STRESS-RELATED ACOUSTIC COMMUNICATION IN THE DOMESTIC PIG (*SUS SCROFA*)

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1. GENERAL INTRODUCTION

1.1 STRESS, COPING, AND ANIMAL WELFARE

Hans Selye, who coined the term 'biological stress', was the first to point out that there are physiological processes common in responses to various noxious agents (Selye 1936). He subsumed these physiological responses to stressors (i.e. stress inducing stimuli) as 'general adaptation syndrome' (GAS; Selye 1978).

Traditionally, living organisms are assumed to strive to maintain a complex equilibrium of their internal states called homeostasis (Cannon 1932). Whenever the organism's homeostasis is disturbed, it responds with physiological and behavioural feedback mechanisms designed to restore homeostasis (Broom & Johnson 1993). Every deviation from homeostasis is considered stress. However, the concept of homeostasis has since been revised, leading to the concept of 'allostasis' (McEwen 1993), meaning stability through change. As opposed to homeostasis allostasis does not consider a steady state or 'set point' to be optimal for the organism, but the ability to adapt to anticipated demands. Thus, permanent changes of the environment are taken account of. The relationship between the well-being of the individual (i.e. animal welfare) and environmental challenges is assumed to form an inverted U-shaped curve. In this concept, hypostimulation (very low level of challenges) as well as hyperstimulation (high level of challenges) will result in impaired welfare, while good welfare is characterised by a high capacity to cope with environmental challenges. Only if an individual is challenged beyond its ability for adaptation, stress and impaired welfare occur (Korte et al. 2007, 2005). Limbic brain areas such as hippocampus, septum, and amygdala are involved in the regulation of allostasis (Korte et al. 2005).

Physiologically, there are two main fundamental endocrine systems involved in the stress response. Firstly, there is the hypothalamic-pituitary-adrenocortical axis (**HPA**; Selye's concept of GAS refers to the actions of this system). The response of the HPA to stressors starts with the release of corticotrophin-releasing hormone (CRH) in the hypothalamus (the paraventricular nucleus of the hypothalamus, PVH, plays a major role here). In the anterior pituitary (also called 'adenohypophysis') CRH elicits the secretion of adrenocorticotropic hormone (ACTH). ACTH is released into the systemic circulation and travels via the bloodstream to the adrenal gland, where it leads to the release of glucocorticoids from the adrenal cortex. Glucocorticoids have been described as crucial mediators of allostasis (Korte et al. 2005). They act on brain areas involved in the regulation of emotions (Korte et al. 2005) as well as organs associated

with energy metabolism (e.g. Nieuwenhuizen & Rutters 2008). Gluconeogenesis (i.e. the *de novo* synthesis of glucose) in the liver is increased by the mobilisation and breakdown of proteins from skeletal muscles. Thus, an adaptive redirection of energy occurs (e.g. Chrousos and Gold 1992, Lovejoy 2005).

Secondly, there is the sympathetic-adrenal medullar system (**SAM**). The SAM is associated with the synthesis and release of catecholamines from the adrenal medulla. The primary catecholamine released on the action of the SAM is adrenaline, while simultaneously the neural branch of the sympathetic system stimulates the release of noradrenaline from nerve terminals impinging on the cardiovascular system (e.g. Lovejoy 2005). As the sympathetic stress responses mostly involve information transport via nerve cells, it is much faster than the HPA response, which is facilitated by ACTH transport via the bloodstream.

The physiological stress response is linked to the systems responsible for reproduction, growth, and immunity (Chrousos and Gold 1992). In general, stress is considered to impair immune responses to pathogens. However, the responses can be stressor-specific. For example, Bowers and colleagues (2008) showed that several commonly used laboratory stressors (handling, restraint, forced swim, isolation, and low ambient temperatures) elicited different responses of corticosterone levels and immune parameters (trafficking of lymphocytes and monocytes) in mice. Moreover, Salak-Johnson and McGlone (2007) suggested that, in addition to specific stressor quality, the social enviroment of an animal can affect immune system activity. Morrow-Tesch and colleagues (1994) showed that socially dominant and submissive pigs are affected more by heat and social stress compared with socially intermediate individuals.

In farm animals, stress is also relevant for meat quality. Pre-slaughter handling has repeatedly been demonstrated to have adverse effects on sensory characteristics of pork (Rosenvold & Andersen 2003, Terlouw et al. 2008). Thus, stress in farm animals plays a role in public perception and acceptance of meat (Verbeke et al. 1999). Furthermore, public concerns are driven by associations of stress and negative emotions (e.g. Broom & Johnson 1993). Emotions can be considered as comprising behavioural, physiological, and subjective components (e.g. Paul et al. 2005). While the subjective component is inaccessible in animals, the physiological and behavioural components can be measured. Through projections to brainstem and spinal autonomic preganglionic neurons as well as to the hypothalamus, the limbic system has an organizing effect on responses to stress (e.g. Lopez et al. 1999, Pacák and Palkovits 2001). As mentioned earlier, the limbic system is also involved in allostasis. The amygdala, which is part of the limbic system, is the central structure underlying fear

acquisition and expression (Davis 1994, 1997; Davis et al. 1997; LeDoux 2000; Davidson 2004; Paré et al. 2004; Phelps 2006). Additionally, brain areas involved in anxiety and pain show high concentrations of CRH-receptors (Symreng & Fishman 2004). Physiological parameters comprise measures of stress hormones such as cortisol or noradrenaline as well as more indirect measures such as heart rate and heart rate variability. Heart rate measurements represent a non-invasive method for the assessment of stress (von Borell et al. 2007). They express the actions of the sympathetic and parasympathetic branch of the autonomous nervous system. Behavioural responses to stress and/or negative emotions comprise effects on locomotory activity and exploratory behaviours, but also vocalisations.

1.2 ACOUSTIC COMMUNICATION

In general, every communicative event requires at least three basic elements: sender, signal, and receiver (Wiley 1983, Bradbury & Vehrencamp 1998). The signal is generally assumed to convey information on the state of the signaller, e.g. its arousal (e.g. Ehret 2005), motivational state (Zahavi 1981), emotional state (e.g. Brudzynski 2007), or physical characteristics (e.g. Harris et al. 2006, Pfefferle & Fischer 2006). Based on this information, the likelihood that the receiver performs a specific behaviour changes, i.e. in most cases a change of behaviour can be observed. There are at least two partly conflicting views on the function of communication (cf. Owings & Leger 1980 for a brief review), the “information hypothesis” (e.g. Smith 1977) and the “manipulation hypothesis” (e.g. Charnov & Krebs 1975, Dawkins & Krebs 1978, Krebs & Dawkins 1984). The information hypothesis proposes that both the sender and receiver benefit from the transfer of information provided by communication. Opposed to that, the manipulation hypothesis states that by communicating, the sender manipulates the receiver in a way that is advantageous for the sender; whether or not the receiver benefits from its response is not crucial (reacting might also be detrimental).

Due to the facility of rapid frequency- and/or amplitude modulations, acoustic signals are highly variable. Therefore, they can be used for communication under various environmental and social circumstances. By changing the amplitude, sound signals can be adapted to high or low levels of background noise and to long- or short-range communication. For example, playback experiments have shown that African elephants (*Loxodonta africana*) transmit information about membership in a certain family or bond group (several families with frequent contact) across distances of up to 2.5 km via infrasonic communication (McComb et al. 2003a, 2003b). Acoustic signals can be more or less easy to locate, depending on time- and frequency-structure. Moreover, sound

production as well as perception does not necessarily interfere with other activities such as feeding (Marler & Hamilton 1966, p. 473). The main problems of sound transmission are attenuation and degradation (e.g. Wiley & Richards 1978, Naguib & Wiley 2001; see also Barnard 2004, pp.559 et seqq.). These are highly dependent on the physical environment (Marten & Marler 1977, Wiley & Richards 1978, Gerhardt 1983). Most acoustically active species make use of different sounds, which can be recorded and arranged in categories according to their general physical structure or the contexts in which they are produced.

1.3 STRESS-RELATED VOCALISATION

In 1872 Charles Darwin pointed out that vocalisations can express emotions. Since then, a broad range of vocalisations associated with emotional states has been described. A prominent example of vocalisation indicative of the emotional state of the sender is ultrasonic vocalisation (USV) in rats (*Rattus norvegicus)*. While so-called 22 kHz-calls are emitted in negative contexts, 50 kHz-calls are supposed to be associated with positive contexts (Brudzynski 2001, Brudzynski 2007, Burgdorf et al. 2007, Panksepp 2007). However, the interpretation of 50 kHz-calls might be more complex (Wöhr et al. 2008). Another focus has been on vocalisation uttered in response to threatening stimuli, i.e. potential stressors. Along with the physiological and behavioural reactions to stress described above, vocalisation has been described as an element of the stress response. Such vocalisations can be highly referential, e.g. vocalisations in the predator context (vervet monkey, *Cercopithecus aethiops*: Struhsaker 1967, Seyfarth et al. 1980; California ground squirrel, *Spermophilus beecheyi*: Owings & Leger 1980; alpine marmot, *Marmota marmota*: Blumstein & Arnold 1995; rat: Litvin et al. 2007). In literature, vocalisations have been described as ‘fear screams’, ‘alarm calls’, or ‘distress calls’. The terms ‘distress call’ and ‘fear scream’ focus on the context of sound production or the emotional state of the sender, while ‘alarm calls’ are interpreted regarding their biological function by examining the receivers’ responses. However, although the approach differs, all these types of vocalisation can be considered indicators of stress perceived by the caller. For example, in rhesus macaques (*Macaca mulatta*) the level of cortisol correlates with the number of alarm calls (Bercovitch et al. 1995).

Similarly, farm animals show vocal responses to stress. For example, cattle vocalisations could be associated with aversive events in commercial slaughter plants (Grandin 2001) and have been suggested as an indicator of welfare problems (Grandin 1998). In domestic pigs (*Sus scrofa*), castration of male piglets causes vocalisations

reflecting experienced pain and suffering (Puppe et al. 2005). These vocalisations can be interpreted as 'honest' signals, giving correct information on the current state of the sender (e.g. Fitch & Hauser 1995, Fitch 1997). Piglets separated from the sow produce vocalisations with higher peak frequency and duration when in a 14°C 'cool' room compared to their littermates isolated in a 30°C 'warm' room (Weary et al. 1997). When piglets are denied access to their teat (i.e. access to milk, as they do not get access to another teat due to competition amongst littermates), their vocalisation rates increase (Appleby et al. 1999). Thus, piglets seem to communicate their need for maternal care. Schrader and Todt (1998) showed that increasing levels of adrenaline correlated positively with the number of 'squeal-grunts', whereas cortisol levels correlated negatively with 'grunts' during the isolation of six-month old pigs.

Thus, vocalisation can be considered indicative of stress and impaired welfare (Manteuffel et al. 2004, Burman et al. 2007). Like indicated earlier, stress leads to impaired animal welfare. Due to the ethical (e.g. Fraser et al. 1997) as well as practical (Terlouw et al. 2008) consequences of poor welfare, reliable indicators of stress need to be found. As stress-related vocalisation seems to convey honest signals in pigs, bioacoustical analyses provide a non-invasive tool for measuring stress (Weary & Fraser 1995b). Such analyses might even be performed on farm in real-time approaches (Schön et al. 2001, 2004).

1.4 THE AIMS OF THIS THESIS

This thesis focuses on stress-related vocalisation in pigs. It comprises theoretical aspects of intraspecific communication, proximate factors connecting emotions and vocalisation on the neuronal level, as well as more praxis-related topics such as the assessment of farm animal welfare.

Current approaches to reduce stress in farm animals and thus increase their welfare are in need of valid, reliable, and non-invasive measures of stress. Vocalisation has been suggested to be an indicator of stress, providing honest signals about the current state of the caller. However, up to now, most studies focused on the responses of single, specific stressors instead of a whole range of praxis-relevant stressors, often using different parameters for sound description. Hardly any effort has been made to define and discriminate between vocal responses to different stressors based on a unified set of parameters. First attempts have been made at developing automated detectors of stress-related vocalisation in pigs (STREMODO: stress monitor and documentation unit; Schön et al. 2001, 2004). However, welfare assessment based on vocalisation would benefit from detailed information on the specific kind of stress

occurring, thus a description of such calls with one set of parameters is crucial to the establishment of this approach. Thus, in the experiments described in the **first chapter** of this thesis, I subjected the animals to various kinds of stressors in order to describe situational specific (i.e. referential) vocalisations based on one set of parameters. I introduced a new analysis which might improve real time analysis of bioacoustic events and subsequent detection of specific stress-related calls. I hypothesised that pigs show stressor-specific vocal responses.

In addition to the context of signalling, the responses of receivers of a communicative signal can give information on the meaning of the signal. Stress-related acoustic communication can be assumed to be honest signalling. Honest signals enable the receiver of such a signal to react appropriately. Such responses might comprise arousal or stress in the receivers, which can be a problem in farming practice (e.g. during transport or at the abattoir). In order to examine the communicative significance of porcine stress-related vocalisation, and to estimate their effect on animal welfare, I performed a playback experiment, which is described in the **second chapter** of this thesis. Juvenile pigs were played conspecific stress-related calls, and their responses were measured both in behaviour and physiology (heart rate variability). My hypothesis here was that stress-related vocalisation elicits arousal or even stress in their conspecifics.

There are studies suggesting an association of negative emotional states and stress-related vocalisation in laboratory animals. Microinjection studies revealed a pathway from the tegmentum to the preoptic area involved in fear related vocalisation in rats. In pigs, such studies have not been performed yet. However, emotional states play a crucial role in public concerns about animal welfare. Therefore, in the **third chapter** I established a method for microinjection studies in pigs. It was used to examine the proximate, neurophysiological factors underlying stress-related vocalisation. The brain areas I focused on were the amygdala and cingulate cortex (both involved in aversive emotions). I hypothesised that these areas, which underlie the regulation of negative emotions, do also underlie stress-related vocalisation. Thus, the neuropharmacological stimulation of these areas should elicit stress-related vocalisation.

2. METHODS OF SOUND ANALYSIS

Sounds are produced when the air pressure changes. In most mammalian vocalisation, these changes of air pressure are created at the vocal folds. Air streams from the lungs through the space between the vocal folds (glottis). The vocal folds vibrate, thus the volume flow of air is modulated. Here, a pure sinus wave, the so-called fundamental frequency, and a series of its multiples, the so-called harmonics, are generated. Subsequently, the signal generated at the vocal folds is modified while trespassing the vocal tract (i.e. trachea, oral, and nasal cavities). This modification depends on the resonance frequencies of the vocal tract. Based on constriction(s) in the vocal tract, some of the harmonic frequencies (or even the fundamental frequency) can be attenuated before the signal leaves the oral cavity. Additionally, turbulence can occur, generating noise (e.g. Stevens 1998).

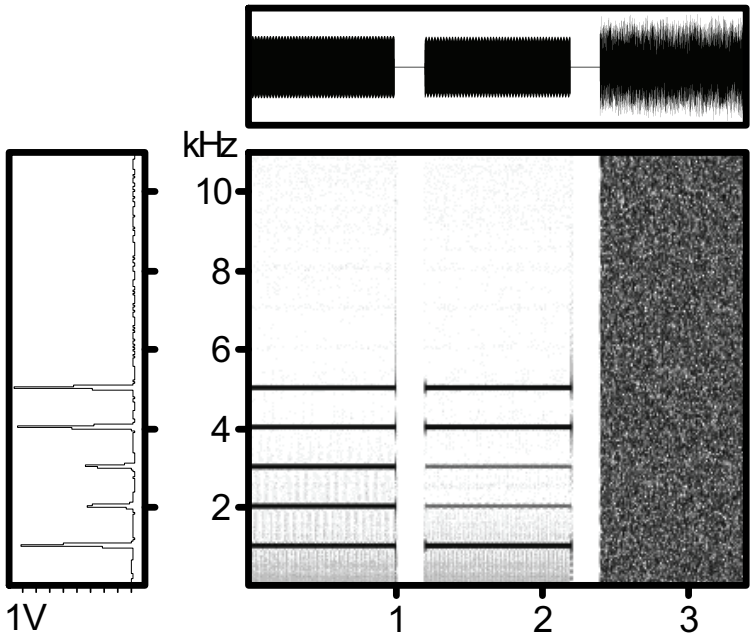

Figure 2.1: Time signal (upper graph), power spectrum (lower left), and spectrogram (lower right) of three artificial sounds. On the left a harmonic sound with a fundamental frequency of 1 kHz and four harmonics of equal amplitude. In the middle the same harmonic sound with attenuated second and third harmonic frequency. On the right so-called white noise, where all frequencies of the spectrum have the same amplitude.

Figure 2.1 shows artificial harmonic sounds with or without attenuation of harmonics, and so-called white noise, which does not show any harmonic elements. Bioacoustical signals cover the whole range from harmonic to non-harmonic sounds (cf. Fitch et al.

2002). The textbook example of sound production is the production of vowels in human language. The vowels contain fundamental and several harmonic frequencies. However, only certain frequencies will not be attenuated. The five vowels are defined by their bands of frequencies called formants (one formant can include more than one harmonic frequency).

Every complex periodic signal can be described as the sum of an infinite number of sinus waves of different amplitude and phase. This procedure is called Fourier transformation. However, calculations of Fourier transformations require too long computation time for practical sound analysis, thus fast Fourier transformations (FFT) are used. The FFT splits a complex periodic signal into a finite number of sinus waves with different amplitude but the same phase (e.g. Burgess 1998). As most natural signals are not periodic, windowing and averaging is applied. These algorithms improve calculations of the frequency spectrum (e.g. Pope 1998).

For analyses, analogue sound signals need to be quantised in time and amplitude, resulting in digital signals. The sampling frequency (sampling = quantisation in time at a uniform rate) determines in which intervals discrete data values are taken from the analogue signal. The higher the sampling frequency, the higher the maximum frequency that can be recovered after sampling. The maximum frequency present in the digital signal (the so-called Nyquist frequency) is half of the sampling rate. Thus, in order to analyse frequencies of up to 22 kHz, a sampling frequency of 44.1 kHz is needed (e.g. Pope 1998).

Based on FFT, several parameters can be derived for the description of sounds. They can be calculated as means across a whole call, or at specific time points within a call. The most frequently used parameter is the peak frequency, i.e. the frequency with the highest amplitude in the frequency spectrum. Other parameters refer to the distribution of energy across the spectrum. For example, the total energy can be divided in quartiles. Thus, 25 percent of the total energy are below the first quartile, 50 percent are below the second quartile, and 75 percent below the third quartile. The difference of the third quartile and the first quartile gives the width of the band in which half of the energy is contained. This value provides information on the pureness of a call. Entropy is another parameter describing energy distribution. High entropy means a low degree of order in the spectrum. It is assumed that a high entropy reflects more unspecific factors affecting vocalisation, whereas entropy is lower when a specific cause underlies call production (e.g. Puppe et al. 2005). The parameters described here can be used alone or in multiparametric analyses (Schrader & Hammerschmidt 1997).

Another approach in sound analysis uses complex algorithms. One such approach is based on linear prediction coding (LPC). LPC analysis extracts characteristics of a call by using a linear forecast model. This procedure models signal alterations instead of the actual signal itself and is a formal equivalent to the source-filter-model of the vocal tract (Fant 1970). The LPC-coefficients derived in this analysis correspond to the filter coefficients of the vocal tract (formant-like structures or resonance frequencies). For the characterisation of pigs' distress calls, 12 LPC-coefficients, equivalent to the first six resonance frequencies, have been shown to be sufficient for call discrimination (Schön et al. 2004).

3. CHAPTER 1

STRESSOR-SPECIFIC VOCALISATION

3.1 INTRODUCTION

The detection and measurement of mental, emotional or motivational states in mammals are of growing interest, especially when animal welfare is concerned. It has been shown in several species that specific mental states can be associated with the production of distinct vocalisations (Panksepp and Burgdorf 2000, Burgdorf and Panksepp 2001, Knutson et al. 2002, Panksepp and Burgdorf 2003, Burman et al. 2007). In this context, stress-related vocalisation is of special interest as an indicator of impaired welfare (Weary and Fraser 1995a, Grandin 1998, 2001; Schön et al. 2004), as it has repeatedly been shown to represent honest signalling (Weary & Fraser 1995a, Puppe et al. 2005). The detailed characterisation of such vocalisations enables the evaluation of stress levels in a non-invasive approach (Manteuffel et al. 2004). Vocalisation of domestic pigs is currently being established as an indicator of stress (von Borell and Ladewig 1992, Schrader and Todt 1998, Weary et al. 1998, Manteuffel et al. 2004, Schön et al. 2004, Puppe et al. 2005), possibly representing a powerful, non-invasive tool for detection and measurement of stressor intensity and/or quality. The meaning of non-invasive methods for assessing (impaired) animal welfare is growing at present. This development calls for a high level of specificity of parameters indicating welfare impairment.

3.1.1 Predictability of the environment

Anticipation is a mental state that occurs when events are predictable, usually because they are announced by a learned cue. Learning of such cues is often based on classical conditioning, where an initially neutral stimulus is repeatedly paired with a biologically significant event and eventually elicits the same set of reactions. The anticipation of emotional events can influence the behaviour of an individual, including its vocalisation (Knutson et al. 1998, Burgdorf et al. 2000).

Predictability of the environment in general, i.e. of positive as well as aversive events, has been suggested to have positive effects on stress levels (reviewed by Bassett and Buchanan-Smith 2007).

Predictable negative events: Fear conditioning

Stressors can influence the affective state of the animal, which, in turn, can affect an individual's responses to environmental stimuli (Harding et al. 2004, Paul et al. 2005). In the context of aversive classical conditioning predictability has been shown to lead to lower levels of anxiety, negative valence and pain intensity (Carlsson et al. 2006),

although the evidence for effects of predictability on stress levels and health in animals is still contradictory (Bassett and Buchanan-Smith 2007). However, predictable aversive stimuli have been preferred over unpredictable stimuli in several studies (reviewed by Weinberg and Levine 1980). Anticipation of an aversive event represents a definite mental state equivalent with the emotion of "fear" (LeDoux 2000, 2003). In recent research, conditioned fear is the focus of studies on emotional learning. It could be shown that the amygdala, a limbic brain structure, plays a crucial role in fear acquisition and expression (Davis 1994, 1997; Davis et al. 1997, LeDoux 2000, Davidson 2004, Paré et al. 2004, Phelps 2006). Vocalisations normally associated with aversive events (commonly termed "distress calls") could be elicited by stimulating the amygdala pharmacologically (Manteuffel et al. 2007), supporting their interpretation as indicators of emotionally aversive states.

In the present study, I focused on the effects of physically and emotionally aversive stimuli on vocalisation in the pig using a classical aversive conditioning paradigm (often termed "fear conditioning") with a moderate electric shock as aversive stimulus (unconditioned stimulus, US) and light as conditioned stimulus (CS). This allowed the separation of influences of mental and physical stressors and, therefore, differentiation between the referring responses: before learning application of the aversive stimulus represents a purely physical stressor, while afterwards presentation of the conditioned stimulus alone imposes mental stress. As a result of the conditioning process, the occurrence of the aversive stimulus becomes predictable, and the presentation of the conditioned cue elicits anticipation. I studied the consequences of the resulting state of fear on the response towards the feared stimulus by applying both CS and US after successful learning. Subsequently, the responses of pigs to the CS alone were tested. I hypothesised that vocal responses to the aversive stimulus before and after learning as well as to the anticipation alone differ due to differing stressor quality and that they can be classified accordingly. Thus, analyses of pigs' vocalisation could serve as an indicator of stressor quality.

Predictable positive events: Anticipation of food

Recent research on predictability of the environment mainly focuses on the anticipation of rewards. It has been shown that the announced behavioural enrichment can reduce the negative effects of weaning stress in piglets more than the same enrichment without announcement (Dudink et al. 2006). Successful coping with a food rewarded learning paradigm reduced general fear responses of pigs in an open field test (Puppe et al. 2007). Analogous to these results, anticipatory behaviours have been suggested to be indicative of animal welfare (Spruijt et al. 2001, van der Harst et al. 2003a,

2003b). However, although predictability of the environment is generally considered positive (Weinberg & Levine 1980), the interpretation of predictable feeding regimes is more complex, as it seems to depend on housing conditions and competition. In group living brown capuchins (*Cebus apella*), a predictable feeding regime was associated with lower cortisol levels, indicating higher welfare (Ulyan et al. 2006), whereas in pigs predictable feeding can also elicit higher levels of aggressive behaviours prior to feeding (Bassett & Buchanan-Smith 2007). Thus, increased competition might counteract the positive effects of predictability.

Pigs start to 'scream' when someone enters the room shortly before their usual feeding time; once the animals have access to food, vocalisation stops (personal observation). Additionally, in a study at the Research Unit Behavioural Physiology of the FBN, domestic pigs showed a peak of vocalisation rate around feeding time (P.C. Schön, personal communication). These vocalisations were detected by STREMODO, an automatic monitor for distress vocalisations of pigs (Schön et al. 2001, 2004). Based on STREMODO, vocalisation at feeding time would be considered indicative of stress. It is likely that these vocalisations directly before pigs get access to food caused the peak of distress calls around feeding times. Most probably, the animals have learned an association between a person (a caretaker) entering the room at specific times and the feeding soon afterwards through classical conditioning.

Thus, the emotional status underlying vocalisation before feeding is difficult to interpret. On the one hand, anticipation of food should represent a positive expectation. However, as the food is not available yet animals might also be frustrated. Competition within the group might cause additional physical stress. Furthermore, anticipation might also cause a rather neutral arousal. Thus, it might be helpful for a classification device for distress calls of pigs to be able to discriminate between vocalisations in anticipation of food and other stress-related vocalisation.

3.2.2 Praxis-relevant stressors

Tail biting

In pig husbandry, several stressors impair animal welfare. Animals housed in barren environments often show stereotypic behaviours (Broom & Johnson 1993). In pigs, stereotypies are often oral behaviours, such as bar gnawing and tail biting. Tail biting can cause severe wounds, especially as usually several group members bite the same individual (personal observation). This can cause inflammations in the tail that might even spread to the spine, leading to lameness of the hind legs (Schrøder-Petersen &

Simonssen 2001, Moinard et al. 2003, Zonderland et al. 2008). Due to these implications for animal health, it is necessary to detect bitten animals as soon as possible. The physical pain associated with being bitten possibly elicits specific vocal responses (Symreng & Fishman 2004, Puppe et al. 2005, Carlson et al. 2006). These could be used as indicators of tail biting.

Restraint

In the wild, animals might be held on to by predators or are stuck in environmental structures. This physical restraint can be considered stressful, as either there is the direct threat imposed by a predator, or the indirect threat of failing to free oneself and starving. Restraint stress has been demonstrated to increase serotonin release in the amygdala (Mo et al. 2008). The activating effect was confined to the central, but not the medial nucleus of the amygdala. The effect was mediated by CRH-receptors, and is thus connected directly to the activation of the HPA. However, Schrader and Todt (1998) found no effects of immobilisation when comparing noradrenaline and cortisol levels of isolated and isolated + immobilised subjects. Opposed to that, Fraser (1975b) showed that suckling piglets vocalise in response to restraint, but this response was attenuated in older piglets (Fraser 1975b). Additionally, restraint prior to castration or during sham castration (Puppe et al. 2005, Weary et al. 1998) caused vocalisations with peak frequencies above 1 kHz.

Social isolation

Living in groups has several advantages for an individual. Especially when animals are living in groups of family members, there are benefits for direct as well as indirect fitness. Therefore, when an animal looses contact to its group, it should strive to re-establish the contact. In order to do so, vocalisations might be produced. In guinea-pigs, suckling pups removed from their mothers produce a specific type of vocalisation. When these are broadcast to their mothers, they approach the loudspeaker and vocalise themselves (Kober et al. 2007). The wild ancestors of the domestic pig live in matrilineal social groups consisting of related females and their offspring. Male offspring leave the group at the onset of adolescence (Gundlach 1968). Social isolation means the loss of benefits provided by the group, representing a stressor. In response to social isolation, suckling piglets emit ‘squealing’ (Fraser 1975a, 1975b). However, even during the suckling period, calling rates decreased with age. Thus, it is unclear whether juvenile pigs still show vocal responses to isolation.

3.1.3 Octave analysis – A new model for bioacoustic signals

There are various ways of describing sounds. These vary from sets of single parameters such as duration, peak frequency and fundamental frequency and harmonics, to mathematical models based on the time- or frequency spectrum. The latter enable a more complex description of natural sounds. However, up to now the use of specific models (as well as some of the more traditional parameters, e.g. fundamental frequency) is reduced to specific sound categories. For example, linear prediction coding (LPC, Schön et al. 2001, 2004) is a valuable method for the description of sounds which are mainly formed by the vocal tract. It gives a measure of the resonance frequencies of the vocal tract. In human speech, these are called formants, with the formants defining the five vowels. In pigs, LPC has proved to be a good method for describing harmonic vocalisations which occur during high levels of distress and/or pain, i.e. screams (Puppe et al. 2005, Düpjan et al. 2008). However, there are several non-harmonic sounds caused by turbulences generated in the vocal tract (Fitch et al. 2002). These cannot be described sufficiently using the LPC approach. For example, in the domestic pig nursing grunts produced by the sow can be described using the so-called cepstrum or the frequency spectrum (Schön et al. 1999).

As there seem to be types of “grunt-like” (i.e. non-harmonic) vocalisations in the pig indicating a certain level of arousal (see section 3.4: social isolation), it would be preferable to have one model describing harmonic as well as non-harmonic vocalisations. This model should reduce the data to allow for real-time classification on (affordable) standard PCs, so it can be implemented in STREMODO (Schön et al. 2001, 2004). To my knowledge, such a model does not yet exist in bioacoustic research.

Therefore, in collaboration with Dr. Schön from the Department of Behavioural Physiology of the FBN (who created an executable software based on LabView®, helped with the processing of the recordings from social isolation, and the training of the neural networks), I introduced a method for modelling bioacoustic signals based on the so-called fractional octave analysis. This model is independent of the underlying mechanisms of sound production, while the amount of data is reduced sufficiently to maybe even enable real time analyses.

The fractional octave analysis is one type of constant percentage bandwidth filters (Pope 1998). It divides the frequency spectrum into frequency bands by setting band pass filters. One octave covers a band with the upper boundary twice the value of the lower one, e.g. from 1 kHz to 2 kHz. The bandwidth increases logarithmically, resulting in higher resolution in the lower frequencies. Thus, it acts similar to human hearing. By

convention, the first full octave chosen for analyses has a centre frequency of 1 kHz, with the other bands resulting from this starting point. In fractional octave analysis, the bands cover less than one octave. For example, in third octave analysis three bands are within one octave. The frequency bands are defined by the highest (f_h) and lowest (f_l) frequency. The filters are set as $f_h = f_l * 2^{1/n}$, where n is the resolution, i.e. 1 for full octave analysis, 3 for third octave analysis, and so on. The geometric mean of each band can be calculated as $\sqrt{f_h * f_l}$. Conventional standards (IEC 1995, ANSI 1986) specify digital filters that have some roll-off. Thus, a single sine-wave signal would be represented in several band adjacent to its own, preventing broader frequency bands from being divided into minor peaks.

In this section I applied third and sixth octave analyses to the data presented in section 3.4, i.e. recordings from tail biting, restraint, and social isolation. In order to evaluate its usability for bioacoustic analyses of pigs' calls I compared octave analyses with the LPC model. As the latter requires resonance frequencies occurring in the signals, discrimination of sound categories (comprising both screams and grunts) should be better based on the octave analyses.

3.1.4 Aims of this chapter

The focus of this chapter is the production of acoustic signals by domestic pigs in different contexts. For this purpose, I observed female, juvenile pigs (German Landrace) in response to different stressors, and analysed their vocalisations. Based on the recordings, principles of stressor-specificity were derived and for part of them, a new classification model was established. The chapter comprises five different experiments in four sections. **Section 3.3** examines the influence of stressor type (physical and/or mental) on vocal responses. This experiment represents a classical fear conditioning study in a laboratory setting. Subsequently (in **section 3.4**), I analysed vocalisations in anticipation of food. The affective background created in this situation is unclear, thus the analysis of vocal responses might shed some light on it. The following three experiments, which will be subsumed in **section 3.5**, focus on stressors likely to occur in daily farming practice. Thus, they are of relevance for the estimation of animal welfare in meat production. In this section, I will describe the vocal responses produced in response to a model of tail biting, restraint, social isolation qualitatively, using conventional parameters of sound analysis. Finally (**section 3.6**), I

will introduce a new model for sound description called fractional octave analysis. This new model applied to the vocalisations produced in response to tail biting, restraint and social isolation.

3.2 GENERAL METHODS

3.2.1 Animals and housing

A total number of 20 subjects was used in fear conditioning, tail biting, restraint, and social isolation, whereas for anticipation of food only 16 subjects were used. Subjects were outbred, female German Landrace pigs at an age of 10 (fear conditioning, anticipation of food, social isolation) or 11 weeks (tail biting, restraint). Prior to experiments, animals were housed in groups of four subjects each for 2 to 2 ½ weeks. The group pen measured 4 by 4 metres, with half solid concrete floor and half fully slatted floor. Subsequently, for all stressors except social isolation subjects were transferred to single pens, providing acoustic contact to the former group mates. The single pens measured 1.0 by 1.9 metres, with half slatted plastic and half solid concrete floor. Four pens, each separated from the neighbouring pens by one vacant pen in between, were arranged in a row. The single housing allowed for stimulation of single animals (fear conditioning) and the transport of a single animal to the recording room without disturbing the other subjects (restraint and tail biting). Food pellets (Ferkelstarter Plus, Trede und von Pein, Itzehoe, Germany) and water were available ad libitum (except for the experimental periods during fear conditioning and social isolation, and for two hours before anticipation of food). A mixture of straw cuttings, wood shavings and hemp pellets was provided as rooting substrate and bedding material.

3.2.2 Acoustic recordings

Vocalisations were recorded with undirected microphones (fear conditioning, anticipation of food: Sennheiser evolution wireless series EW 112 G2: microphone: ME2, sender: SK 100 G2, receiver: EM 112 G2; tail biting, restraint, social isolation: Sennheiser ME64/K6). During fear conditioning and anticipation of food, sounds were recorded by a program based on LabView® (National Instruments Corporation, Austin, Texas, USA). The recording programme included an amplitude threshold in order to reduce data by excluding random ambient noises of low amplitude. An electronic protocol was generated giving date, time, delay between onset of stimulation and first recorded acoustic event and, subsequently, intervals between acoustic events.

Vocalisations produced in response to tail biting, restraint, and social isolation were recorded with a digital audio tape recorder (Sony TCD-D100). Based on previous experiences in the Department of Behavioural Physiology, sampling rates of 22.05 kHz have been shown to be sufficient for the analysis of porcine calls (e.g. Schön et al. 2004, Puppe et al. 2005). Thus, the recording program used in fear conditioning and anticipation of food operated with a sampling frequency of 22.05 kHz (accuracy: 16 bit, mono). However, the octave analysis described in section 3.5 requires a sampling rate of 2.5 to 3 times the maximum frequency analysed. Thus, for the further experiments (tail biting, restraint, social isolation) a sampling frequency of 44.1 kHz was used.

3.3 FEAR CONDITIONING

3.3.1 Methods

Experimental procedure

Conditioning took place in the subjects' home pens. Thus, influences of additional stress due to transportation and/or contextual learning (association of aversive stimulus and training environment) were avoided (Langbein et al. 2004), while sufficient recording conditions were provided.

For classical conditioning, light and a moderate electric shock were used as conditioned stimulus (CS) and unconditioned stimulus (US), respectively. The visual conditioned stimulus allowed for separation of CS and the expected vocal response (conditioned response, CR).

Light (duration: 2.5 s) was provided by halogen spots built-in on the pen doors. They illuminated all pen walls, so the subject could perceive the light stimulus irrespective of its orientation in the pen, while no subject could see another one's light due to the single housing with separating vacant pens.

The electric shock was applied by a battery operated livestock prod (Torero 2000, Elektroistallation Boeck, Buchbach Oberbay, Germany; U~2000 V, I<1 mA, duration: 500 ms; supplied with an additional remote control unit) with a delay of 2 s after the onset of the CS (delay conditioning). It was placed in a belt the subjects were wearing continuously from the day before first training onwards. Copper wires (length ca. 12 cm, distance ca. 4 cm) connected to the prod and leading underneath the belt conducted the current to the subject's skin between and slightly below the shoulder blades.

Conditioning comprised six sessions with five stimulations per animal. The stimulations were separated by intervals of at least 15 min within subjects and approximately 5 min between subjects. Sessions were performed on three consecutive days with two sessions each day (morning: 8-10 a.m.; afternoon: 13-15 p.m.). During the first three sessions, the subjects were presented both US and CS at each stimulation. On the last three sessions, only the first stimulation comprised both US and CS (to avoid extinction due to repeated non-appearance of the electric shock), while on the subsequent four stimulations only CS was presented.

Application of stimuli and recording of vocal responses was controlled by a program based on LabView® (National Instruments Corporation, Austin, Texas, USA). Stimulation was started manually, which triggered the US/CS-sequence as well as an automatic sound recording programme. The microphone was placed centrally in front of the row of experimental pens at a height of approximately 1.9 m. Sounds were stored to the hard disk drive of the control PC. Additionally, behavioural reactions before onset of US (i.e. reactions associated with noise due to movement/startle) and interferences by non-focus animals were recorded in writing.

Acoustic and statistical analyses

The vocal responses comprised high-frequency elements (HF) and low-frequency elements (LF), the former occurring in time with the US and shortly afterwards as well as at the onset of vocal responses to the CS. HF can thus be considered the direct responses towards electrical stimulation, i.e. the physical stressor, while the cause and meaning of LF is less clear. Hence, only HF were considered in the analyses. High-frequency calls have been suggested to be reliable indicators of distress in pigs (Weary and Fraser 1995a, Manteuffel et al. 2007).

During conditioning, the subjects learned the association of light and electric shock. The resulting anticipation was indicated by behavioural responses towards CS before US onset, comprising startle and vocal responses.

Three stages were distinguished: the first stage comprised stimulations with CS + US without anticipation of US (non-anticipated aversive stimulus, NS); the second stage included CS + US stimulations with response to CS, i.e. anticipation of US (anticipated aversive stimulus, AS); stage three comprised all stimulations with CS only (anticipation of aversive stimulus, A). In AS only vocal responses from US onset onwards were considered for evaluation in order to analyse the response to anticipated physical stimulation. Due to different learning progress between subjects NS and AS were identified individually. Three stimulations per subject and stage including HF were selected to provide a more balanced dataset. Subjects with less than three responses

in one or more of the stages were regarded as of low general reactivity and therefore were excluded. For subsequent analyses HF were extracted using STREMODO, an automatic system for HF-call detection in pigs based on a general model of porcine distress call characteristics using linear prediction coding (LPC) and a neuronal network (for a detailed description of STREMODO see Schön et al. 2004). Twelve LPC coefficients were estimated for time windows of 92.88 ms, constituting one parameter vector per time window (see also Schön et al. 2001).

In order to analyse the effects of varying stressors on vocal responses, a comparison of the means of LPC-vectors originating from the three different stages was conducted using multivariate analysis of variance (MANOVA, F statistic for Wilk's lambda) with the twelve LPC-coefficients as dependent variables. Additionally, analyses of variance (ANOVA) were performed for univariate testing of the class means for each variable (i.e. LPC-coefficient). These allowed for the identification of LPC-coefficients showing most pronounced effects. By inversing the LPC algorithm, we calculated the resonance frequencies referring to these LPC-coefficients. For both MANOVA and ANOVA the SAS procedure DISCRIM (SAS® 9.1, SAS Institute Inc., Cary, NC, USA) was employed by setting the corresponding options. Subsequently, means of those LPC-coefficients showing significant differences ($p<0.05$) in the ANOVA underwent post hoc testing (Tukey-Kramer) with the SAS procedure MIXED, taking repeated measurements into account.

Furthermore, the classification of parameter vectors from the LPC-model into the three different classes (i.e. stages) was done with the SAS procedure DISCRIM by a non-parametric discriminant analysis (DA) using the k-nearest-neighbour method ($k=2$). The probability of misclassification (i.e. assigning the parameter vector to the wrong class) was estimated by cross validation. This method excludes a single parameter vector of the classification model, calculates a new classification model without the excluded parameter vector, and the excluded parameter vector is then tested concerning the class membership. The procedure is repeated stepwise for each vector in the dataset. Thus, the DA provides information about whether distress calls can be classified reliably regarding stressor quality, which is the basic prerequisite for automated stress measurements based on bioacoustic analyses.

Discriminant function analyses were made for each of the subjects separately and the median classification probabilities were calculated.

Habituation can cause continuous decreases or increases of response parameters along stimulations. We controlled for habituation by calculating MANOVAs comparing the LPC coefficients of the three calls selected for each subject within a stage. Additionally, we calculated non-parametric repeated measures ANOVAs for the

number of HF vectors (i.e. number of 92.88 ms time-windows) for the three consecutive calls within each stage.

3.3.2 Results

The learning process as measured by the number of stimulations (CS+US) until first reaction to the CS was individually different (Fig. 3.3.2.1). It ranged from 5 (3 subjects) to 14 (one subject) with a median of 9 stimulations. Both MANOVAs of LPC vectors and non-parametric repeated measures ANOVAs of number of HF per call did not reveal any systematic changes over time within each period, i.e. we found no effects of habituation. The amount of HF decreased over the three stages, though whether this was due to habituation or the varying stressors cannot be differentiated.

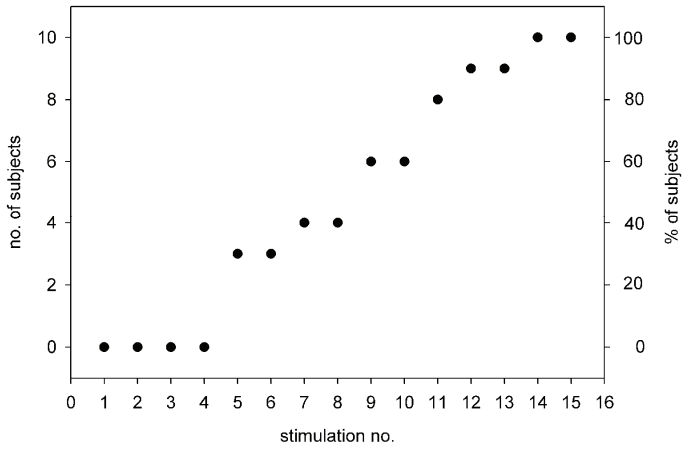

Figure 3.3.2.1: Learning curve. The sums of subjects having shown response towards the light until a given stimulation are given. Total number of stimulations was 15.

The subjects reacted to electric shock and light in AS and A exposing both startle responses (i.e. spontaneous movements) and vocalisation. The response latencies to light varied within subjects, indicating varying attentiveness to this stimulus. The vocal responses comprised high frequency elements (HF), which could be extracted reliably based on the general model of porcine distress calls of STREMODO, as well as low-frequency elements (LF). Typical responses of NS, AS and A are depicted in Fig. 3.3.2.2. HF occurred during electrical shocks as well as in response to CS after learning (AS and A), while LF typically occurred after electrical stimulation or as a response to the light in A.

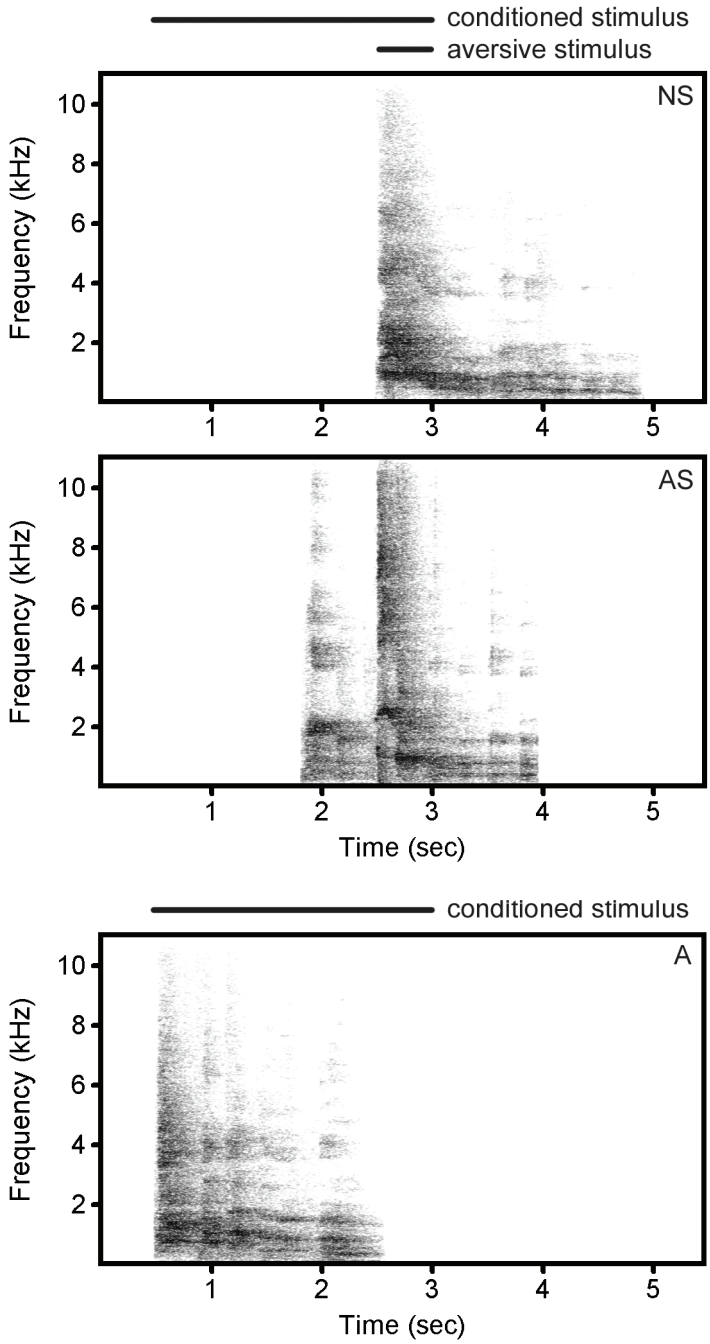

Figure 3.3.2.2: Spectrograms of typical vocal responses in the three periods. Bars above the spectrograms indicate stimulus presentation.

A total number of 10 subjects showing less than three responses including HF in any of the three stages were excluded from the dataset. These included two subjects that hardly ever reacted even to the electric shock, suggesting technical causes.

MANOVA showed highly significant differences between stages (n=1138; F statistic for Wilk's lambda: $F=13.44$, $DF_{num}=24$, $DF_{den}=2248$, $p<0.001$), indicating pronounced effects of stressor quality on vocal responses. The ANOVAs for the twelve LPC-coefficients revealed most prominent effects ($F>12$, $p<0.001$) for the first two LPC-

coefficients, which together represent the first resonance frequency (coefficient 1: F=30.39, DF_{num}=2, DF_{den}=1135; coefficient 2: F=12.99, DF_{num}=2, DF_{den}=1135). Post-hoc tests suggest that these are based on differences between NS and AS (Tukey-Kramer; coefficient 1: t=-3.72, p<0.001; coefficient 2: t=2.55, p<0.05) and NS and A (Tukey-Kramer; coefficient 1: t=-3.12, p<0.01; coefficient 2: t=2.53, p<0.05), while AS and A showed no significant differences. The referring first resonance frequencies based on the given mean LPC-coefficients in NS, AS, and A, were 1097 Hz, 945 Hz, and 973 Hz, respectively.

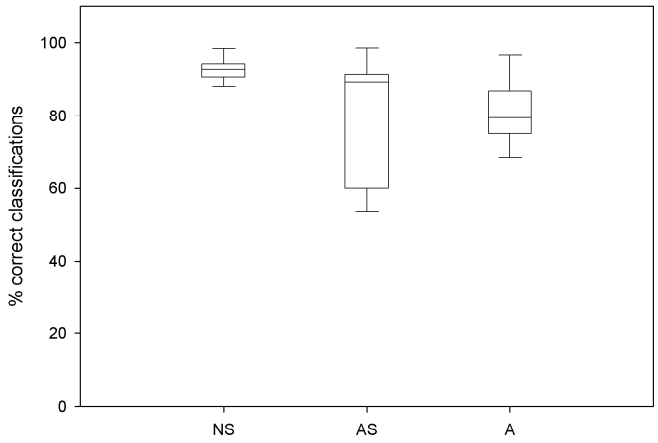

Figure 3.3.2.3: Percentage of correct classifications of LPC-vectors from the three groups (median and quartiles). Classification results are higher than 80% for each of the periods.

The subsequent discriminant function analysis revealed a high level of correct classifications of vocal responses regarding stage in all subjects, varying between 50.00% and 100%. The median percentage of correct classifications was 92.76, 89.24, and 80.91 for NS, AS, and A, respectively (Fig. 3.3.2.3). In each stage, misclassifications were attributed to both incorrect stages unsystematically.

3.4 ANTICIPATION OF FOOD

3.4.1 Methods

Experimental procedure

Recordings were performed in four consecutive groups of four individually housed subjects each. All subjects had been fed directly before the feeding troughs were removed from the pens for two hours. After the two hours the feeding troughs were refilled. Three out of the four subjects were fed directly, while one subject (the focus animal) received its food approximately two minutes later. Thus, for these last two

minutes only the focus animal vocalised. I tried to record vocalisations from each focus animal at least once.

Acoustic analyses

Vocalisations without noise interference were isolated from the recordings. For all further analyses, I performed fast fourier transformations (FFT) using Avisoft-SASLab Pro software (version 4.40; Avisoft Bioacoustics, Berlin, Germany). I used a FFT-length of 1024, frame size of 100%, and Hamming window with 50% overlap; these settings lead to a temporal resolution of 23.2 ms and a frequency resolution of 22 Hz. Based on the FTT, the following parameters were measured for each of the calls: duration, peak frequency, quartiles, and entropy. All parameters were measured as means across the whole call. Means ± standard deviation were calculated for all parameters.

3.4.2 Results

A total number of 137 vocalisations from 8 subjects (i.e. 50% of the subjects) were analysed. They were long, harmonic sounds (Fig 3.4.2.1). The mean duration was 1.77±0.98 s; peak frequency was 1021±644 Hz. 72 of the 137 calls (i.e. 53%) had a mean peak frequency above 1 kHz. The entropy was 0.67±0.10, and the Q75-Q25 value was 3437±447 Hz.

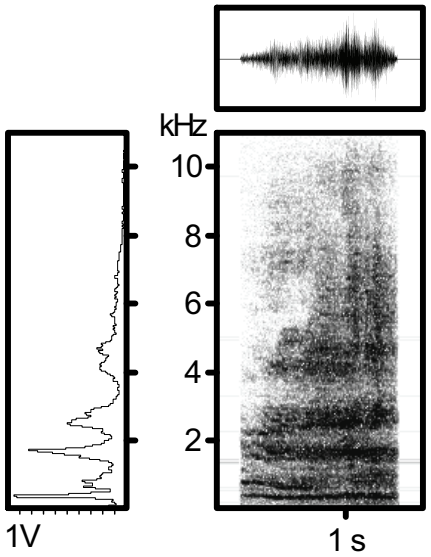

Figure 3.4.2.1: Time signal (upper graph), mean power spectrum (lower left) and spectrogram (lower right) of a typical vocalisation produced in anticipation of food. The spectrogram shows that the call starts with a low amplitude, which increases slowly and decreases relatively abruptly at the end of the call. In the spectrogram several frequency bands with only slight modulations become obvious.

3.5 TAIL BITING, PHYSICAL RESTRAINT AND SOCIAL ISOLATION

3.5.1 Methods

Experimental procedure

Tail biting

Recordings were made in a room prepared for acoustic recordings with a sound dampening rubber floor and sound dampening walls. Subjects were placed in a scale (approx. 1.5 by 0.6 m) reducing the degree of movement. However, they were still able to walk and turn in the scale to a degree reducing restriction-related distress to a minimum. In order to simulate tail biting by a conspecific the pigs' tails were squeezed with a pair of pipe tongs. The tongs' jaws were wrapped with tape in order to avoid skin wounds. Additionally, the minimum distance of the jaws was fixed to approximately 2 centimetres. The pigs' tails were squeezed approximately 5 to 10 centimetres from the tail root. The procedure was repeated five times per subject. The microphone was placed 2 metres in front of the subjects at a height of 1.7 metres.

Physical restraint

Recordings were made in the same room used for tail biting (see above). Subjects were supplied with a dogs' harness and transferred to this room individually. There, they were restrained in an experimental stand allowing for only a small degree of movement (see Fig. 3.5.1.1). The dogs' harness allowed for the fixation at the back and both sides. Bars blocked the way back out of the stand. Subjects remained in the stand for five minutes and were then returned to their home pens immediately. The microphone was placed 1.5 metres in front of the subjects at a height of 1.7 metres.

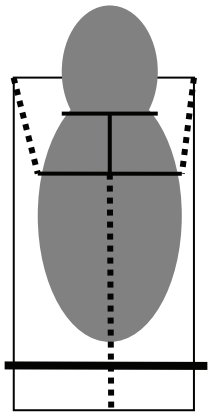

Figure 3.5.1.1: Schematic view of the restriction stand from above. The grey shape represents the pig with the dogs' harness on (solid black lines). The dashed lines represent metal chains fixed to the harness on the one side and the stand on the other side (one to each side and on to the back). The solid line on the back represents a removable metal bar blocking the way back out of the stand.

Social isolation

Preliminary experiments with four piglets showed that total isolation, i.e. visual and acoustic, in a sound dampened room (unfamiliar to the subjects, i.e. novel environment) did hardly elicit any vocal responses. The only exception was when a screaming pig from a nearby room could be heard, thus a so-called 'audience effect' (Zuberbühler 2008) can be assumed. Hence, I performed social isolation in the home pen of the subjects with the remaining group members in a second pen within the same room. An opaque wall that allowed for acoustic contact separated the two pens.

Before isolation of the first member of a group, the whole group was moved to the second pen and remained there for two minutes. Then, the first subject was returned to the home pen and stayed there for 15 minutes. Subsequently, the subject was brought back to the second pen, and the next subject was isolated after two additional minutes. The procedure was repeated with the remaining two subjects of the group, each time with an interval of two minutes. The order of the four subjects within each group was randomised. The microphone was placed centrally above the home pen in a height of approximately 1.7 metres.

Acoustic analyses

All recordings were transferred to a PC (sampling rate: 44.1 kHz, 16 bit accuracy, mono). Vocalisations without any disturbing noise were isolated from the recordings. For all further analyses, I performed fast fourier transformations (FFT) using Avisoft-SASLab Pro software (version 4.40; Avisoft Bioacoustics, Berlin, Germany). I used a FFT-length of 1024, frame size of 100%, and Hamming window with 50% overlap; these settings lead to a temporal resolution of 11.6 ms and a frequency resolution of 43 Hz. Based on the FTT, the following parameters were measured for each of the calls: duration, peak frequency, quartiles, and entropy. All parameters were measured as means across the whole call. Means ± standard deviation were calculated for all parameters.

3.5.2 Results

Tail biting

Of the 20 subjects, only one did not vocalise in response to the 'tail biting'; one was vocalising permanently, probably because of a high level of arousal elicited by the experimental situation. Thus, 90 vocalisations (5 per subject) could be analysed. Subjects only showed vocal responses when the pressure was applied quickly.

Vocal responses (for a typical example see Fig. 3.5.2.1) to tail biting were short (0.29±0.16 s) with a mean peak frequency of 2591±1742 Hz (95.5% above 1 kHz). Mean entropy was 0.65±0.08, mean Q75-Q25 was 5639±830 Hz.

Physical restraint

All subjects vocalised in response to physical restraint. I selected the first 10 vocalisations without disturbing noises for analysis (n=200). Vocalisations (Fig. 3.5.2.2) were long (mean duration 2.02±1.00 s) with a mean peak frequency of 2383±1251 Hz (98.5% above 1 kHz). Mean entropy was 0.71±0.06.

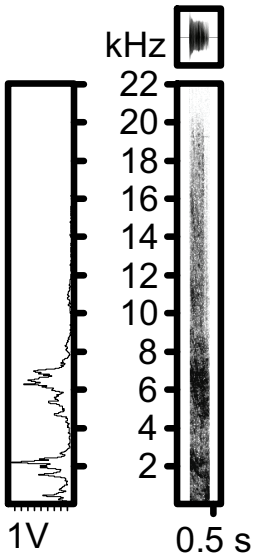

Figure 3.5.2.1: Time signal (upper graph), mean power spectrum (lower left) and spectrogram (lower right) of a typical vocalisation produced in response to tail biting. The time signal shows that the call starts and ends abruptly. The spectrogram and power spectrum show two broad frequency bands. These are present in all single spectra (data not presented here).

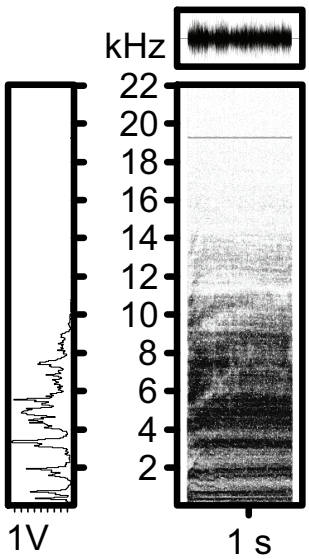

Figure 3.5.2.2: Time signal (upper graph), mean power spectrum (lower left) and spectrogram (lower right) of a typical vocalisation produced in response to physical restraint. The time signal shows that the call is long, and starts and ends abruptly. In the spectrogram, several frequency bands with only slight modulations become obvious.

Social isolation

During social isolation different types of vocalisation occurred. Based on their general characteristics I divided them into single grunts (Gs; Fig. 3.5.2.3), and grunt-squeals (GR/SQ; Fig. 3.52.4), and two types of groans (G1, G2; Fig. 3.5.2.3).

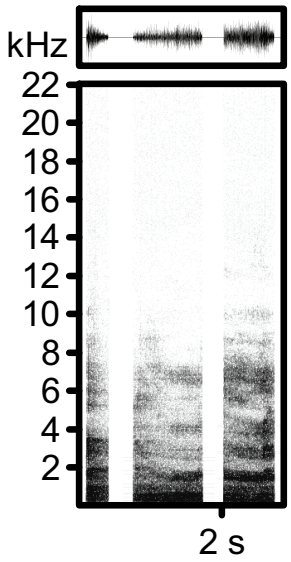

Figure 3.5.2.3: Time signal (upper graph) and spectrogram (lower graph) of typical grunts produced in response to physical restraint (left: Gs, middle: G1, right: G2; see text).

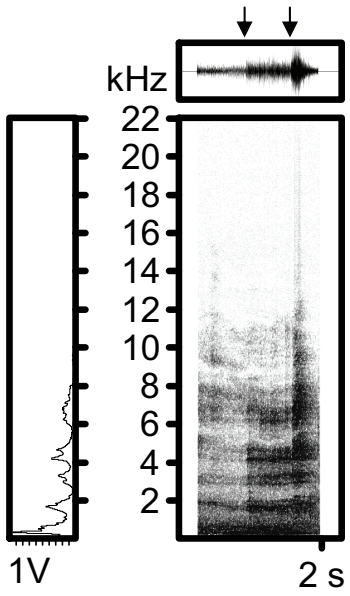

Figure 3.5.2.4: Time signal (upper graph) and spectrogram (lower graph) of a grunt-squeal in response to isolation. It seems to start with a type 1 groan, followed by a type 2 groan, and ends with a squeal, which has a higher amplitude. The three sections can be seen in the time signal (changes marked by arrows), with the type 1 grunt showing the lowest amplitude and the squeal showing the highest amplitude. The grunt sections are not amplitude modulated, while the squeal is. More usual were grunt-squeals with only one type of non-harmonic call.

The grunt-squeals consisted of a non-harmonic section followed by a harmonic section, whereas the other categories all represent non-harmonic calls. The non-harmonic section of the grunt-squeals resembled the groans rather than the grunts. However, the term is commonly used in literature on porcine vocalisation (e.g. Fraser 1975a, 1975b;

Schrader & Todt 1998). Very rarely (four subjects), single vocalisations which can be described as screams occurred. Due to the small sample size, they were not analysed. The groans had either one clear frequency band (G1), or more than one clear frequency band (G2). For analyses, I separated the grunt-squeals into the grunt-sections (GR) and squeal-sections (SQ). As the numbers of calls of different types varied between subjects and due to interfering noises, not all vocalisation types could be analysed for all subjects. Table 3.5.2.1 shows the numbers of calls per category, the number of subjects, and the mean number of calls analysed from each of these subjects. Of the harmonic calls produced during isolation, 83.7% had peak frequencies above 1 kHz, while only 15.3% of the non-harmonic calls had peak frequencies above this threshold. Results of duration, entropy and Q75-Q25 are subsumed in Fig. 3.5.2.5.

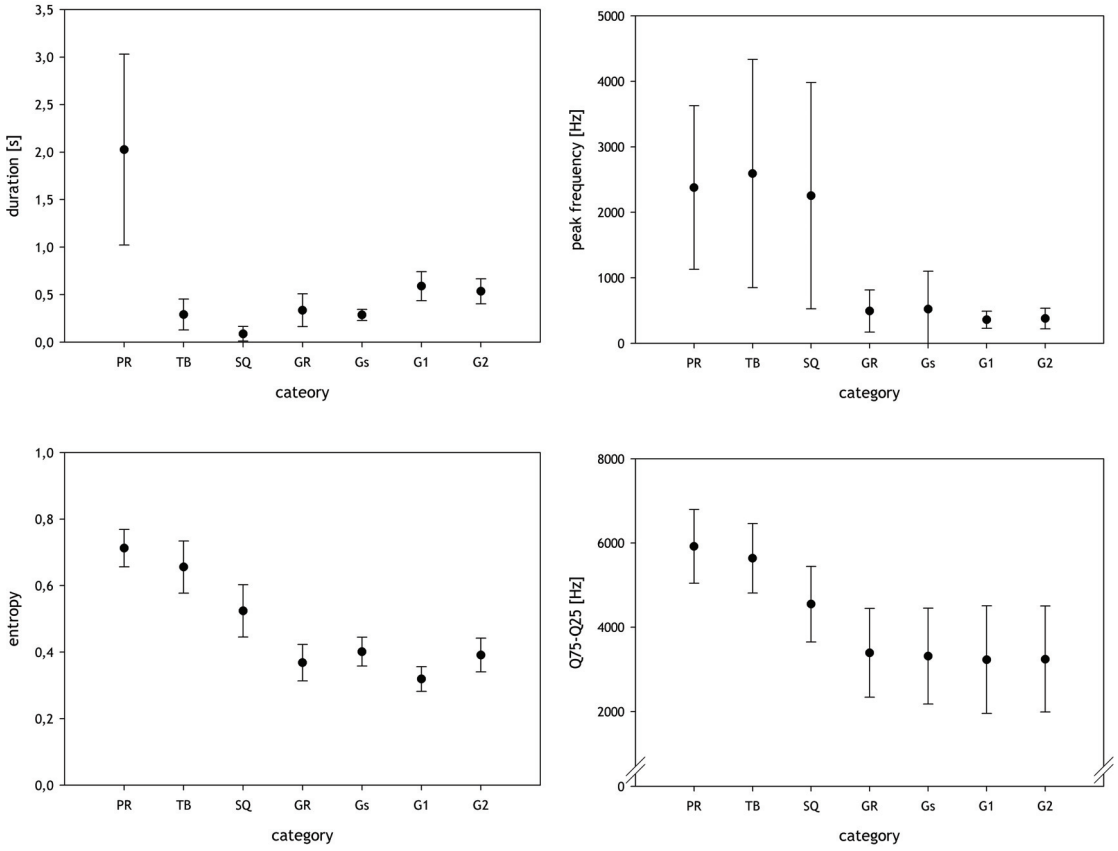

Figure 3.4.3: Mean (±s.d.) duration, peak frequency, entropy, and Q75-Q25 of the vocalisations produced during physical restraint (PR, n=200), tail biting (TB, n=90), and social isolation (grunt-squeals: GR/SQ, n=245; short grunts: Gs, n=64; long grunts with one frequency band: G1, n=156; long grunts with more than one frequency band: G2, n=66).

Table 3.5.2.1: Distribution of data analysed from social isolation (number of calls per subject±s.d.). Short grunts and groans type 2 were relatively rare, while grunt-squeals were most common.

	total number	number of subjects	number of calls per subject
squeal (SQ)	245	9	17.6±15.9
grunt (GR)	245	14	14.5±15.8
short grunt (Gs)	64	10	6.4±4.1
groan type 1 (G1)	156	13	12.0±13.0
groan type 2 (G2)	66	14	7.3±7.2

3.6 OCTAVE ANALYSIS - A NEW MODEL FOR BIOACOUSTIC SIGNALS

3.6.1 Methods

I performed fractional octave analyses (third and sixth octave) for the data gathered from the recordings during tail biting, restraint, and social isolation. Additionally, a linear prediction coding (LPC) approach was used.

All parameters were estimated for time windows of 92.88 ms and constituted one parameter vector per time window. For the characterisation of pigs' distress calls, 12 LPC-coefficients, equivalent to the first six resonance frequencies, were extracted. (see also Schön et al. 2001). The third octave analysis comprised 26 frequency bands, and the sixth octave analysis comprised 51 frequency bands. The parameters constituted parameter vectors (one vector with 12, 26, or 51 dimensions for each time window), which were then subjected to further analyses.

In order to analyse the effects of varying stressors on vocal responses, comparisons of the parameter vectors originating from the different sound categories were conducted using multivariate analysis of variance (MANOVA, F statistic for Wilk's lambda) for each type of analysis.

The classification of parameter vectors derived from the different models into the seven different sound categories was done with the SAS procedure DISCRIM. I performed a non-parametric discriminant analysis (DA) using the k-nearest-neighbour method (k=2). The probability of misclassification (i.e. assigning the parameter vector to the wrong class) was estimated by cross validation (cf. p. 23).

Furthermore, self-organizing neural networks were trained with the data. We used Kohonen networks with 100 by 100 neurons, and an initial learning radius of 20 neurons for the complete data set and 80 by 80 (initial learning radius: 20) neurons for the data sets including either harmonic or non-harmonic calls only. Networks were trained twice in order to improve classification. This kind of self-organizing neural network delivers a visualisation of the goodness of discrimination between different categories of data (Janata 2001). Similar data, whose vectors have low euclidian distances, gather in spatially restricted areas (thus 'self-organizing'). In case of several different categories, several areas with low within-category distances and high between-category distances will appear. When there are overlapping categories, no clear spatial separation will occur.

Table 3.6.1.1: Total number of parameter vectors analysed in this section, number of subjects and mean±s.d. number of parameter vectors per subject.

	total number	number of subjects	number of data per subject
physical restraint	320	15	21.3±10.8
tail biting	269	15	17.9±4.1
squeak	295	15	22.7±12.5
grunt	323	13	24.8±11.3
grunt type 1	163	9	18.1±10.3
grunt type 2	336	13	25.8±11.9
grunt type 3	313	9	34.8±29.2

We did not analyse all available vocalisations, as the DA and neuronal networks require comparable total time (i.e. number of vectors) of the different call categories. As some calls are longer than others, this results in different numbers of calls. Thus, the data shown here refer to the subset of data described in Table 3.6.1.1. As in grunt-squeals the grunts were markedly longer than the squeals, I could not analyse complete grunt-squeals. In all further categories, whole vocalisations were measured. The aim was to analyse approximately 300 parameter vectors per category, except for category G1, where only 163 were available. All responses to tail biting were analysed, while for physical restraint only the first vocalisation without interfering noise was selected. For the data from social isolation, calls were selected randomly from all

available data. Thus, the analysed calls were from all 15 minutes of the isolation procedure.

3.6.2 Results

MANOVAs revealed highly significant differences between call categories based on all three analyses (third octave analysis, sixth octave analysis, and LPC analysis) and in all subsets of vocalisation (all, harmonic only, non-harmonic only; see Table 3.6.2.1). The results of the discriminant function analyses based on the data derived are depicted in Fig. 3.6.2.1.

Table 3.6.2.1: Statistical results of octave- and LPC-analysis based MANOVAs.

	LPC		1/3 octave		1/6 octave	
	F	p	F	p	F	p
all	69.45	<0.001	43.23	<0.001	23.05	<0.001
harmonic	75.11	<0.001	27.45	<0.001	16.22	<0.001
non-harmonic	20.65	<0.001	9.20	<0.001	5.98	<0.001

In the neuronal networks, harmonic sound categories discriminated well, while the grunts, especially the type 1 grunts, created no clearly separated areas (see Fig. 3.6.2.2; see appendix for neural networks based on LPC and sixth octave analyses). Only type 2 grunts formed a relatively large area. When harmonic (restraint, tail biting, squeals) and non-harmonic (grunts, groans) vocalisations were analysed alone, the pattern remained similar (Fig. 3.6.2.3). Harmonic calls are arranged in clearly separated areas, whereas the non-harmonic calls are more diffuse.

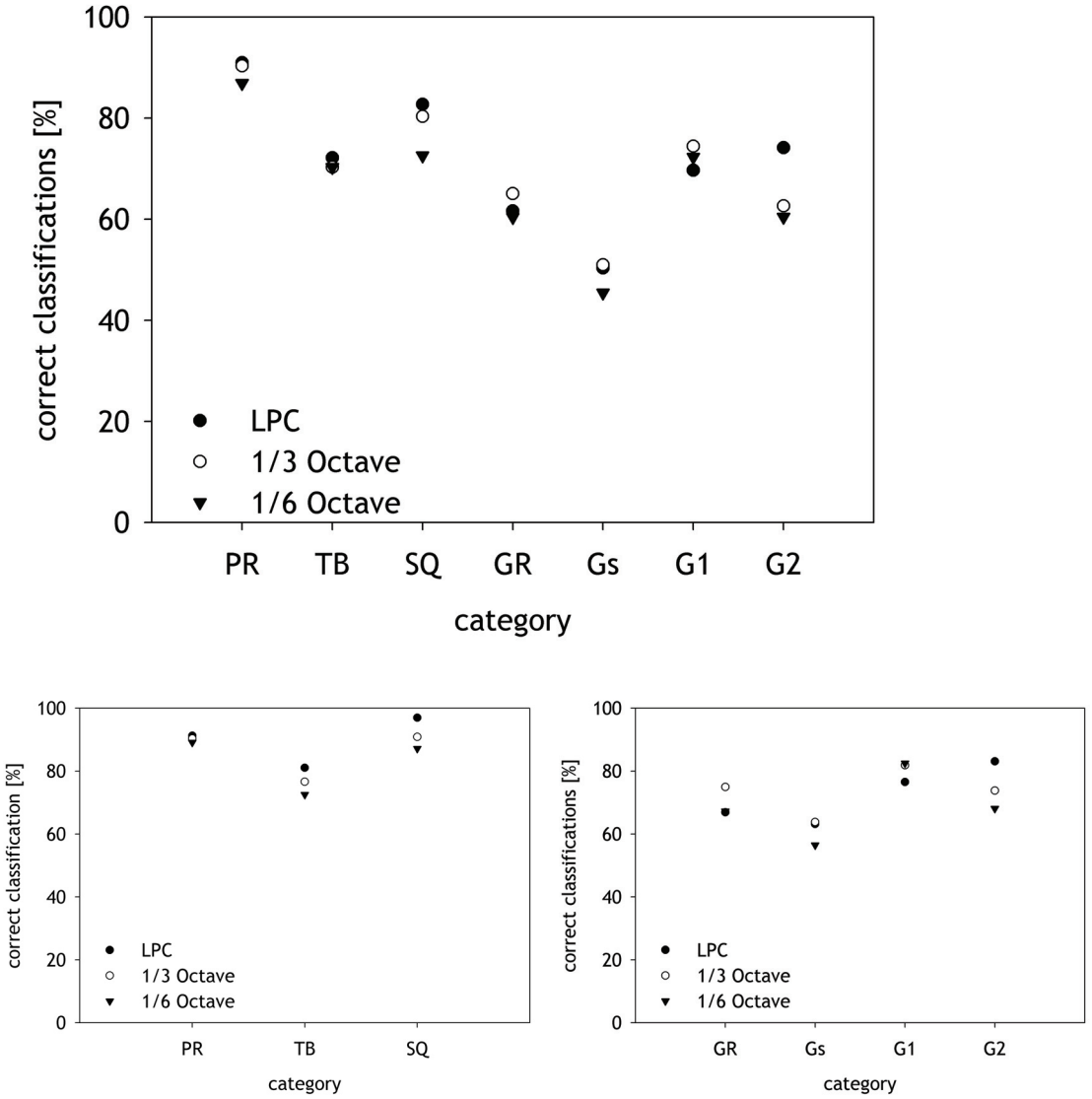

Figure 3.6.2.1: Classification results of the discriminant function analyses based on LPC, third octave, and sixth octave analyses including all (upper graph), only harmonic (lower left graph), and only non-harmonic vocalisations (lower right graph) produced during social isolation. Overall, classifications were better when calls were separated into harmonic and non-harmonic vocalisations.

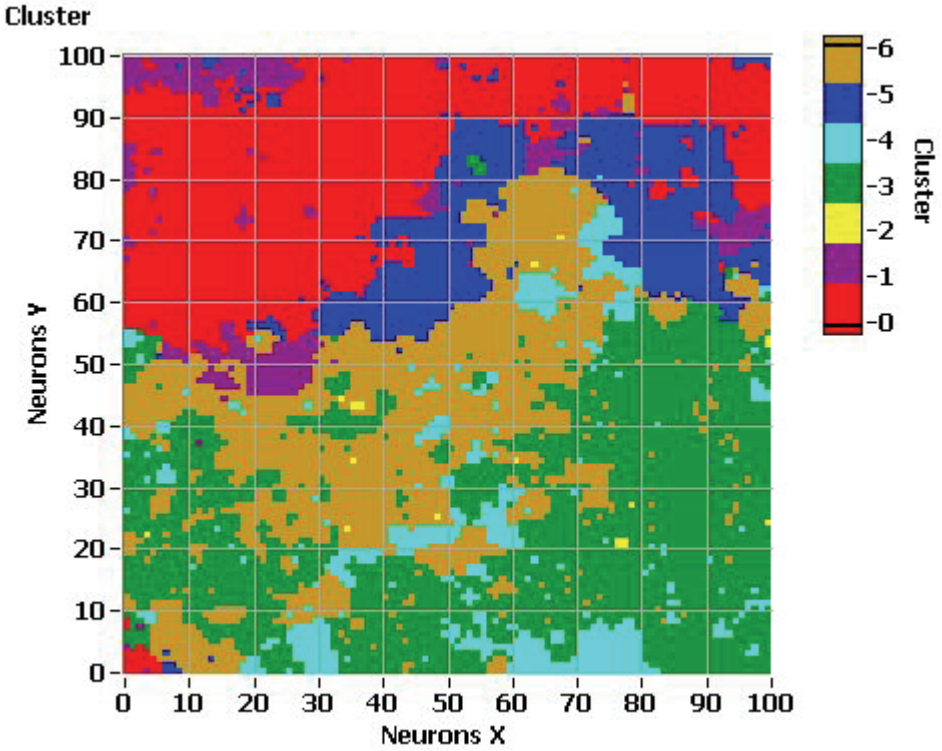

Figure 3.6.2.2: Third octave analysis-based cluster result of the Kohonen network trained with all types of vocalisation after second learning. Different colours represent the seven categories (ochre: tail biting; red: physical restraint; dark blue: squeal; purple: grunt; yellow: short grunt; green: groan type 1; light blue: groan type 2).

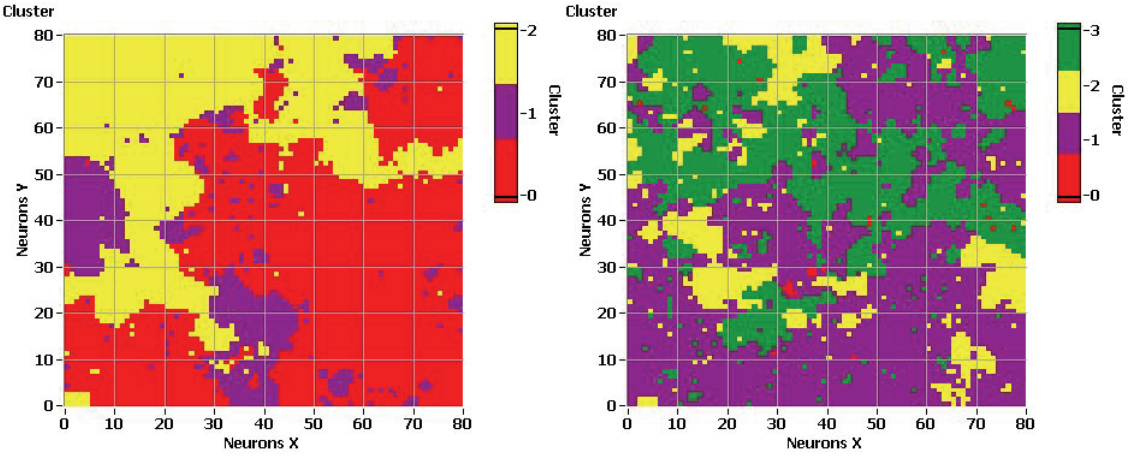

Figure 3.6.2.3: Third octave analysis-based cluster result of the Kohonen network trained with only harmonic (left graph) and non-harmonic (right graph) vocalisations after second learning. Different colours represent the different categories (left: yellow: tail biting; red: physical restraint; purple: squeal/ right: red: grunt; purple: short grunt; yellow: groan type 1; green: groan type 2).

3.7 DISCUSSION

Fear conditioning

Vocal responses to a non-anticipated electric shock (NS), an anticipated electric shock (AS), and to anticipation of an electric shock (i.e. fear; A) differed in their particular frequency structure as described by the LPC model. These results show that pigs produce different vocal responses to mental and physical stressors. This implicates that the anticipation of a stressor has a marked effect on the vocal response towards it, which can be interpreted as an effect of predictability of the aversive stimulus (Knutson et al. 1998, Burgdorf et al. 2000). However, the vocalisations recorded during all three stages were well within the range of general porcine distress vocalisations (Schön et al. 2001, 2004). Thus, the state of fear induced by a classical aversive conditioning paradigm represents a stressor in itself.

Our data revealed highly individual differences in learning speed and varying response latencies to the CS within subjects. We suggest these differences to be caused, mainly, by the varying attentiveness to the visual CS. However, although the use of this sensory modality might have been detrimental for learning speed in our experiment, it was favoured as it avoided interferences between CS and vocal responses. Varying attentiveness to the visual cue is also one possible reason for the exclusion of eight of the subjects from our analyses, as it might have led to low responsiveness directly in A (exclusion due to too few responses) or indirectly by influencing learning speed in AS (exclusion due to too few stimulations after learning). However, low vocal responsiveness might also have been caused by subjects simply not expressing their perceived stress vocally while still experiencing it. Our data do not allow for a differentiation between these alternative explanations, stressing the importance of controlling for attentiveness in future studies.

The resonance frequencies as described by the linear prediction coding (LPC) model (Schön et al. 2001, 2004) represent the filter characteristics of the vocal tract caused by tensional changes. These formant-like structures have been suggested to play a crucial role in honest signalling (Fitch 1997). Thus, they are a potential indicator of the current state of the animal, e.g. its stress level. This approach is strongly supported by the data presented here, which indicate different types or intensities of experienced stress referring to different resonance frequencies.

In conclusion, in a classical aversive conditioning paradigm, stressor quality affected vocal responses of pigs. I suggest that this is based on different internal evaluations of physical and mental stressors.

Anticipation of food

After approximately two hours without food, subjects vocalised after hearing the sound of their troughs being filled. Weary and Fraser (1995a) suggested that pigs' vocalisations with peak frequencies above 1 kHz can be considered as distress calls, while lower peak frequencies indicate non-stress contexts. This threshold is based on the bimodal distribution of peak frequency shown in their data, which could also be found by Puppe and colleagues (2005). The mean peak frequency of vocalisations elicited by anticipation of food was slightly above this threshold. However, considering the variance of the data, several of the calls might not have passed as distress-related calls, for their mean peak frequencies were below 1 kHz. Thus, the general characteristics of the calls do not necessarily indicate that the anticipation of food causes distress in pigs. Studies examining predictability of positive events such as food (Ulyan et al. 2006) or enrichment (Dudink et al. 2006) showed lowered stress levels in anticipation of the rewarding stimulus. Additionally, I cannot exclude that prior experience with aggressive competition for food caused the vocalisations. Subjects might have learned the association of feeding and aggressive encounters, i.e. an aversive event. Thus, anticipation of food is not necessarily anticipation of a positive event.

Tail biting, physical restraint and social isolation

The different stressors that the subjects were subjected to in this experiment caused different vocalisations. Based on the general acoustic parameters, a differentiation of call categories can be derived. However, it becomes obvious that the rather simple parameters described here can describe the vocalisations only roughly, especially as there are overlaps of parameter values in single parameters (partly due to huge standard deviations). Vocalisations during restraint are obviously the longest. Both vocalisations described from pre-castration restraint (Puppe et al. 2005) and screams in social isolation with or without restraint (Schrader & Todt 1998) had almost half the mean duration than vocalisation measured here. Durations might have been lower in the castrated piglets due to smaller volume of the lungs. However, Schrader and Todt (1998) used even older subjects, but they did not discriminate between vocalisations from social isolation alone and isolation with additional restraint. Thus, the affective background might have been different. Moreover, peak frequencies show great variance within categories, especially in the harmonic vocalisations (physical restraint, tail biting, isolation squeals). Peak frequencies of the harmonic calls are higher than described for screams as well as squeals, while those of the non-harmonic grunts and groans are higher than described by Schrader and Todt (1998). These differences

might reflect ontogenetic changes of the sound producing system. Longer vocal folds allow for deeper frequencies, while the filter characteristics of the vocal tract change as well.

Another effect of age was probably shown in the preliminary experiments, where subjects did hardly vocalise when isolated visually as well as acoustically. Younger piglets do vocalise in comparable situations (Fraser 1975 a, 1975b; personal observations). These differences might reflect differences in their need to re-establish contact to their group.

Octave analysis - A new model for bioacoustic analyses

Based on fractional octave analysis, harmonic vocalisations caused by different stressors could be discriminated well. Analyses of variance showed highly significant differences when all categories of stress calls were analysed together, as well as when harmonic and non-harmonic vocalisations were analysed separately. However, the classifications based on both discriminant function analyses and Kohonen networks were relatively low for different types of non-harmonic vocalisation. The more detailed sixth octave analyses did not increase the level of correct classifications, but rather decrease it. Thus, the third octave analysis proved to be both more reliable and requiring less calculations. The latter is important for the application in real-time analyses. The LPC model is an arithmetic equivalent of the source-filter-model of sound production (Fant 1970) describing the resonance frequencies of the vocal tract. However, non-harmonic sounds are assumed not to show formant-like structures. Thus, they have been described using different approaches before (Schön et al. 1999). However, the LPC model seems to be very robust. At least, classification results were not lower than those based on third octave analyses.

Whether the non-harmonic calls, which could not be discriminated sufficiently, indicate different affective states remains to be tested. In addition, the grunt-sections of the grunt-squeals seem to resemble the groans. Thus, classification can be expected to be low. Classification might be better when categories like the two ‘groans’ and the grunt-sections of grunt-squeals would be comprised in one category instead.

Stressor-specific vocalisation

In this chapter I described vocal responses to different stressors and suggested a new model for the description of such calls. Some of the vocalisations were stressor-specific, while others seemed to form overlapping categories.

Even though I did not measure whether predictability of the electric shock in fear conditioning reduced or enhanced physiological parameters of stress, a reduction of

the physiological stress response is implied by recent literature. In a functional magnetic resonance imaging study in human subjects, Carlsson et al. (2006) showed that the predictability of a mild electric shock lead to lower levels of anxiety, negative valence and pain intensity, while unpredictability was correlated to activity in posterior parietal cortex and lateral cortex, indicating states of enhanced alertness and sustained attention. My data showed that the first resonance frequency was highest when the aversive stimulus was not anticipated by the subjects. Given that high frequencies are regarded as indicators of high levels of stress (Weary and Fraser 1995a), this can be interpreted as indicating that subjects were more stressed when they could not predict the shock than when they were able to predict it.

Effects of predictability of aversive events on stress levels have been proposed to be based on decreased general alertness and the occurrence of distinct “safe periods” (Weinberg and Levine 1980, Wiepkema and Koolhaas 1993, Carlsson et al. 2006, Bassett and Buchanan-Smith 2007). My study supports the assumption that such effects can also be found in conditions where the stressor cannot be controlled or avoided (see also Weinberg and Levine 1980, Bassett and Buchanan-Smith 2007). This might bear implications for future farm animal husbandry, where some stressors are predictable as they are announced by reliable cues (e.g. specific times of the day or persons), while others are not. Thus, it has been suggested that stressors occurring regularly in farm animal maintenance should be announced by reliable cues (Bassett and Buchanan-Smith 2007).

Anticipation of food also caused vocalisations. Although the peak frequencies were relatively low compared with restraint or tail biting, their length was comparable to restraint calls, and the frequency structure was harmonic. I suggest that the subjects were not in the positive anticipation of a reward. More likely, they were either frustrated, as the food was not available yet, or they had learned to associate feeding with aggressive competition. Thus, the emotional background was rather negative. However, in order to confirm these interpretations, parameters of the physiological stress response should be measured simultaneously to recordings of vocalisation in anticipation of food. Pigs did not vocalise every time, which might be caused by varying states of hunger. Therefore, future studies should measure indicators of distress, such as heart rate variability, as well as indicators of hunger, such as blood glucose levels, during the recording of vocalisation. Such an approach would allow for a clear correlation of internal state and acoustic signal.

Entropy of responses to tail biting and restraint resembled values described for all three phases of castration (before, during, and after; Puppe et al. 2005). It was lower in squeals. It is generally assumed that entropy is higher when there are unspecific

factors affecting vocalisation, while when one specific factor becomes prominent entropy decreases (Puppe et al. 2005).

During isolation, vocal responses mostly comprised different types of non-harmonic calls. I suggest that they can also be indicative of stress. In order to prove this, physiological measures of stress (hormones such as adrenaline/noradrenaline or heart rate measurements) should be correlated with these vocalisations. Additionally, I suggest that there is a specific order of the types of vocalisations. This order might indicate increasing distress, starting with short grunts (Gs), followed by the longer groans with one frequency band (G1), long groans with more than one frequency bands (G2), grunt-squeals (GR/SQ), and in some rare cases calls which might be called squeals or screams. The sequence of vocalisation types was only obvious during the direct observations during isolation. However, in the acoustic recordings the different types of vocalisation are mixed up. I assume that this sequence of vocalisations was interrupted due to irregular noises from the subject's group mates or from outside the experimental room. However, as I did not record these disturbances, this interpretation remains to be proven. In order to do so, an acoustically isolated recording room would be needed in addition to precise protocols on disturbing events (either uncontrolled or controlled ones). Based on such experiments, possible correlations of non-harmonic vocalisations and stress could be revealed. Based on my observations I assume that groans indicate higher levels of stress. They do resemble the non-harmonic sections of the so-called grunt-squeals. Assuming that during social isolation stress levels rise, and that high-frequency, harmonic vocalisations are indicative of high stress levels, this goes along with the sequence of call categories suggested above. The non-harmonic calls come first, indicating low stress levels. Subsequently, as arousal rises, groans occur. When stress levels rise even more, the non-harmonic groans switch to grunt-squeals (which might better be called groan-squeals). Finally (but only rarely in my experiments) purely harmonic calls are produced. Thus, the grunt-squeals might be indicative of increased arousal as a transient state between 'no stress' and 'stress'.

The effect of age on vocalisation proved to be an important factor to be considered when using bioacoustic analyses for welfare assessment. On the one hand, due to growth the characteristics of the vocal folds and tract change, affecting frequency characteristics of the produced calls. On the other hand, the need for communication might change, as the animals get more independent. However, Schrader and Todt (1998) showed that social isolation elicits vocal responses in six-months-old pigs. Fattening pigs are usually slaughtered not long after that, so in farming praxis such vocalisation might still play a role.

Classification of the non-harmonic vocalisations produced during isolation (groans and especially the grunts) was relatively low. In the grunt-squeals, the grunt sections showed similarities to both types of long single grunts, thus the separation into different categories might be incorrect. The spectrogram of a grunt-squeal presented in section 3.5.2 (Fig. 3.5.2.4, p. 31) supports this interpretation. There the grunt-component shows similarities to type 2 and type 3 grunts. Misclassifications of the short grunts might reflect effects of sample size. Unfortunately, I had only approximately half the amount of data available for short grunts compared to all other categories. However, the problem of varying sample size was accounted for in the discriminant function analysis and should not state a problem.

The introduction of arithmetic models increased the ability to discriminate between underlying stressors (section 3.3) and call types (section 3.6). However, the fractional octave analysis, which was firstly applied to bioacoustical signals, did not allow for clear classifications of all call types. Additionally, the LPC model, which has been applied to porcine stress calls before (Schön et al. 2001), showed similarly good discrimination of non-harmonic calls. When harmonic and non-harmonic calls were classified separately, the amount of correct classifications increased slightly in both groups (section 3.6). The LPC model showed better discrimination of harmonic calls, while third octave analysis was better for non-harmonic calls. Harmonic and non-harmonic calls seem to show pronounced differences in peak frequency (sections 3.4 and 3.5), thus maybe a combination of peak frequency detection followed by either LPC (Peak frequency > 1 kHz) or third octave analysis (peak frequency < 1 kHz) would result in optimal detection of different call types. Anyway, the association of non-harmonic vocalisation types and physiological parameters of the stress response needs to be confirmed first. Maybe not all the grunt-like sounds indicate welfare relevant stress levels, thus their detection would be unnecessary. My data did not comprise ‘neutral’ (e.g. during feeding or during locomotion in the home pen) or positive vocalisations (e.g. nurse grunting). Such non-harmonic calls might be clearly separable from the arousal-associated vocalisations produced during social isolation.

Research on communicative significance of pigs’ acoustic signals is rare and the potential receivers of these signals are not known for all subtypes of vocalisation (Weary and Fraser 1995a, 1995b; Weary et al. 1997, Appleby et al. 1999), but honest signalling in pigs has been shown to occur in the mother-offspring context (Weary and Fraser 1995a). In order to further establish analyses of vocalisation as a tool to measure animal distress, the information derived from it should be both sophisticated and highly reliable. The classifiability of different distress calls shown in my data

supports such efforts. Detailed information is the basic prerequisite for any complex automated system of stress measurement based on acoustic analyses.

In conclusion, domestic pigs produce referential acoustic signals in response to various stressors. Thus, vocalisations reflect characteristics of the stressors (length, intensity, quality: mental and/or physical), and can be used as a differentiated, reliable indicator of the internal state of animals.

4. CHAPTER 2

BEHAVIOURAL AND CARDIOVASCULAR RESPONSES TOWARDS CONSPECIFIC DISTRESS CALLS

4.1 INTRODUCTION

As already stated in the general introduction, communication includes (at least) three basic elements: sender, signal, and receiver (Wiley 1983, Hasson 1991, Bradbury & Vehrencamp 1998). Sender and signal were the focus of the first part of this thesis, where I described stress-related vocalisations in different contexts, which could be categorized based on their structure. Such signals can be assumed to convey information on the state of the signaller, e.g. its arousal (e.g. Ehret 2005), motivational state (Zahavi 1981), emotional state (e.g. Brudzynski 2007), or physical characteristics (e.g. Harris et al. 2006, Pfefferle & Fischer 2006). However, although the context of vocalisation gives a good idea about the motives of calling, the meaning of such calls as signals for intraspecific (or interspecific) communication can only be evaluated based on both context and responses of the receivers (e.g. Macedonia & Evans 1993, Seyfarth & Cheney 2003). Therefore, in the following chapter I will focus on the receiver. It is generally agreed upon that the transmission of a signal from a sender shall influence the receiver's behaviour, i.e. the probability that certain behaviour will be shown increases while other behaviours become less likely. This way, the predictability of the receiver's behaviour increases, which is advantageous for the sender of the signal (Wiley 1983). The responses of receivers to specific signals can be studied in so-called playback experiments, where vocalisations are broadcast either in a laboratory setting or in the wild (Hurlbert 1984, Kroodsma 2001). These kind of experiments have, for example, led to the interpretation of certain types of vocalisations as alarm calls, which are produced upon the sight of a predator and elicit flight responses in nearby conspecifics (or even members of different species) (e.g. Leger & Owings 1978, Schwagmeyer & Brown 1981, Harris et al. 1983, Fichtel & Kappeler 2002, Platzen & Magrath 2004, 2005). We can assume that the calling subjects are in a state of stress when seeing the approaching predator, while the reactions of the receivers of such calls can be interpreted as displays of distress themselves. Therefore, stress-related acoustic communication can cause a transmission of distress from the sender to the receiver(s). Another well-studied category of vocalisation is isolation calls of young directed towards their parent(s) (e.g. Weary et al. 1997, Kober et al. 2007, Chaloupková et al. 2008, Illmann et al. 2008). Liu and colleagues (2006) could show cortical entrainment to infant calls in mice. Infant isolation calls occur in numerous species, and induce parental care, which can be accompanied by increased arousal and therefore represent a state of stress, too. In domestic pigs, piglets show vocal responses to separation from the sow (Fraser 1975a, 1975b; Weary et al. 1997). The strength of responses depends on their need parental care (Weary & Fraser

1995a, Weary et al. 1996, Weary et al. 1997). Thus, they can be considered so-called 'honest signals' (cf. Fitch & Hauser 1995) providing true information on the state of the sender.

Domestic pigs are highly social and have a complex system of acoustic communication. To time many studies have examined vocalisations produced in the context of stress in pigs (Manteuffel et al. 2004, Puppe et al. 2005). They correlate with behavioural as well as physiological indicators of distress (Schrader & Todt 1998, Manteuffel et al. 2004). However, the information conveyed by some of those vocalisations remains unclear. In pig production, there are controversial reports on the spreading of stress-related vocalisations across a whole group. On the one hand, there are reports on non-responding pigs standing by a screaming conspecific, e.g. during castrations. On the other hand, a spreading of stress responses is reported: once one single animal starts screaming in an abattoir, other individuals nearby will join in. However, a simultaneous response to the commonly shared situation might be more likely to elicit the simultaneous vocalisation. In the case of distress transmission via vocalisation, the negative effects of a stressor might not only affect the initially and directly affected animal, but also the whole group. This represents a problem for both animal welfare and productivity in meat production (Wiepkema & Koolhaas 1993, Manteuffel et al. 2004, Burman et al. 2007, Salak-Johnson & McGlone 2007).

In the present study, I tested the effects of conspecific distress calls on juvenile pigs using a playback paradigm. In an open field setting (i.e. a novel environment without hiding places) conspecific calls produced during restraint (cf. chapter 1, section 3.4/3.5) were broadcast and the responses in both behaviour and heart rate/heart rate variability were observed. This allowed for a detailed analysis of overt as well as covert reactions including responses of the autonomous nervous system. Screams caused by restraint are harmonic vocalisations (see chapter 1, section 3.4). They might serve to express fear. In the wild, they might signal an attack by a predator and hence induce flight behaviour (e.g. Fichtel & Kappeler 2002). Therefore, subjects should show avoidance or alert in response to playback of screams. On the other hand, a scream could serve no intraspecific function, but be a so-called 'pursuit-deterrent signal' directed towards a predator (Högstedt 1983, Hasson 1991) and therefore induce no behavioural changes in conspecifics.

The analysis of heart rate and heart rate variability parameters together provides insight into the working of the two branches of the autonomous nervous system, the sympathetic and parasympathetic (vagal) system. Those two are classically considered opponents, but throughout the last years, this view had to be revised (von Borell et al.

2007). I performed analyses of heart rate variability in the time domain. The parameters provided by these analyses were the SDNN (standard deviation of successive interbeat intervals), which represents both the sympathetic and parasympathetic branch, and the RMSSD (root of the mean squares of successive differences of interbeat intervals), which represents the parasympathetic system alone.

A decrease in heart rate can be induced by both an activation of the parasympathetic system and a decrease in sympathetic activity and vice versa. In case of a simultaneous activation of both the sympathetic and parasympathetic systems, there might be no observable effect on heart rate. Such an effect was shown in several studies, which points out the importance of considering the heart rate variability, too (Désiré et al. 2004, von Borell et al. 2007).

The majority of studies show that stressors elicit increases of heart rate caused by an activation of the sympathetic system. However, a drop of heart rate can associate freezing behaviour, and attentive behaviour can be accompanied by an activation of the parasympathetic system, as represented by an increase in RMSSD (Désiré et al. 2004). Therefore, a prediction of the direction of a heart rate response in the context of the perception of conspecific distress calls is not easy. In case of a “fight or flight” response, an increase in sympathetic activity and, thus, heart rate is expected. However, when attention is elicited, an increase in parasympathetic activity would be likely to occur, which might cause a decrease in heart rate.

As mentioned before, it is often reported that in everyday situations pigs show hardly any response to a conspecific’s distress calls. Some pigs, however, will show attention to the calls. Therefore, I did expect rather subtle behavioural responses to broadcasted distress calls. Anyway, theory does not necessarily predict pronounced behavioural responses to such calls. In the wild, any pronounced behavioural response might increase the receiver’s conspicuousness to predators. Here, the simultaneous measurements of heart rate could improve the analyses of subjects’ responses.

I hypothesised that conspecific distress calls would elicit both subtle behavioural and more pronounced physiological responses in juvenile pigs, leading to a state of attention and maybe distress in the receivers of such acoustic signals.

4.2 METHODS

4.2.1 Animals and housing

Subjects were 24 female, 6-weeks old German Landrace pigs from different litters. They were housed in flat decks (3 by 2 metres with fully slatted plastic floor except for a

heated area in the middle) in groups of approximately 10 animals of the same age. All subjects had been weaned at 28 days of age, thus they were in the current groups for approximately two weeks. While all subjects of each trial were housed in the same room, only up to five were housed in the same pen. Thus, we reduced the disturbances caused by the experiments (i.e. mostly the capturing of the subjects).

4.2.2 Stimuli

Distress calls used for playbacks were taken from the recordings performed during immobilisation stress by first tethering in an experimental stand in experiment 1 (see figure 3.5.2.2, p. 30). All recordings were performed in the same room, with optimal recording conditions due to noise dampening walls. Stimulus animals were strangers to playback subjects, but were of the same age. Each playback contained twice a sequence of one minute of vocalisations derived from ten minutes of recording. Sections of more than 2 seconds of silence were removed, and one minute of vocalisation used to create playback sequences. We had recordings of 32 stimulus animals, and as 12 of the recordings did not comprise a total of one minute of calling, 20 playback sequences could be created. Four of these had to be broadcast to two subjects, while the remaining 16 were only used once. The stimuli used twice were selected prior to the last experimental run. They were before presented to subjects whose datasets were incomplete (no number of vocalisations in one case and high rate of artefacts in heart rate measurements in the others). This way the possible effects of pseudoreplication were minimised (Hurlbert 1984, Kroodsma 2001).

For the control stimuli, playbacks with a 500 Hz signal were generated. This frequency was well below the peak frequency of the conspecific sound (section 3.5). Control stimuli were generated for each individual distress call stimulus, with the temporal pattern of sound equal to the referring distress call recordings. Therefore, in both treatments the number of sounds as well as the durations were exactly the same, providing for a high level of comparability.

All stimuli were broadcast with an average amplitude of 74 dB at a distance of 1 m and 0.5 m height.

4.2.3 The open field

The open field was placed in a room dampened for sound reflections and measured 2.8 by 2.8 metres, with a height of 1.4 metres (see figure 4.2.3.1). The walls were made of wood and the floor of sound reflection dampening rubber, which also allowed for a good grip. Outside one of the corners of the open field, an elevated chair was placed to enable direct observations of the whole area. A personal computer supplied

with The Observer software (Version 3.0, Noldus, Wageningen, The Netherlands) was placed next to it. Two speakers were placed centrally on one wall directed slightly downwards. The open field was divided into three areas of equal size by equidistant chalk lines parallel to this wall (areas 1, 2, and 3, with area 3 closest to the speakers).

The open field was cleaned roughly between experiments and thoroughly after each experimental day. The chalk marks were drawn before the first experiment of the day, and had to be redrawn between experimental days. There was no need to refresh them between experiments.

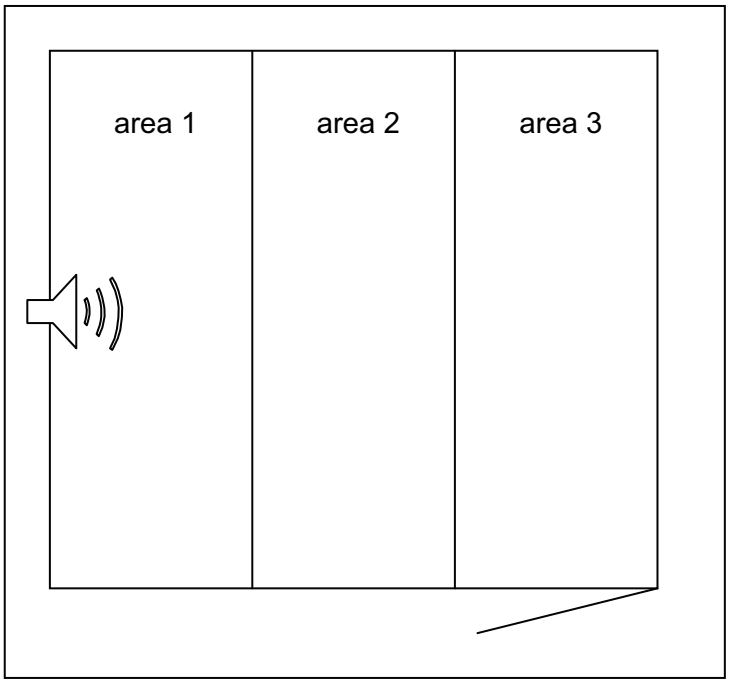

Figure 4.2.3.1: Sketch of the experimental room and open field. Observations were made from an elevated chair beside the corner depicted on the lower left. On the lower right corner, the door is indicated.

4.2.4 Experimental procedure

On the day before the first experiments, each subject was transported to the experimental room once and stayed in the open field for 4 minutes. During that time, I was sitting on the observation chair and typed codes into the keyboard of the PC like later on during the experiments in order to get the subjects used to these sounds, too.

Subjects were supplied a heart rate measurement belt (details see below) in their home pens and the transmission of signals to the monitor checked. They were then brought to the open field in a closed, wheeled wooden box to reduce their spatial orientation. They were then supplied the heart rate monitor which was fixed on their backs with self adherent medical tape (Fixomull® stretch, BSN medical, Hamburg, Germany) and transferred to a wooden enclosure placed in the middle of the open field. The enclosure measured 1 by 1 metres and was 50 centimetres high to prevent

subjects from seeing where I left the open field (in a preliminary study subjects favoured the area in front of the exit). The enclosure was then lifted to a height of 2 metres (lower edge to floor) and the observation and the playback started simultaneously. After three minutes (one minute of familiarisation, followed by two minutes of pre-stimulus observations) the distress calls or control sounds were broadcast, respectively. These ended after two minutes and were followed by another two minutes of post-stimulus observation. Both the pre- and post-stimulus observations were accompanied by the broadcasting of artificially generated silence in order to keep possible noises of the speakers identical throughout the experiments.

4.2.5 Behavioural data

I observed the behaviour of the subjects based on six categories (locomotion, standing/sitting, lying, wall contact, elimination, flight; see Table 4.2.5.1 for definitions), with the area in which they were performed as a modifier (continuous sampling). Additionally, vocalisations were recorded (all were non-harmonic, i.e. 'grunts'). Total durations of all behavioural categories and number of vocalisations were calculated for two minutes pre-, during, and post-stimulus presentation.

Table 4.5.2.1: Definitions of observed behaviours. All behaviours were recorded as states.

behaviour	definition
locomotion	moving with at least three feet
standing/ sitting	standing with at least two feet steady/ touching the ground with the complete hind legs
lying	touching the ground with all four legs and stomach
wall contact	manipulation of the walls with the snout
elimination	urination and defecation
flight	jumping against the wall

4.2.6 Heart rate measurements

Heart rate measurements were performed with the Polar system (Monitor: S810i; Polar Electro Oy, Kempele, Finland). The belts were fixed directly behind the front legs with the electrodes both on the left side of the animal and the transmitter in the "armpit". Ultrasound transmission gel (Henry Schein, Melville, NY, USA) was used to improve

contact to the skin. This procedure showed lowest rates of artefacts in preliminary tests. R-R intervals were recorded, i.e. intervals between each heartbeat, allowing for analyses of heart rate variability.

Data were corrected for artefacts under visual control (settings: sensitivity very low, peak detection, minimal protection zone: 20). Data with more than 10% artefacts were excluded (cf. von Borell et al. 2007). Mean heart rate and heart rate variability parameters were calculated for the two minutes of pre-, during, and post-stimulus presentation. Heart rate variability parameters derived in the time domain were the standard deviation of inter beat intervals (SDNN), and the root mean of squared distances of subsequent interbeat intervals (RMSSD). The RMSSD was calculated as follows:

$$\text{RMSSD} = \sqrt{\sum_{k=1}^{n} \frac{\left(i_n - i_{n-1}\right)^2}{(n-1)}}$$

with i_1, i_2 = consectutive interbeat intervals,

n = total number of interbeat intervals measured

All time domain parameters were calculated using an Excel-Macro, which automatically calculates the referring parameters from a dataset of inter beat intervals for any given number and length of intervals from a given starting point onwards (programmed by K. Siebert).

Additionally, mean heart rate as well as SDNN and RMSSD were calculated for ten second sections throughout the pre-, during, and post-stimulus periods.

4.2.7 Statistical analyses

Firstly, all parameters were calculated for the two minute periods before, during and after playbacks. Behavioural data were summed up for all three areas. All analyses had subject as repeated factor.

The behavioural data were analysed using a fixed effect model with period, stimulus, day, and interactions of all factor pairs as fixed effects. The times spent in the three areas were transformed (sums of all behaviours in each area) into a single score value by adding the total duration spent in the area next to the speakers, the duration spent in the middle area multiplied by two and the time spent in the area farthest away from the speakers multiplied by three. This weighting considers the basic assumption that the animals should avoid the source of stress-related calls (cf. Kober et al. 2007).

Prior to the analyses of heart rate measurements, I calculated Spearman correlations of locomotion and heart rate, SDNN and RMSSD. Subsequently, mixed effect models

were calculated for the heart rate/heart rate variability parameters with period, stimulus, experimental day, and the interactions of all factor pairs as fixed effects, and total locomotion (sum of durations in all areas) as covariate. Post hoc comparisons were calculated using t-tests with Tukey-Kramer adjustment of p-values due to multiple testing.

Additionally, heart rate measurements were calculated for sections of 10 seconds. Based on these, pair wise comparisons of all sections were calculated (t-tests) a) between stimuli for each section, and b) within stimuli, between the last section preceding start or end of playbacks and all sections during or after playbacks, respectively.

All statistical tests were calculated using the procedure MIXED in SAS (SAS® 9.1, SAS Institute Inc., Cary, NC, USA).

4.3 RESULTS

4.3.1 Behaviour

Piglets showed high levels of locomotion during experiments. Lying, flight, and wall contact were rare and, therefore, were excluded from further analyses. None of the behavioural measurements differed between treatments in the two minutes before playbacks. In addition, none showed a significant effect of the interactions of stimulus and day or period and day (Table 4.3.1.1).

Stimulus

Analyses of variance revealed a significant effect of stimulus on vocalisation, locomotion, score, standing, but not on elimination. Vocalisation rates were higher during controls (n=23, t=4.39, p<0001; Fig. 4.3.1.1). Locomotion decreased in the interval with playback, both in the distress call and the control situation (distress calls: n=24, t=2.83, p=0.059; control: n=24, t=2.99, p=0.039; Fig. 4.3.1.1).

Period

Main effects of period (before, during and after playbacks) could be found in vocalisation, locomotion, standing, and elimination, but not score (Table 4.3.1.1).

Across stimuli, vocalisation rates decreased after the onset of playbacks (n=23, t=6.57, p<0.001), and increased again after the end of playbacks (n=23, t=-3.30, p=0.004). However, vocalisation rates after playbacks were still significantly lower than before (n=23, t=3.27, p=0.004). During broadcasting of distress calls subjects vocalised

significantly less than during controls (n=23, t=4.39, p<0.001; Fig. 4.3.11). After playbacks, vocalisation rates still tended to be higher in controls (n=23, 2.72, p=0.079; Fig. 4.3.1.1).

Experimental day

Additionally, there were significant effects of experimental day on vocalisation, locomotion and standing/sitting (see table 2.2). In general, locomotion and vocalisation decreased from day one to day two (locomotion: n=24, t=2.85, p=0.005; vocalisation: n=23, t=3.18, p=0002). Simultaneously, mean heart rate decreased as well (see below). All this indicates a habituation process occurring from day one to day two.

Table 4.3.1.1: Statistical results of the behavioural data. (n=24 for all data except vocalisation, where n=23).

	stimulus		period		day	
	F	p	F	p	F	p
score	5.69	0.019	0.63	0.532	0.78	0.380
vocalisation	30.18	<0.001	21.55	<0.001	10.01	0.002
locomotion	8.08	0.005	8.51	<0.001	8.14	0.005
stand/sit	12.14	<0.001	18.00	<0.001	5.52	0.021
elimination	1.39	0.241	7.99	<0.001	1.99	0.161

	stimulus * period		stimulus * day		period * day	
	F	p	F	p	F	p
score	1.24	0.293	1.69	0.207	0.23	0.795
vocalisation	1.10	0.335	0.36	0.553	1.49	0.229
locomotion	2.47	0.089	1.90	0.182	0.75	0.476
stand/sit	2.04	0.135	1.52	0.231	0.10	0.908
elimination	0.11	0.893	0.73	0.402	1.18	0.312

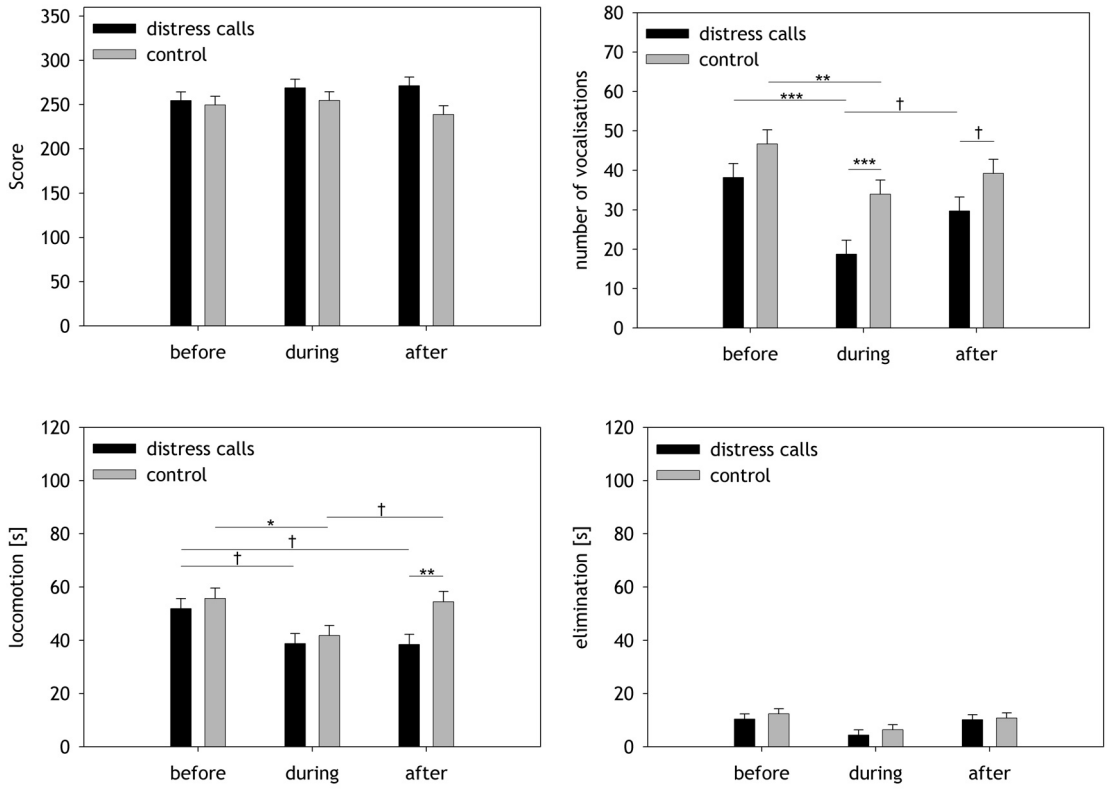

Figure 4.3.1.1: Least square means±s.e. of location score, vocalisation, locomotion and elimination. Statistical differences within period (before, during, after playbacks) and within stimuli are given (*** = p<0.001; ** = p<0.01; * = p<0.05; † = p<0.1).

4.3.2 Heart rate measurements

Overall, the amount of locomotion in each 2 minute interval correlated with heart rate measurements, therefore it was included as a random factor (see Table 4.3.2.1). None of the parameters showed differences in the two minutes before playbacks (Fig. 4.3.2.1).

Table 4.3.2.1: Correlation of locomotion (total duration in s) and heart rate measurements.

	heart rate	SDNN	RMSSD
r_s	0.51	-0.34	-0.49
p	<0.001	<0.001	<0.001

Stimulus

Stimulus had significant effects on all three heart rate measurements (see Table 4.3.2.1). However, only SDNN showed significant differences between the two stimuli when compared within periods: it was significantly higher during controls (n=24, t=3.58, p=0.007; Fig. 4.3.2.1).

Period

Period had a significant effect on heart rate, but not SDNN and RMSSD (Table 4.3.2.1). There was a significant decrease in heart rate from before to after playbacks across stimuli (n=24, t=3.24, p=0.005). When analysing the data within stimuli, this difference was only present between before and after distress call playbacks (n=24, t=3.36, p=0.014; Fig. 4.3.2.1).

Experimental day

Experimental day had significant effects on heart rate and RMSSD. Heart rate was significantly lower and RMSSD significantly higher on day two (heart rate: n=24, t=5.26, p<0.001; RMSSD: n=24, t=-2.46, p=0.016), indicating habituation.

Table 4.3.2.1: Statistical results of the mixed effect models analysing the heart rate measurements.

	stimulus		period		day	
	F	p	F	p	F	p
heart rate	4.25	0.042	5.29	0.007	27.65	<0.001
SDNN	7.66	0.007	0.08	0.924	2.44	0.122
RMSSD	4.71	0.033	1.69	0.190	6.04	0.016
	stimulus * period		stimulus * day		period * day	
	F	p	F	p	F	p
heart rate	1.21	0.302	1.60	0.221	0.82	0.443
SDNN	2.55	0.083	1.33	0.262	0.95	0.390
RMSSD	0.33	0.719	1.76	0.200	0.87	0.423

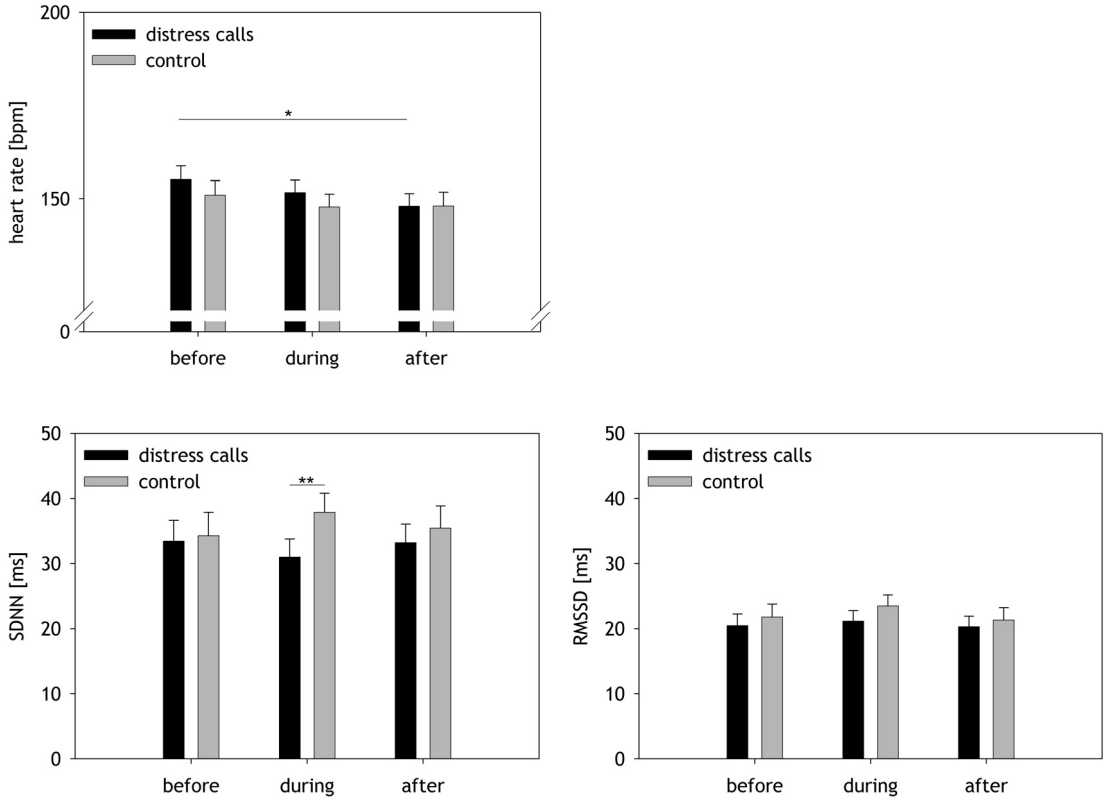

Figure 4.3.2.1: Least square means of heart rate, SDNN and RMSSD. Statistical differences within period (before, during, after playbacks) and within stimuli are given (*** = p<0.001; ** = p<0.01; * = p<0.05; † = p<0.1).

Ten second sections

The three periods before, during and after playbacks were split down into 12 sections of 10 seconds each. Therefore, sections 1 to 12 were before, sections 13 to 24 during, and sections 25 to 36 after playbacks.

While heart rate decreased at the onset of playbacks both in distress calls and controls (Table 4.3.2.2), it was higher in response to distress calls than to controls during the first thirty seconds after the onset of playbacks (only a tendency in section 14). The minimum heart rates were reached in section 14, i.e. 11 to 20 seconds after onset of the stimuli. Then, they reached the basic value of the last section before playbacks again after 40 and 70 seconds during distress calls and control, respectively (Table 4.3.2.2). SDNN showed a peak during both conditions in the first section after onset of the playbacks (Fig. 4.3.2.3, Table 4.3.2.2). From section 14 onwards, levels had returned to the values of section 12. RMSSD tended to differ between stimuli in section 13, i.e. directly after onset of the playbacks (figure 2.4). It remained significantly higher than directly before playbacks for thirty and forty seconds during distress calls and controls, respectively (Table 4.3.2.2).

After playbacks ended, there were no changes in heart rate after controls, but after distress calls (Table 4.3.2.2). These caused differences between distress call and control condition (sections 26 and 27; Fig. 4.3.2.2). SDNN and RMSSD showed no significant differences between stimuli after playbacks (Fig. 4.3.2.3). However, SDNN was significantly higher in the first section after playbacks of distress calls, while RMSSD tended to be higher during the first two sections after distress calls, and was significantly higher ten seconds after controls.

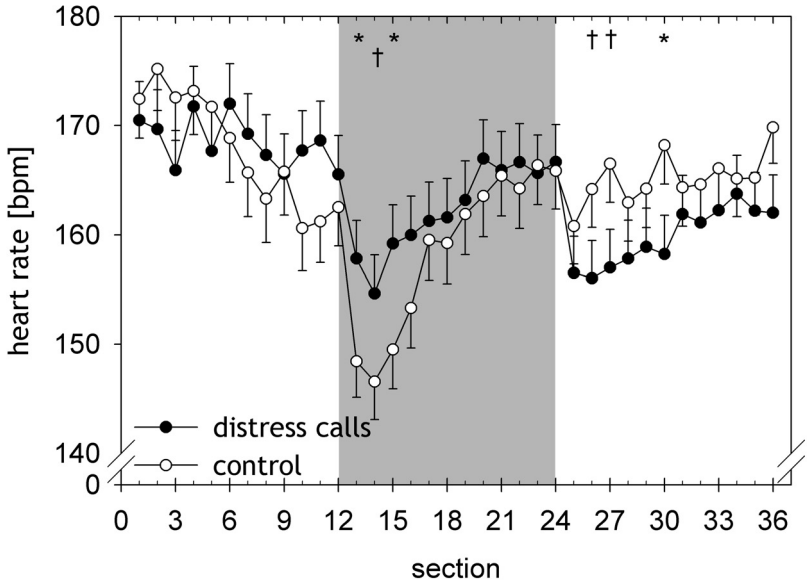

Figure 4.3.2.2: Mean heart rate before, during and after playbacks in 10 second sections. Vertical dotted lines indicate start and end of the playbacks. Asterisks indicate differences between stimuli (*** = p<0.001; ** = p<0.01; * = p<0.05; † = p<0.1). The grey area marks the stimulus presentation.

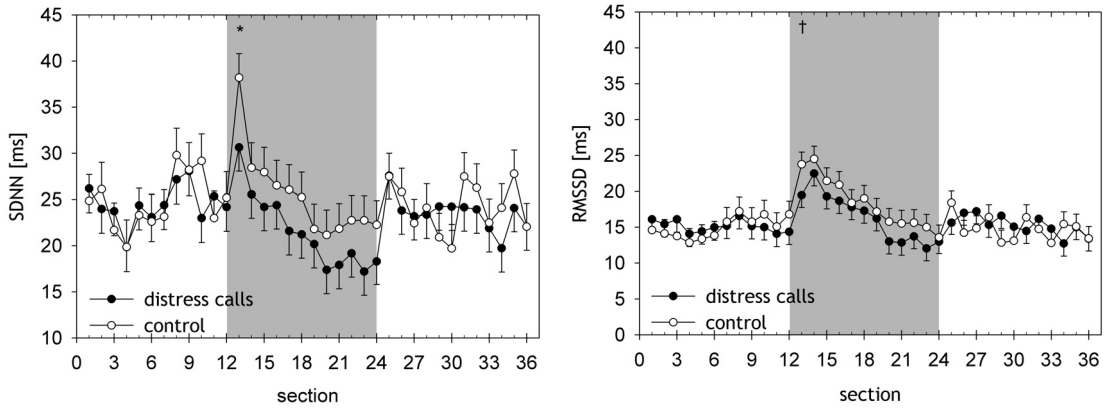

Figure 4.3.2.3: SDNN (left) and RMSSD (right) before, during and after playbacks in 10 second sections. Asterisks indicate differences between stimuli (*** = p<0.001; ** = p<0.01; * = p<0.05; † = p<0.1). The grey areas mark the stimulus presentation.

Table 4.3.2.2: Statistical results. Comparisons between the twelve sections during and after playbacks and the last section of the previous period (i.e. section 12 vs. 13-24 and section 24 vs. 25-36; *** = p<0.001; ** = p<0.01; * = p<0.05; † = p<0.1; - = not significant).

	heart rate		*SDNN*		*RMSSD*	
section	*distress calls*	*control*	*distress calls*	*control*	*distress calls*	*control*
13	*	***	*	***	***	**
14	***	***	-	-	***	***
15	*	***	-	-	*	*
16	†	***	-	-	†	*
17	-	†	-	-	-	-
18	-	*	-	-	-	-
19	-	-	-	-	-	-
20	-	-	*	-	-	-
21	-	-	†	-	-	-
22	-	-	-	-	-	-
23	-	-	*	-	-	-
24	-	-	-	-	-	-
25	**	-	**	-	-	*
26	**	-	-	-	†	-
27	**	-	-	-	†	-
28	**	-	-	-	-	-
29	*	-	†	-	-	-
30	*	-	†	-	†	-
31	-	-	†	-	-	-
32	-	-	†	-	-	-
33	-	-	-	-	-	-
34	-	-	-	-	-	-
35	-	-	†	-	-	-
36	-	-	-	-	-	-

4.4 DISCUSSION

In response to the broadcasting of conspecific distress calls as well as a neutral control sound, juvenile piglets showed a decrease in locomotion and vocalisation, as well as a short-time decrease of heart rate.

The decrease in vocalisation was higher in response to the distress calls. This hints at a higher level of attention, probably in order to explore the cause of the vocalisation. In the wild, it would make sense to react on a warning signal by trying to detect its cause and gather more information. In many alarm calling species, alarm calls inform group members about an approaching predator, while the direction from which this predator approaches can only be derived from contextual information such as orientation of the caller (Leger et al. 1979, Schwagmeyer & Brown 1981, Blumstein 1995, Fichtel & Kappeler 2002). Thus, the receiver can evaluate the threat imposed on itself in more detail than when reacting on the conspecific signal alone, and act accordingly.

However, the decrease in heart rate was higher in response to the control stimulus, which was a surprising result. Considering that the control stimulus was artificial and, therefore, unfamiliar, the stronger response might be caused by the adding effects of suddenness and novelty (Désiré et al. 2004). The referring behavioural data are contradictory: significantly lower vocalisation rates during distress call playbacks indicate higher attention to distress calls, while locomotion showed no differences between stimuli during stimulus presentation at all. The decrease of heart rate was most probably caused by an activation of the parasympathetic branch of the autonomous nervous system, as indicated by the increase of RMSSD (von Borell et al. 2007). Activation of the parasympathetic system has previously been shown in response to novel stimuli (Désiré et al. 2004). However, Désiré and colleagues did not find a heart rate change in response to novelty alone, indicating a simultaneous activation of the sympathetic system. Additionally, they found an activation of the sympathetic system in response to suddenness, along with an increase in heart rate. Heart rate variability parameters cannot determine sympathetic activity, but if such an increase occurred it was much smaller than the parasympathetic effects leading to a net decrease in heart rate.

To my knowledge, responses after the end of a playback stimulus have not been shown before. In the present study, the heart rate response at the end of distress call playbacks was similar to that shown at the onset of stimulus presentation. However, this decrease of heart rate at the end of distress calls could not be assigned to an activation of the parasympathetic or inactivation of the sympathetic system. On the one

hand, the RMSSD, i.e. parasympathetic activity, tends to be higher for 20 seconds, but on the other hand, the SDNN is significantly higher in the first ten seconds. In the social isolation of the open field setting I chose for the experiments, the conspecific calls might have represented some kind on contact to conspecifics. The withdrawal of this contact might have caused the observed effects. In social animals, it is important to keep in contact with the group, thus loss of this contact should induce attention and searching.

One reason for the low levels of responses could be that the subjects used were not the addressees of the vocalisations. Vocalisations produced during restraint might be remnants of a mother-young communication, directed from piglet in danger of being crushed by their mother in order to stop her form lying down. Broadcasting the screams of piglets to nursing sows induces postural changes in the sows during the first hours post partum (Chaloupková et al. 2008, Illmann et al. 2008). Thus, the distress calls used here might only be relevant for mothers, but not for unfamiliar juvenile conspecifics. The latter might only use the information conveyed by such vocalisations as ‘eavesdroppers’, and show no response apart from increased attention whenever they do not perceive a threat imposed on themselves.

Additionally, the subjects used in this study might not have experienced an association between stress-calls and any actual harm to themselves. Hence, two explanations for the low levels of responses are possible: Firstly, responses to conspecific stress calls must be learned, which my subjects never did. Secondly, an innate response to conspecific stress calls might have been overridden by habituation. Both alternatives point at the importance of learning in the given environment. Such learning processes might depend on the husbandry system an individual is subjected to, as these may differ in overall vocalisation rates (and thus the opportunities to learn).

In preliminary studies, I used a shuttle-box design for playback experiments. The open field was divided into halves, and a light barrier in the middle registered the movement of the subjects from one side to the other. Each subject was played one type of stimulus on the one side, and another type on the other side (replay of stimuli was triggered by the light barrier). One stimulus was always conspecific stress calls from an unfamiliar, restrained animal, whereas on the other side silence, sounds of feeding pigs (including grunts), or nursing grunts from an unfamiliar sow were presented. In order to correct for side preferences, half of the subjects were assigned the stress stimulus on the side where the other half of the subjects was played the control. However, within subjects the assignment of a stimulus to a side remained constant throughout the experiment. We expected that the subjects would try to avoid the conspecific stress calls and thus learn to stay within the control side after several

sessions in the shuttle-box. However, in none of the stimulus pairings such an avoidance reaction occurred. As I could not clearly decide whether the animals did not avoid the distress calls because they had not motivation to do so, or simply did not learn that they could avoid them, I decided to use a paradigm which did not require learning. However, the lack of a strong response in the following, more simplistic playback experiments implies that subjects had no need to avoid the stress calls.

Although I used four of the playback stimuli twice, pseudoreplication is not likely to have influenced the data (Hurlbert 1984, Searcy 1989, Kroodsma 2001).

In conclusion, juvenile pigs show responses to sudden noises in general, with differences in the response to conspecific stress-related vocalisations and artificial, unfamiliar sounds. However, distress calls only elicit a short-term response, which indicates that they do not play a role as stressors. Therefore, hearing conspecific distress calls will most probably not affect animal welfare.

5. CHAPTER 3

NEUROLOGICAL UNDERPINNINGS OF STRESS-RELATED VOCALISATION

5.1 INTRODUCTION

Vocalisations have been shown to correlate with emotional states in various species, including the domestic pig (see chapter 1). Current research considers emotions to comprise three basic elements, namely a physiological, a behavioural, and a subjective component (so-called 'componential view') (Paul et al. 2005). However, the conscious component (the 'feeling') cannot be measured in animals.

In an anthropomorphic approach certain types of vocalisation ('screaming') can be considered an indicator of distress, as it occurs in situations likely to be stressful. Furthermore, this distress can be considered to be associated with negative affect. However, such interpretations need to be validated by correlations of vocalisation and both physiological and behavioural indicators of distress. Schrader and Todt (1998) could show that specific vocalisations are associated with high levels of adrenaline. Additionally, Puppe and colleagues (2005) could show that vocalisations during castration of male piglets show parameters affected by putatively painful manipulations of the caller. In my experiment, however, I chose a different approach. Specific brain systems are known to play a crucial role in emotions. The limbic system comprises areas responsible for the emotional evaluation of incoming stimuli, as well as areas regulating the behavioural and/or physiological component of emotion (Isaacson 1982, Lopez et al. 1999, Pacák and Palkovits 2001). In case there is a direct association between specific types of vocalisation and emotion, the brain areas responsible for the regulation of emotions should be involved in the regulation of vocalisation as well. This could be shown for 50 kHz vocalisations in rats, which are considered indicative of positive emotional states (Fendt et al. 2006, Thompson et al. 2006). My thesis focuses on stress-related vocalisations; hence this experiment focuses on areas supposed to underlie negative emotions. The **amygdala** has been shown to be involved in the regulation of fear, especially learned fear. It has repeatedly been described as the central structure regulating every aspect of conditioned fear, from the learning to the evocation of the learned fear (Davis 1994, 1997; Davis et al. 1997, LeDoux 2000, Davidson 2004, Paré et al. 2004, Phelps 2006), and also plays a role in contextual learning (Perez-Villalba et al. 2008). Amygdala activation could also be shown to correlate with heart rate during emotion processing in humans (Yang et al. 2007). Brudzynski and colleagues (1995) could not elicit an emotional aversive response in the amygdala of the cat; however, they used carbachol, a muscarinic (i.e. cholinergic) agonist, which might not bind to the subpopulations of cholinergic receptors expressed in the amygdala. Brudzynski (2001) reviewed microinjection studies performed in rats. These could describe a row of brain areas reaching from the tegmentum to the preoptic

area and the septum, collectively termed 'cholinoceptive vocalisation strip'. Vocalisations pharmacologically induced in this strip could be shown to resemble naturally induced 20 kHz calls (Brudzynski et al. 1991). Associated to the amygdala, the anterior cingulate cortex is involved in amygdala dependent fear learning (Bissière et al. 2008), emotion processing in general (Bush et al. 2000), and the voluntary control of vocalisation (Jürgens 1998). The cholinoceptive vocalisation strip has been defined based on microinjection studies with acetylcholine (or cholinergic agonists). CRH and noradrenaline (or adrenergic agonists), both being produced as part of the physiological stress response, may also play an important role in the production of stress-related vocalisations. Binding site densities of NMDA, $GABA_A$, but not AMPA-receptors correlated with fear sensitised acoustic startle and elevated plus maze behaviour in mice (Yilmazer-Hanke et al. 2003). Furthermore, NMDA plays a crucial role in long-term potentiation (LTP). In LTP, the stimulation of afferent pathways can cause an increased responsiveness of cells within the amygdala. This mechanism is suppossed to underlie emotional learning (Fendt & Fanselow 1999).

The aim of this experiment was to establish an experimental design to examine, whether the amygdala and cingulate cortex are involved the regulation in the domestic pig. No one has done this kind of experiments before in pigs, therefore every detail from handling surgery, and finally the experiments, had to be determined. I used various stimulating agents in order to cover a large array of underlying neural circuits.

Hence, I performed three consecutive sets of experiments. Experimental procedures were modified based on the experiences from the preceding sets, resulting in one suggested design in the last set of experiments.

5.2 METHODS

5.2.1 Animals and housing

A total of 39 subjects were used; 17 in the first set, 10 in the second set, and 12 in the third set. All subjects were outbred female German Landrace pigs. At the beginning of the experiments, subjects were six weeks old. They were housed in single pens measuring 2 by 1 metres with half solid and half fully slatted floor. Water and food pellets (Ferkelstarter Plus, Trede und von Pein, Itzehoe, Germany) were available ad libitum.

5.2.2 Experimental procedure

Each experimental trial took three weeks, approximately one for presurgical training, one for postsurgical reconvalescence, and one for the experiments.

In the first set I injected stimulants into the amygdala or the anterior cingulate cortex. In the last two subjects of the first set of experiments, plasma catecholamine levels were measured to get information on the physiological stress response. As this proved to be problematic, I used heart rate measurements in the second and third set of experiments. Furthermore, the volume injected into the brain sites was reduced in the second set of experiments, while the injection time was maintained, slowing down injection speed. Thus, a more focused stimulation was achieved. Injections in the second set of experiments aimed at the basolateral and central nucleus of the amygdala. The number of substances was reduced to noradrenaline and acetylcholine in order to enable repetitions of experiments within subjects. Finally, in the third set of experiments, AMPA and two types of artificial adrenergic agonists were tested along with acetylcholine (seperately and in combinations). In this last set, the injected volume was increased again to 15μl, while injection speed was maintained.

Training

An intensive training was necessary to habituate subjects to the experimental situation. Specifically, subjects had to be trained to move between home pen and the experimental room and to lie down in a restriction apparatus in that experimental room. In each trial three animals were trained, but only two were used as subjects. Thus, in case of health problems or insufficient habituation to the experimental procedure I could still perform experiments with two animals (which was the maximum number possible per trial). Each subject was trained for at least twice an hour each day (one hour in the morning, one in the afternoon). They were first habituated to human contact and fitted a dogs' harness, which they were wearing permanently from the first or second day of training onwards. Then, they learned to walk from their home pens to the experimental room and back. In the next step, animals were introduced to the experimental stand, where they could be restricted during experiments. Subjects successively habituated to staying in this stand for up to one hour. At the end of training the subjects would calmly go to the experimental room, lie down in the restriction stand and tolerate being touched on their heads. Training was continued during the week after surgery. Then, treatment of the wounds was done in the experimental situation.

Surgery

Subjects were anesthesised with intramuscular injections of Ketamine (Ursotamin®, Serumwerke Bernburg, Bernburg, Germany) and Xylazin (Rompun®, Bayer, Leverkusen, Germany) intramuscular, or Pitramid (Dipidolor™, Janssen-Cilag, Neuss, Germany) (the latter was used when jugular vein catheters were added) in the first set of experiments. In the second and third set, a combination of medetomidine (Domitor®, Pfizer, Zurich, Switzerland) and atipamezole (Antisedan®, Pfizer, Zurich, Switzerland) was used (Antisedan ends anaesthesia, thus the critical wakening phase is shortened dramatically from up to 10 hours to 2-4 hours). The subjects were then fixed in a stereotaxic head holder for growing pigs (ASI Instruments, Warren, MI, USA). Permanent guide cannulae penetrating the dura were implanted and fixed to the skull using a modified introducer for brain probes (Integra, Plainsboro, NJ, USA). I used bregma as the marker from which the coordinates (rostral and lateral) of the burr holes were determined (based on the stereotaxic atlas of the pig brain [Félix et al. 1999] first, and experience later). For thee days post surgery animals were treated with intramuscular injections of antibiotic (Trimetox®, Veyx Pharma, Berlin, Germany) and analgetic (Metapyrin®, Medistar, Holzwickede, Germany). Temperature was checked twice daily, and in case of values above 40° Celsius (Hannon et al. 1990) animals were treated with Metapyrin. The institute's veterinarian (Dr. Olaf Bellmann), who was also responsible for all medical treatments did anasthesia.

Injections

Subjects were transferred to the experimental room and tethered at least 5 min before injection cannulae (Kapillar-Stahlrohr, Innovative Laborsysteme, Stützerbach, Germany: stainless steel, outer diameter: 0.64 mm, inner diameter: 0.15 mm, length: 100 mm) were inserted and fixed. Recordings started directly after insertion of the cannulae. Injections started five minutes after that, and the experiment ended after a total time of 15 minutes. However, only responses occurring within 3 minutes after injections can be considered to be elicited in the target area of injection.

Confirmation of injection sites

After the last experiment, subjects were killed by the veterinarian lege artis (T61, Intervet, Unterschleißheim, Germany), and cresylviolet solution was injected (1% cresylviolet(acetate), Merck, Darmstadt, Germany, in $aqua_{dest}$; set1: 20μl; set 2: 10μl; set 3: 5μl). In the first set of experiments, the whole brain was removed and fixated and stored in formaldehyde for at least two weeks. Afterwards, brains were dissected using a microtome blade starting from approximately 5 mm rostral from injection sites. For

orientation the injection points of the guide cannulae on top of the brain could be used; sometimes the cresylviolet solution could be seen from the outside.

In the second and third set of experiments, a slice (approx. 2 cm) of the brains including the injection sites was removed and frozen on dry ice. They were stored at -80° Celsius, and dissected on the microtome (20μm). Slices including the injection sites were dyed with Nissl stain.

Stimulants

In the first set of experiments, stimulants were dissolved in 20μl of vehicle (artificial liquor cerebrospinalis: 7.71 g NaCl, 0.22 g KCl, 0.14 g $CaCl_2$, 0.238 g $MgCl_2 \cdot 6\ H_2O$, 0,036 g NaH_2PO_4, 0.170 g Na_2HPO_4 in 1 L; pH: 7.02). The vehicle alone served as control. Each animals was subjected to a maximum of 8 injections on 4 days, i.e. 2 injections per day with at least 4 hours in between. Stimulants were injected from microliter-syringes (set 1: 100 μl total volume; sets 2 and 3: 50μl total volume; for details on stimulants see appendix). Injection cannulae were connected to the syringes via silicon tubes to allow for movements of the subject. The syringe itself and the tube were filled with neutral oil (walnut). Stimulants and their amount are listed in tables 5.2.2.1 (first set of experiments) and 5.2.2.2 (second and third set of experiments). The second set of experiments was started with injections of 10μl in one minute. However, in order to increase the amount of substances applied, the volume was increased to 15μl in 1.5 minutes after 6 trials (resulting in 5 trials using 15μl). In the first set of experiments, I knew which substance was injected, while afterwards I was blind to the stimulant.

Table 5.2.2.1: Stimulants used in the first set of microinjection experiments.

name	action	amount in 20μl
noradrenaline-bitartrate	adrenergic	800 nmol
Acetylcholine (ACh)	cholinergic	5,500 nmol
NMDA	glutaminergic	2,000 nmol
CRH	HPA-axis hormone	8 nmol
Bicucullin	$GABA_A$-antagonist	250 nmol

Table 5.2.2.2: Stimulants used in the first set of microinjection experiments.

name	action	amount in 10μl	amount in 15μl
noradrenaline	adrenergic	4,560 nmol	6,840 nmol
acetylcholine	cholinergic	5,500 nmol	8,250 nmol
isoproterenol	adrenergic (β1,β2)	5,500 nmol	8,250 nmol
phenylephrine	adrenergic (α1)	5,500 nmol	8,250 nmol
AMPA	glutaminergic	2 nmol	3 nmol

Acoustic recording

An undirected microphone (Sennheiser ME64/K6) was placed about 1 m in front of the subject at a height of 1.7 metres. Signals were transmitted to a digital audio tape recorder (Sony TCD-D100) in the first set of experiments and to a portable hard disk recorder (Marantz PMD 680) where the recordings were stored (sampling rate: 44.1 kHz, 16 bit accuracy, mono).

First recordings were made at first tethering on the experimental room (first 5 min). In the first set of experiments, I recorded the last tethering before surgery and the first tethering after surgery. As hardly any subject vocalised around surgery any more, from the second set of experiments on the second tethering was recorded, instead (first 15 min). Subsequently, recordings were made from 5 min before injection to 10 min after start of injections. A protocol was written for all injection experiments, giving the exact time, site, substance, and volume of injection. Additionally, I registered whether subjects showed any observable responses to injections (vocalisation or other behaviours), and whether they showed general signs of arousal. Hence, only vocal responses exceeding vocal activity before injections were considered responses.

Catecholamines

The last two subjects of the first set of experiments were additionally supplied with jugular vein catheters. Thus, blood could be sampled and catecholamine concentrations in the plasma were measured via HPLC (high pressure liquid chromatography; protocol cf. Otten et al. 2004). Blood samples were taken a) in the pen, approximately 5 minutes before transfer to the experimental room, b) directly before injection, directly after injection, c) 4 min after the end of injection, and d) 8 min after the end of injection.

Heart rate measurements

In the second set of experiments, I introduced heart rate measurements in addition to the acoustic recordings (procedure see section 4.2.6, p. 51 et seq.). R-R intervals, i.e. intervals between each heart beat, were recorded, allowing for analyses of heart rate variability.

I measured heart rate during the second tethering in the experimental stand and during experiments (from five minutes before to ten minutes after start of injections).

Data were corrected for artefacts under visual control (sensitivity: very low, peak detection activated, minimal protection zone: 20). Data with more than 10% artefacts had to be excluded from the set (cf. von Borell et al. 2007). Mean heart rate as well as heart rate variability parameters were calculated for ten second sections for three minutes before and after start of injections (see section 4.2.6, p.51 et seq.).

5.2.3 Acoustic analyses

Vocalisations elicited in response to injections were isolated. In order to eliminate noise caused by the chains used to fix the animal in the stand, sample rate was decreased to 22.05Hz (antialiasing was applied), and fast fourier transformations (FFT) were performed using Avisoft-SASLab Pro software (version 4.40; Avisoft Bioacoustics, Berlin, Germany). I used a FFT-length of 1024, frame size of 100%, and Hamming window with 50% overlap; these settings lead to a temporal resolution of 23.2 ms and a frequency resolution of 22 Hz. Based on the FTT, the following parameters were measured for each of the calls: duration, peak frequency, quartiles, and entropy. All parameters were measured as means across the whole call.

5.3 RESULTS

First, the correct coordinates for the injection sites had to be found, which took three trials. In general, all data from experiments with injection sites other than the amygdala or cingulate cortex were excluded from further analyses.

In the **first set of experiments**, 14 of the 17 animals were subjected to experiments. In two of them the cingulate cortex was unsuccessfully aimed at in one hemisphere (amygdala in the other), while the remaining 12 subjects received cannulae to he amygdala bilaterally. Of these 24 injection sites, 13 could be verified as lying within the amygdala (5 subjects bilaterally, 3 subjects unilaterally). However, only one of those subjects showed vocal responses to injections. To be precise, that subject (subject 27) vocalised after unilateral injection of acetylcholine, but showed no responses to

injection of both CRH or control. The amplitude of vocal responses to left amygdala injections were too low for analyses (recording level was adjusted to the responses to first tethering, which turned out to be much louder), but in the right amygdala vocalisations of sufficient amplitude were elicited. Mean (±s.d.) duration was 2.7±0.8 seconds (n=9). Peak frequency, entropy, and Q75-Q25 are depicted in Fig. 5.3.1. For comparison, 20 vocalisations recorded during the first tethering in the experimental stand and 15 vocalisations from tethering one day after surgery were analysed (see Fig. 5.3.2 for spectrograms).

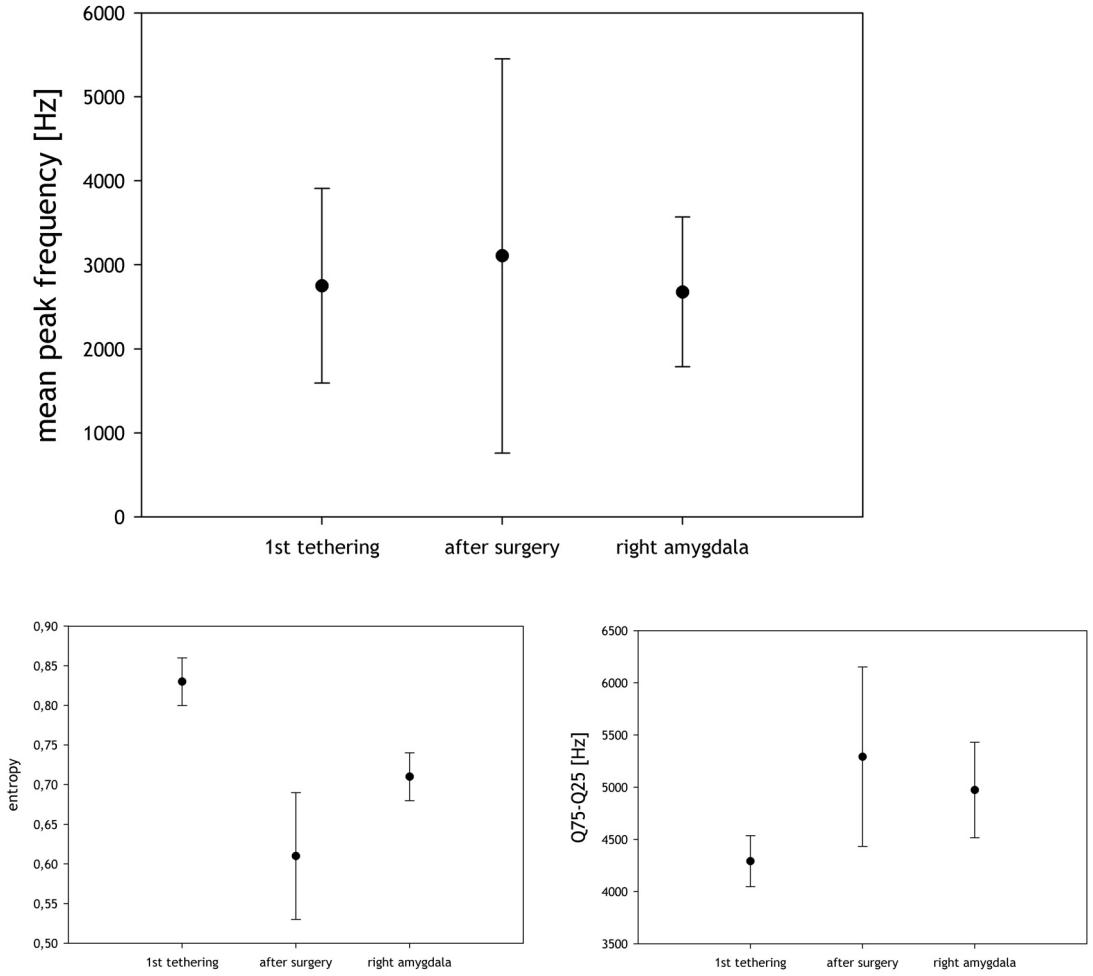

Figure 5.3.1: Mean±s.d. peak frequency, entropy, and Q75-Q25 of subject 27 during tethering (first and after surgery) and after injection of acetylcholine in the right amygdala. (n: 1st tethering= 20; after surgery=15; acetylcholine-induced=9)

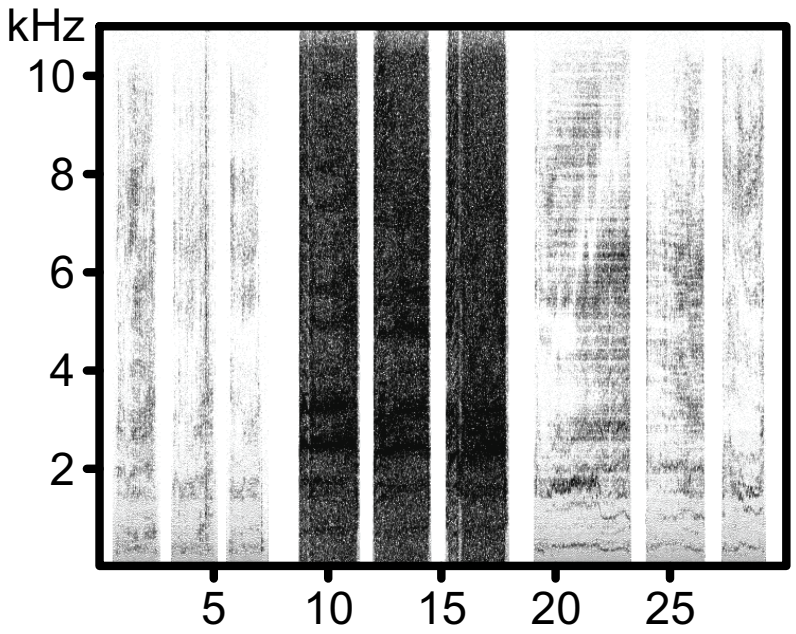

Figure 5.3.2: Time signals (upper graph) and spectrograms (lower graph) of three calls emitted by subject 27 after injection of acetylcholine to the right amygdala (first set of calls), first tethering (second set of calls), and tethering after surgery (last set of calls).

This subject was supplied with a jugular venous catheter. The results of catecholamine analysis are depicted in Fig. 5.3.3. The injections are presented in the actual order of experiments. Apart from a peak of noradrenaline in the pens for the first three experiments (which can be explained by an inflammation of stitches fixing the catheter on the subject's skin), there were additional peaks of both noradrenaline and adrenaline after the bilateral injection of acetylcholine. Additionally, directly after injection of acetylcholine in the left amygdala the levels of both noradrenaline and adrenaline were higher than directly before. As there were some problems with sampling blood (the subject seemed to have been lying on the internal catheter tube), some samples are missing. Injections of control (left, right, and bilaterally) and CRH (bilaterally) did not cause increases of noradrenaline or adrenaline levels (Fig. 5.3.3). In the same trial, the second subject (subject 25) was supplied with a catheter. The injections sites could not be confirmed within the amygdala but the hypothalamic area in both hemispheres. This subject did not show vocal responses to any of the injections (control: right, left, bilaterally; ACh: bilateral; CRH: bilateral; NA: bilateral), and only injection of noradrenaline bilaterally caused a short-term peak of noradrenaline (but not adrenaline; Fig. 5.3.4).

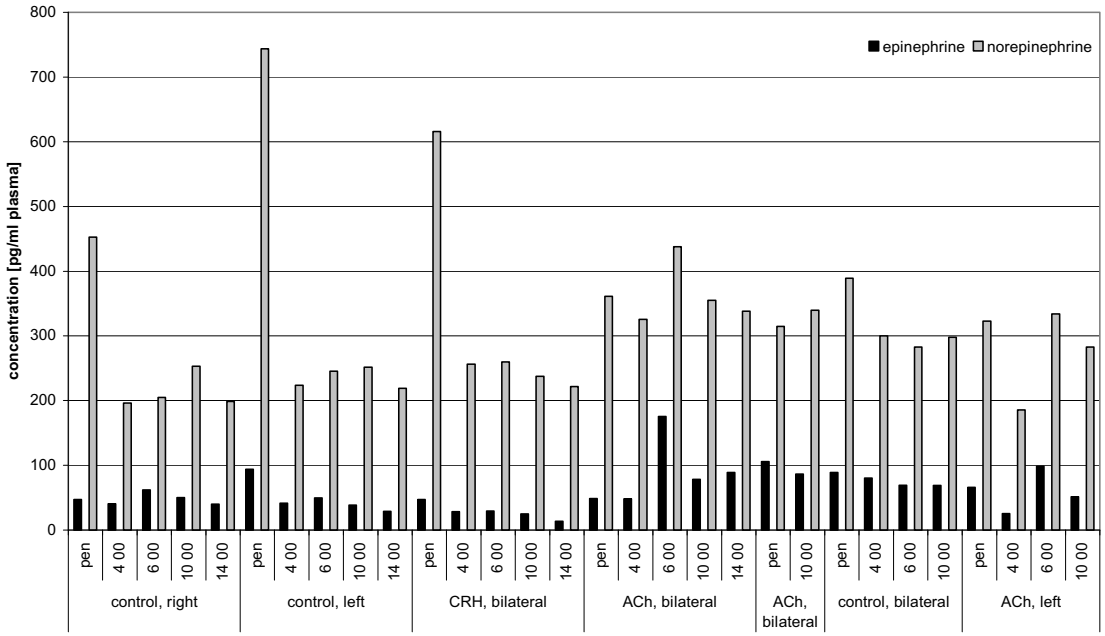

Figure 5.3.3: Catecholamine levels of subject 27. The amygdala was successfully stimulated in both hemispheres of this subject, causing vocal responses. Injections were at 5:00.

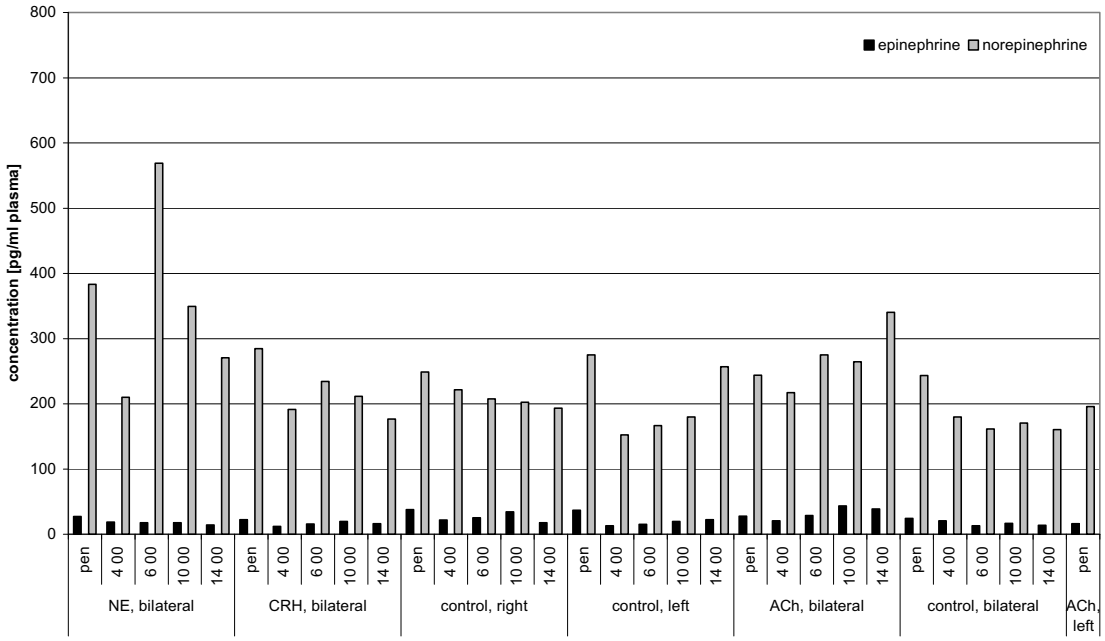

Figure 5.3.4: Catecholamine levels of subject 25, which did not vocalise. In this subject, both injection sites lay outside the amygdala in the hypothalamic area. Injections were at 5:00.

In the **second set of experiments**, 6 out of 15 injection sites were within the amygdala (central: 2; basal: 1; central/medial: 1; medial: 1; lateral: 1), and 3 sites were

in areas between amygdala and neighbouring nuclei (nucleus caudatus: 2; hippocampus: 1). None of the subjects showed vocal responses to injections.

In the **third set of experiments**, 6 subjects (i.e. all subjects in the first three trials) were supposed to be stimulated in the cingulate cortex in one hemisphere and amygdala in the other hemisphere (bilateral stimulations of the cingulate cortex are not possible, as the two areas are too close to fix two guide cannulae). The cingulate cortex was verified as the injection site 5 times; however, a leakage of stimulant into the liquor cerebrospinalis could not be prevented due to its position (medial, only approximately 1.5 centimetres below the skull). Therefore, the cingulate cortex was not focused on any further. Of the 18 sites, 16 sites were within or next to amygdalar nuclei (basolateral: 5; basomedial: 2; basal: 2; basolateral amygdala/piriform cortex: 4; lateral amygdala/piriform cortex: 2; basomedial amygdala/piriform cortex: 1). Thus, only 12.5% of the injection sites were incorrect in the third set of experiments.

However, only one subject (subject 54) seemed to show any response to injections. During injection of AMPA to the left, lateral amygdala this subject produced vocalisations. However, these did hardly resemble screams but were of low amplitude and had a ‘gurgling’ sound. The subject showed no response of heart rate (Fig. 5.3.5) or heart rate variability (Fig. 5.3.6) to the injection.

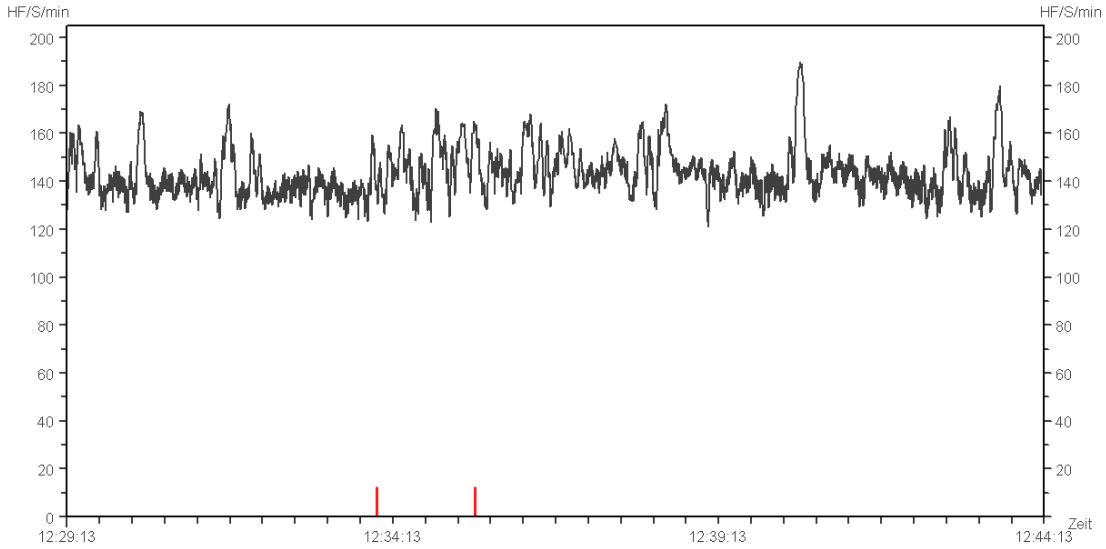

Figure 5.3.5: Heart rate curve (beats per minute) of subject 54 at injection of AMPA to the left amygdala. The lines at the time axis mark the start and end of injection.

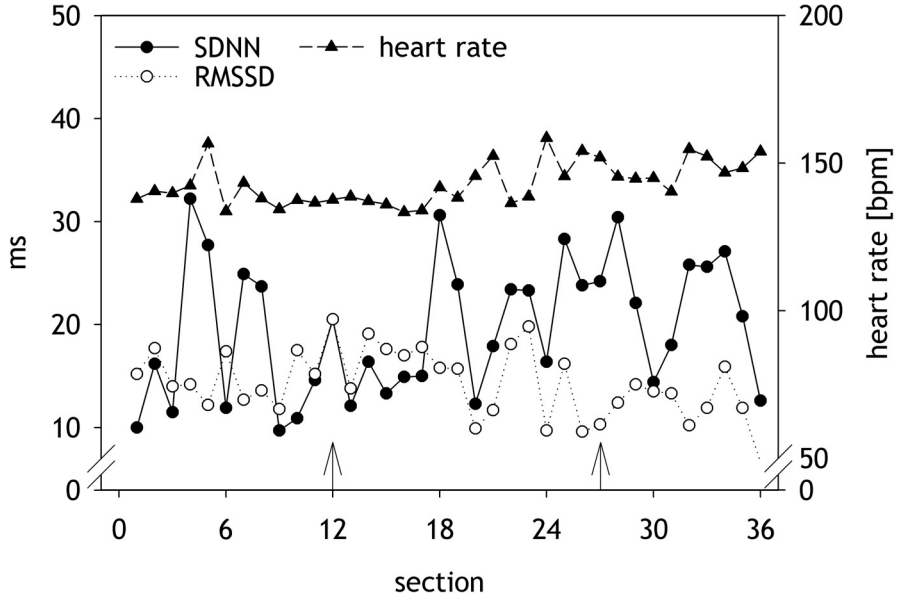

Figure 5.3.6: Heart rate and heart rate variability parameters 3 min before and after onset of AMPA injection to the left amygdala of subject 54. Injection started with section 18 and ended with section 27 (indicated by arrows).

5.4 DISCUSSION

In the course of these experiments, an experimental design could be developed that allows for focused injections of stimulants to the pig brain.

Coordinates were adjusted, hence in the last set of experiment only 12.5% of the injection sites lay outside our focus area. From the second set of experiments onwards, experiments were performed in the 'Tiertechnikum' of the FBN, where more sterile conditions can be achieved. There, much less health problems occurred, indicated by fewer cases of high temperature and better wound healing. Additionally, the length of incisions during surgery was reduced. Thus, only the minimum space needed for fixing the permanent guide cannulae on the skull was revealed. Furthermore, the microtome sections of the brain to confirm injection sites improved accuracy.

Vocalisations elicited by injection of acetylcholine to the amygdala resembled vocalisation produced during tethering after surgery instead of first tethering. This indicates a moderate instead of a high levels of distress. This is also represented by the catecholamine concentrations (which showed only weak peaks). The weak vocal response of subject 54 to injection of AMPA to the left amygdala (which could clearly be distinguished from stress-related calls by hearing) was not associated with heart rate or heart rate variability changes. Hence, the interpretation of the calls as stress-related calls can be doubted.

Only a small number of animals responded vocally to injections of stimulants to the amygdala. It is striking that the most responsive animal showed a high overall responsiveness in the experimental stand. Most subjects did not vocalise any more on the days before and after surgery. This might indicate a high level of habituation. Thus, the final design (set 3) comprises recordings at first, second, and third tethering in the experimental stand. I assume that these recordings demonstrate the decreasing stress elicited in this situation due to habituation. With increasing habituation, however, the responsiveness to react to injections might have decreased, as well. Manteuffel and colleagues (2007) suggested in a pilot study to this one that the resting potential of cells in the central nucleus of the amygdala was reduced due to habituation. Hence, the required stimulation needed in order to reach the firing threshold of those cells was increased. Analogously, there might be a 'stress threshold' for vocal responses, which might act by summing up all current stressors. Only when the stress level exceeds the threshold, vocal responses are produced. In the pilot study, habituation might have been lower, resulting in a higher general stress level in the experiments. However, pigs need to be habituated to this kind of experiments. Several times, injection cannulae were destroyed when subjects moved their heads during insertion (I always prepared two sets of syringe and cannula). With less habituation, pigs tended to flinch whenever their heads were touched from above, thus the insertion of a cannula would have been impossible.

Moreover, as mentioned earlier, several studies did not find vocal responses to neuropharmacological stimulation of the amygdala (Brudzynski et al.1995, Brudzynski 2001). Allikmets and colleagues (1969) could only demonstrate aggressive behaviour, seizures, and salivation as well as behavioural inhibition and sleep in response to acetylcholine injections in the amygdala. Noradrenaline elicited no response.

Furthermore, most currently the interpretation of the amygdala as a brain area related to negative emotions only begins to change. Contrary to former results the amygdala also seems to be involved in processing of positive reward and reinforcement (Murray 2007). In a meta-analysis of functional neuroimaging studies, Sergerie and colleagues (2008) showed that effect size was larger in response to positive than negative stimuli, and Costafreda and colleagues (2008) suggested that amygdala activation might be an indicator of arousal rather than emotional valence.

In conclusion, I established a method to perform microinjection studies in pigs. Whether stress-related vocalisations are elicited by activity in the amygdala cannot be confirmed. However, the data presented here provide first hints at the involvement of the amygdala in the regulation of the vocal stress responses.

6. GENERAL DISCUSSION

In my experiments, I demonstrated that female juvenile piglets (German Landrace) a) produce stressor-specific, i.e. referential, stress-related vocalisations; b) showed short-term responses to such conspecific, stress-related vocalisations; and that c) the amygdala might be involved in the regulation of stress-related calls.

Different types of stressors elicited specific vocal responses. Therefore, I could demonstrate that pigs use referential stress-related signals, conveying detailed information for the receivers. Most probably, such vocalisations in response to stressors represent honest signals (Fitch & Hauser 1995). Thus, vocalisation can be used for the real-time assessment of animal health and welfare in different phases of meat production, or in different husbandry systems.

Referential and honest signals enable the receiver to react appropriately. However, what exactly an 'appropriate response' to stress-related vocalisations might be is not clear. In the playback experiments (chapter 2), I found short-term responses of piglets to conspecific stress calls. Both behavioural and physiological parameters indicate attention to the conspecific calls, but the control sound elicited a similar response. Thus, pigs showed attention for spontaneous noises in general. Whether my results could be replicated in different housing conditions remains unclear. Anyway, in the conventional husbandry system at the FBN, stress responses do not spread from a single, stressed animal over a whole group via acoustic signals. Therefore, not every stressor acting on an individual impairs the welfare of the whole group. This needs to be considered when vocalisation is used as an indicator of stress.

The conspecific stress calls used as playback stimuli in chapter 2 had been recorded during physical restraint (chapter 1, chapter 3). In the wild, signallers' situation might be similar to situations where an animal is captured by a predator. Whether this predator would represent a threat to another individual is uncertain, as the predator it is currently dealing with the caller. This makes clear that the appropriate response to referential signals can also be not to change behaviour. That way, ongoing behaviours are not disturbed unnecessarily. Another explanation for the low response to conspecific stress-related calls is that these vocalisations are not directed at conspecifics, but rather at the predator. Stress-related calls produced during restraint are loud, and thus they can possibly distract or startle the predator (e.g. Högstedt 1983), increasing the prey's chance of an escape.

In addition to stressor-specific vocalisation, I could show that the same stressor can elicit different responses in different individuals of different age. The age dependent

differences occurred in the social isolation in the open field, i.e. during visual as well as acoustic isolation. In chapter 1, subjects (10-weeks-old) did hardly show vocal responses to this setting, while during playbacks (chapter 2) subjects (6-weeks-old) grunted frequently except for when playback stimuli called for their attention. The differences in vocalisation rates and call types shown by 10- and 6-weeks old piglets might reflect their different states of need when isolated (cf. Weary & Fraser 1995a, 1995b; Weary et al. 1997). I suggest that with growing age pigs show a decrease in their tendency to respond vocally to isolation. However, longer isolation might have elicited responses, as the duration of a stressor plays an important role in the ability of the individual to cope with it (Korte et al. 2007).

Moreover, experience was shown to influence vocal responses to stress. In the microinjection studies (chapter 3), the subjects were tethered in the same stand as for restraint in chapter 1. However, in chapter 3 subjects were restrained repeatedly. Vocal responses changed during the subsequent restraints, both in amplitude and call rates (data not presented). Most subjects did not show vocal responses to tethering after four or five trials. This can be considered indicative of habituation. Subjects learned that no harm is associated with the experimental stand, and thus most probably also their physiological stress response decreased. I could show that vocalisation which was cholinergically induced in the amygdala resembled vocalisation after some habituation to tethering in the experimental stand rather than at first tethering. Whether higher doses of stimulants would elicit vocalisation bearing closer resemblance to calls produced during first tethering cannot be decided. However, neurochemically induced vocalisation did not resemble sounds produced in response to one of the other stressors tested in chapter 1. Therefore, the neuropharmacological stimulation of the 'fear centre' amygdala can be suggested to increase the tendency to respond emotionally to the current situation rather than producing a stress response independent of the present context. Thus, activation of the amygdala might give the impulse to react on contextual cues. However, the neural mechanisms underlying the production of stress-related calls seem to involve brain circuits regulating aversive emotional states. These results give support to public concerns on animal welfare, as vocal stress responses can be considered indicative of negative emotional states, as well.

A classificator of stress-related calls needs to be validated by correlating vocalisation with parameters of the physiological stress response. Heart rate and heart rate variability provide non-invasively measured parameters characterising pigs' stress responses. This approach is supported by the data presented in this thesis. However, in small piglets (which have higher base levels of heart rate) electrocardiograms should

be preferred over the Polar system used in this study. The latter cannot evaluate severe stress in young animals. The maximum possible number of heartbeats is fixed at 240 beats per minute, thus whenever heart rates exceed 240, the system considers this an error and only counts every second heartbeat. An alternative to heart rate measurements during acute stress would be the measurement of catecholamines or glucocorticoids in the blood plasma. However, these cannot be collected without additional stress by setting catheters (repeated samples) or restraining of the subjects (single samples). Additionally, group housing is problematic in catheterised animals.

Modern animal farming praxis involves changing environments. The animals have to cope with challenges such as early weaning, transportation, and other handling procedures, all of which might cause stress (e.g. Stephens & Perry 1990, Lawrence et al. 1991). The non-invasive measurement of vocal responses to such stressors is of interest due to both ethical and financial concerns. While the public is mainly concerned about meat quality and animal well-being (Broom & Johnson 1993, Rosenvold & Andersen 2003, Terlouw et al. 2008), the farmers are also concerned about impaired animal health and productivity (Verbeke et al. 1999). Stress-related vocalisation in pigs could be described in several different situations. Emotionally relevant brain circuits seem to underlie their production. Thus, vocalisations represent a referential indicator of stress and negative emotional states, and it can be used to measure impaired welfare in a non-invasive approach (Manteuffel et al. 2004). In this thesis, I could describe stress-related porcine calls using classical parameters as well as complex models. As mentioned earlier, up to now there has been no approach to describe vocal responses to several stressors with a unified set of parameters in pigs. My results showed that either fractional octave analysis or linear prediction coding provide a basis for such a set of parameters. Most stressors elicited vocal responses showing high variability both within and between individuals. An automatic detector of such vocalisation must be able to handle these variances. Based on my data I conclude that neural networks can probably solve this problem (Schön et al. 2001, 2004). The data gathered in chapter 1 can help to improve the automated classificator for pigs' stress call (STREMODO, Schön et al. 2001, 2004). STREMODO only classifies two categories of sounds: stress and non-stress. However, it would be good to discriminate within the stress-category. For example, anticipation of food might cause a peak of vocalisation around feeding times (P.C. Schön, personal communication). In case of aggressive competition for food, additional vocalisations might be produced in response to physical pain. Animal welfare can be improved by decreasing such aggression. Thus, a classificator separating calls elicited by physical pain can be used to evaluate different feeding regimes. Additionally, maybe some non-

harmonic calls produced by isolated pigs should be included enabling the detection of animals who lost contact to their group.

In conclusion, the data presented in this thesis broaden our knowledge of porcine stress-related vocalisation. I introduced new techniques of acoustic analysis and for neurophysiological studies in pigs, which might be helpful for future research. My results will further establish the non-invasive evaluation of welfare status in pigs based on the analysis of vocalisation.

7. SUMMARY

This thesis focused on stress-related vocalisation in the domestic pig. Vocalisation has been suggested as an indicator of emotional states in several species. It can be used as an indicator of stress, and thus of impaired animal welfare. In order to establish vocalisation as a reliable, valid indicator of stress in domestic pigs, I examined a) whether pigs produce stressor-specific vocalisations, b) which communicative significance they have for conspecifics, and c) whether the production of stress-related vocalisation is associated with brain areas underlying the regulation of aversive emotions.

In the experiments subsumed in the first chapter, pigs were subjected to several stressors, comprising laboratory settings as well as praxis-relevant situations. Using a classical fear conditioning approach I could show that vocal responses to mental and physical stressors can be differentiated based on linear prediction coding (LPC) analysis. Not only the anticipation of aversive events in fear conditioning caused vocalisation, but also the anticipation of food. However, this might be caused by the association of feeding and aggressive competition. Vocalisation produced in response to restraint and tail biting could be described, as well as several call categories produced during social isolation. In the latter, harmonic as well as non-harmonic calls were produced. While the harmonic vocalisations (restraint, tail biting, squeals from isolation) could be clearly discriminated, the non-harmonic sounds did not segregate well in discriminant function analyses and neural networks. However it remains to be examined whether the suggested sound categories refer to different underlying emotional states. The introduction of fractional octave analysis for sound description did not improve classification results remarkably.

In the second chapter, I describe experiments in which the responses both in behaviour and in heart rate variability to replay of conspecific stress-related (restraint) calls was tested. Subjects showed attention to sudden sounds in general, i.e. to both conspecific calls and a neutral control sound. This was shown by lower vocalisation rates as well as an activation of the parasympathetic system (represented by heart rate variability). However, these responses were short-term and ended during the two minutes of playbacks. Thus, the meaning of conspecific stress-related calls is not clear yet. Juvenile pigs might not be the addressees of such calls, but rather the sow. Furthermore, my subjects may never have learned an association of a conspecific's

scream and a threat to themselves. The constant lack of such an association could also lead to habituation, eliminating an inherited response tendency.

In the third chapter, I established a method for brain microinjection studies in pigs. It was applied to an experiment investigating the neural basis of stress-related calls. It is generally assumed that stress is associated with negative emotions. I hypothesised that brain areas involved in the regulation of such negative emotions are also the basis of stress-related vocalisation. First results support this interpretation. At least one subject showed vocalisation and a simultaneous increase of the stress hormone adrenaline in response to cholinergic activation of the amygdala, the limbic area crucial for fear acquisition and expression. The vocalisations resembled calls produced in response to a moderate level of stress. However, several subjects stimulated in the amygdala did not vocalise. Most probably this was due to a too good or full habituation to the experimental situation.

In conclusion, I could prove that pigs do show stressor-specific vocal responses. These responses elicit short-term alertness in conspecifics unfamiliar with the caller. First results from a microinjection study implied that the amygdala, i.e. the crucial centre for fear acquisition and expression, is involved in the generation of stress-related calls. These results will further establish the non-invasive evaluation of welfare status in pigs based on the analysis of vocalisation.

8. ZUSAMMENFASSUNG

Die vorliegende Arbeit befasste sich mit der stressbezogenen Lautgebung beim Hausschwein. In verschiedenen Spezies wird deren Vokalisation als ein möglicher Indikator für emotionale Zustände betrachtet. Akustische Signale können Stress damit beeinträchtigtes Wohlbefinden anzeigen. Um Vokalisation als zuverlässigen, aussagekräftigen Indikator für Stress beim Hausschwein zu etablieren, untersuchte ich a) ob Schweine stressorspezifische Vokalisationen produzieren, b) welche kommunikative Bedeutung sie für Artgenossen haben, und c) ob die Produktion stressbezogener Laute mit Hirnarealen assoziiert ist, die an der Regulation aversiver Emotionen beteiligt sind.

In den im ersten Kapitel beschriebenen Versuchen wurden Schweine verschiedenen Stressoren ausgesetzt. Diese umfassten sowohl Laborversuche als auch praxisrelevante Situationen. In einer klassischen Angstkonditionierung konnte ich zeigen, dass vokale Antworten auf mentale und physische Stressoren mittels linearer Vorhersagemodelle (LPC) differenziert werden können. Nicht nur die Erwartung eines aversiven Stimulus in der Angstkonditionierung, sondern auch die Erwartung von Futter löste Vokalisationen aus. Diese könnten jedoch durch die gelernte Assoziation von Fütterungszeiten mit aggressiven Auseinandersetzungen (Konkurrenz) ausgelöst sein. Lautantworten auf Bewegungsrestriktion und simuliertes Schwanzbeißen konnten ebenso beschrieben werden wie verschiedene Lauttypen, die während sozialer Isolation auftraten. Letztere enthielten sowohl harmonische als auch unharmonische Laute. Während die harmonischen Vokalisationen (Bewegungsrestriktion, Schwanzbeißen, ‘Quieker’ aus der Isolation) deutlich unterschiedlich waren, konnten die unharmonischen Laute weder in der Diskriminanzanalyse noch mit Hilfe neuronaler Netzwerke differenziert werden. Die hier erstmalige Verwendung von Oktavanalysen zur Beschreibung bioakustischer Signale ist eine geeignete Analysemethode, konnte die Klassifizierung jedoch nicht deutlich verbessern.

Im zweiten Kapitel beschreibe ich Versuche, in deren Verlauf sowohl Verhaltens- als auch Herzfrequenzreaktionen auf die Präsentation arteigener Stresslaute untersucht wurden. Die Versuchstiere zeigten erhöhte Aufmerksamkeit bei plötzlich auftretenden Geräuschen im Allgemeinen, d.h. bei arteigenen Lauten sowie bei einem neutralen Kontrollton. Dies drückte sich in geringeren Vokalisationsraten sowie der Aktivierung des parasympathischen Systems (abgebildet in der Herzfrequenzvariabilität) aus. Diese Reaktionen waren jedoch nur kurzfristig und endeten noch während der

zweiminütigen Playbacks. Folglich ist die kommunikative Bedeutung arteigener, stressbezogener Vokalisation noch nicht geklärt. Juvenile Schweine sind unter Umständen nicht die Adressaten solcher Laute, sondern eher die Sau. Zudem hatten die Versuchstiere eventuell nie die praktische Erfahrung einer Assoziation von arteigenen Stressschreien und einer Bedrohung für sie selbst gemacht. Das dauerhafte Ausbleiben einer solchen Verknüpfung kann möglicherweise zu einer Habituierung führen, wodurch eine angeborene Reaktionstendenz aufgehoben wird.

Im dritten Kapitel etablierte ich eine Methode für Mikroinjektionsstudien im Gehirn beim Schwein. Sie wurde in einem Versuch angewendet, der die neuronale Grundlage stressbezogener Lautgebung untersuchte. Es wird allgemein angenommen, dass Stress mit negativen Emotionen verknüpft ist. Meine Hypothese war, dass Hirnareale, die in die Steuerung solcher negativer Emotionen involviert sind, auch der Steuerung stressbezogener Vokalisation zugrunde liegen. Erste Ergebnisse stützen diese Interpretation. Zumindest ein Versuchstier zeigte bei cholinerger Aktivierung des Mandelkerns (Amygdala), welcher eine zentrale Rolle bei der Steuerung von Angst spielt, Vokalisationen sowie einen simultanen Anstieg des Stresshormons Adrenalin. Die Laute entsprachen solchen, wie sie bei moderater Stressbelastung geäußert worden waren. Mehrere Versuchstiere, welche in der Amygdala stimuliert wurden, zeigten keine Lautäußerungen. Dies ist jedoch vermutlich auf eine starke Gewöhnung an die Versuchssituation zurückzuführen.

Es lässt sich zusammenfassend schlussfolgern, dass Schweine stressorspezifische Lautantworten zeigen. Diese Laute lösen kurzfristig Aufmerksamkeit bei unbekannten Artgenossen aus. Erste Ergebnisse aus einer Mikroinjektionsstudie legen nahe, dass die Amygdala, das ‚Angstzentrum' des Gehirns, an der Produktion stressbezogener Vokalisationen beteiligt ist. Die Ergebnisse der vorliegenden Arbeit tragen dazu bei, die nicht-invasive Beurteilung des Wohlbefindens bei Schweinen auf Grundlage von Vokalisationsanalysen weiter zu verbessern und damit als Instrument in der Tierhaltung nutzbar zu machen.

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So tell him, with th' occurrents, more and less,
Which have solicited ... the rest is silence.

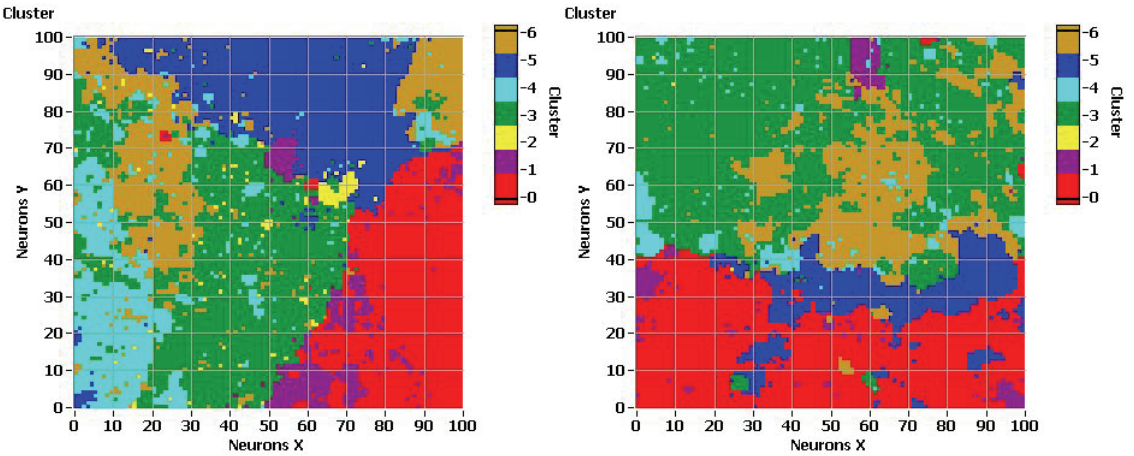
William Shakespeare, *Hamlet*, 5.2

APPENDIX

1. ADDITIONAL RESULTS - KOHONEN NETWORKS

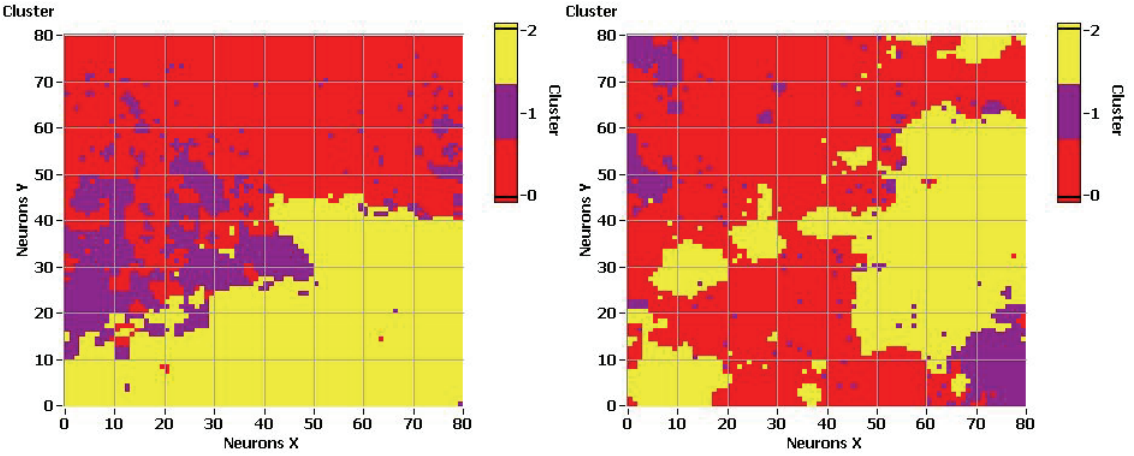
1.1 Cluster results

1.1.1 All vocalisations



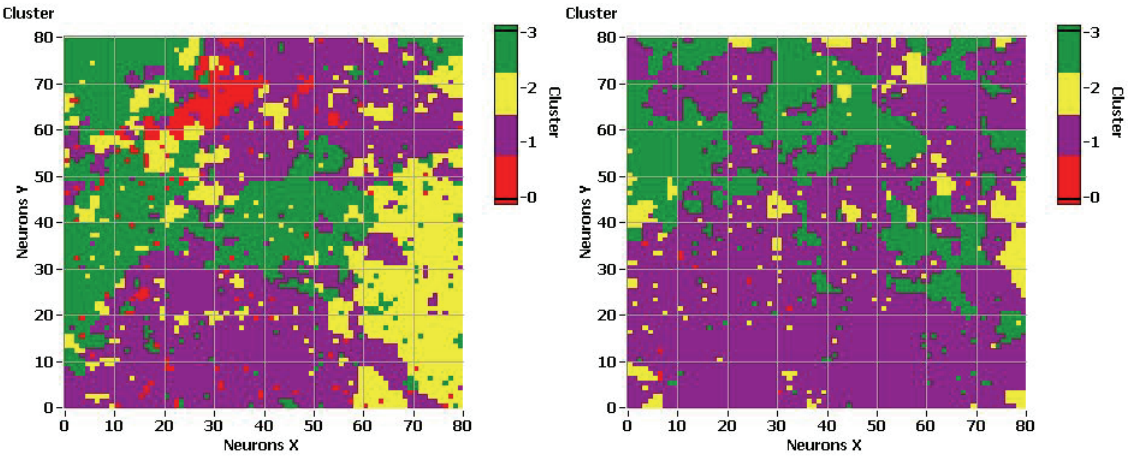
LPC (left) and sixth octave analysis-based (right) cluster results of the Kohonen networks trained with all types of vocalisation after second learning. Different colours represent the seven categories (ochre: tail biting; red: physical restraint; dark blue: squeal; purple: grunt; yellow: short grunt; green: groan type 1; light blue: groan type 2).

1.1.2 Harmonic vocalisations



LPC (left) and sixth octave analysis-based (right) cluster results of the Kohonen networks trained with harmonic vocalisations after second learning. Different colours represent the different categories (yellow: tail biting; red: physical restraint; purple: squeal).

1.1.3 Non-harmonic vocalisations



LPC (left) and sixth octave analysis-based (right) cluster results of the Kohonen networks trained with harmonic vocalisations after second learning. Different colours represent the different categories (red: grunt; purple: short grunt; yellow: groan type 1; green: groan type 2).

1.2 Evaluation of network training

Mean distance and maximum distance of vectors in the Kohonen networks. Both were smaller after the second trainings, indicating better classifications.

no. of classes	model	no. of parameters	neurons X	neurons Y	no. trainings	mean distance	maximum distance
7	LPC	12	100	100	1	0,199	1,84
7	LPC	12	100	100	2	0,127	1,106
7	OCT3	26	100	100	1	0,2	0,663
7	OCT3	26	100	100	2	0,129	0,458
7	OCT6	51	100	100	1		
7	OCT6	51	100	100	2	0,198	0,617
4	LPC	12	80	80	1	0,209	1,305
4	LPC	12	80	80	2	0,131	0,708
4	OCT3	26	80	80	1	0,164	0,689
4	OCT3	26	80	80	2	0,104	0,39
4	OCT6	51	80	80	1	0,26	0,775
4	OCT6	51	80	80	2	0,166	0,72
3	LPC	12	80	80	1	0,143	1,349
3	LPC	12	80	80	2	0,085	0,742
3	OCT3	26	80	80	1	0,186	0,532
3	OCT3	26	80	80	2	0,114	0,509
3	OCT6	51	80	80	1	0,273	0,659
3	OCT6	51	80	80	2	0,166	0,466

2. STIMULANTS USED FOR MICROINJECTIONS

name	batch molecular formula	manufacturer	city	coutry
(RS)-AMPA	$C_7H_{10}N_2O_4$	Biotrend Chemicals AG	Wangen/Zurich	Switzerland
Acetylcholine-chloride	$C_7H_{16}NO_2Cl$	Sigma-Aldrich	Steinheim	Germany
L-Norepinephrine (+) bitartrate salt	$C_8H_{11}NO_3 \cdot C_4H_6O_6 \cdot H_2O$	Sigma-Aldrich	Steinheim	Germany
(-) Isoproterenol (+) bitartrate	$C_{11}H_{17}NO_3 \cdot C_4H_6O_6$	Sigma-Aldrich	Steinheim	Germany
L-Phenylephrine hydrochloride	$C_9H_{13}NO_2 \cdot ClH$	Sigma-Aldrich	Steinheim	Germany
(-)-Bicuculline methobromide	$C_{21}H_{20}BrNO_6 \cdot H_2O$	Sigma-Aldrich	Steinheim	Germany
N-Methyl-D-Aspartic Acid	$C_5H_9NO_4$	Sigma-Aldrich	Steinheim	Germany
Corticotropin Releasing Factor	$C_{208}H_{344}N_{60}O_{63}S_2$	CalBiochem	Schwalbach/Ts.	Germany

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Publications

Düpjan, S., Schön, P.C., Puppe, B., Tuchscherer, A., Manteuffel, G. 2008: Differential vocal responses to physical and mental stressors in domestic pigs (*Sus scrofa*). Applied Animal Behaviour Science 114, 105-115.

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Teaching experience

In 2003 and 2004 I was tutor of courses on animal behaviour, evolution and ecology at the University of Bielefeld, Department of Animal Behaviour. Subsequently, I tutored students of the University of Rostock during courses at the Research Institute for the Biology of Farm Animals, Research Unit Behavioural Physiology.

Conferences

Talks

Düpjan, S., Schön, P.C., Puppe, B., Tuchscherer, A., Manteuffel, G.: Stressor-spezifische Lautgebung beim Schwein. Annual meeting of the DGfZ/GfT, 2007.

Düpjan, S.: Do pigs communicate stress? On emotional correlates of pigs' distress calls in senders and receivers. Graduate meeting of the DZG and the German Ethological Society, 2005.

Posters

Düpjan, S., Puppe, B., Tuchscherer, A., Schön, P.C., Manteuffel, G.: Die Bedeutung arteigener Stresslaute beim juvenilen Hausschwein – Auswirkungen auf Verhalten und Herzschlagmessungen. Annual meeting of the DVG, section „Applied Ethology", 2008.

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Düpjan, S., Schön, P.C., Puppe, B., Manteuffel, G.: Vocalisations associated with fear conditioning in the domestic pig. Biannual meeting of the ISAE, 2006.

ERKLÄRUNG

Ich erkläre, dass ich die eingereichte Dissertation selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die den benutzten Werken wörtlich oder inhaltlich übernommenen Stellen als solche kenntlich gemacht habe.

Dummerstorf, 28.11.2008