premoli *et al*., 2021). Using these software solutions will not only allow for more efficient, objective and accurate assessment, but also allow us to examine complex relationships and patterns in the behavioural data which a human is simply incapable of. This capability was evidenced by Lawson *et al*. (2021), where the employment of DeepSqueak, a machine learning-driven tool (Coffey *et al*., 2019), revealed hidden qualities in the data which were not seen during manual coding of USV categories. However, the novelty of such technologies means that they require validation before we can widely implement them in experimental conditions. Thus, while the future lies in these new technologies, it must be accompanied by the active participation of researchers experienced in the framework of behavioural assessment and well-designed validation experiments.

The application of Qualitative Behavioural Assessment to rats

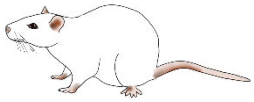
The analysis of behaviour using quantitative approaches is central to the assessment of rodent welfare (Jirkof *et al*., 2019). However, the use of behaviour to investigate welfare is not restricted to a strict ethological approach, *i.e.,* the measurement of specific behavioural elements. Qualitative Behavioural Assessment (QBA) is a unique approach to the assessment of animal welfare that bridges the gap that traditionally exists between subjective judgments and scientific approaches (Wemelsfelder *et al*., 2001, 2000). This approach is described as ‘whole body approach’ in which the ‘expressive quality’ of an animal’s dynamic interaction with the environment is assessed. It is centred on the ability of humans to incorporate information of not only *what* animals are doing but *how* they are doing it (Wemelsfelder *et al*., 2001, 2000). Instead of measuring the frequency and duration of certain behaviours, human scorers are asked to describe the expression of the animal based on body language in self-generated terms such as ‘content’ or ‘anxious’ (Wemelsfelder *et al*., 2001, 2000). In the 20 years and more since the first experimental paper on QBA was published (Wemelsfelder *et al*., 2000), several studies have provided evidence for the validity of this method. QBA not only shows strong correlations with behavioural and physiological measures

(Ceballos *et al*., 2021; Hintze *et al*., 2017; Wickham *et al*., 2012) but has been validated using pharmacological manipulation (Rutherford *et al*., 2012). In relation to the assessment of PAW, QBA is particularly promising as it does not rely on the expression of a single marker behaviour to infer a positive affective state.

Whilst QBA has been applied to a wide range of species (*e.g.,* dogs; Shaw *et al*., 2022, horses; Hintze *et al*., 2017, salmon; Jarvis *et al*., 2021, dairy cows; Ceballos *et al*., 2021 and giraffes; Patel *et al*., 2019), it is surprisingly yet to be applied to rats. In light of this novelty and its promise for the assessment of PAW, the development of QBA for rats was one of my initial directions for this thesis. I specifically aimed to investigate whether human-scored QBA judgements of behavioural expression change when viewing footage of juvenile laboratory rats whose affective state had been altered using playback of positively-valenced 50-kHz USVs compared with the presentation of Background Noise. To do this I would compare the behavioural data, including USVs, with the QBA data to establish if QBA picks up the treatment effects. The aim of this initial investigation was specific to the playback of acoustic stimuli as there was a potential confound introduced by including a human-rat interaction. As the videos collected during the experiments in Chapter 4 were not of sufficient quality for QBA (due to the use of red light to reduce stress and maximise behavioural output of nocturnal animals), I would need to conduct another experiment and complete the steps in Chapter 4 to determine the affective state of the rats. As such, the aim to apply QBA for the first time to rats was out with the scope of this thesis, yet remains a promising direction for future work.

Concluding remarks

The two aims of this doctoral research were to develop methods of inducing positive affective state and to investigate whether play is sensitive to this positive affective state as a promising indicator of PAW. In relation to the first aim, this work provides support for the use of two innovative methods to induce a positive affective state in rats, achieved through a total of six independent experiments. This work also contributes to the currently limited body of evidence that suggests that positive affect stimulates play. Of most significance is the fundamental evidence provided by this work for the capacity of rats to experience positive affective states and the relative ease in which positive affective states can be induced in this species. This further highlights our responsibility to incorporate PAW for rats as a prerequisite for their use.



References

Abbott, A., 2009. Return of the rat. Nature 460, 788. https://doi.org/10.1038/460788a

Adriaense, J.E.C., Koski, S.E., Huber, L., Lamm, C., 2020. Challenges in the comparative study of empathy and related phenomena in animals. Neurosci Biobehav Rev 112, 62–82. https://doi.org/10.1016/j.neubiorev.2020.01.021

Ahloy-Dallaire, J., Espinosa, J., Mason, G., 2017. Play and optimal welfare: does play behaviour indicate the presence of positive affective states? Behav Process 156, 3–15. https://doi.org/10.1016/j.beproc.2017.11.011

Alvarez, E.O., Alvarez, P.A., 2008. Motivated exploratory behaviour in the rat: The role of hippocampus and the histaminergic neurotransmission. Behav Brain Res 186, 118–125. https://doi.org/10.1016/j.bbr.2007.07.038

American Psychiatric Association, 2013. Diagnostic and statistical manual of mental disorders: DSM-5. American Psychiatric Publishing, Washington, D.C.

Anderson, C., Keyserlingk, M. von, Lidfors, L., Weary, D., 2020. Anticipatory behaviour in animals: A critical review. Anim Welfare 29, 231–238. https://doi.org/10.7120/09627286.29.3.231

Anderson, J.W., 1954. The Production of Ultrasonic Sounds by Laboratory Rats and Other Mammals. Science 119, 808–809. https://doi.org/10.1126/science.119.3101.808

Antoniuk, S., Bijata, M., Ponimaskin, E., Wlodarczyk, J., 2018. Chronic Unpredictable Mild Stress for Modeling Depression in Rodents:Meta-analysis of Model Reliability. Neurosci Biobehav Rev 99, 101–116. https://doi.org/10.1016/j.neubiorev.2018.12.002

Arifin, W., Zahiruddin, W., 2017. Sample Size Calculation in Animal Studies Using Resource Equation Approach. Malays J Medical Sci 24, 101–105. https://doi.org/10.21315/mjms2017.24.5.11

Atsak, P., Orre, M., Bakker, P., Cerliani, L., Roozendaal, B., Gazzola, V., Moita, M., Keysers, C., 2011. Experience Modulates Vicarious Freezing in Rats: A Model for Empathy. PLoS ONE 6, e21855. https://doi.org/10.1371/journal.pone.0021855

Auger, A.P., Olesen, K.M., 2009. Brain Sex Differences and the Organisation of Juvenile Social Play Behaviour. J Neuroendocrinol 21, 519–525. https://doi.org/10.1111/j.1365-2826.2009.01871.x

Axelrod, J., Reisine, T.D., 1984. Stress Hormones: Their Interaction and Regulation. Science 224, 452–459. https://doi.org/10.1126/science.6143403

Bailey, C.S., Hsiao, S., King, J.E., 1986. Hedonic reactivity to sucrose in rats: Modification by pimozide. Physiol Behav 38, 447–452. https://doi.org/10.1016/0031-9384(86)90409-9

Bailey, K.R., Crawley, J.N., 2009. Anxiety-Related Behaviors in Mice. In: Buccafusco JJ, editor. Methods of Behavior Analysis in Neuroscience. 2nd ed. Boca Raton (FL): CRC Press/Taylor & Francis; . PMID: 21204329.

Barfield, R., Geyer, L., 1972. Sexual behavior: Ultrasonic postejaculatory song of the male rat 176, 1349–50. https://doi.org/10.1126/science.176.4041.1349

Barker, D.J., 2018. Chapter 24 - Ultrasonic Vocalizations as an Index of Positive Emotional State, in: Brudzynski, S.M. (Ed.), Handbook of Behavioral Neuroscience. Elsevier, pp. 253–260. https://doi.org/10.1016/b978-0-12-809600-0.00024-x

Bateson, P., Martin, P., 2014. Play, playfulness, creativity and innovation. Cambridge University Press, Cambridge. https://doi.org/10.1017/cbo9781139057691

Bayne, K., Würbel, H., 2014. The impact of environmental enrichment on the outcome variability and scientific validity of laboratory animal studies. Revue Sci Et Technique Int Office Epizootics 33, 273–80. https://doi.org/10.20506/rst.33.1.2282

Begni, V., Sanson, A., Pfeiffer, N., Brandwein, C., Inta, D., Talbot, S.R., Riva, M.A., Gass, P., Mallien, A.S., 2020. Social isolation in rats: Effects on animal welfare and molecular markers for neuroplasticity. PLOS One 15, e0240439. https://doi.org/10.1371/journal.pone.0240439

Bekoff, M., 2001. The Evolution of Animal Play, Emotions, and Social Morality: On Science, Theology, Spirituality, Personhood, and Love. Zygon 36, 615–655. https://doi.org/10.1111/0591-2385.00388

Bekoff, M., 1984. Social Play Behavior. Bioscience 34, 228–233. https://doi.org/10.2307/1309460

Berridge, K.C., 2000. Measuring hedonic impact in animals and infants: microstructure of affective taste reactivity patterns. Neurosci Biobehav Rev 24, 173–198. https://doi.org/10.1016/s0149-7634(99)00072-x

Berridge, K.C., 1996. Food reward: Brain substrates of wanting and liking. Neurosci Biobehav Rev 20, 1–25. https://doi.org/10.1016/0149-7634(95)00033-b

Berridge, K.C., Kringelbach, M.L., 2015. Pleasure Systems in the Brain. Neuron 86, 646–664. https://doi.org/10.1016/j.neuron.2015.02.018

Berridge, K.C., Robinson, T.E., 1998. What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? Brain Res Rev 28, 309–369. https://doi.org/10.1016/s0165-0173(98)00019-8

Bialy, M., Rydz, M., Kaczmarek, L., 2000. Precontact 50-kHz Vocalizations in Male Rats During Acquisition of Sexual Experience. Behav Neurosci 114, 983–990. https://doi.org/10.1037/0735-7044.114.5.983

Bigelow, L.J., Fiset, C., Jarvis, J.H.M., Macleod, S., Wöhr, M., Benke, T.A., Bernard, P.B., 2022. Early-life seizures modify behavioral response to ultrasonic vocalization playback in adult rats. Epilepsy Behav 127, 108494. https://doi.org/10.1016/j.yebeh.2021.108494

Binder, M.S., Pranske, Z.J., Lugo, J.N., 2021. Evaluating the DeepSqueak and Mouse Song Analyzer vocalization analysis systems in C57BL/6J, FVB.129, and FVB neonates. J Neurosci Meth 364, 109356. https://doi.org/10.1016/j.jneumeth.2021.109356

Boissy, Alain, Manteuffel, G., Jensen, M.B., Moe, R.O., Spruijt, B., Keeling, L.J., Winckler, C., Forkman, B., Dimitrov, I., Langbein, J., Bakken, M., Veissier, I., Aubert, A., 2007. Assessment of positive emotions in animals to improve their welfare. Physiol Behav 92, 375–397.

Bombail, V., Barret, B., Raynaud, A., Jerôme, N., Saint-Albin, A., Ridder, C., Collin, A., Leterrier, C., Guilloteau, L.A., Nielsen, B.L., 2018. In search of stress odours across species: Behavioural responses of rats to faeces from chickens and rats subjected to various types of stressful events. Appl Anim Behav Sci 205, 216–226. https://doi.org/10.1016/j.applanim.2017.10.013

Bombail, V., Brown, S.M., Hammond, T.J., Meddle, S.L., Nielsen, B.L., Tivey, E.K.L., Lawrence, A.B., 2021. Crying With Laughter: Adapting the Tickling Protocol to Address Individual Differences Among Rats in Their Response to Playful Handling. Frontiers Vet Sci 8, 1–5. https://doi.org/10.3389/fvets.2021.677872

Bombail, V., Jerome, N., Lam, H., Muszlak, S., Meddle, S., Lawrence, A., Nielsen, B., 2019. Odour conditioning of positive affective states: Rats can learn to associate an odour with being tickled. Plos One 14, e0212829. https://doi.org/10.1371/journal.pone.0212829

Bracke, M.B.M., Hopster, H., 2006. Assessing the Importance of Natural Behavior for Animal Welfare. J Agric Environ Ethics 19, 77–89. https://doi.org/10.1007/s10806-005-4493-7

Brambell, F., 1965. Report of the Technical Committee to Enquire Into the Welfare of Animals Kept Under Intensive Livestock Husbandry Systems. HMSO, London, p. 85.

Brennan, K., Roberts, D., Anisman, H., Merali, Z., 2001. Individual differences in sucrose consumption in the rat: motivational and neurochemical correlates of hedonia. Psychopharmacology 157, 269–276. https://doi.org/10.1007/s002130100805

Briefer, E.F., 2018. Vocal contagion of emotions in non-human animals. Proc Royal Soc B Biological Sci 285, 20172783. https://doi.org/10.1098/rspb.2017.2783

Briefer, E.F., 2012. Vocal expression of emotions in mammals: mechanisms of production and evidence. J Zool 288, 1–20. https://doi.org/10.1111/j.1469-7998.2012.00920.x

Briefer, E.F., Sypherd, C.C.-R., Linhart, P., Leliveld, L.M.C., Torre, M.P. de la, Read, E.R., Guérin, C., Deiss, V., Monestier, C., Rasmussen, J.H., Špinka, M., Düpjan, S., Boissy, A., Janczak, A.M., Hillmann, E., Tallet, C., 2022. Classification of pig calls produced from birth to slaughter according to their emotional valence and context of production. Sci Rep-uk 12, 1–10. https://doi.org/10.1038/s41598-022-07174-8

Broom, D.M., 1993. Assessing the welfare of modified or treated animals. Livest Prod Sci 36, 39–54. https://doi.org/10.1016/0301-6226(93)90136-6

Broom, D.M., Johnson, K.G., 2019. Stress and Animal Welfare, 2nd ed. Springer, Berlin, Germany.

Browning, J.R., Browning, D.A., Maxwell, A.O., Dong, Y., Jansen, H.T., Panksepp, J., Sorg, B.A., 2011. Positive affective vocalizations during cocaine and sucrose self-administration: A model for spontaneous drug desire in rats. Neuropharmacology 61, 268–275. https://doi.org/10.1016/j.neuropharm.2011.04.012

Brudzynski, S., 2013. Ethotransmission: communication of emotional states through ultrasonic vocalization in rats. Curr Opin Neurobiol 23, 310–317. https://doi.org/10.1016/ j.conb.2013.01.014

Brudzynski, S.M., 2007. Ultrasonic calls of rats as indicator variables of negative or positive states: Acetylcholine–dopamine interaction and acoustic coding. Behav Brain Res 182, 261–273. https://doi.org/10.1016/j.bbr.2007.03.004

Brudzynski, S.M., Pniak, A., 2002. Social contacts and production of 50-kHz short ultrasonic calls in adult rats. J Comp Psychol 116, 73. https://doi.org/10.1037/0735-7036.116.1.73

Burgdorf, J., Ghoreishi-Haack, N., Cearley, C., Kroes, R., Moskal, J., 2019. Rat ultrasonic vocalizations as a measure of the emotional component of chronic pain. Neuroreport 30, 863–866. https://doi.org/10.1097/wnr.0000000000001282

Burgdorf, J., Knutson, B., Panksepp, J., 2000. Anticipation of Rewarding Electrical Brain Stimulation Evokes Ultrasonic Vocalization in Rats. Behav Neurosci 114, 320–327. https://doi.org/10.1037/0735-7044.114.2.320

Burgdorf, J., Knutson, B., Panksepp, J., Ikemoto, S., 2001. Nucleus Accumbens Amphetamine Microinjections Unconditionally Elicit 50-kHz Ultrasonic Vocalizations in Rats. Behav Neurosci 115, 940–944. https://doi.org/10.1037/0735-7044.115.4.940

Burgdorf, J., Kroes, R.A., Moskal, J.R., Pfaus, J.G., Brudzynski, S.M., Panksepp, J., 2008. Ultrasonic Vocalizations of Rats (Rattus norvegicus) During Mating, Play, and Aggression: Behavioral Concomitants, Relationship to Reward, and Self-Administration of Playback. J Comp Psychol 122, 357–367. https://doi.org/10.1037/a0012889

Burgdorf, J., Panksepp, J., 2001. Tickling induces reward in adolescent rats. Physiol Behav 72, 167–173. https://doi.org/10.1016/s0031-9384(00)00411-x

Burgdorf, J., Panksepp, J., Brudzynski, S.M., Beinfeld, M.C., Cromwell, H.C., Kroes, R.A., Moskal, J.R., 2009. The effects of selective breeding for differential rates of 50-kHz ultrasonic vocalizations on emotional behavior in rats. Dev Psychobiol 51, 34–46. https://doi.org/10.1002/dev.20343

Burgdorf, J., Panksepp, J., Brudzynski, S.M., Kroes, R., Moskal, J.R., 2005. Breeding for 50-kHz Positive Affective Vocalization in Rats. Behav Genet 35, 67–72. https://doi.org/10.1007/s10519-004-0856-5

Burgdorf, J., Panksepp, J., Moskal, J.R., 2011. Frequency-modulated 50kHz ultrasonic vocalizations: a tool for uncovering the molecular substrates of positive affect. Neurosci Biobehav Rev 35, 1831–1836. https://doi.org/10.1016/j.neubiorev.2010.11.011

Burgdorf, J., Wood, P.L., Kroes, R.A., Moskal, J.R., Panksepp, J., 2007. Neurobiology of 50-kHz ultrasonic vocalizations in rats: Electrode mapping, lesion, and pharmacology studies. Behav Brain Res 182, 274–283. https://doi.org/10.1016/j.bbr.2007.03.010

Burghardt, G., 2005. The genesis of animal play: Testing the limits. The MIT Press. https://doi.org/10.7551/mitpress/3229.001.0001

Burke, C.J., Kisko, T.M., Swiftwolfe, H., Pellis, S.M., Euston, D.R., 2017. Specific 50-kHz vocalizations are tightly linked to particular types of behavior in juvenile rats anticipating play. PLOS One 12, e0175841. https://doi.org/10.1371/journal.pone.0175841

Burman, O., Ilyat, A., Jones, G., Mendl, M., 2007. Ultrasonic vocalizations as indicators of welfare for laboratory rats (Rattus norvegicus). Appl Anim Behav Sci 104, 116–129. https://doi.org/10.1016/j.applanim.2006.04.028.

Burn, C.C., 2008. What is it like to be a rat? Rat sensory perception and its implications for experimental design and rat welfare. Appl Anim Behav Sci 112, 1–32. https://doi.org/10.1016/j.applanim.2008.02.007

Cait, J., Cait, A., Scott, R.W., Winder, C.B., Mason, G.J., 2022. Conventional laboratory housing increases morbidity and mortality in research rodents: results of a meta-analysis. Bmc Biol 20, 15. https://doi.org/10.1186/s12915-021-01184-0

Carbone, L., 2021. Estimating mouse and rat use in American laboratories by extrapolation from Animal Welfare Act-regulated species. Sci Rep-uk 11, 493. https://doi.org/10.1038/s41598-020-79961-0

Carrillo, M., Han, Y., Migliorati, F., Liu, M., Gazzola, V., Keysers, C., 2019. Emotional Mirror Neurons in the Rat’s Anterior Cingulate Cortex. Curr Biol 29, 1301–1312. https://doi.org/10.1016/j.cub.2019.05.064

Carrillo, M., Migliorati, F., Bruls, R., Han, Y., Heinemans, M., Pruis, I., Gazzola, V., Keysers, C., 2015. Repeated Witnessing of Conspecifics in Pain: Effects on Emotional Contagion. PLOS One 10, e0136979. https://doi.org/10.1371/journal.pone.0136979

Ceballos, M.C., Góis, K.C.R., Sant’Anna, A.C., Wemelsfelder, F., Costa, M.P. da, 2021. Reliability of qualitative behavior assessment (QBA) versus methods with predefined behavioral categories to evaluate maternal protective behavior in dairy cows. Appl Anim Behav Sci 236, 105263. https://doi.org/10.1016/j.applanim.2021.105263

Chabot, D., Gagnon, P., Dixon, E.A., 1996. Effect of predator odors on heart rate and metabolic rate of wapiti (Cervus elaphus canadensis). J Chem Ecol 22, 839–868. https://doi.org/10.1007/bf02033590

Champeil-Potokar, G., Crossouard, L., Jérôme, N., Ouali, C., Darcel, N., Davidenko, O., Rampin, O., Bombail, V., Denis, I., 2021. Diet Protein Content and Individual Phenotype Affect Food Intake and Protein Appetence in Rats. J Nutrition 151, 1311–1319. https://doi.org/10.1093/jn/nxaa455

Chartrand, T.L., Bargh, J.A., 1999. The Chameleon Effect: The Perception–Behavior Link and Social Interaction. J Pers Soc Psychol 76, 893–910. https://doi.org/10.1037/0022-3514.76.6.893

Chiara, G.D., Imperato, A., 1988. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc National Acad Sci 85, 5274–5278. https://doi.org/10.1073/pnas.85.14.5274

Clarkson, J.M., Dwyer, D.M., Flecknell, P.A., Leach, M.C., Rowe, C., 2018. Handling method alters the hedonic value of reward in laboratory mice. Sci Rep-uk 8, 2448. https://doi.org/10.1038/s41598-018-20716-3

Cloutier, Sylvie, Baker, C., Wahl, K., Panksepp, J., Newberry, R.C., 2013. Playful handling as social enrichment for individually- and group-housed laboratory rats. Appl Anim Behav Sci 143, 85–95. https://doi.org/10.1016/j.applanim.2012.10.006

Cloutier, S., LaFollette, M.R., Gaskill, B.N., Panksepp, J., Newberry, R.C., 2018. Tickling, a Technique for Inducing Positive Affect When Handling Rats. J Vis Exp Jove 57190. https://doi.org/10.3791/57190

Cloutier, S., Newberry, R.C., 2008. Use of a conditioning technique to reduce stress associated with repeated intra-peritoneal injections in laboratory rats. Appl Anim Behav Sci 112, 158–173. https://doi.org/10.1016/j.applanim.2007.07.003

Coffey, K.R., Marx, R.G., Neumaier, J.F., 2019. DeepSqueak: a deep learning-based system for detection and analysis of ultrasonic vocalizations. Neuropsychopharmacol 44, 859–868. https://doi.org/10.1038/s41386-018-0303-6

Corsini, R., 2002. The Dictionary of Psychology. Routledge, New York. https://doi.org/10.4324/9781315781501-10

Davitz, J.R., Mason, D.J., 1955. Socially facilitated reduction of a fear response in rats. J Comp Physiol Psych 48, 149–151. https://doi.org/10.1037/h0046411

Dawson, L.C., Meagher, R., Ahloy-Dallaire, J., Mason, G., 2022. Practice does not make perfect: Juvenile object play does not improve the predation skills of adult mink in “simulated prey” tests. Dev Psychobiol 64, e22268. https://doi.org/10.1002/dev.22268

Deussing, J.M., 2007. Animal models of depression. Drug Discov Today Dis Model 3, 375–383. https://doi.org/10.1016/j.ddmod.2006.11.003

Driel, K.S. van, Talling, J.C., 2005. Familiarity increases consistency in animal tests. Behav Brain Res 159, 243–245. https://doi.org/10.1016/j.bbr.2004.11.005

Edgar, J.L., Mullan, S.M., Pritchard, J.C., McFarlane, U.J.C., Main, D.C.J., 2013. Towards a ‘Good Life’ for Farm Animals: Development of a Resource Tier

Framework to Achieve Positive Welfare for Laying Hens. Animals 3, 584–605. https://doi.org/10.3390/ani3030584

Ellenbroek, B., Youn, J., 2016. Rodent models in neuroscience research: is it a rat race? Dis Model Mech 9, 1079–1087. https://doi.org/10.1242/dmm.026120

Elliot, A.J., 2013. Handbook of Approach and Avoidance Motivation. Psychology Press. https://doi.org/10.4324/9780203888148

Elliot, A.J., Thrash, T.M., 2002. Approach-Avoidance Motivation in Personality: Approach and Avoidance Temperaments and Goals. J Pers Soc Psychol 82, 804–818. https://doi.org/10.1037/0022-3514.82.5.804

Finlayson, K., Lampe, J., Hintze, S., Wurbel, H., Melotti, L., 2016. Facial indicators of positive emotions in rats. PLOS One 11, e0166446. https://doi.org/10.1371/journal.pone.0166446

Flaherty, C.F., Riley, E.P., Spear, N.E., 1973. Effects of sucrose concentration and goal units on runway behavior in the rat. Learn Motiv 4, 163–175. https://doi.org/10.1016/0023-9690(73)90028-3

Forbes, E.E., Dahl, R.E., 2005. Neural systems of positive affect: Relevance to understanding child and adolescent depression? Dev Psychopathol 17, 827–850. https://doi.org/10.1017/s095457940505039x

Forbes, N.F., Stewart, C.A., Matthews, K., Reid, I.C., 1996. Chronic Mild Stress and Sucrose Consumption: Validity as a Model of Depression. Physiol Behav 60, 1481–1484. https://doi.org/10.1016/s0031-9384(96)00305-8

Franks, B., Champagne, F.A., Higgins, E.T., 2013. How Enrichment Affects Exploration Trade-Offs in Rats: Implications for Welfare and Well-Being. PLOS One 8, e83578. https://doi.org/10.1371/journal.pone.0083578

Fraser, D., 2008. Understanding animal welfare, in: The Role of the Veterinarian in Animal Welfare. Animal Welfare: Too Much or Too Little? The 21st Symposium of the Nordic Committee for Veterinary Scientific Cooperation (NKVet). Acta Veterinaria Scandinavica, Værløse, Denmark. https://doi.org/10.1186/1751-0147-50-s1-s1

Fraser, D., Duncan, I.J.H., 1998. ‘Pleasures’, ‘Pains’ and Animal Welfare: Toward a Natural History of Affect. Animal Welfare 4, 383–396.<no doi available.

Garner, J.P., 2005. Stereotypies and Other Abnormal Repetitive Behaviors: Potential Impact on Validity, Reliability, and Replicability of Scientific Outcomes. Ilar J 46, 106–117. https://doi.org/10.1093/ilar.46.2.106

Gärtner, K., Büttner, D., Döhler, K., Friedel, R., Lindena, J., Trautschold, I., 1980. Stress response of rats to handling and experimental procedures. Lab Anim 14, 267–274. https://doi.org/10.1258/002367780780937454

Gorwood, P., 2008. Neurobiological mechanisms of anhedonia. Dialogues Clin Neurosci 10, 291–9.

Goussha, Y., Bar, K., Netser, S., Cohen, L., Hel-Or, Y., Wagner, S., 2022. HybridMouse: A Hybrid Convolutional-Recurrent Neural Network-Based Model for Identification of Mouse Ultrasonic Vocalizations. Front Behav Neurosci 15, 810590. https://doi.org/10.3389/fnbeh.2021.810590

Green, T., Mellor, D., 2011. Extending ideas about animal welfare assessment to include ‘quality of life’ and related concepts. New Zeal Vet J 59, 263–271. https://doi.org/10.1080/00480169.2011.610283

Greenberg, R., 2003. The role of neophobia and neophilia in the development of innovative behaviour of birds, in: Reader, S.M., Laland, K.N. (Eds.), Animal Innovation. Oxford University Press, pp. 175–196. https://doi.org/10.1093/acprof:oso/9780198526223.003.0008

Haemisch, A., Guerra, G., Furkert, J., 1999. Adaptation of corticosterone-but not β-endorphin-secretion to repeated blood sampling in rats. Lab Anim 33, 185–191. https://doi.org/10.1258/002367799780578309

Hammond, T., Bombail, V., Nielsen, B.L., Meddle, S.L., Lawrence, A.B., Brown, S.M., 2019. Relationships between play and responses to tickling in male juvenile rats. Appl Anim Behav Sci 104879. https://doi.org/10.1016/j.applanim.2019.104879

Han, Y., Sichterman, B., Carrillo, M., Gazzola, V., Keysers, C., 2020. Similar levels of emotional contagion in male and female rats. Sci Rep-uk 10, 2763. https://doi.org/10.1038/s41598-020-59680-2

Harmon, K.M., Cromwell, H.C., Burgdorf, J., Moskal, J.R., Brudzynski, S.M., Kroes, R.A., Panksepp, J., 2008. Rats selectively bred for low levels of 50 kHz ultrasonic vocalizations exhibit alterations in early social motivation. Dev Psychobiol 50, 322–331. https://doi.org/10.1002/dev.20294

Harst, J.E. van der, Baars, A.-M., Spruijt, B.M., 2003. Standard housed rats are more sensitive to rewards than enriched housed rats as reflected by their anticipatory behaviour. Behav Brain Res 142, 151–156. https://doi.org/10.1016/s0166-4328(02)00403-5

Hatfield, E., Cacioppo, J.T., Rapson, R.L., 1993. Emotional Contagion. Cambridge University Press, Cambridge. https://doi.org/10.1017/cbo9781139174138.007

Held, S.D.E., Špinka, M., 2011. Animal play and animal welfare. Anim Behav 81, 891–899. https://doi.org/10.1016/j.anbehav.2011.01.007

Hernandez-Lallement, J., Gómez-Sotres, P., Carrillo, M., 2020. Towards a unified theory of emotional contagion in rodents—A meta-analysis. Neurosci Biobehav Rev 132, 1229–1248. https://doi.org/10.1016/j.neubiorev.2020.09.010

Heyse, N.C., Brenes, J.C., Schwarting, R.K.W., 2015. Exercise reward induces appetitive 50-kHz calls in rats. Physiol Behav 147, 131–140. https://doi.org/10.1016/j.physbeh.2015.04.021

Himmler, B., Kisko, T., Euston, D., Kolb, B., 2014. Are 50-kHz calls used as play signals in the playful interactions of rats? I. Evidence from the timing and context of their use. Behav Processes 106, 60–66.

Hinchcliffe, J.K., Mendl, M., Robinson, E.S.J., 2020. Rat 50 kHz calls reflect graded tickling-induced positive emotion. Curr Biol 30, R1034–R1035. https://doi.org/10.1016/j.cub.2020.08.038

Hintze, S., Murphy, E., Bachmann, I., Wemelsfelder, F., Würbel, H., 2017. Qualitative Behaviour Assessment of horses exposed to short-term emotional treatments. Appl Anim Behav Sci 196, 44–51. https://doi.org/10.1016/j.applanim.2017.06.012

Hoffman, K.L., 2016. 2 - What can animal models tell us about depressive disorders?, in: Modeling Neuropsychiatric Disorders in Laboratory Animals. pp. 35–86. https://doi.org/10.1016/b978-0-08-100099-1.00002-9

Hogg, S., 1996. A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. Pharmacol Biochem Behav. 54, 21–30.

Hori, M., Hayashi, T., Nakagawa, Y., 2009. Positive emotion-specific changes in the gene expression profile of tickled rats. Mol Med Rep 2, 157–61. https://doi.org/10.3892/mmr_00000077

Hori, M., Shimoju, R., Tokunaga, R., Ohkubo, M., 2013. Tickling increases dopamine release in the nucleus accumbens and 50 kHz ultrasonic vocalizations in adolescent rats. Neuroreport 24, 241–245. https://doi.org/10.1097/wnr.0b013e32835edbfa

Hori, M., Yamada, K., Ohnishi, J., Sakamoto, S., Furuie, H., 2014. Tickling during adolescence alters fear-related and cognitive behaviors in rats after prolonged isolation. Physiology & Behavior 131, 62–67. https://doi.org/10.1016/j.physbeh.2014.04.008

Hubbard, J.W., Cox, R.H., Sanders, B.J., Lawler, J.E., 1986. Changes in cardiac output and vascular resistance during behavioral stress in the rat. Am J Physiology-regulatory Integr Comp Physiology 251, R82–R90. https://doi.org/10.1152/ajpregu.1986.251.1.r82

Hubrecht, R.C., 2014. The Welfare of Animals Used in Research: Practice and Ethics, UFAW/Wiley-Blackwell Animal Welfare Book Series. John Wiley & Sons, Incorporated.

Huff, J.K., Davies, M.I., 2002. Microdialysis monitoring of methylphenidate in blood and brain correlated with changes in dopamine and rat activity. J Pharmaceut Biomed 29, 767–777. https://doi.org/10.1016/s0731-7085(02)00196-6

Ishiyama, S., Brecht, M., 2016. Neural correlates of ticklishness in the rat somatosensory cortex. Science 354, 757–760. https://doi.org/10.1126/science.aah5114

Jarvis, S., Ellis, M.A., Turnbull, J.F., Planellas, S.R., Wemelsfelder, F., 2021. Qualitative Behavioral Assessment in Juvenile Farmed Atlantic Salmon (Salmo salar): Potential for On-Farm Welfare Assessment. Frontiers Vet Sci 8, 702783. https://doi.org/10.3389/fvets.2021.702783

Jensen, M.B., Studnitz, M., Halekoh, U., Pedersen, L.J., Jørgensen, E., 2008. Pigs' preferences for rooting materials measured in a three-choice maze-test. Appl Anim Behav Sci 112, 270–283. https://doi.org/10.1016/j.applanim.2007.07.012

Jirkof, P., Rudeck, J., Lewejohann, L., 2019. Assessing Affective State in Laboratory Rodents to Promote Animal Welfare—What Is the Progress in Applied Refinement Research? Animals 9, 1026. https://doi.org/10.3390/ani9121026

Jones, A.C., Gosling, S.D., 2014. "Individual Differences in Approach and Avoidance Motivation in Animals, in: Elliot, A.J. (Ed.), Handbook of Approach and Avoidance Motivation. Psychology Press, pp. 165–185. https://doi.org/10.4324/9780203888148.ch10

Kaliste, E., Mering, S., 2007. The Welfare of Laboratory Animals. Anim Welfare 153–180. https://doi.org/10.1007/978-1-4020-2271-5_8

Kane SP. 2018. ClinCalc. At https://clincalc.com/stats/Power.aspx.

Keeling, L., Evans, A., Forkman, B., Kjaernes, U., 2013. Welfare Quality® principles and criteria, in: Improving Farm Animal Welfare. Wageningen Academic Publishers, Wageningen, pp. 91–114. https://doi.org/10.3920/978-90-8686-770-7_5

Kennedy, J.S., Thorpe, W.H., 1964. Learning and Instinct in Animals. J Animal Ecol 33, 378. https://doi.org/10.2307/2644

Kerkhof, L., Damsteegt, R., Trezza, V., Voorn, P., Vanderschuren, L., 2013. Social play behavior in adolescent rats is mediated by functional activity in medial prefrontal cortex and striatum. Neuropsychopharmacology 38, 1899–1909. https://doi.org/10.1038/npp.2013.83

Kikusui, T., Winslow, J.T., Mori, Y., 2006. Social buffering: relief from stress and anxiety. Philosophical Transactions Royal Soc B Biological Sci 361, 2215–2228. https://doi.org/10.1098/rstb.2006.1941

Kirkden, R.D., Pajor, E.A., 2006. Using preference, motivation and aversion tests to ask scientific questions about animals’ feelings. Appl Anim Behav Sci 100, 29–47. https://doi.org/10.1016/j.applanim.2006.04.009

Klein, Z.A., Padow, V.A., Romeo, R.D., 2010. The effects of stress on play and home cage behaviors in adolescent male rats. Dev Psychobiol 52, 62–70. https://doi.org/10.1002/dev.20413

Knapska, E., Mikosz, M., Werka, T., Maren, S., 2010. Social modulation of learning in rats. Learn Mem 17, 35–42. https://doi.org/10.1101/lm.1670910

Knutson, B., Burgdorf, J., Panksepp, J., 2002. Ultrasonic vocalizations as indices of affective states in rats. Psychol Bull 128, 961–77. https://doi.org/10.1037/0033-2909.128.6.961

Knutson, B., Burgdorf, J., Panksepp, J., 1999. High-Frequency Ultrasonic Vocalizations Index Conditioned Pharmacological Reward in Rats. Physiol Behav 66, 639–643. https://doi.org/10.1016/s0031-9384(98)00337-0

Knutson, B., Burgdorf, J., Panksepp, J., 1998. Anticipation of play elicits high-frequency ultrasonic vocalizations in young rats. J Comp Psychol 112, 65–73. https://doi.org/10.1037/0735-7036.112.1.65

Kõiv, K., Metelitsa, M., Vares, M., Tiitsaar, K., Raudkivi, K., Jaako, K., Vulla, K., Shimmo, R., Harro, J., 2016. Chronic variable stress prevents amphetamine-elicited 50-kHz calls in rats with low positive affectivity. Eur Neuropsychopharm 26, 631–643. https://doi.org/10.1016/j.euroneuro.2016.02.011

Korte, S.M., Boer, S.F.D., 2003. A robust animal model of state anxiety: fear-potentiated behaviour in the elevated plus-maze. Eur J Pharmacol 463, 163–175. https://doi.org/10.1016/s0014-2999(03)01279-2

Kremer, L., Holkenborg, S.E.J.K., Reimert, I.S., Bolhuis, J.E., Webb, L.E., 2020. The nuts and bolts of animal emotion. Neurosci Biobehav Rev 113, 273–286. https://doi.org/10.1016/j.neubiorev.2020.01.028

Kurt, L., 1936. A dynamic theory of personality-selected papers. Read Books Ltd. https://doi.org/10.1097/00005053-193611000-00051

LaFollette, M.R., O'Haire, M.E., Cloutier, S., Blankenberger, W.B., Gaskill, B.N., 2017. Rat tickling: A systematic review of applications, outcomes, and moderators. PLOS One 12, e0175320. https://doi.org/10.1371/journal.pone.0175320

LaFollette, Megan R., O'Haire, M.E., Cloutier, S., Gaskill, B.N., 2018a. A Happier Rat Pack: The Impacts of Tickling Pet Store Rats on Human-Animal Interactions and Rat Welfare. Appl Anim Behav Sci 203, 92–102. https://doi.org/10.1016/j.applanim.2018.02.006

LaFollette, Megan R , O'Haire, M.E., Cloutier, S., Gaskill, B.N., 2018b. Practical rat tickling: determining an efficient and effective dosage of heterospecific play. Appl Anim Behav Sci. https://doi.org/10.1016/j.applanim.2018.08.005

Lampe, J.F., Burman, O., Wurbel, H., Melotti, L., 2017. Context-dependent individual differences in playfulness in male rats. Dev Psychobiol 59, 460–472. https://doi.org/10.1002/dev.21509

Laricchiuta, D., Petrosini, L., 2014. Individual differences in response to positive and negative stimuli: endocannabinoid-based insight on approach and avoidance behaviors. Frontiers Syst Neurosci 8, 238. https://doi.org/10.3389/fnsys.2014.00238

Laurijs, K.A., Briefer, E.F., Reimert, I., Webb, L.E., 2021. Vocalisations in Farm Animals: a Step Towards Positive Welfare Assessment. Appl Anim Behav Sci 236, 105264. https://doi.org/10.1016/j.applanim.2021.105264

La-Vu, M., Tobias, B.C., Schuette, P.J., Adhikari, A., 2020. To Approach or Avoid: An Introductory Overview of the Study of Anxiety Using Rodent Assays. Front Behav Neurosci 14, 145. https://doi.org/10.3389/fnbeh.2020.00145

Lawrence, A.B., 2016. Applied animal behaviour science and animal welfare: seeking the best balance between our science and its application. Appl Anim Behav Sci 115. https://doi.org/10.3920/978-90-8686-828-5_6

Lawrence, A.B., 1987. Consumer demand theory and the assessment of animal welfare. Anim Behav 35, 293–295. https://doi.org/10.1016/s0003-3472(87)80236-1

Lawrence, A.B., Newberry, R.C., Špinka, M., 2018. 15 Positive welfare What does it add to the debate over pig welfare?, in: Špinka, M. (Ed.), Advances in Pig Welfare, Woodhead Publishing Series in Food Science, Technology and Nutrition. Woodhead Publishing, pp. 415–444. https://doi.org/10.1016/b978-0-08-101012-9.00014-9

Lawrence, A.B., Vigors, B., Sandøe, P., 2019. What Is so Positive about Positive Animal Welfare?—A Critical Review of the Literature. Animals 9, 783. https://doi.org/10.3390/ani9100783

Lawson, K.A., Flores, A.Y., Hokenson, R.E., Ruiz, C.M., Mahler, S.V., 2021. Nucleus Accumbens Chemogenetic Inhibition Suppresses Amphetamine-Induced Ultrasonic Vocalizations in Male and Female Rats. Brain Sci 11, 1255. https://doi.org/10.3390/brainsci11101255

Lever, C., Burton, S., O’Keefe, J., 2006. Rearing on Hind Legs, Environmental Novelty, and the Hippocampal Formation. Rev Neuroscience 17, 111–134. https://doi.org/10.1515/revneuro.2006.17.1-2.111

Makowska, I.J., Weary, D.M., 2020. A Good Life for Laboratory Rodents? Ilar J 60, 373–388. https://doi.org/10.1093/ilar/ilaa001

Makowska, I.J., Weary, D.M., 2013. Assessing the emotions of laboratory rats. Appl Anim Behav Sci 148, 1–12. https://doi.org/10.1016/j.applanim.2013.07.017

Mällo,, T., Matrov, D., Herm, L., Koiv, K., Eller, M., Rinken, A., Harro, J., 2007. Tickling-induced 50-kHz ultrasonic vocalization is individually stable and predicts behaviour in tests of anxiety and depression in rats. Behav Brain Res 184, 57–71. https://doi.org/10.1016/j.bbr.2007.06.015

Manser, C.E., Elliott, H., Morris, T.H., Broom, D.M., 1996. The use of a novel operant test to determine the strength of preference for flooring in laboratory rats. Lab Anim 30, 1–6. https://doi.org/10.1258/002367796780744974

Manteuffel, G., Puppe, B., Schön, P.C., 2004. Vocalization of farm animals as a measure of welfare. Appl Anim Behav Sci 88, 163–182. https://doi.org/10.1016/j.applanim.2004.02.012

Marchant, J.N., Broom, D.M., Rudd, A.R., Mendl, M.T., 1995. The effect of agonistic social interactions on the heart rate of group-housed sows. Proc Br Soc Animal Sci 46, 197–197. https://doi.org/10.1017/s0308229600029627

Martin, P., Bateson, P., 1993. Measuring Behaviour: An Introductory Guide (2nd ed.) Cambridge University Press. https://doi.org/10.1017/cbo9781139168342

Marx, G., Leppelt, J., Ellendorff, F., 2001. Vocalisation in chicks (Gallus gallus dom.) during stepwise social isolation. Appl Anim Behav Sci 75, 61–74. https://doi.org/10.1016/s0168-1591(01)00180-0

McCall, R.B., Lester, M.L., Corter, C.M., 1969. Caretaker effect in rats. Dev Psychol 1, 771–771. https://doi.org/10.1037/h0028199

McHugh, M.L., 2012. Interrater reliability: the kappa statistic. Biochem Medica 22, 276–282. https://doi.org/10.11613/bm.2012.031

Mead, R., 1988. The design of experiments: statistical principles for practical applications. Cambridge University Press.

Mellor, D., 2014a. Positive animal welfare states and encouraging environment-focused and animal-to-animal interactive behaviours. New Zeal Vet J 63, 9–16. https://doi.org/10.1080/00480169.2014.926800

Mellor, D., 2014b. Enhancing animal welfare by creating opportunities for positive affective engagement. New Zeal Vet J 63, 3–8. https://doi.org/10.1080/00480169.2014.926799

Mellor, D., 2012. Animal emotions, behaviour and the promotion of positive welfare states. New Zeal Vet J 60, 1–8. https://doi.org/10.1080/00480169.2011.619047

Mellor, D., Beausoleil, N., 2015. Extending the “Five Domains” model for animal welfare assessment to incorporate positive welfare states. Anim Welfare 24, 241–253. https://doi.org/10.7120/09627286.24.3.241

Melotti, L., Bailoo, J. D., Murphy, E., Burman, O., & Würbel, H., 2014. Play in rats: Association across contexts and types, and analysis of structure. Animal Behav and Cog, 1(4), 489-501. https://doi.org/10.12966/abc.11.06.2014

Mendl, M., Burman, O.H.P., Paul, E.S., 2010. An integrative and functional framework for the study of animal emotion and mood. Proc Royal Soc B Biological Sci 277, 2895–2904. https://doi.org/10.1098/rspb.2010.0303

Mendl, M., Paul, E.S., 2020. Assessing affective states in animals, in: McMillan, F. (Ed.), Mental Health and Well-Being in Animals. CABI Publishing, pp. 328–344. https://doi.org/10.1079/9781786393401.0328

Meyer, P.J., Ma, S.T., Robinson, T.E., 2012. A cocaine cue is more preferred and evokes more frequency-modulated 50-kHz ultrasonic vocalizations in rats prone to attribute incentive salience to a food cue. Psychopharmacology 219, 999–1009. https://doi.org/10.1007/s00213-011-2429-7

Morley-Fletcher, S., Rea, M., S, M., G, L., 2003. Environmental enrichment during adolescence reverses the effects of prenatal stress on play behaviour and HPA axis reactivity in rats. Eur J Neurosci 18, 3367–3374. https://doi.org/10.1111/j.1460-9568.2003.03070.x.

Mulvihill, K.G., Brudzynski, S.M., 2018. Individual behavioural predictors of amphetamine-induced emission of 50 kHz vocalization in rats. Behav Brain Res 350, 80–86. https://doi.org/10.1016/j.bbr.2018.05.009

Nakahashi, W., Ohtsuki, H., 2015. When is emotional contagion adaptive? J Theor Biol 380, 480–488. https://doi.org/10.1016/j.jtbi.2015.06.014

Nathiya, V.C., Vanisree, A.J., 2010. Investigations on light induced stress model and on the role of Phyllanthus amarus in attenuation of stress related depression with focus on 5HT2Am- RNA expression. Ann Neurosci 17, 167–175. https://doi.org/10.5214/ans.0972.7531.1017405

National Research Council (US) Committee, 2008. Recognition and Alleviation of Distress in Laboratory Animals. Washington DC: National Academies Press US. https://doi.org/10.17226/11931

Neville, V., Lecorps, B., Mendl, M., 2022. Good science requires better animal welfare. Science 376, 809–809. https://doi.org/10.1126/science.abq7198

Noirot, E., 1968. Ultrasounds in young rodents. II. Changes with age in albino rats. Anim Behav 16, 129–134. https://doi.org/10.1016/0003-3472(68)90123-1

Norscia, I., Palagi, E., 2011. When play is a family business: adult play, hierarchy, and possible stress reduction in common marmosets. Primates 52, 101–104. https://doi.org/10.1007/s10329-010-0228-0

Oliveira-Maia, A.J., Roberts, C.D., Walker, Q.D., Luo, B., Kuhn, C., Simon, S.A., Nicolelis, M.A.L., 2011. Intravascular Food Reward. PLOS One 6, e24992. https://doi.org/10.1371/journal.pone.0024992

Olsson, I.A.S., Nevison, C.M., Patterson-Kane, *E.G.,* Sherwin, C.M., Weerd, H.A.V. de, Würbel, H., 2003. Understanding behaviour: the relevance of ethological approaches in laboratory animal science. Appl Anim Behav Sci 81, 245–264. https://doi.org/10.1016/s0168-1591(02)00285-x

Olszyński, K.H., Polowy, R., Małż, M., Boguszewski, P.M., Filipkowski, R.K., 2020. Playback of Alarm and Appetitive Calls Differentially Impacts Vocal, Heart-Rate, and Motor Response in Rats. Iscience 23, 101577. https://doi.org/10.1016/j.isci.2020.101577

Padgett, D.A., Glaser, R., 2003. How stress influences the immune response. Trends Immunol 24, 444–448. https://doi.org/10.1016/s1471-4906(03)00173-x

Panksepp, J., 2005. Beyond a Joke: From Animal Laughter to Human Joy? Science 308, 62–63. https://doi.org/10.1126/science.1112066

Panksepp, J. (1998). Affective Neuroscience: The Foundations of Human and Animal Emotions. Oxford: Oxford University Press.

Panksepp, J., 1981. The ontogeny of play in rats. Dev Psychobiol 14, 327–332. https://doi.org/10.1002/dev.420140405

Panksepp, J., Burgdorf, J., 2010. Laughing Rats? Playful Tickling Arouses High-Frequency Ultrasonic Chirping in Young Rodents. Am J Play 2, 357–372. https://doi.org/10.1016/s0031-9384(03)00159-8

Panksepp, J., Burgdorf, J., 2003. “Laughing” rats and the evolutionary antecedents of human joy? Physiol Behav 79, 533–547. https://doi.org/10.1016/s0031-9384(03)00159-8

Panksepp, J., Burgdorf, J., 2000. 50-kHz chirping (laughter?) in response to conditioned and unconditioned tickle-induced reward in rats: effects of social housing and genetic variables. Behav Brain Res 115, 25–38. https://doi.org/10.1016/s0166-4328(00)00238-2

Panksepp, J., Gordon, N., Burgdorf, J., 2001. Empathy and the action-perception resonances of basic socio-emotional systems of the brain. Behav Brain Sci 25, 43–44. https://doi.org/10.1017/s0140525x0247001x

Papini, M.R., 2020. Comparative Psychology. Comparative Psychology. In: Davis, S.F. (Ed.), Handbook of Research Methods in Experimental Psychology. John Wiley & Sons. pp. 409–454. https://doi.org/ 10.1002/9780470756973.ch10

Papp, M., Willner, P., Muscat, R., 1991. An animal model of anhedonia: attenuation of sucrose consumption and place preference conditioning by chronic unpredictable mild stress. Psychopharmacology 104, 255–259. https://doi.org/10.1007/bf02244188

Parsana, A.J., Moran, E.E., Brown, T.H., 2012. Rats learn to freeze to 22-kHz ultrasonic vocalizations through autoconditioning. Behav Brain Res 232, 395–399. https://doi.org/10.1016/j.bbr.2012.03.031

Patel, F., Wemelsfelder, F., Ward, S.J., 2019. Using Qualitative Behaviour Assessment to Investigate Human-Animal Relationships in Zoo-Housed Giraffes (Giraffa camelopardalis). Animals 9, 381. https://doi.org/10.3390/ani9060381

Paul, E.S., Harding, E.J., Mendl, M., 2005. Measuring emotional processes in animals: the utility of a cognitive approach. Neurosci Biobehav Rev 29, 469–491. https://doi.org/10.1016/j.neubiorev.2005.01.002

Paul, E.S., Mendl, M.T., 2018. Animal emotion: Descriptive and prescriptive definitions and their implications for a comparative perspective. Appl Anim Behav Sci 205, 202–209. https://doi.org/10.1016/j.applanim.2018.01.008

Paul, E.S., Sher, S., Tamietto, M., Winkielman, P., Mendl, M.T., 2019. Towards a comparative science of emotion: Affect and consciousness in humans and animals. Neurosci Biobehav Rev. https://doi.org/10.1016/j.neubiorev.2019.11.014

Pellis, Field, E., Smith, L., Pellis, V., 1997. Multiple differences in the play fighting of male and female rats. Implications for the causes and functions of play. Neurosci Biobehav Rev 21, 105-120. https://doi.org/10.1016/0149-7634(95)00060-7

Pellis, S., Pellis, V., 2013. The playful brain: venturing to the limits of neuroscience. Simon and Schuster. 10.1016/0149-7634(95)00060-7

Pellis, S.M., 2002. Sex differences in play fighting revisited: traditional and nontraditional mechanisms of sexual differentiation in rats. Arch Sex Behav 31, 17–26. https://doi.org/10.1023/A:1014070916047

Pellis, S.M., McKenna, M.M., 1992. Intrinsic and extrinsic influences on play fighting in rats: effects of dominance, partner's playfulness, temperament and neonatal exposure to testosterone propionate. Behav Brain Res 50, 135–145. https://doi.org/10.1016/s0166-4328(05)80295-5

Pellis, S.M., Pellis, V.C., 1991. Role reversal changes during the ontogeny of play fighting in male rats: Attack vs. defense. Aggressive Behav 17, 179–189. https://doi.org/10.1002/1098-2337(1991)17:3<179::aid-ab2480170306>3.0.co;2-q

Pellis, S.M., Pellis, V.C., 1983. Locomotor-rotational movements in the ontogeny and play of the laboratory rat Rattus norvegicus. Dev Psychobiol 16, 269–286. https://doi.org/10.1002/dev.420160403

Pérez-Manrique, A., Gomila, A., 2021. Emotional contagion in nonhuman animals: A review. Wiley Interdiscip Rev Cognitive Sci 13, e1560. https://doi.org/10.1002/wcs.1560

Pfaus, J.G., Kippin, T.E., Centeno, S., 2001. Conditioning and Sexual Behavior: A Review. Horm Behav 40, 291–321. https://doi.org/10.1006/hbeh.2001.1686

Phillips, C., 2008. Animal welfare: A construct of positive and negative affect? Vet J 175, 291–292. https://doi.org/10.1016/j.tvjl.2007.05.015

Pitchers, K.K., Balfour, M.E., Lehman, M.N., Richtand, N.M., Yu, L., Coolen, L.M., 2010. Neuroplasticity in the Mesolimbic System Induced by Natural Reward and Subsequent Reward Abstinence. Biol Psychiat 67, 872–879. https://doi.org/10.1016/j.biopsych.2009.09.036

Premoli, M., Baggi, D., Bianchetti, M., Gnutti, A., Bondaschi, M., Mastinu, A., Migliorati, P., Signoroni, A., Leonardi, R., Memo, M., Bonini, S.A., 2021. Automatic classification of mice vocalizations using Machine Learning techniques and Convolutional Neural Networks. Plos One 16, e0244636. https://doi.org/10.1371/journal.pone.0244636

Proctor, H.S., Carder, G., 2015. Measuring positive emotions in cows: Do visible eye whites tell us anything? Physiol Behav 147, 1–6. https://doi.org/10.1016/j.physbeh.2015.04.011

Proctor, H.S., Carder, G., Cornish, A.R., 2013. Searching for Animal Sentience: A Systematic Review of the Scientific Literature. Animals 3, 882–906. https://doi.org/10.3390/ani3030882

Raoult, C.M.C., Gygax, L., 2018. Valence and Intensity of Video Stimuli of Dogs and Conspecifics in Sheep: Approach-Avoidance, Operant Response, and Attention. Animals Open Access J Mdpi 8, 121. https://doi.org/10.3390/ani8070121

Ratuski, A.S., Weary, D.M., 2022. Environmental Enrichment for Rats and Mice Housed in Laboratories: A Metareview. Animals 12, 414. https://doi.org/10.3390/ani12040414

Rault, J.L., Hintze, S., Camerlink, I., Yee, J.R., 2020. Positive Welfare and the Like: Distinct Views and a Proposed Framework. Frontiers Vet Sci 7, 370. https://doi.org/10.3389/fvets.2020.00370

Rault, J.L., Sandøe, P., Sonntag, Q., Stuardo, L., 2022. Positive Animal Welfare: Bridging the Gap or Raising Inequalities Worldwide? Frontiers Animal Sci 3, 825379. https://doi.org/10.3389/fanim.2022.825379

Reimert, Inonge, Bolhuis, J.E., Kemp, B., Rodenburg, T.B., 2013. Indicators of positive and negative emotions and emotional contagion in pigs. Physiol Behav 109, 42–50. https://doi.org/10.1016/j.physbeh.2012.11.002

Richter, S.H., Garner, J.P., Würbel, H., 2009. Environmental standardization: cure or cause of poor reproducibility in animal experiments? Nat Methods 6, 257–261. https://doi.org/10.1038/nmeth.1312

Riley, V., 1981. Psychoneuroendocrine Influences on Immunocompetence and Neoplasia. Science 212, 1100–1109. https://doi.org/10.1126/science.7233204

Robinson, T.E., Kolb, B., 1999. Alterations in the morphology of dendrites and dendritic spines in the nucleus accumbens and prefrontal cortex following repeated treatment with amphetamine or cocaine. Eur J Neurosci 11, 1598–1604. https://doi.org/10.1046/j.1460-9568.1999.00576.x

Rodgers, R., Cao, B., Dalvi, A., Holmes, A., 1997. Animal models of anxiety: an ethological perspective. Braz J Med Biol Res 30, 289–304. https://doi.org/10.1590/s0100-879x1997000300002

Ross, M., Menzler, S., Zimmermann, E., 2008. Rapid facial mimicry in orangutan play. Biol Letters 4, 27–30. https://doi.org/10.1098/rsbl.2007.0535

Rowe, E., Mullan, S., 2022. Advancing a “Good Life” for Farm Animals: Development of Resource Tier Frameworks for On-Farm Assessment of Positive Welfare for Beef Cattle, Broiler Chicken and Pigs. Animals 12, 565. https://doi.org/10.3390/ani12050565

Rushen, J., 1986. Some problems with the physiological concept of “stress.” Aust Vet J 63, 359–361. https://doi.org/10.1111/j.1751-0813.1986.tb02896.x

Russell, J.A., 2003. Core Affect and the Psychological Construction of Emotion. Psychol Rev 110, 145–172. https://doi.org/10.1037/0033-295x.110.1.145

Rutherford, K.M.D., Donald, R.D., Lawrence, A.B., Wemelsfelder, F., 2012. Qualitative Behavioural Assessment of emotionality in pigs. Appl Anim Behav Sci 139, 218–224. https://doi.org/10.1016/j.applanim.2012.04.004

Rygula, R., Pluta, H., Popik, P., 2012. Laughing Rats Are Optimistic. PLOS One 7, e51959. https://doi.org/10.1371/journal.pone.0051959

Sadanana, M., Wöhr, M., Schwarting, R., 2008. Playback of 22-kHz and 50-kHz ultrasonic vocalizations induces differential c-fos expression in rat brain. Neurosci Lett 435, 17–23. https://doi.org/10.1016/j.neulet.2008.02.002

Saito, Y., Yuki, S., Seki, Y., Kagawa, H., Okanoya, K., 2016. Cognitive bias in rats evoked by ultrasonic vocalizations suggests emotional contagion. Behav Process 132, 5–11. https://doi.org/10.1016/j.beproc.2016.08.005

Scheggi, S., Montis, M.G.D., Gambarana, C., 2018. Making sense of rodent models of anhedonia. Int J Neuropsychoph 21, 1049–1065. https://doi.org/10.1093/ijnp/pyy083

Schneirla, T.C., 1965. Aspects of stimulation and organization in approach/withdrawal processes underlying vertebrate behavioral development. Adv Stud Behav 1, 1–74. https://doi.org/10.1016/s0065-3454(08)60055-8

Schwarting, R.K.W., 2018. Ultrasonic vocalization in juvenile and adult male rats: A comparison among stocks. Physiol Behav 191, 1–11. https://doi.org/10.1016/j.physbeh.2018.03.023

Schwarting, R.K.W., Jegan, N., Wöhr, M., 2007. Situational factors, conditions and individual variables which can determine ultrasonic vocalizations in male adult Wistar rats. Behav Brain Res 182, 208–222. https://doi.org/10.1016/j.bbr.2007.01.029

Schwarting, R.K.W., Kisko, T.M., Wöhr, M., 2018. Chapter 34 Playback of Ultrasonic Vocalizations to Juvenile and Adult Rats: Behavioral and Neuronal Effects, in: Brudzynski, S.M. (Ed.), Handbook of Behavioral Neuroscience, Handbook of Behavioral Neuroscience. pp. 357–369. https://doi.org/10.1016/b978-0-12-809600-0.00034-2

Schwarting, R.K.W., Wöhr, M., 2012. On the relationships between ultrasonic calling and anxiety-related behavior in rats. Braz J Med Biol Res 45, 337–348. https://doi.org/10.1590/s0100-879x2012007500038

Sclafani, A., Ackroff, K., 2003. Reinforcement value of sucrose measured by progressive ratio operant licking in the rat. Physiol Behav 79, 663–670. https://doi.org/10.1016/s0031-9384(03)00143-4

Seffer, D., Schwarting, R.K.W., Wöhr, M., 2014. Pro-social ultrasonic communication in rats: Insights from playback studies. J Neurosci Meth 234, 73–81. https://doi.org/10.1016/j.jneumeth.2014.01.023

Seidisarouei, M., Gurp, S. van, Pranic, N.M., Calabus, I.N., Wingerden, M. van, Kalenscher, T., 2021. Distinct Profiles of 50 kHz Vocalizations Differentiate

Between Social Versus Non-social Reward Approach and Consumption. Front Behav Neurosci 15, 693698. https://doi.org/10.3389/fnbeh.2021.693698

Sengupta, P., 2012. The Laboratory Rat: Relating Its Age With Human's. Int J Prev Medicine 4, 624–30.<no doi available.

Shaw, N., Wemelsfelder, F., Riley, L.M., 2022. Bark to the future: The welfare of domestic dogs during interaction with a positively reinforcing artificial agent. Appl Anim Behav Sci 249, 105595. https://doi.org/10.1016/j.applanim.2022.105595

Siviy, S.M., Panksepp, J., 1987. Sensory modulation of juvenile play in rats. Dev Psychobiol 20, 39–55. https://doi.org/10.1002/dev.420200108

Špinka, M., 2012. Social dimension of emotions and its implication for animal welfare. Appl Anim Behav Sci 138, 170–181. https://doi.org/10.1016/j.applanim.2012.02.005

Špinka, M., Palečková, M., Řeháková, M., 2016. Metacommunication in social play: the meaning of aggression-like elements is modified by play face in Hanuman langurs (Semnopithecus entellus). Behaviour 153, 795–818. https://doi.org/10.1163/1568539x-00003327

Spoor, J.R., Kelly, J.R., 2004. The Evolutionary Significance of Affect in Groups: Communication and Group Bonding. Group Process Interg 7, 398–412. https://doi.org/10.1177/1368430204046145

Spruijt, B.M., Bos, R. van den, Pijlman, F.T.A., 2001. A concept of welfare based on reward evaluating mechanisms in the brain: anticipatory behaviour as an indicator for the state of reward systems. Appl Anim Behav Sci 72, 145–171. https://doi.org/10.1016/s0168-1591(00)00204-5

Starcevic, A., Petricevic, S., Radojicic, Z., Djulejic, V., Ilankovic, A., Starcevic, B., Filipovic, B., 2016. Glucocorticoid levels after exposure to predator odor and chronic psychosocial stress with dexamethasone application in rats. Kaohsiung J Medical Sci 32, 235–240. https://doi.org/10.1016/j.kjms.2016.04.011

Strekalova, T., Spanagel, R., Bartsch, D., Henn, F.A., Gass, P., 2004. Stress-Induced Anhedonia in Mice is Associated with Deficits in Forced Swimming and Exploration. Neuropsychopharmacol 29, 2007–2017. https://doi.org/10.1038/sj.npp.1300532

Strouthes, A., 1971. Thirst and saccharin preference in rats. Physiol Behav 6, 287–292. https://doi.org/10.1016/0031-9384(71)90157-0

Taylor, G.T., 1981. Fear and affiliation in domesticated male rats. J Comp Physiol Psych 95, 685–693. https://doi.org/10.1037/h0077817

Thor, D.H., Holloway, W.R., 1984. Developmental analyses of social play behavior in juvenile rats. B Psychonomic Soc 22, 587–590. https://doi.org/10.3758/bf03333916

Tooby, J., Cosmides, L., 1990. The past explains the present Emotional adaptations and the structure of ancestral environments. Ethol Sociobiol 11, 375–424. https://doi.org/10.1016/0162-3095(90)90017-z

Trezza, V., Baarendse, P.J.J., Vanderschuren, L.J.M.J., 2010. The pleasures of play: pharmacological insights into social reward mechanisms. Trends Pharmacol Sci 31, 463–469. https://doi.org/10.1016/j.tips.2010.06.008

Turner, P.V., 2019. Moving Beyond the Absence of Pain and Distress: Focusing on Positive Animal Welfare. Ilar J 60, 366–372. https://doi.org/10.1093/ilar/ilaa017

UK Home Office, 2016. Statistics of Scientific Procedures on living animals, Great Britain. At https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/627284/annual-statistics-scientific-procedures-living-animals-2016.pdf (2017).

Vanderschuren, L.J.M.J., Trezza, V., 2013. The Neurobiology of Childhood. Curr Top Behav Neurosci 189–212. https://doi.org/10.1007/978-3-662-45758-0_268

Vedhara, K., Miles, J., Bennett, P., Plummer, S., Tallon, D., Brooks, E., Gale, L., Munnoch, K., Schreiber-Kounine, C., Fowler, C., Lightman, S., Sammon, A., Rayter, Z., Farndon, J., 2003. An investigation into the relationship between salivary cortisol, stress, anxiety and depression. Biol Psychol 62, 89–96. https://doi.org/10.1016/s0301-0511(02)00128-x

Vere, A.J. de, Kuczaj, S.A., 2016. Where are we in the study of animal emotions? Wiley Interdiscip Rev Cognitive Sci 7, 354–362. https://doi.org/10.1002/wcs.1399

Vielle, C., Montanari, C., Pelloux, Y., Baunez, C., 2021. Evidence for a vocal signature in the rat and its reinforcing effects: a key role for the subthalamic nucleus. Proc Royal Soc B 288, 20212260. https://doi.org/10.1098/rspb.2021.2260

Vigors, B., 2019. Citizens' and Farmers' Framing of 'Positive Animal Welfare' and the Implications for Framing Positive Welfare in Communication. Animals 9, 147. https://doi.org/10.3390/ani9040147

Waal, F.B.M. de, 2008. Putting the Altruism Back into Altruism: The Evolution of Empathy. Annu Rev Psychol 59, 279–300. https://doi.org/10.1146/annurev.psych.59.103006.093625

Waal, F.B.M. de, 2007. The "Russian doll" model of empathy and imitation, in: Bråten, S. (Ed.), On Being Moved: From Mirror Neurons to Empathy. John Benjamins Publishing Company, pp. 49–69. https://doi.org/10.1075/aicr.68.06waa

Waiblinger, S., Boivin, X., Pedersen, V., Tosi, M.-V., Janczak, A.M., Visser, K.E., Jones, R., 2006. Assessing the human–animal relationship in farmed species: A critical review. Appl Anim Behav Sci 101, 185–242. https://doi.org/10.1016/j.applanim.2006.02.001

Walf, A.A., Frye, C.A., 2007. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat Protoc 2, 322–328. https://doi.org/10.1038/nprot.2007.44

Watts, J.M., Stookey, J.M., 2000. Vocal behaviour in cattle: the animal's commentary on its biological processes and welfare. Appl Anim Behav Sci 67, 15–33. https://doi.org/10.1016/s0168-1591(99)00108-2

Webb, L.E., Veenhoven, R., Harfeld, J.L., Jensen, M.B., 2019. What is animal happiness? Ann Ny Acad Sci 1438, 62–76. https://doi.org/10.1111/nyas.13983

Webber, E.S., Harmon, K.M., Beckwith, T.J., Peña, S., Burgdorf, J., Panksepp, J., Cromwell, H.C., 2012. Selective breeding for 50kHz ultrasonic vocalization emission produces alterations in the ontogeny and regulation of rough-and-tumble play. Behav Brain Res 229, 138–144. https://doi.org/10.1016/j.bbr.2012.01.012

Wemelsfelder, F., Hunter, E.A., Mendl, M.T., Lawrence, A.B., 2000. The spontaneous qualitative assessment of behavioural expressions in pigs: first explorations of a novel methodology for integrative animal welfare measurement. Appl Anim Behav Sci 67, 193–215. https://doi.org/10.1016/s0168-1591(99)00093-3

Wemelsfelder, F., Hunter, T.E.A., Mendl, M.T., Lawrence, A.B., 2001. Assessing the ‘whole animal’: a free choice profiling approach. Anim Behav 62, 209–220. https://doi.org/10.1006/anbe.2001.1741

Whishaw, I.Q., Burke, C.J., Pellis, S.M., 2021. Does play shape hand use skill in rats? Exp Brain Res 239, 1895–1909. https://doi.org/10.1007/s00221-021-06097-6

Wickham, S.L., Collins, T., Barnes, A.L., Miller, D.W., Beatty, D.T., Stockman, C., Blache, D., Wemelsfelder, F., Fleming, P.A., 2012. Qualitative behavioral assessment of transport-naive and transport-habituated sheep. J Anim Sci 90, 4523–35. https://doi.org/10.2527/jas.2010-3451

Willner, P., 2016. Reliability of the chronic mild stress model of depression: A user survey. Neurobiology Stress 6, 68–77. https://doi.org/10.1016/j.ynstr.2016.08.001

Willner, P., 1997. Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. Psychopharmacology 134, 319–329. https://doi.org/10.1007/s002130050456

Willner, P., Muscat, R., Papp, M., 1992. Chronic mild stress-induced anhedonia: A realistic animal model of depression. Neurosci Biobehav Rev 16, 525–534. https://doi.org/10.1016/s0149-7634(05)80194-0

Willner, P., Towell, A., Sampson, D., Sophokleous, S., Muscat, R., 1987. Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. Psychopharmacol 93, 358–364. https://doi.org/10.1007/bf00187257

Wilmouth, C.E., Spear, L.P., 2009. Hedonic sensitivity in adolescent and adult rats: Taste reactivity and voluntary sucrose consumption. Pharmacol Biochem Be 92, 566–573. https://doi.org/10.1016/j.pbb.2009.02.009

Winder, C.B., LeBlanc, S.J., Haley, D.B., Lissemore, K.D., Godkin, M.A., Duffield, T.F., 2017. Clinical trial of local anesthetic protocols for acute pain associated with caustic paste disbudding in dairy calves. J Dairy Sci 100, 6429–6441. https://doi.org/10.3168/jds.2017-12724

Wöhr, M., 2017. Ultrasonic communication in rats: appetitive 50-kHz ultrasonic vocalizations as social contact calls. Behav Ecol Sociobiol 72, 14. https://doi.org/10.1007/s00265-017-2427-9

Wöhr, M., Houx, B., Schwarting, R.K., Spruijt, B., 2008. Effects of experience and context on 50-kHz vocalizations in rats. Physiol Behav 93, 766–776. https://doi.org/10.1016/j.physbeh.2007.11.031

Wöhr, M., Schwarting, R., 2013. Affective communication in rodents: ultrasonic vocalizations as a tool for research on emotion and motivation. Cell Tissue Res 354, 81–97. https://doi.org/10.1007/s00441-013-1607-9

Wöhr, M., Schwarting, R., 2009. Ultrasonic communication in rats: Effects of morphine and naloxone on vocal and behavioral responses to playback of 50-kHz vocalizations. Pharmacol Biochem Behav 94, 285–295. https://doi.org/10.1016/j.pbb.2009.09.008

Wöhr, M., Schwarting, R.K., 2007. Ultrasonic Communication in Rats: Can Playback of 50-kHz Calls Induce Approach Behavior? PLOS One 2, e1365. https://doi.org/10.1371/journal.pone.0001365

Wöhr, M., Schwarting, R.K.W., 2012. Testing social acoustic memory in rats: Effects of stimulus configuration and long-term memory on the induction of social approach behavior by appetitive 50-kHz ultrasonic vocalizations. Neurobiol Learn Mem 98, 154–164. https://doi.org/10.1016/j.nlm.2012.05.004

Wöhr, M., Seffer, D., Schwarting, R.K.W., 2016. Studying Socio-Affective Communicationin Rats through Playback of Ultrasonic Vocalizations. Current Protocols in Neuroscience 75, 8–35. https://doi.org/10.1002/cpns.7

Wright, Jennifer M , Gourdon, J.C., Clarke, P.B., 2010. Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context. Psychopharmacology 211, 1–13. https://doi.org/10.1007/s00213-010-1859-y

Yamamuro, T., Hori, M., Nakagawa, Y., Hayashi, T., Sakamoto, S., Ohnishi, J., Takeuchi, S., Mihara, Y., Shiga, T., Murakami, K., Urayama, O., 2013. Tickling stimulation causes the up-regulation of the kallikrein family in the submandibular gland of the rat. Behav Brain Res 236, 236–243. https://doi.org/10.1016/j.bbr.2012.09.001

Yamamuro, T., Senzaki, K., Iwamoto, S., Nakagawa, Y., Hayashi, T., Hori, M., Sakamoto, S., Murakami, K., Shiga, T., Urayama, O., 2010. Neurogenesis in the dentate gyrus of the rat hippocampus enhanced by tickling stimulation with positive emotion. Neurosci Res 68, 285–289. https://doi.org/10.1016/j.neures.2010.09.001

Yeates, J.W., Main, D.C.J., 2008. Assessment of positive welfare: A review. Vet J 175, 293–300. https://doi.org/10.1016/j.tvjl.2007.05.009

Zentall, T.R., 2001. Imitation in animals: evidence, function, and mechanisms. Cybernet Syst 32, 53–96. https://doi.org/10.1080/019697201300001812

Zupan, M., Buskas, J., Altimiras, J., Keeling, L.J., 2016. Assessing positive emotional states in dogs using heart rate and heart rate variability. Physiol Behav 155, 102–111. https://doi.org/10.1016/j.physbeh.2015.11.027