universität wien

DISSERTATION / DOCTORAL THESIS

Titel der Dissertation / Title of the Doctoral Thesis

„Tame Voices:
An investigation into human-animal communication and the effects of artificial selection pressures on vocal production in a domesticated mammal“

verfasst von / submitted by

Raffaela Lesch, BSc, MSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Doctor of Philosophy (PhD)

Wien, 2020 / Vienna 2020

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 794 685 437
Dissertationsgebiet lt. Studienblatt / field of study as it appears on the student record sheet:	Biology
Betreut von / Supervisor:	Univ.-Prof. Tecumseh Fitch, PhD
Betreut von / Supervisor:	ao. Univ.-Prof. i.R. Mag. Dr. Kurt Kotrschal

To my mum and dad!

Acknowledgements

I want to thank my supervisor Prof. W. Tecumseh Fitch for offering me this PhD position. Thank you for giving me the opportunity and freedom to choose the topics and species I wanted work with. During my PhD I had a lot of questions and you always took the time to answer them, I will always be incredibly grateful for that! Finally I want to thank you for your continuous support throughout this whole journey, it was one of the most formative times of my life. And last but not least thank you for helping me develop and improve my scientific skills and accepting my failures with humour and advice!

Next, I want to thank my co-supervisor Prof. Kurt Kotrschal. I'm incredibly grateful for your support throughout the last six years! Thank you so much for all the times you spent discussing ideas and thoughts with me. I will always be incredibly grateful for your honest, empathic and humours way of looking at problems, manuscripts and life.

My sincere gratitude to Prof. Betty Zimmerberg. Working with you has been an absolute joy! You've been the most generous host during my time at Williams College. My time working with you and tickling rats was one of the highlights of this journey. Thank you for the amazing time and fun I had working with you!

A huge thank you to Dan Bowling and Cliodhna Quigley, both post-docs at the Department of Cognitive Biology! You both were incredibly kind and helped me through many difficult situations with words of advice and experience that I will always be grateful for!

A massive thank you to Philipp Maier, Nadja Kavcik-Graumann, Alexandra Bohmann, Petra Pesak and Sarah Deventer! You've always been there for me when I needed help, thank you! I also want to thank the entire CogBio family for their support and help throughout this journey! I learned a lot from all of you and will always look back at this time fondly!

I also want to thank my parents! I would not be in this privileged position today without your support and help. You both sacrificed so much to give me this opportunity, I will forever be grateful for that! I also want to thank my brother Fabi and my best friend Isa. Fabi, your humour and calm helped me through so many difficult moments and decisions. Isa, you always manage to lift my mood and keep my spirits up no matter what. Thank you both!

Finally, I want to thank my partner and husband Dan. You are the voice of reason and calm in moments I feel lost. You always find ways to make me smile and laugh when I most need it and you never lose faith in me. You are the most brilliant, loving, hilarious, genius and humble person I ever met! I couldn't have done this without you! Thank you!

“[…]
if man goes on selecting, and thus augmenting, any peculiarity, he will almost certainly unconsciously modify other parts of the structure
[…]”

Charles Darwin,
The origin of species 1859

Table of Contents

General introduction

Domestication

Domesticated animals have lived with humans since the process of domestication began with wolves 20,000 to 40,000 years ago (Botigué *et al.*, 2017). Since then, domesticated animals have become an increasingly important part of our history, society and lifestyle (Hemmer, 1990b; Herre and Röhrs, 1988c; Diamond, 2002). Humankind owes a lot of our success to domesticated animals which (in addition to metalworking and domesticated plants) helped induce the Neolithic revolution, permanently changing human history (Hemmer, 1990c; Diamond, 2002; Francis, 2015a).

Nevertheless, defining the term "domestication" has proven to be difficult (Bökönyi, 1989; Ducos, 1989). Zeder (2015) offered a definition that would at the same time be broad yet specific enough to encompass all aspects of domestication:

> "*Domestication is a sustained multigenerational, mutualistic relationship in which one organism assumes a significant degree of influence over the reproduction and care of another organism in order to secure a more predictable supply of a resource of interest, and through which the partner organism gains advantage over individuals that remain outside this relationship, thereby benefitting and often increasing the fitness of both the domesticator and the target domesticate."*

Darwin's observations on domesticated animal breeds led to his description of natural selection. He also suggested splitting artificial selection into two types: unconscious and methodical artificial selection (Darwin, 1868; Gregory, 2008). The understanding of the term artificial selection nowadays would be in line with what Darwin called methodical artificial selection, here defined as a human-led conscious process of selectively breeding for desired traits. Darwin defined unconscious artificial selection as the result of a process of continuous preservation of more valued individuals (Gregory, 2008). The definition of natural selection I'll use here is that natural selection is a continuous selection pressure that leads to the most well adapted individuals increased survival and reproduction. I place the term "unconscious

artificial selection" between my definition of artificial and natural selection since it incorporates aspects of both.

The distinction between natural and an artificial selection processes becomes blurry in the light of domestication (Kohane and Parsons, 1988; Francis, 2015a). Although the domestication process is traditionally described and viewed as an artificial selection process, Darwin considered it an analogous process to natural selection (Darwin, 1868; Clutton-Brock, 1992; Francis, 2015a). This blurred distinction is especially evident when we think about dog domestication. Dogs were domesticated in a process from cohabitation and toleration to active interaction, which doesn't have to be artificial in the sense of conscious (Herre and Röhrs, 1988a; Zeder, 2006). It might have started out as a commensal relationship, benefitting both humans and dogs equally, and over time morphing into its modern form which cannot be described as symbiotic by any means (Herre and Röhrs, 1988b; Bökönyi, 1989). In contrast, modern dog breeds emerged through a very clear artificial selection pressure applied by humans within the last two centuries (Hemmer and Röhrs, 1988; Parker *et al.*, 2004). Francis argues that the process of domestication could be seen as an accelerated form of evolution (Francis, 2015a).

Tameness is a key component in the process of animal domestication (Belyaev, 1979). During the early stages of domestication there was most likely an exchange and influx of tamed animals to and from the existing stock of wild-living animals (Brentjes, 1975). To establish such a stock, young animals were, for example, taken from the wild and raised. To thrive under human care, these animals were probably under both active and passive selection for reduced stress responses and tameness even before they were actively bred (Brentjes, 1975; Kohane and Parsons, 1988).

Belyaev's domestication experiment

Based on the idea of tameness being a crucial factor in the process of domestication, Belyaev and his team started a daring experiment in the 1950s (Trut, Oskina and Kharlamova, 2009; Francis, 2015b): attempting to domesticate a species within a human lifetime. They began selecting fur farm foxes based solely on tameness towards humans, and within six generations they saw additional, unselected changes in behaviour (e.g. tail wagging) and morphology (e.g. curly tails). After generation 42, traits typical for

domesticated dogs were ubiquitous among the fox population (Kukekova, Trut and Acland, 2014).

This experiment of selection for tameness has since been successfully repeated with rats (*Rattus norvegicus*), again resulting in unselected changes to the behaviour, physiology and neurochemistry (Albert *et al.*, 2008). This further supports the idea that selection for tameness alone is enough to kickstart the domestication process (Trut, Oskina and Kharlamova, 2009).

Neural crest and domestication syndrome

Not only did domesticated animals' behaviour change by living in close proximity with humans, but this new living situation also affected their morphology and anatomy (Hemmer, 1990a; Kukekova, Trut and Acland, 2014). Domesticated animals typically have huge variability in appearance, but in general, white spots, floppy ears, smaller cranial capacity, shorter muzzles, smaller teeth and curled tails are common traits across domesticated mammals. This collection of traits has been observed and described by many scientists, including Darwin, and recently has been dubbed the "domestication syndrome" by Wilkins et al. and Hare et al. (Darwin, 1868; Hare, Wobber and Wrangham, 2012; Wilkins, Wrangham and Fitch, 2014). Although these changes have been well documented, a unified explanation for their common occurrence was missing. Wilkins et al. describe the first hypothesis to unify all these traits under one mechanistic explanation, suggesting that a mild deficiency in neural crest cell development and migration leads to all traits described under the domestication syndrome (Wilkins, Wrangham and Fitch, 2014; Pendleton *et al.*, 2018). Neural crest cells emerge during early stages of embryogenesis and disperse throughout the body. Neural crest cells are specific to vertebrates, and act as precursors to a variety of cell types while also being connected to the development of others (Trainor, 2014): most of these tissues and cell types are affected in the domestication syndrome.

Neural crest cells and the origins of the larynx

The neural crest cells are not only relevant for traits of the "domestication syndrome" described above, but are also crucial in the formation of the sound producing structures (Tabler *et al.*, 2017). The larynx is made up of the thyroid, cricoid, paired arytenoids, and epiglottic cartilages, hyoid bones, muscle and tissue derived from the branchial arches throughout evolution (Schneider, 1964; Harrison, 1995b). According to Harrison, it is probable that the evolutionary origin of the larynx is found in the second to seventh branchial arch (Harrison, 1995a). In a recent review, Danowitz and colleagues describe branchial arches 2 and 3 as forming the hyoid, and branchial arches 4 to 6 making up the thyroid and other laryngeal cartilages (Danowitz *et al.*, 2016). Tabler et al argue that the thyroid cartilage is mostly formed from neural crest cells (NCCs). In addition to knowing the origins of the larynx at this developmental level, we know that NCCs are crucial for its formation e.g., an overly large number of NCCs results in a degenerated larynx (Tabler *et al.*, 2017).

This direct relationship between neural crest cells, and both domestication and the development of the larynx, brings up major questions at the intersection of bioacoustics and domestication. Did the domestication process itself change the larynx (and thus the voices) of domesticated animals? And if the domestication process affected the larynx, were there artificial or unconscious selection pressures from humans responsible for changes in vocal output?

Prior to attempting to answer any of these big questions, I ask in this thesis if strong artificial selection pressures in a domesticated species can change vocal behaviour and production?

Research investigating such changes during the domestication process found that the vocal behaviour of domesticated foxes changed in comparison to their wild-type cousins (Gogoleva *et al.*, 2008). Two further well-known examples for vocalisations, considered typical for domesticated animals, are cat meows and dog barks. Cat meows are mostly used in cat-human interactions in domestic cats and are typically not found in cat-cat interactions after infancy (Bradshaw and Cameron-Beaumont, 2000; Nicastro, 2004). Dogs produce barks in a wide variety of different contexts and scenarios, while in wolves, the bark has a very specific meaning in threatening contexts and is not as ubiquitous (Faragó, Townsend and Range, 2013). With cat meows and dog barks, we only see the end product and can only speculate about the causes that might have led to these peculiarities. While barks are heavily

associated with dogs, an ancient dog breed of basenjis is known for not being able to bark but rather "yodel" (Ashdown and Lea, 1979). We know little about the selection process that led to this phenomenon, but research on basenji laryngeal structure indicated that the ventricles are shallow in comparison to other dog breeds. This change in ventricle depth may be a reason for the basenji's inability to produce explosive bark vocalizations (Ashdown and Lea, 1979). Apart from basenjis' inability to produce barks, New Guinea singing dogs are also known for their strongly modulated howl and a high frequency pulsed trill. These vocalizations may be produced by rapid vibration of a rudimentary uvula not found in other canids (Koler-Matznick *et al.*, 2003). As with dog barks, basenji yodels and cat meows, we don't know if these traits were artificially selected for by humans or if they are a by-product of domestication combined with any other type of selection pressure.

Bioacoustics under artificial selection pressures

My core question in this thesis is whether and how strong artificial selection pressures in a domesticated mammal can affect their vocal behaviour and production. The impact of artificial and natural selection pressures on bioacoustic signals so far mainly have been studied in songbirds. They are an easily accessible and interesting group due to their vocal learning abilities and song. Songbird species have been successfully domesticated and kept in human care for centuries, facilitating research on how natural and artificial selection pressures change their song.

In canaries, a passerine bird originating from the Canary islands, a study compared 3 different lineages: one bred for their morphology, one for their song, and the wild ancestors (Güttinger, 1985). These canaries offered the opportunity to study both the general impact of domestication and its natural selection pressures on the birds song production, as well as the impact selective breeding for a trait (artificial selection) has on the song. This study found that the duration of tours and proportion of single utterances changed in the course of domestication, but also argued that selective breeding favours the augmentation of traits that are already present due to domestication (Güttinger, 1985). In the case of canaries, this means that domesticated birds that only have been bred for morphology still show the same trends observed in domesticated birds that have been bred for their song.

Another songbird highly relevant in investigating bioacoustic changes due to domestication is the Bengalese finch. The Bengalese finch originates from the white-rumped munia, a passerine bird endemic to southeast Asia. It has been bred in captivity for over 240 years and since then has been domesticated to its current form of the Bengalese finch (Okanoya, 2004). In the process of domestication these finches have not been selected for their song; yet their song changed from a very simple song found in white-rumped munias to a more complex song in the Bengalese finches (Okanoya, 2017). A hypothesis explaining this change in complexity is that the domestication process loosened or extinguished predatory selection pressures. The missing selection pressures by predators allowed female choice to have its full effect, pushing the song to more complex and attractive states that would have been detrimental in the wild due to predation (Okanoya, 2017). With the example of the Bengalese finch it seems that domestication in this case may have led to a relaxation of certain constraints, which in turn allowed for other selection pressures (in this case female choice) to take over.

A study on guinea pigs and their progenitors, wild cavies, investigated how domestication changed the repertoire and structure of calls. In the course of domestication guinea pigs have been selected for increased body size. This increase in body size was favoured due to an increase in meat production and it seems there was no direct selection pressures on vocalizations (Monticelli and Ades, 2011). The study's authors argue that this artificial selection for size might have indirectly altered the vocal tract which in turn could potentially affect temporal changes to the alarm and courtship calls (Monticelli and Ades, 2011).

No clear and scientifically supported record exists for any domesticated mammal that unambiguously shows whether changes to their vocalisations were selected for by humans during domestication or if they are a by-product of the domestication process. The only solid and traceable case of a mammalian species undergoing changes to their vocal behaviour as a clear by-product of domestication are the domesticated Siberian foxes (Gogoleva et al., 2008).

The fox farm domestication experiment, started by Belyaev in the 1950s, allowed for close observations of the selection pressures these animals underwent (Trut, 1999). The selection for tameness towards the human experimenter lead to numerous changes, but one among those changes was a change in vocal behaviour of tame foxes. Compared to wild/aggressive foxes, domesticated foxes produce a series of explosive vocalizations to greet humans (Gogoleva et al., 2011); a crossbreeding experiment further hinted that these human-

directed vocalizations may be a discrete phenotypic trait of tame foxes (Gogoleva et al., 2009).

Thus, compared to birds, we have little knowledge on the effects artificial selection pressures can have on the voice and sound producing structures of domesticated mammals. Güttinger suggested in his work on canaries that traits can only be selected during artificial selection if they are already present to a minor degree in the originally domesticated group (Güttinger, 1985). Evans further summed up Darwin's view of the relevance of domesticated animals and breeds as a window into natural selection processes (Evans, 1984). Both Güttinger's suggestion and Darwin's view on domesticated animals supports the core idea of this thesis, that investigating the impact of artificial selection pressures on the vocal production structures in domesticated mammals can increase our understanding of similar processes found in nature (Güttinger, 1985; Price, 2002).

Rats and domestication

Domesticated rats stem from the wild progenitor species of brown rats (*Rattus norvegicus).* In the initial stages of domestication, rats were caught and bred for blood sports or sold as fancy rats to aristocrats (Castle, 1947; Francis, 2015b). While domesticated rats have a growing fanbase that enjoys breeding and keeping them as pets, they have been used in large numbers in laboratory settings for decades.

Rats have a communication range from 1-100kHz, in which the majority of vocal communication is above the human hearing range, leading to the misconception of these animals being quiet (Brudzynski, 2018). High frequency, ultrasonic vocalizations (USVs), are used by rats for short-range communication and communicate different messages depending on the frequency range (Brudzynski and Fletcher, 2010): Calls at 22kHz communicate negative affective states whereas calls centered at around 50kHz communicate positive affective states (Portfors, 2007). Rat pups mostly use fundamental frequencies around 40kHz but can go up as high as 100kHz (Brunelli, Shair and Hofer, 1994). To achieve this range of communication rats use different strategies for vocal production.

While low frequency vocalizations are most likely produced by ordinary vocal fold vibration, as described in other mammals, ultrasonic calls are produced by a whistle mechanism (Roberts, 1975; Riede *et al.*, 2011; Mahrt et al 2016; Herbst *et al.*, 2012). There is

still debate regarding the precise workings of this whistle mechanism, but both a planar impinging jet and an edge-tone mechanism have been suggested (Mahrt *et al.*, 2016; Riede, Borgard and Pasch, 2017). The existence of two different production mechanisms for low frequency and ultrasonic calls make the rat an exceptional model species within domesticated animals, to investigate how artificial selection might affect the voice source and vocal output.

Companion animal-directed speech

In addition to the question of how domestication affected our domestic animals' vocal production mechanism, another aspect of domestication is how *humans* communicate with tame and domesticated animals. Domestication not only led to behavioural and morphological changes in dogs, but close interaction and proximity to humans also leads dogs to be very much in tune with human emotions, facial expressions and gestures (Hare *et al.*, 2002; Hare and Tomasello, 2005; Virányi *et al.*, 2008; Kotrschal, 2016). Apart from these special adjustments to living in close proximity with humans, dogs have also developed the ability to tap into the human attachment/caregiving system, evoking caregiving responses from "their" humans (Archer, 1997).

The human caregiving system is oriented towards a quick caregiving response to infants and young children to provide comfort (Bowlby, 1980; Cassidy and Shaver, 1999). Infant-directed speech is one of these caregiving responses, and not only supports the child in learning how to speak, but it is used to communicate affection and alleviate stress (Burnham and Francis, 1998; Hoff, 2009). Even though our animals don't learn how to speak, we nonetheless use a similar type of speech, "companion animal-directed speech", with our companion animals (Burnham and Francis, 1998; Burnham, Kitamura and Vollmer-Conna, 2002; Xu *et al.*, 2013).

Focus of the thesis and overview of chapters

The goal of this thesis is twofold: In the first chapter, I further our understanding of how humans interact with domesticated animals at the acoustic level. In the second and third chapters, I explore how artificial selection pressures can change a domesticated animal's

vocal behaviour and underlying vocal production mechanisms. To access these two different aspects at the intersection of domestication and bioacoustics, I used a variety of methods, including acoustic analysis, µCT scans, histology and elemental analysis. With my research in this thesis, I hope to illustrate 1) how stress and human personality might affect companion animal directed speech, 2) how artificial selection pressures can permanently change vocal behaviour, and 3) how artificial selection pressures can change the laryngeal structures crucial for sound production.

In chapter 1, I discuss the influence that owner personality and stress have on vocal parameters of companion animal-directed speech. My colleagues and I used a standardized strange situation procedure to elicit stress responses in dogs which, in turn, provoked caregiving behaviour and companion animal-directed speech in the owner. I discuss the differential use of frequency range and mean fundamental frequency in companion animal-directed speech, and describe how owner personality and gender impacts both. The goal of this study was to deepen research on animal-directed speech by taking into account if, and how, stress and individual personality differences might shape how humans communicate with companion animals.

In chapter 2 and 3, I investigate the influence of selective breeding on the vocal output and larynx morphology in rats (*Rattus norvegicus domestica*). My colleagues and I worked with rats that had been bred for over 20 years to produce low or high numbers of ultrasonic calls as pups during a separation paradigm. I analysed the changes in adult acoustic behaviour and the adult larynx caused by this artificial selection on an infantile trait. I found that the strong artificial selection pressure for pup USVs lastingly changes both the adult laryngeal structure as well as adult low frequency, but not ultrasonic, vocalisations.

With the studies collected in this PhD thesis, I hope to create a basis on which to continue research on how the process of domestication itself might have shaped and changed the larynx and thus vocal communication.

References

Albert, F. W. et al. (2008) 'Phenotypic differences in behavior, physiology and neurochemistry between rats selected for tameness and for defensive aggression towards humans', Hormones and Behavior, 53(3), pp. 413–421. doi: 10.1016/j.yhbeh.2007.11.010.

Archer, J. (1997) 'Why do people love their pets?', Evolution and Human Behavior, 18(4), pp. 237–259. doi: 10.1016/S0162-3095(99)80001-4.

Ashdown, R. R. and Lea, T. (1979) 'The larynx of the Basenji dog', Journal of Small Animal Practice, 20(11), pp. 675–679. doi: 10.1111/j.1748-5827.1979.tb06680.x.

Belyaev, D. (1979) 'Destabilizing selection as a factor in domestication', The Journal of Heredity, 70, pp. 301–308.

Bökönyi, S. (1989) 'Definitions of animal domestication', in The Walking Larder: Patterns of Domestication, Pastoralism and Predation, pp. 22–26.

Botigué, L. R. et al. (2017) 'Ancient European dog genomes reveal continuity since the Early Neolithic', Nature Communications, 8(May). doi: 10.1038/ncomms16082.

Bowlby, J. (1980) 'Attachment and Loss', Key Concepts in Family Studies, 54(1980), pp. 9–12. doi: 10.4135/9781446250990.n2.

Bradshaw, J. and Cameron-Beaumont, C. (2000) 'The signalling repertoire of the domestic cat and its undomesticated relatives', in The Domestic Cat: The Biology of Its Behaviour, pp. 67–93.

Brentjes, B. (1975) Die Erfindung des Haustieres, Die Erfindung des Haustieres. Leipzig.

Brudzynski, S. M. (2018) Handbook of Ultrasonic Vocalization: A Handbook into the Emotional Brain. 1st edn. London: Levy, Niki.

Brudzynski, S. M. and Fletcher, N. H. (2010) Rat ultrasonic vocalization: Short-range communication, Handbook of Behavioral Neuroscience. Elsevier B.V. doi: 10.1016/B978-0-12-374593-4.00008-5.

Brunelli, S. A., Shair, H. N. and Hofer, M. A. (1994) 'Hypothermic vocalizations of rat pups (*Rattus norvegicus*) elicit and direct maternal search behavior.', Journal of comparative psychology, 108(3), pp. 298–303. doi: 10.1037/0735-7036.108.3.298.

Burnham, D. and Francis, E. (1998) 'Are you my little pussy-cat? Acoustic, phonetic and affective qualities of infant-and pet-directed speech', in Proc. 5th International Conference on Spoken Language Processing (ICSLP 98).

Burnham, D., Kitamura, C. and Vollmer-Conna, U. (2002) 'What's new, pussycat? On talking to babies and animals', Science, 296(5572), p. 1435. doi: 10.1126/science.1069587.

Cassidy, J. and Shaver, P. R. (1999) The nature of the child's ties. Handbook of attachment: Theory, research, and clinical applications. New York: The Guilford Press.

Castle, W. E. (1947) 'The domestication of the rat', Proceedings of the National Academy of Sciences, 33(5), pp. 109–117.

Clutton-Brock, J. (1992) 'The process of domestication', Mammal Review, 22(2), pp. 79–85. doi: 10.1111/j.1365-2907.1992.tb00122.x.

Danowitz, M. et al. (2016) 'A combined approach of teaching head development using embryology and comparative anatomy', Edorium J Anat Embryo, 3, pp. 17–27. doi: 10.5348/A04-2016-11-RA-3.

Darwin, C. (1868) The variation of animals and plants under domestication.

Diamond, J. (2002) ‘Evolution, consequences and future of plant and animal domestication’, Nature, 418(6898), pp. 700–707. doi: 10.1038/nature01019.

Ducos, P. (1989) ‘Defining domestication: a clarification’, in The Walking Larder: Patterns of Domestication, Pastoralism and Predation, pp. 28–30.

Evans, L. T. (1984) ‘Darwin’s use of the analogy between artificial and natural selection’, Journal of the History of Biology, 17(1), pp. 113–140. doi: 10.1007/BF00397504.

Faragó, T., Townsend, S. and Range, F. (2013) ‘The Information Content of Wolf (and Dog) Social Communication’, in Biocommunication of Animals, pp. 41–62.

Francis, R. (2015a) ‘Preface’, in Domesticated: Evolution in a Man-Made World, pp. 1–6.

Francis, R. (2015b) ‘Rodents’, in Domesticated: Evolution in a Man-Made World, pp. 234–255.

Gogoleva, S. S. et al. (2008) ‘To bark or not to bark: Vocalizations by red foxes selected for tameness or aggressiveness toward humans’, Bioacoustics, 18(2), pp. 99–132. doi: 10.1080/09524622.2008.9753595.

Gogoleva, S. S. et al. (2009) ‘Kind granddaughters of angry grandmothers: The effect of domestication on vocalization in cross-bred silver foxes’, Behavioural Processes, 81(3), pp. 369–375. doi: 10.1016/j.beproc.2009.03.012.

Gogoleva, S. S. et al. (2011) ‘Explosive vocal activity for attracting human attention is related to domestication in silver fox’, Behavioural Processes. Elsevier B.V., 86(2), pp. 216–221. doi: 10.1016/j.beproc.2010.12.001.

Gregory, T. R. (2008) ‘Artificial Selection and Domestication: Modern Lessons from Darwin’s Enduring Analogy’, Evolution: Education and Outreach, 2(1), pp. 5–27. doi: 10.1007/s12052-008-0114-z.

Güttinger, H. R. (1985) ‘Consequences of domestication on the song structures in the canary’, Behaviour, 95(3/4), pp. 254–278.

Hare, B. et al. (2002) ‘The domestication of social cognition in dogs’, Science, 298(5598), pp. 1634–1636. doi: 10.1126/science.1072702.

Hare, B. and Tomasello, M. (2005) ‘Human-like social skills in dogs?’, Trends in Cognitive Sciences, 9(9), pp. 439–444. doi: 10.1016/j.tics.2005.07.003.

Hare, B., Wobber, V. and Wrangham, R. (2012) ‘The self-domestication hypothesis: Evolution of bonobo psychology is due to selection against aggression’, Animal Behaviour, 83(3), pp. 573–585. doi: 10.1016/j.anbehav.2011.12.007.

Harrison, D. F. N. (1995a) ‘Framework, cartilages, ligaments and related joints’, in The anatomy and physiology of the mammalian larynx, pp. 56–85.

Harrison, D. F. N. (1995b) The anatomy and physiology of the mammalian larynx. Cambridge: Cambridge University Press.

Hemmer, H. (1990a) ‘Changes in behaviour’, in Domestication: The decline of environmental appreciation, pp. 81–92.

Hemmer, H. (1990b) ‘Overall synopsis’, in Domestication: The decline of environmental appreciation, pp. 187–191.

Hemmer, H. (1990c) ‘Why are domesticated animals kept?’, in Domestication: The decline of environmental appreciatione, pp. 1–12.

Hemmer, H. and Röhrs, M. (1988) ‘Über Rassen von Haustieren’, in Haustiere - zoologisch gesehen, pp. 135–204.

Herbst, C. T. et al. (2012) ‘How low can you go? Physical production mechanism of elephant infrasonic vocalizations’, Science, 337(6094), pp. 595–599. doi: 10.1126/science.1219712.

Herre, W. and Röhrs, M. (1988a) ‘Die Domestikation der einzelnen Arten’, in Haustiere - zoologisch gesehen, pp. 95–125.

Herre, W. and Röhrs, M. (1988b) ‘Über das Wesen der Domestikation unnd den Begriff Haustier’, in Haustiere - zoologisch gesehen, pp. 8–16.

Herre, W. and Röhrs, M. (1988c) ‘Warum Haustierforschung in zoologischer Sicht?’, in Haustiere - zoologisch gesehen, pp. 3–7.

Hoff, E. (2009) Language Development. 5th edn. Wadsworth: Jon-David Hague.

Kohane, M. J. and Parsons, P. A. (1988) ‘Domestication: Evolutionary Change under Stress’, Evolutionary Biology, 23, pp. 31–48.

Koler-Matznick, J. et al. (2003) ‘An updated description of the New Guinea singing dog (*Canis hallstromi*, Troughton 1957)’, Journal of Zoology, 261(2), pp. 109–118. doi: 10.1017/S0952836903004060.

Kotrschal, K. (2016) Hund-Mensch. Das Geheimnis der Seelenverwandtschaft. Wien: Brandstätter.

Kukekova, A. V., Trut, L. N. and Acland, G. M. (2014) Genetics of Domesticated Behavior in Dogs and Foxes. Second Edi, Genetics and the Behavior of Domestic Animals. Second Edi. Elsevier Inc. doi: 10.1016/B978-0-12-394586-0.00010-X.

Mahrt, E. et al. (2016) ‘Mice produce ultrasonic vocalizations by intra-laryngeal planar impinging jets’, Current Biology. Elsevier, 26(19), pp. R880–R881. doi: 10.1016/j.cub.2016.08.032.

Monticelli, P. F. and Ades, C. (2011) ‘Bioacoustics of domestication: Alarm and courtship calls of wild and domestic cavies’, Bioacoustics, 20(2), pp. 169–191. doi: 10.1080/09524622.2011.9753642.

Nicastro, N. (2004) ‘Perceptual and acoustic evidence for species-level differences in meow vocalizations by domestic cats (*Felis catus*) and African wild cats (*Felis silvestris lybica*)’, Journal of Comparative Psychology, 118(3), pp. 287–296. doi: 10.1037/0735-7036.118.3.287.

Okanoya, K. (2004) ‘The Bengalese finch: A window on the behavioral neurobiology of birdsong syntax’, Annals of the New York Academy of Sciences, 1016, pp. 724–735. doi: 10.1196/annals.1298.026.

Okanoya, K. (2017) ‘Sexual communication and domestication may give rise to the signal complexity necessary for the emergence of language: An indication from songbird studies’, Psychonomic Bulletin & Review. Psychonomic Bulletin & Review, 24(1), pp. 1–5. doi: 10.3758/s13423-016-1165-8.

Parker, H. G. et al. (2004) ‘Genetic Structure of the Purebred Domestic Dog’, Science, 304(5674), pp. 1160–1164. doi: 10.1126/science.1097406.

Pendleton, A. L. et al. (2018) ‘Comparison of village dog and wolf genomes highlights the role of the neural crest in dog domestication’, BMC Biology. BMC Biology, 16(1), pp. 1–21. doi: 10.1186/s12915-018-0535-2.

Portfors, C. V (2007) ‘Types and functions of ultrasonic vocalizations in laboratory rats and mice.’, Journal of the American Association for Laboratory Animal Science : JAALAS, 46(1), pp. 28–34.

Price, E. O. (2002) ‘Artificial selection’, in Animal domestication and behavior, pp. 43–51.

Riede, T. et al. (2011) ‘Elasticity and stress relaxation of a very small vocal fold’, Journal of Biomechanics, 44(10), pp. 1936–1940. doi: 10.1016/j.jbiomech.2011.04.024.

Riede, T., Borgard, H. L. and Pasch, B. (2017) ‘Laryngeal airway reconstruction indicates that rodent ultrasonic

vocalizations are produced by an edge-tone mechanism', Royal Society Open Science, 4(11). doi: 10.1098/rsos.170976.

Roberts, L. H. (1975) 'The functional anatomy of the rodent larynx in relation to audible and ultrasonic cry production', Zoological Journal of the Linnean Society, 56(3), pp. 255–264. doi: 10.1111/j.1096-3642.1975.tb00268.x.

Schneider, R. (1964) 'Der Larynx der Säugetiere', Handbuch der Zoologie. Berlin, 5(7), pp. 1–128.

Tabler, J. M. et al. (2017) 'Cilia-mediated Hedgehog signaling controls form and function in the mammalian larynx', pp. 1–26. doi: 10.7554/eLife.19153.

Trainor, P. A. (2014) Neural Crest Cells: Evolution, Development and Disease. Amsterdam: Academic Press.

Trut, L. (1999) 'Early Canid Domestication: The Farm-Fox Experiment: Foxes bred for tamability in a 40-year experiment exhibit remarkable transformations that suggest an interplay between behavioral genetics and development', American Scientist, 87(2), pp. 160–169. doi: 10.2307/27857815.

Trut, L., Oskina, I. and Kharlamova, A. (2009) 'Animal evolution during domestication: The domesticated fox as a model', BioEssays, 31(3), pp. 349–360. doi: 10.1002/bies.200800070.

Virányi, Z. et al. (2008) 'Comprehension of human pointing gestures in young human-reared wolves (*Canis lupus*) and dogs (*Canis familiaris*)', Animal Cognition, 11(3), pp. 373–387. doi: 10.1007/s10071-007-0127-y.

Wilkins, A. S., Wrangham, R. W. and Fitch, W. T. (2014) 'The "domestication syndrome" in mammals: A unified explanation based on neural crest cell behavior and genetics', Genetics, 197(3), pp. 795–808. doi: 10.1534/genetics.114.165423.

Xu, N. et al. (2013) 'Vowel hyperarticulation in parrot-, dog- and infant- directed speech', Anthrozoos, 26(3), pp. 373–380. doi: 10.2752/175303713X13697429463592.

Zeder, M. A. (2006) 'Archaeological approaches to documenting animal domestication', in Zeder, M. A. et al. (eds) Documenting Domestication: New Genetic and Archaeological Paradigms, pp. 171–180.

Zeder, M. A. (2015) 'Core questions in domestication research', Proceedings of the National Academy of Sciences of the United States of America, 112(11), pp. 3191–3198. doi: 10.1073/pnas.1501711112.

Chapter 1|

Talking to Dogs: Companion Animal-Directed Speech in a Stress Test

This chapter is published as an article in a peer-reviewed journal.

Citation: **Lesch, R.**; Kotrschal, K.; Schöberl, I.; Beetz, A.; Solomon, J.; Fitch, W.T. Talking to Dogs: Companion Animal-Directed Speech in a Stress Test. Animals 2019, *9*, 417. doi:10.3390/ani9070417

animals

MDPI

Article

Talking to Dogs: Companion Animal-Directed Speech in a Stress Test

Raffaela Lesch [1,*], Kurt Kotrschal [2], Iris Schöberl [2], Andrea Beetz [2,3], Judith Solomon [2] and W. Tecumseh Fitch [1,*]

1 Department of Cognitive Biology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria
2 Department of Behavioral Biology, Konrad Lorenz Research Station, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria
3 Department of Special Education, Institut für Sonderpädagogische Entwicklungsförderung und Rehabilitation, University of Rostock, August-Bebel-Str. 28, 18055 Rostock, Germany
* Correspondence: raffaela.lesch@univie.ac.at (R.L.); tecumseh.fitch@univie.ac.at (W.T.F.)

Received: 3 June 2019; Accepted: 27 June 2019; Published: 4 July 2019

Simple Summary: Companion animal-directed speech is a current topic of research, interesting due to its similarity to infant-directed speech. Dog owners seem to almost subconsciously use this high-pitched and repetitive way of speaking, slightly adapted, for dogs. The aim of this study was to investigate dog-directed speech in different contexts and examine whether owner personality and relationship quality affect it. We found that owners' personality and gender affect their dog-directed speech. The majority of the modifications of dog-directed speech could be explained by a differential use of voice pitch and range. Our study supports the idea that voice pitch was used to communicate affect, whereas pitch range was used as an attention-getting strategy. Based on our results, we conclude that dog-directed speech is adjusted depending on context, gender, and personality. Societal value in this study consists of its contribution to basic knowledge of how we talk to animals, which may help in preventing accidents (e.g., dog bites) as well as improving animal training.

Abstract: Companion animal-directed speech (CADS) has previously been investigated in comparison to infant-directed speech and adult-directed speech. To investigate the influence of owner caregiving, attachment pattern, and personality on CADS, we used the Ainsworth strange situation procedure. It allowed us to assess voice source parameters of CADS across different contexts. We extracted speech parameters (voicing duration, voice pitch, pitch range, and jitter) from 53 dog owners recorded during the procedure. We found that owner personality and gender but not caregiving/attachment behavior affect their voice's pitch, range, and jitter during CADS. Further, we found a differential and context-specific modification of pitch and range, consistent with the idea that pitch communicates affect, whereas range is more of an attention-getting device. This differential usage, and the increased pitch, emphasize and support the parallels described between CADS and infant-directed speech. For the first time, we also show the effect of personality on CADS and lay the basis for including jitter as a potentially useful measure in CADS.

Keywords: companion animal-directed speech; dog-directed speech; attachment; caregiving; Ainsworth strange situation; pet-directed speech

1. Introduction

It has long been known that the human voice carries substantial cues to the emotional state of the owner, completely independent of any verbal or linguistic content [1]. With the exception of a few familiar words (e.g., their name, "good", "bad", etc.), animals listening to human speech presumably

rely primarily upon such nonverbal information to interpret speaker state and intentions [2]. However, the specific forms of information available in companion animal-directed speech (CADS) remain understudied. In this study, we analyzed dog-directed speech in different situations to gain insight into this question. We focused attention on source characteristics, particularly voice fundamental frequency (f0, often colloquially termed "voice pitch"), which has reliably been found to vary with emotional state, though often in individual-specific manners [3,4]. Based on this previous research, we hypothesized that voice pitch variables would increase in increasingly arousing situations. In addition to summary statistics on f0 itself (mean, range, duration), we also analyzed voice perturbation using jitter, which quantifies local period-to-period deviation in the length of successive f0 periods. Measures of voice pitch irregularities (jitter) have a long history in voice emotion analysis, and high jitter has been suggested to correlate with high stress or arousal [3,5]. However, inconsistencies across studies have been argued to reflect individual personality differences [1] and may vary with coping style (e.g., for inhibited vs. outgoing children [6]). We therefore had only a weak a priori prediction that jitter should increase with arousal, perhaps in individual- or personality-specific ways.

Research trying to identify information encoded in CADS has used infant-directed speech (IDS) as a comparison. When compared to ordinary speech, CADS has a higher fundamental frequency, higher pitch range, and is more repetitive; these characteristics have also been found in IDS [7–9]. CADS may be similar to IDS in quality but differs from IDS in an apparent lack of hyper-articulation and lack of a language learning context [7,10,11]. These similarities and subtle differences make the comparison all the more intriguing and fascinating. Not only do people use CADS with dogs, but dogs also seem to pay attention to it. CADS has been evaluated for its attention-getting quality with adult dogs and seems to draw adult dogs' attention more than adult-directed speech [12]. It appears that we adapt CADS (e.g., omitting hyper articulation), knowing that our four-legged friends will never be able to speak, but we still use it in navigating our relationship with them. One of the most important functions within relationships is giving and receiving emotional social support. How we give and receive this emotional and social support depends on and varies with arousal level and attachment representations [13,14]. Humans biophilic nature combined with dogs' adaptation to humans (domestication) allows both sides to use and respond to the aforementioned human offspring–caregiver attachment behavior [15,16].

Any attachment relationship is characterized by maintaining proximity, separation distress, and using the caregiver as a secure base to explore from, and as a safe haven to return to [17,18]. Mary Ainsworth developed a classification tool to diagnose the quality of an infant's attachment [19,20], the Ainsworth strange situation procedure (ASSP). The ASSP is designed to assess attachment patterns by increasing the subjects' mental stress load and arousal over consecutive episodes [21,22]. It is created to increasingly activate attachment behavior on the side of the child or dog; this behavior is usually reciprocated by the caregiver in a complementary manner [23], which means the experience of mental stress has the subject turn towards support and comfort, which is found in the form of social support [19]. This is why we want to investigate CADS in this setup: Social communication has a highly relevant function in providing emotional and social support in any relationship. Both attachment theory and the ASSP have previously been applied to human–dog dyads, with promising results [24–27]. Since literature suggests that CADS can be influenced by owner gender and context [28,29], the ASSP is an excellent tool for testing CADS in varying contexts. To date, analyses of CADS did not take into account important factors in social communication, such as attachment pattern, caregiving behavior or personality. Here, we want to investigate their potential impact on the voice source during CADS.

2. Materials and Methods

2.1. Subjects

A total of 59 human–dog teams participated in this study. Six out of the 59 teams had to be excluded from the analysis due to recording equipment malfunctions. The remaining 53 human–dog teams (28 women, 25 men, 28 female dogs, 25 male dogs) in balanced combinations of male–male,

female–female, male–female, and female–male were a subset of 132 human–dog dyads who had already participated in an experimental study of interaction styles and human–dog relationships [30,31]. The recruitment for our study was based on voluntary participation of teams from the pool of 132 dyads. Our subset of dyads represents those who responded positively to our request for participation in this study. All dogs lived with the owner who was also the main attachment figure from puppyhood onward. All dogs were intact, i.e., were neither spayed nor neutered. Their mean age was 4 years ± 1.5 SD and their mean weight was 30 kg ± 13.2 SD. Mean owner age was 46.2 years ± 10.2 SD. All dyads were recruited from Vienna and surrounding areas in Austria.

2.2. Owner Personality Axis

Dog owners were asked to fill in the German version of the NEO Five-Factory Inventory evaluating their own personality [32,33], a 60-item psychometric instrument designed to evaluate nonclinical adult personality structures along five major dimensions: neuroticism, extraversion, openness, agreeableness, and conscientiousness.

2.3. Ainsworth Strange Situation Procedure

The Ainsworth strange situation procedure [20] was adapted to assess the human–dog relationship. Within this assessment, dog attachment behavior is activated via increasing stress during the procedure, which in turn potentially activates caregiving behavior in the owner. Before testing, the room was prepared by placing two color-tagged chairs next to each other in the middle of the room, closing the windows and shades, and depositing several toys between and in front of the chairs. The dog owners were guided through the procedure by the experimenter in the following fixed order:

0. Controls (~10 s): Two control settings (reading and speaking/adult-directed speech) are recorded in the waiting room, adjacent the experimental room: First, the owner is asked to read a predefined text in presence of the dog out loud. (" . . . der beste Freund des Schäferhundes Rex ist die Ente Oskar . . . "—" . . . the German shepherd Rex's best friend is the duck Oscar . . . "). For the second control, the experimenter engages the owner in small talk about their dog.

1. Introduction (~20 s): The experimenter leads the owner–dog dyad into the room, introduces them to the surroundings, asks the owner to unleash the dog for the duration of the procedure, and leaves the room again.

2. Exploration (3 min): Both dog and owner can move around freely and explore the room and the toys. The owner is free to interact and talk with the dog normally.

3. Encounter (1 + 2 min): A person previously unknown to the dog ('the stranger') enters the room and asks the owner to take their designated seat if they were not sitting yet. Other than this, there is no further interaction initially: For one minute, the stranger sits down on the other chair, remaining motionless. In the second minute, the stranger initiates small talk with the dog owner. In the third and last minute of this first encounter, the stranger tries to interact with and elicit play from the dog. At the end of minute three, the stranger asks the dog owner to leave the room.

4. Separation (3 min): The dog stays alone in the experimental room with the stranger. The stranger tries to engage the dog by playing and interacting.

5. Call (~5 s): The experimenter instructs the dog owner to stand in front of the experimental room door and to loudly call the dog's name.

6. Reunion (3 min): The owner re-enters the room and reunites with the dog. The owner is free to interact and to play with the dog. The stranger quietly leaves the room. At the end of minute three, a vibrating phone indicates that the owner should leave the experimental room.

7. Separation (3 min): The dog remains alone in the experimental room for three minutes.

8. Encounter (3 min): The stranger from stage 3 re-enters and the dog remains in the experimental room with the stranger. The stranger tries to comfort the dog by playing and interacting.

9. Call (~5 s): The experimenter again instructs the dog owner to stand in front of the experimental room door and loudly call the dog's name.

10. Reunion (3 min): The owner enters the room, reunites with the dog, and provides at least one physical contact. The owner is free to interact and play with the dog. The stranger quietly leaves the room. At the end of minute three, the experimenter enters the room and ends the procedure.

The procedure is illustrated graphically in Figure 1, marking all episodes (0, 2, 3, 5, 6, 9, 10) where the owner's utterances were analyzed with the owner icon.

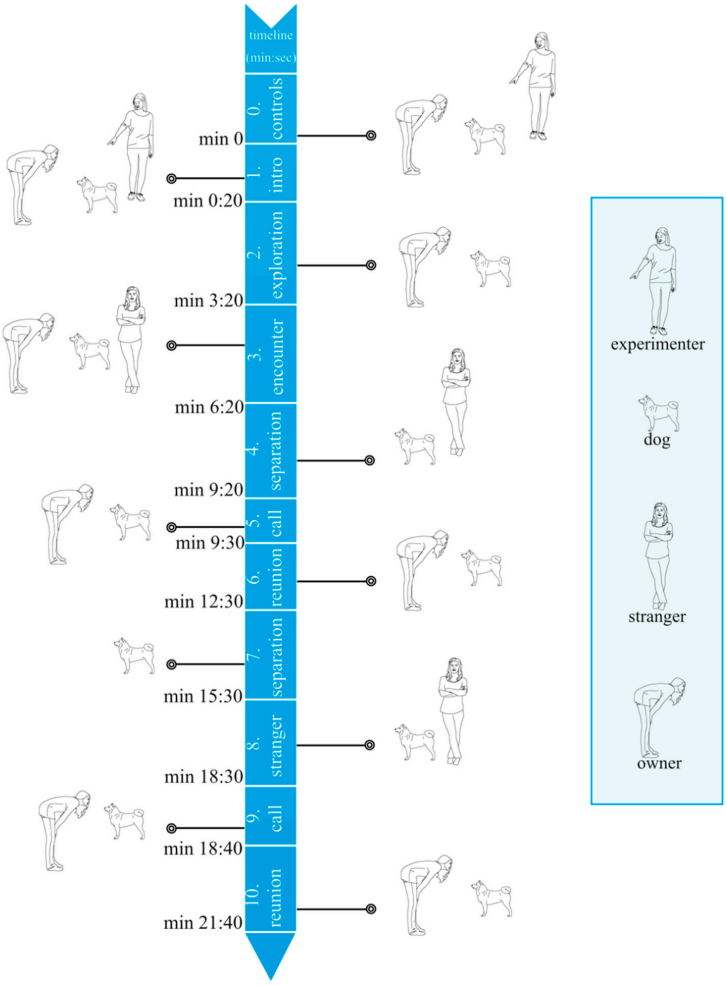

Figure 1. The Ainsworth strange situation (ASS) procedure visualized in the form of a timeline. The icons represent all parties of this study. The dog was present at all times and is therefore marked as such in each episode. The episodes marked with the owner icon were used for audio analysis.

2.4. Video Recording, Surveillance and Animal Welfare

The test room was equipped with a wide-angle lens video surveillance system (Canon Inc., Canon Austria GmbH, 1100 Vienna, Austria) to record and monitor the proceedings throughout from outside the room. The experimenter monitored the situation directly outside of the test room to ensure the dog and dyad's safety and to intervene if needed. The dog owners could terminate the experiment at

any point without giving any reason and were permitted to monitor the dog after exiting the room from the video monitor outside, with the experimenter. In cases where any of the dogs experienced extensive stress, the experiment would have been stopped by the experimenter independently of the dog owner; however, this was never the case. As judged by behavioral parameters, none of the dogs experienced stress levels outside of ordinary levels, so in no case did the owner or experimenter intervene to terminate the test situation. All participants were asked to sign consent forms and were informed about their voluntary participation and their right to stop the experiment at any time without providing a reason (see Section 2.11).

2.5. Dog Attachment Classification

The dog's attachment classification, based on the adapted ASSP, was analyzed according to methods detailed in Schöberl et al. and Solomon et al. [30,34]. Based on the video recordings of the ASSP, coauthors AB and JS, two psychologists trained in attachment categorization of human toddlers, together classified the dogs (with 89% interrater reliability) into five categories: secure, insecure avoidant, insecure ambivalent, insecure disorganized, and unclassifiable. KK and IS assisted with their expertise in dog behavior. Sample sizes and criteria were as follows:

A total of 27 out of 53 dogs were classified as 'securely attached': They eagerly approached their owners during the reunion and actively searched for and tolerated physical contact, while also showing interest in exploring the room. Three dogs were classified as 'insecure-avoidant', showing little tendency to approach their owner during the reunion but spending much time exploring the room and the toys. Six dogs were classified as 'insecure-ambivalent' based on their tendency to not explore their environment after the reunion at all but instead remaining in close proximity to their owner. Nine dogs were categorized as 'insecure-disorganized' who showed odd behavioral elements not being part of the normal attachment repertoire, such as freezing, staring or evident stereotypies. For eight dogs, no consensus could be reached; they were therefore labelled 'unclassifiable'.

2.6. Owner Caregiving Rating

The owner caregiving rating was developed by Solomon et al. [34] in the context of previous studies [30,31] and is based on Ainsworth's maternal sensitivity scale [20] and the 'supportive presence' scale [35]. The scale was designed to capture the caregiver's responsiveness and sensitivity to the dog's needs in a threatening situation [34]. During the threat task, an unfamiliar person entered the test room wearing a black coat, hat, and ski mask (with only the eyes visible). The unfamiliar person took three steps (in an interval of three seconds) towards the tethered dog while staring at its face. This process was repeated twice. After the second encounter, the unfamiliar person de-escalated the threat by stepping back, taking off the disguise, talking to the dog in a calming matter and offering cheese. The dog owner was present in only one encounter with the unfamiliar threat. The order of the owner's presence or absence during the encounters was randomized for each dyad. The experimenter (and in one test scenario, also the owner) observed both conditions on monitors outside the room. The dyad participated in the mild threat task four weeks to a year prior to entering the ASS procedure. Caregiving behavior by owners was rated based on the threat task on a seven-point caregiving scale for all dyads. The highest score of seven was given if the owner showed a consistent, quick, and flexible response in their caregiving behavior. The minimum score of one was given if the dog owner did not respond at all to the dog, or if they responded in a negative or punishing way. The ethics of the caregiving scales development was reviewed by the 'Faculty of Life Sciences' at the University of Vienna (case number: 2014-015).

2.7. Audio Recording

The dog owners' vocalizations were recorded during the entire ASSP with an H4N recorder connected to a small Sennheiser (ew 100 G3) microphone attached to the clothing in the chest area. The sampling frequency was 48 kHz with 16-bit quantization, and the sensitivity was adjusted prior to

recording to prevent clipping. The recording was stopped by the experimenter shortly after entering the room after episode ten.

2.8. Audio Treatment and Analysis

All 53 recordings had an adequate signal-to-noise ratio for audio analysis. The audio files of all dyads were prepared for semi-automated, acoustic analysis by hand editing with Audacity (Version 2.1.1; www.audacityteam.org), removing ASSP episodes and episode parts where either a talking second person was in the test room with the owner, or where the dog owner quietly waited outside of the test room (Figure 1). Following this procedure, episodes 1, 4, 7, and 8 as well as minutes two to three of episode 3 were excluded with Audacity (1: experimenter talking in room; 3: strange person talking in room; 4, 7, and 8: owner waiting outside). The remaining episodes 0, 2, 3, 5, 6, 9, and 10 were run through a semi-automated analysis pipeline in Praat (Version 6.0.23; www.fon.hum.uva.nl/praat/). All analysis was conducted using five custom written Praat scripts.

I. A: Normalization and prefiltering: Each audio file was adjusted to a maximum amplitude peak of 0.99 and treated with a spectral subtraction background noise filter using Praat commands 'Scale peak' and 'Remove noise'.

I. B: Speech segmentation: Praat's 'To TextGrid (silences)' was used to label those portions of the audio containing the owners' speech. The thresholds for the speech stream segmentation were based on silence in between intervals with a threshold of −35 dB silence, a minimum duration of 0.5 s for silent intervals, and a minimum duration of 0.07 s for spoken intervals. This process labelled the relevant spoken intervals automatically by marking them in the Praat TextGrid. The analyst then verified these automatic labels and made manual accuracy adjustments if necessary, in case of bursts of sound created by the dog vocalizing or playing with the toys.

II: Pitch contour tracking: All spoken intervals were saved, and their pitch contours were tracked and extracted using the Praat command 'Extract visible pitch contour'. The Praat internal commands 'Get mean', 'Get minimum', and 'Get maximum' were used to measure the acoustic parameters mean, minimum, and maximum of each individual pitch contour.

III: Episode matching: A TextGrid tier with episode identifiers was added to match each spoken interval with the corresponding episode. The start and end time of all spoken intervals were extracted.

IV: Jitter measurements: The command 'To PointProcess' was used to create a point process object out of the pitch contour extracted in script II. Those 'PointProcesses' and the command 'Get jitter (local)' were used to measure voice's jitter. The default settings were used except for the 'Longest period (s)', where 0.033 (minimum frequency measured) was input.

2.9. Analyzed Parameters

Four summary parameters of the f0 ('voice pitch') track were used to describe the owner's speech in spoken intervals throughout the test procedure (Figure 2): voiced duration, mean, range, and jitter. The semiautomated system measured each speech parameter below for every spoken interval. Intervals labelled as spoken but without measurable f0 were automatically excluded from further analysis. For readability, the fundamental frequency (f0) is referred to as 'pitch' throughout the rest of the paper.

Duration: Voiced utterance duration measured in seconds for each interval and calculated by subtracting the start time from the end time.

Mean Pitch: Mean f0 of the spoken interval in hertz.

Pitch Range: The minimum f0 measured within the interval, subtracted from the f0 maximum.

Jitter: Jitter was measured using Praat's algorithm 'To Jitter (local)'. This parameter is used as a measure of voice quality and is 'the average absolute difference between consecutive periods, divided by the average period' (Version 6.0.23; http://www.fon.hum.uva.nl/praat/manual/PointProcess__Get_jitter__local____.html).

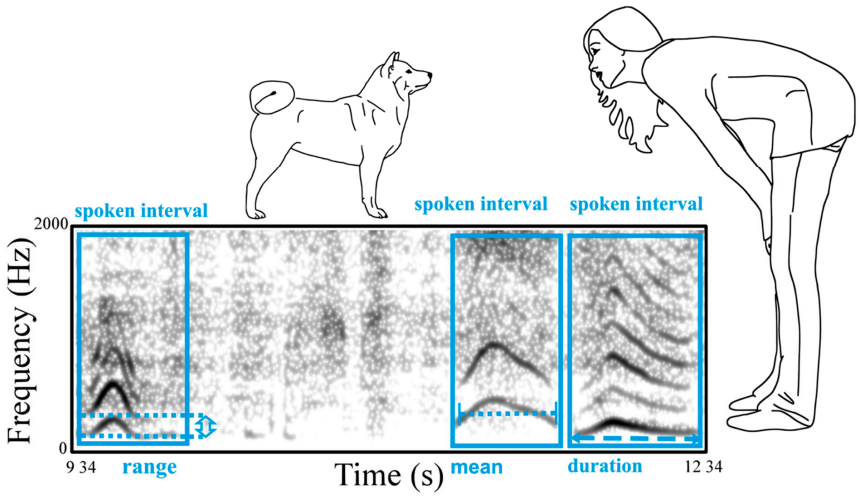

Figure 2. Spectrogram of a female owner talking to her dog during a reunion of the ASS procedure with illustrations of the spoken intervals and the measured variables mean f0 (average f0), voiced duration, and f0 range.

2.10. Statistical Analysis

Statistical analyses were done using R (Version 3.3.3; www.r-project.org/) and R-Studio (Version 1.0.13; www.rstudio.com/) and the packages ggthemes, ggplot2, psych, data.table, visreg, dplyr, doBy, xlsx, usdm, tidyverse, G.Gally, stats, lme4, car, nlme, lme4, cowplot, multcomp, MuMIn, piecewiseSEM, sjstats, and MASS. The data set for this study is available in the Supplementary Materials (Spreadsheet S1). Of all 53 dyads included in this study, over 4100 measurements were analyzed for each of the four response variables. Prior to running detailed analysis, all fixed factors tested negatively for multicollinearity. After visual inspection of the residuals of each response variable, the basic assumption of linear mixed models that the residuals follow a normal distribution could not be confirmed for jitter, mean pitch, pitch range, and voiced duration. Therefore, generalized linear mixed models (glmm) fit using maximum likelihood were calculated for all these four variables. The best distribution fit for each individual model's response variable was established by investigating the residuals' histogram and plotting the full models fitted residuals over the estimated residuals. Since the four response variables jitter, mean pitch, pitch range, and voiced duration were continuous and positively skewed towards the right, a Gamma distribution with a log link was used. Dyad was included as random effect to control for individual variances in each model, and the centered and scaled NEO FFI character traits (agreeableness, conscientiousness, extraversion, openness, and neuroticism), centered and scaled caregiving rating, attachment pattern, and owner gender were added as fixed factors and episode as a covariate of the full model. Because voice pitch varies by roughly an octave between adult men and women, gender was highly important to include as a fixed factor for each model. All attachment categories, except for the secure group, were collapsed into the nonsecure attachment group. This was a necessary simplification to reduce the number of levels within categories and facilitate model convergence. Null models included the covariate episode and the random effect dyad. All full models, except the duration model, tested to be a significantly better fit than the null model. Based on the results of the full-null model testing, duration was thus excluded from further analysis. Up to this point, standard practice of fitting linear mixed models was followed. Further decisions for the statistical analysis require some explanation.

A widely-used traditional but criticized approach for finding the best fitting model is a method called stepwise model reduction [36]. This process excludes one variable at a time from the full model based on *p*-values or AICc scores until reaching the null model. Apart from the problems arising from the usage of *p*-values, another issue arises in this context [37]: This stepwise reduction prevents an overview of all possible combinations of fixed factors explaining the response variable. To circumvent the problems and limitations arising through stepwise reduction, we used the function 'dredge' of the MuMIn package. This function creates every single model possible out of the fixed factors of the full model with (in our case) AICc scores. The model with the lowest AICc score is picked to provide a baseline, and within a delta of 2 upwards, all models are considered mathematically equally good fits [38]. The models within this range are put through model averaging to create averaged coefficients. This process allows evaluation of the influence all fixed factors have on the response variable without the restriction of *p*-values. For compatibility with the described approach of model averaging based on AICc values, a confidence interval of 85% is used to judge each factor's impact on the speech parameters [39]. Factors with a confidence interval not including 0 were chosen to be of importance in predicting voice parameters. Relative importance, a measure describing each factor's relative importance compared to the most valuable factor within the averaged coefficients, is used as a second measure and confirmation of the confidence intervals.

This approach of comparing all models within the delta 2 of the lowest AICc through model averaging was used to gain insight into the complex framework of CADS and its interactions with human personality and the human–dog attachment/caregiving system, without the unnecessary restriction of eliminating valuable comparisons from step one. For a review on null hypotheses significance testing and information theory based approaches and their possible combinations as used here, see Mundry 2011 [40].

2.11. Ethics

All participants were asked to sign consent forms, were informed about the procedure, and could terminate participation at any time. The ethics regarding human participation in the ASS procedure was reviewed and approved by the German Society for Psychology (Deutsche Gesellschaft für Psycholgie, AB 07_2011). All human/animal data collection and analysis was done in accordance with the declaration of Helsinki and the EU Directive 2010/63/EU for animal experiments. The ethics for this study was reviewed and approved by the 'Faculty of Life Sciences' animal welfare committee at the University of Vienna (case number: 2014-015).

3. Results

3.1. Mean Pitch

We found owner gender to affect pitch across episodes (Table 1). CADS was consistently higher in voice pitch than read speech (male mean: 121 ± 20 Hz SD; female mean: 203 ± 33 Hz SD) or conversational speech (male mean: 119 ± 19 Hz SD; female mean: 204 ± 38 Hz SD; Figure 3). Both male and female owners' highest median pitch was recorded during the call episodes (male mean: 176 ± 32 Hz SD; female mean: 300 ± 57 Hz SD). In both genders, the reunions, exploration, and encounter had a similar mean pitch. No effect of personality traits on CADS mean pitch was observed for male and female owners.

3.2. Pitch Range

We found gender and openness to affect pitch range across episodes (Table 2). Pitch range in CADS and conversation was reduced relative to reading. Both male and female owners showed the highest median pitch range in the reading control condition (Figure 4A). The median pitch range in the reunions, exploration, and encounter episodes in both men and women was lower than the speaking (male mean: 73 ± 48 Hz SD; female mean: 124 ± 84 Hz SD; Figure 4A) and reading controls (male

mean: 84 ± 42 Hz SD; female mean: 146 ± 54 Hz SD; Figure 4A). Female but not male owners high in openness showed an increased frequency range (Figure 4B).

Table 1. Model averaged coefficients for mean pitch models. Only variables with a minimum relative importance of 1 and a confidence interval not ranging over 0 are taken into consideration for model interpretation.

Parameter	Estimate	Std. Error	Confidence Interval (85%)		Relative Importance
			0.075	0.925	
(Intercept)	4.7658	0.0429	4.7041	4.8276	
speaking control	−0.005	0.0249	−0.0409	0.0309	1
exploration	0.1904	0.0196	0.1621	0.2187	´´
stranger	0.1859	0.0263	0.148	0.2237	´´
call (1)	0.3863	0.0353	0.3355	0.4371	´´
reunion (1)	0.1822	0.0194	0.1544	0.2101	´´
call (2)	0.3756	0.0348	0.3255	0.4257	´´
reunion (2)	0.1453	0.0197	0.117	0.1736	´´
gender (female)	0.5513	0.0537	0.4739	0.6286	1
caregiving	−0.0027	0.0121	−0.0596	0.0197	0.13
extraversion	0.002	0.0101	−0.0186	0.0506	0.13
attachment (non-secure)	−0.0035	0.0093	−0.0301	−0.0259	0.13
neuroticism	0.0015	0.0106	−0.0281	0.0545	0.12
openness	0.0009	0.0099	−0.033	0.0502	0.11
agreeableness	0.0008	0.0089	−0.0299	0.0451	0.11

Intercept includes reading control, secure attachment, and owner gender male.

Table 2. Model averaged coefficients for frequency range models. Only variables with a minimum relative importance of >0.8 and a confidence interval not ranging over 0 are taken into consideration for model interpretation.

Parameter	Estimate	Std. Error	Confidence Interval (85%)		Relative Importance
			0.075	0.925	
(Intercept)	4.3426	0.0911	4.2114	4.4738	
speaking control	−0.1656	0.1013	−0.3114	−0.0199	1
exploration	−0.6098	0.0799	−0.7248	−0.4948	´´
stranger	−0.7518	0.1072	−0.9062	−0.5974	´´
call (1)	−0.2962	0.1436	−0.5028	−0.0895	´´
reunion (1)	−0.5974	0.0787	−0.7107	−0.4841	´´
call (2)	−0.3351	0.1415	−0.5388	−0.1315	´´
reunion (2)	−0.618	0.079	−0.7317	−0.5042	´´
gender (female)	0.788	0.0782	0.6755	0.9006	1
openness	0.0613	0.0436	0.0179	0.127	0.85
extraversion	0.0157	0.0303	−0.0016	0.0997	0.32
agreeableness	0.0038	0.0159	−0.0158	0.0858	0.11
neuroticism	0.0059	0.0217	−0.0259	0.0945	0.17
attachment (non-secure)	0.0053	0.0281	−0.0489	0.1668	0.09
conscientiousness	0.0018	0.012	−0.0293	0.0751	0.08
caregiving	−0.0006	0.0099	−0.0624	0.0435	0.07

Intercept includes reading control, secure attachment, and owner gender male.

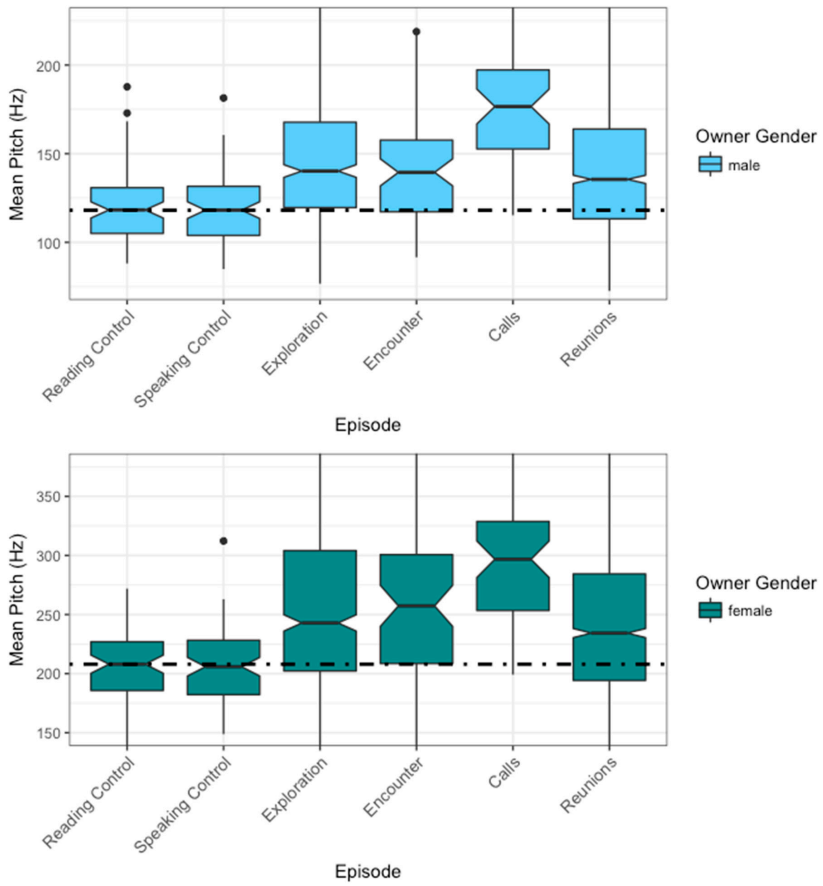

Figure 3. Change in mean pitch by gender over the ASS procedure episodes. The male owners are represented in light blue and the female owners in dark blue. Due to physiological differences, pitch (f0) is separated by about an octave between men and women. Therefore, results are presented in separate graphs (mean pitch plotted by gender over ASS procedure (ASSP) episodes, with the dashed line representing the reading controls median. The two calls and reunions are visualized combined as "calls" and "reunions".) n = 53; number of observations = 4121.

3.3. Jitter

Jitter results in the CADS condition were highly variable. We found owner gender and openness to influence jitter across episodes (Table 3). The median jitter was lowest in the encounter (male mean: 0.014 ± 0. 009 % SD; female mean: 0.01 ± 0.007 % SD) and the call episodes (male mean: 0.009 ± 0.004 % SD; female mean: 0.007 ± 0.003 % SD) in both male and female owners. The speaking control condition had the highest median percentage of jitter in both men (male mean: 0.018 ± 0.008 % SD) and women (female mean: 0.012 ± 0.005 % SD). Male owners' reunion and exploration episodes had a lower percentage of voice jitter than their speaking control condition; women's percentage of jitter in the same episodes had similar levels to the speaking control (Figure 5A). Male owners' openness values scaled positively with the percentage of jitter in their vocalizations; this effect was comparatively weaker in female owners (Figure 5B).

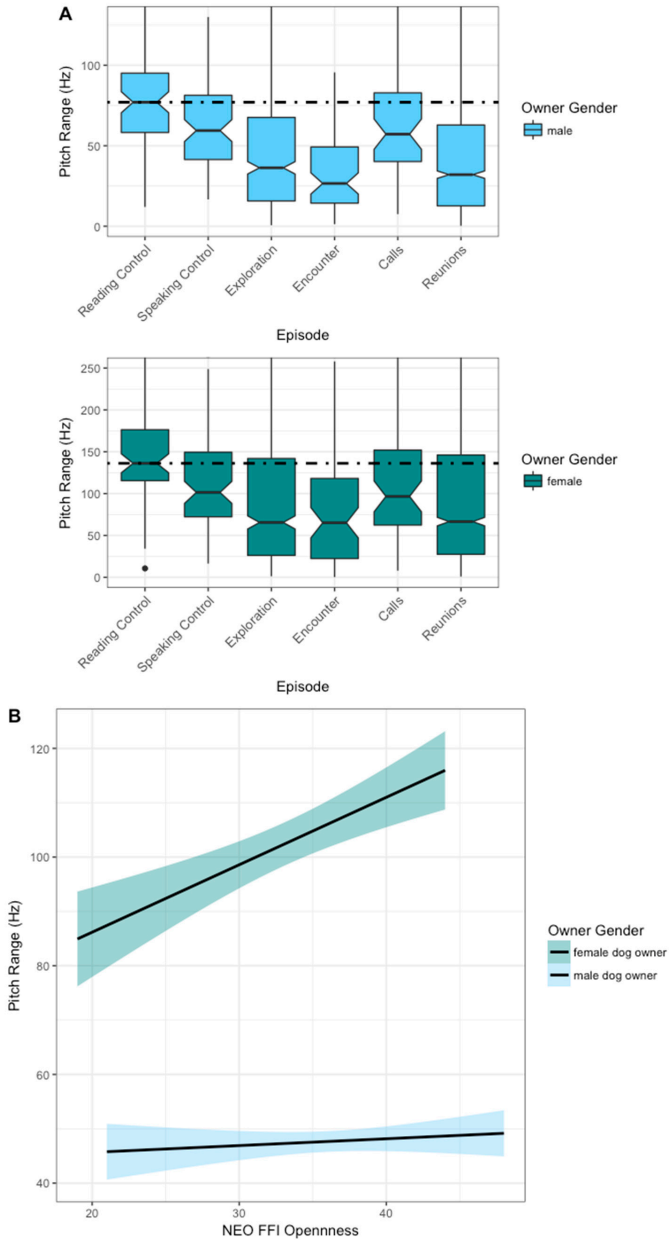

Figure 4. Pitch range by gender over the NEO FFI character trait openness and throughout the ASSP. The light blue line represents male and the dark blue line represents female owner. (**A:** Pitch range plotted by gender over ASSP episodes with the dashed line representing the reading controls median. The two calls and reunions are visualized combined as "calls" and "reunions". **B:** Pitch range plotted by gender over the NEO FFI openness score.) $n = 53$, number of observations = 4121.

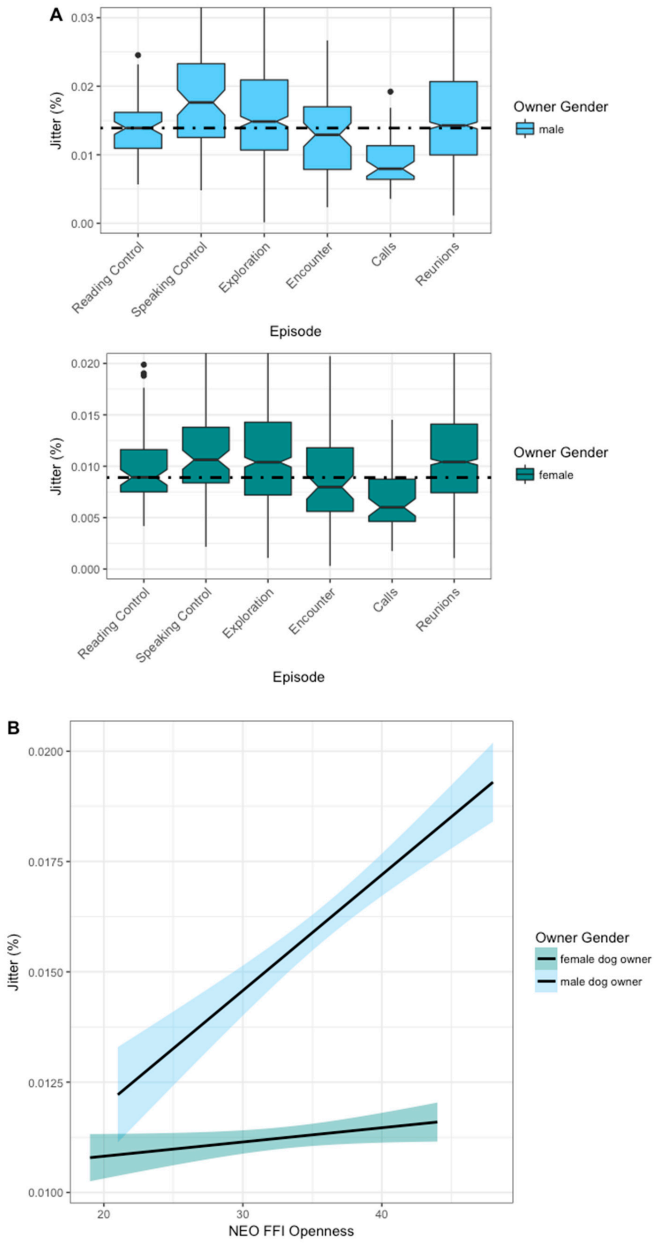

Figure 5. Percentage of jitter by gender over the dog owners' NEO FFI character trait openness and throughout the ASSP. The light blue line represents male and the dark blue line represents female owner. (**A:** Jitter plotted by gender over ASSP episodes with the dashed line representing the reading controls median. The two calls and reunions are visualized combined as "calls" and "reunions". **B:** Jitter plotted by gender over the NEO FFI openness score.) n = 53; number of observations = 4121.

Table 3. Model averaged coefficients for jitter models. Only variables with a minimum relative importance of >0.8 and a confidence interval not ranging over 0 are taken into consideration for model interpretation.

Parameter	Estimate	Std. Error	Confidence Interval (85%)		Relative Importance
			0.075	0.925	
(Intercept)	−4.3029	0.0599	−4.389	−4.2167	
speaking control	0.2193	0.0532	0.1427	0.2959	1
exploration	0.1795	0.0418	0.1193	0.2397	´´
stranger	0.0309	0.0563	−0.0501	0.1119	´´
call (1)	−0.4104	0.0754	−0.5189	−0.3019	´´
reunion (1)	0.146	0.0413	0.0865	0.2055	´´
call (2)	−0.2983	0.0746	−0.4057	−0.191	´´
reunion (2)	0.1679	0.0415	0.1082	0.2276	´´
gender (female)	−0.3087	0.0652	−0.4025	−0.2148	1
openness	0.0531	0.0358	0.0156	0.1063	0.87
caregiving	0.0037	0.0145	−0.0181	0.0686	0.15
extraversion	−0.0023	0.0115	−0.0575	0.0212	0.13
agreeableness	0.001	0.01	−0.0331	0.0509	0.11
attachment (non-secure)	−0.0013	0.0207	−0.1025	0.0784	0.1
neuroticism	−0.0004	0.0103	−0.0496	0.0422	0.1

Intercept includes reading control, secure attachment, and owner gender male.

4. Discussion

We analyzed vocal parameters in dog-directed speech during a series of controlled encounters in dog–human dyads and compared these to normal speech (a read passage, or between-human conversation). The staged encounters were designed to elicit arousal in the dogs and caregiving from their owners. In general, we found that CADS was higher in f0 ("voice pitch") than normal speech but showed a narrower pitch range. Results concerning pitch perturbation (jitter) were quite variable and showed no clear effect of arousal; the most pronounced effect was a considerable decrease in jitter associated with the owner calling the dog's name in the two "call" episodes.

Compared to earlier work on CADS, our results support the findings of an increased pitch but fail to support the previously described broader pitch range. Jeannin et al. [29] partially used an approach similar to the ASSP and found voice pitch in dog-directed speech to increase in reunion episodes compared to adult-directed speech. Our results showed the exact same pattern, with a strong increase in voice pitch during the reunion episodes. Gergely et al. [28] reported a broader frequency range in CADS in mothers compared to fathers. We found the same effect in women to using a broader frequency range during CADS than men did. A well-described phenomenon in IDS and CADS is a differential use of voice pitch and frequency range. Pitch is said to communicate affect, whereas the range is used more in attention getting [10,28,41–43]. This different function of pitch and range might also be the explanation as to why we did not find a broader pitch range. Pitch range might simply be adjusted strongly in accordance with context. Namely, if the owner wants to draw the dogs' attention away from something, a broad pitch range could be used. The opposite would be true if the owner already has the dog's attention and is trying to soothe and calm it [41]. This would explain why the owners used a broader pitch range and a high pitch while calling their dogs and therefore hoping to draw their attention while also communicating positive intentions. The pitch in exploration, encounter, and the reunion episodes was lower in comparison to the call episodes but still increased compared to the read and adult-directed speech. This elevated pitch coincided with the narrowest pitch ranges in the same episodes. Exploration, encounter, and both reunions share in common that most dogs had already directed their attention towards their owner (in a 2013 study, 85% of the dogs followed their owners around in the reunion and encounter episodes [44]); no broad pitch range was needed to keep their focus. The owners continued to use an elevated pitch to communicate positive affect and a nonthreatening situation.

The results regarding jitter were less clear and more variable. Both males' and females' adult-directed speech had the highest percentage of jitter compared to the lowest in the call episodes. Female owners' median jitter slowly returns to the levels of the reading control throughout the exploration, encounter, and reunions. Male owners' jitter stays at an elevated level during the exploration and reunion episodes. Interestingly, jitter was highest in the adult-directed speech and behaved contrary to what we would have expected. An explanation for this might be found in the hypothesis that an increase in the speakers' stress leads to a decreased jitter due to higher tension on the vocal folds [1,4]. This might be a parabola-like phenomenon with relaxed and extremely stressed speakers producing the highest jitter values. This hypothesis would explain why we would see jitter to be lowest when the speaker is stressed but not overly so; enough to cause tension (in the body and the vocal folds) yet mild enough to be dealt with. This hypothesis fits best with our results, but further research must be done to empirically assess this claim.

Our work also illustrates the importance of owner gender and owner personality on CADS. Not only does our study support the idea that CADS is gender-specific and variable enough among dyads to constitute an important component of human–dog caregiving and attachment strategy, we also found openness to drive CADS modulation within this ASSP setup. The influence of personality on CADS seems to be gender-specific and may be considered part of gender-specific performance. Men's openness was positively correlated with utterance jitter, while women showed almost no correlation. Higher scores in openness correlated with a higher pitch range in women but showed no correlation in men. The limited literature regarding personality in the context of CADS restricts us in doing more than speculating as to why openness might influence jitter and pitch range. There might be one intriguing hypothesis explaining the influence of this one personality axis. The ASSP is designed to evoke arousal and a stress response (and therefore cortisol excretion) in the dogs, which in turn causes a similar stress response in owners. Research suggests a link between cortisol stress response, personality, and gender [45,46]. We propose this link to be reflected in the vocal parameters of jitter and pitch range. This possible interaction of personality, cortisol, and CADS might be a potentially interesting field of further research.

5. Conclusions

To summarize, our data partially support and partially contradict our initial hypothesis of an increase in voice source variables with increasingly aroused situations. We did find voice pitch to increase in the ASSP setup in comparison to normal speech, but we found the opposite to be true for pitch range. The decrease in pitch range might be caused by the differential use and function of mean pitch and range. With the narrower range, the owners tried to calm the dogs in this stressful setting. Due to limited reports in literature and close to no precedent, our predictions and hypothesis on jitter were less clear. The idea of voice perturbation to increase with arousal was not supported by our data, but it fit the hypothesis of being (at least partially) personality-dependent.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2615/9/7/417/s1, Spreadsheet S1: data set.

Author Contributions: Conceptualization, R.L.; Data curation, I.S.; Formal analysis, R.L.; Funding acquisition, K.K. and W.T.F.; Investigation, I.S.; Methodology, R.L., K.K., I.S., A.B., J.S., and W.T.F.; Project administration, I.S.; Resources, K.K. and W.T.F.; Software, R.L.; Supervision, K.K. and W.T.F.; Validation, I.S., A.B., and J.S.; Visualization, R.L.; Writing—original draft, R.L.; Writing—review and editing, K.K. and W.T.F.

Funding: The study was funded by the Austrian Science Fund (FWF), Project P23345, by Waltham grant (FA 566001) (both to K.K.), by the PhD program Cognition and Communication 1 and 2 from the Austrian Science Fund (FWF): Project W1234-G17 (to K.K./I.S.), Project W1262-B29 (to R.L./W.T.F.) and by a J. William Fulbright award (to J.S.). The funding agencies were not involved in the collection, analysis, interpretation of data, writing the report, and decision to submit the article.

Acknowledgments: We would like to thank all owners and their dogs for their participation in our study. We thank Manuela Wedl for her important impact during the developmental stage of this project and all students, who were involved: Evi Myska, Sarah Prettner, Philipp Stöger, Sigrid Amon, and Verena Ziemen. We thank Dan C. Mann for support with Praat and Matthias Loretto for input on statistical analysis. Further, we thank Arik

Kershenbaum and one anonymous reviewer for comments and suggestions of improvement on a previous version of this manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The sponsors had no role in the design, execution, interpretation, or writing of the study.

References

1. Protopapas, A.; Lieberman, P. Fundamental frequency of phonation and perceived emotional stress. *J. Acoust. Soc. Am.* **1997**, *101*, 2267–2277. [CrossRef] [PubMed]
2. Siniscalchi, M.; D'Ingeo, S.; Fornelli, S.; Quaranta, A. Lateralized behavior and cardiac activity of dogs in response to human emotional vocalizations. *Sci. Rep.* **2018**, *8*, 77. [CrossRef] [PubMed]
3. Williams, C.E.; Stevens, K.N. Emotions and Speech: Some Acoustical Correlates. *J. Acoust. Soc. Am.* **1972**, *52*, 1238–1250. [CrossRef] [PubMed]
4. Scherer, K. Vocal affect expression: A review and model for further research. *Psychol. Bull.* **1986**, *99*, 143–165. [CrossRef] [PubMed]
5. Smith, G.A. Voice analysis for the measurement of anxiety. *Br. J. Med. Psychol.* **1977**, *50*, 367–373. [CrossRef] [PubMed]
6. Kagan, J.; Reznick, J.S.; Snidman, N. Biological bases of childhood shyness. *Science* **1988**, *240*, 167–171. [CrossRef]
7. Burnham, D.; Kitamura, C.; Vollmer-Conna, U. What's new, pussycat? On talking to babies and animals. *Science* **2002**, *296*, 1435. [CrossRef]
8. Burnham, D.; Francis, E. Are you my little pussy-cat? Acoustic, phonetic and affective qualities of infant-and pet-directed speech. In Proceedings of the 5th International Conference on Spoken Language Processing (ICSLP 98), Sydney, Australia, 30 November–4 December 1998.
9. Ben-Aderet, T.; Gallego-abenza, M.; Reby, D.; Mathevon, N. Dog-directed speech: Why do we use it and do dogs pay attention to it? *Proc. R. Soc. B Biol. Sci.* **2017**, *284*, 20162429. [CrossRef]
10. Xu, N.; Burnham, D.; Kitamura, C.; Vollmer-Conna, U. Vowel hyperarticulation in parrot-, dog- and infant-directed speech. *Anthrozoos* **2013**, *26*, 373–380. [CrossRef]
11. Hoff, E. *Language Development*; Jon-David Hague: Wadsworth, OH, USA, 2009.
12. Jeannin, S.; Gilbert, C.; Amy, M.; Leboucher, G. Pet-directed speech draws adult dogs' attention more efficiently than Adult-directed speech. *Sci. Rep.* **2017**, *7*, 4980. [CrossRef]
13. Julius, H.; Beetz, A.; Kotrschal, K.; Turner, D.C.; Uvnäs-Moberg, K. *Attachment to Pets*; Hogrefe: Göttingen, Germany, 2012.
14. Siniscalchi, M.; d'Ingeo, S.; Quaranta, A. Orienting asymmetries and physiological reactivity in dogs' response to human emotional faces. *Learn. Behav.* **2018**, *46*, 574–585. [CrossRef] [PubMed]
15. Archer, J. Why do people love their pets? *Evol. Hum. Behav.* **1997**, *18*, 237–259. [CrossRef]
16. Kotrschal, K. *Hund-Mensch. Das Geheimnis der Seelenverwandtschaft*; Brandstätter: Wien, Austria, 2016.
17. Cassidy, J.; Shaver, P.R. The Nature of the Child's Ties. In *Handbook of Attachment: Theory, Research, and Clinical Applications*; The Guilford Press: New York, NY, USA, 1999; Volume XVII.
18. Bowlby, J. Attachment and Loss. *Key Concepts Fam. Stud.* **1980**, *54*, 9–12.
19. Ainsworth, M. Attachments Beyond Infancy. *Am. Psychol.* **1989**, *44*, 709–716. [CrossRef] [PubMed]
20. Ainsworth, M.D.S.; Blehar, M.C.; Waters, E.; Wall, S. *Patterns of Attachment: A Psychological Study of the Strange Situation*; Lawrence Erlbaum: Oxford, UK, 1978.
21. Palestrini, C.; Prato Previde, E.; Custance, D.M.; Spiezio, C.; Sabatini, F.; Verga, M. Heart rate and behavioural responses in dogs (*Canis familiaris*) in the Ainsworth's Strange situation stress: A pilot study. In Proceedings of the 3th International Congress on Veterinary Behavioural Medicine, Vancouver, BC, Canada, 7–8 August 2001; Volume 94, pp. 89–91.
22. Main, M.; Solomon, J. Procedures for idenifying infants as disorganaized/disoriented during the Ainsworth Strange Situation. In *Attachment in the Preschool Years: Theory, Research, and Intervention*; University of Chicago Press: Chicago, IL, USA, 1990; pp. 161–182.
23. Ainsworth, M.D.S.; Wittig, B.A. Attachment and the exploratory behavior of one-year olds in a strange situation. *Determ. Infant Behav.* **1969**, *4*, 111–136.

24. Gácsi, M.; Maros, K.; Sernkvist, S.; Miklósi, Á. Does the owner provide a secure base? Behavioral and heart rate response to a threatening stranger and to separation in dogs. *J. Vet. Behav. Clin. Appl. Res.* **2009**, *4*, 90–91. [CrossRef]
25. Palmer, R.; Custance, D. A counterbalanced version of Ainsworth's Strange Situation Procedure reveals secure-base effects in dog-human relationships. *Appl. Anim. Behav. Sci.* **2008**, *109*, 306–319. [CrossRef]
26. Prato-Previde, E.; Custance, D.M.; Spiezio, C.; Sabatini, F. Is the dog-human relationship an attachment bond? An observational study using Ainsworth's strange situation. *Behaviour* **2003**, *140*, 225–254. [CrossRef]
27. Topál, J.; Miklósi, Á.; Csányi, V.; Dóka, A. Attachment Behavior in Dogs (*Canis familiaris*): A New Application of Ainsworth's (1969) Strange Situation Test. *J. Comp. Psychol.* **1998**, *112*, 219–229. [CrossRef]
28. Gergely, A.; Faragó, T.; Galambos, Á.; Topál, J. Differential effects of speech situations on mothers' and fathers' infant-directed and dog-directed speech: An acoustic analysis. *Sci. Rep.* **2017**, *7*, 13739. [CrossRef]
29. Jeannin, S.; Gilbert, C.; Leboucher, G. Effect of interaction type on the characteristics of pet-directed speech in female dog owners. *Anim. Cogn.* **2017**, *20*, 499–509. [CrossRef] [PubMed]
30. Schöberl, I.; Beetz, A.; Solomon, J.; Wedl, M.; Gee, N.; Kotrschal, K. Social factors influencing cortisol modulation in dogs during a strange situation procedure. *J. Vet. Behav. Clin. Appl. Res.* **2016**, *11*, 77–85. [CrossRef]
31. Schöberl, I.; Wedl, M.; Beetz, A.; Kotrschal, K. Psychobiological factors affecting cortisol variability in human-dog dyads. *PLoS ONE* **2017**, *12*, e0170707. [CrossRef] [PubMed]
32. Costa, P.T.; McCrae, R.R. *Revised NEO Personality Inventory (NEO-PI-R) and NEO Five-Factor*; The Sage Handbook of Personality Theory and Assessment; Psychological Assessment Resources: Odessa, Ukraine, 1992.
33. Borkenau, P.; Ostendorf, F. *NEO-Fünf-Faktoren Inventar:(NEO-FFI); nach Costa und McCrae*; Hogrefe: Göttingen, Germany, 1993.
34. Solomon, J.; Beetz, A.; Schöberl, I.; Gee, N.; Kotrschal, K. Attachment security in companion dogs: Adaptation of Ainsworth's strange situation and classification procedures to dogs and their human caregivers. *Attach. Hum. Dev.* **2018**, *21*, 389–417. [CrossRef] [PubMed]
35. Matas, L.; Arend, R.A.; Sroufe, L.A. Continuity of Adaptation in the Second Year: The Relationship between Quality of Attachment and Later Competence. *Child Dev.* **1978**, *49*, 547–556. [CrossRef]
36. Grueber, C.E.; Nakagawa, S.; Laws, R.J.; Jamieson, I.G. Multimodel inference in ecology and evolution: Challenges and solutions. *J. Evol. Biol.* **2011**, *24*, 699–711. [CrossRef] [PubMed]
37. Amrhein, V.; Korner-Nievergelt, F.; Roth, T. The earth is flat ($p > 0.05$): Significance thresholds and the crisis of unreplicable research. *PeerJ* **2017**, *5*, e3544. [CrossRef] [PubMed]
38. Jørgensen, S.E. Model Selection and Multimodel Inference. *Ecol. Model.* **2004**, *172*, 96–97. [CrossRef]
39. Arnold, T.W. Uninformative Parameters and Model Selection Using Akaike's Information Criterion. *J. Wildl. Manag.* **2010**, *74*, 1175–1178. [CrossRef]
40. Mundry, R. Issues in information theory-based statistical inference—A commentary from a frequentist's perspective. *Behav. Ecol. Sociobiol.* **2011**, *65*, 57–68. [CrossRef]
41. Trainor, L.J.; Austin, C.M.; Desjardins, N. Is infant-directed speech prosody a result of the vocal expression of emotion? *Psychol. Sci.* **2000**, *11*, 188–195. [CrossRef] [PubMed]
42. Singh, L.; Morgan, J.L.; Best, C.T. Infants' Listening Preferences: Baby Talk or Happy Talk? *Infancy* **2002**, *3*, 365–394. [CrossRef]
43. Kitamura, C.; Burnham, D. Pitch and communicative intent in mother's speech: Adjustments for age and sex in the first year. *Infancy* **2009**, *4*, 85–110. [CrossRef]
44. Siniscalchi, M.; Stipo, C.; Quaranta, A. "Like owner, like dog": Correlation between the owner's attachment profile and the owner-dog bond. *PLoS ONE* **2013**, *8*, e78455. [CrossRef] [PubMed]
45. Oswald, L.M.; Zandi, P.; Nestadt, G.; Potash, J.B.; Kalaydjian, A.E.; Wand, G.S. Relationship between cortisol responses to stress and personality. *Neuropsychopharmacology* **2006**, *31*, 1583–1591. [CrossRef] [PubMed]
46. Schöberl, I.; Wedl, M.; Bauer, B.; Day, J.; Möstl, E.; Kotrschal, K. Effects of owner-dog relationship and owner personality on cortisol modulation in human-dog dyads. *Anthrozoos* **2012**, *25*, 199–214. [CrossRef]

Chapter 2|

Selection on ultrasonic call rate in neonatal rats affects low frequency, but not ultrasonic, vocalizations in adults

This chapter is published as an article in a peer-reviewed journal.

Citation: **Lesch, R.**; Orozco, A.; Shilling, M.; Zimmerberg, B.; Fitch, W.T. Selection on ultrasonic call rate in neonatal rats affects low frequency, but not ultrasonic, vocalizations in adults. Ethology 2020, 126, 1007–1018.


NOVEMBER 2020
VOL. 126 (11), 1007–1078
ethology
international journal of behavioural biology
Check for updates
WILEY

Received: 13 February 2020 | Revised: 14 May 2020 | Accepted: 22 May 2020

DOI: 10.1111/eth.13075

RESEARCH PAPER

ethology WILEY

Selection on ultrasonic call rate in neonatal rats affects low frequency, but not ultrasonic, vocalizations in adults

Raffaela Lesch[1] | Andrea Orozco[2] | Margaret Shilling[2] | Betty Zimmerberg[2] | W. Tecumseh Fitch[1]

[1]Department of Behavioral and Cognitive Biology, University of Vienna, Vienna, Austria

[2]Department of Psychology, Williams College, Williamstown, MA, USA

Correspondence
Raffaela Lesch, Department of Behavioral and Cognitive Biology, University of Vienna, Althanstrasse 14, Vienna 1090, Austria.
Email: raffaela@lesch.at

Tecumseh Fitch, Department of Behavioral and Cognitive Biology, University of Vienna, Althanstrasse 14, Vienna 1090, Austria.
Email: tecumseh.fitch@univie.ac.at

Funding information
RL was funded by the FWF DK grant Communication and Cognition to WTF (FWF project W1262-B29). BZ was not funded externally. AO and MS were undergraduate students at Williams College. Austrian Science Fund W1262-B29.

Editor: Wolfgang Goymann

Abstract

In this experiment, we studied a rodent model selected over 57 generations for high or low rates of ultrasonic vocalizations (USVs) during maternal separation as pups. We investigated the influence of this breeding on the adult animals' subsequent vocal output, comparing acoustic variables across developmental stages. We hypothesized that selection on pup USV rate would impact adult USV production without affecting lower frequency calls. Contrary to this hypothesis, we found neither number of USV calls or other acoustic variables to differ among selected adult lines. Instead, we found that pup USV selection mainly affected adults' low-frequency (human-audible) calls. Furthermore, low-frequency vocalizations did not fully fit a predicted correlation between body weight and fundamental frequency: high line males, although the heaviest on average, did not produce the lowest fundamental frequencies. Our findings suggest that selection for early ultrasonic vocal behaviour pleiotropically results in changes in anatomical production mechanisms and/or neural control affecting low-frequency calls.

KEYWORDS

development, domestication, human–animal interaction, play, pleiotropic effects, selection, ultrasonic vocalization, vocal communication

1 | INTRODUCTION

Pleiotropy is a pervasive phenomenon in evolution. Its relevance and existence were recognized early in medical syndromes, but the term "pleiotropy" was first coined by Ludwig Plate in 1910 (Stearns, 2010). Pleiotropy is an important concept with many different facets and definitions, but generally describes "the phenomenon of a single gene affecting multiple traits" (Paaby & Rockman, 2013). Here, we investigated the emergence of unselected by-products coupled to an originally selected for trait, including across developmental stages, as a possible example of pleiotropy, but we make no claims about genetics (Paaby & Rockman, 2013). Coherent development throughout developmental stages is essential for functionality in morphological structures (Cheverud, 1996), and pleiotropic traits can thus be representative of adaptive developmental processes that form and shape structures in organisms (Klingenberg, 2008).

A potential example of a pleiotropic effect can be found in dogs and other domesticated mammals. Human selection for tameness in these animals has been suggested to lead to downregulation of neural crest cell development and migration (Wilkins, Wrangham,

Betty Zimmerberg and W. Tecumseh Fitch shared last author.

& Fitch, 2014). Neural crest cells have multiple functions in embryonic development, generating pigmentation, sympathetic nervous tissues, craniofacial cartilages and bones, and interact in the formation of adrenal glands. A reduced functionality in neural crest cells is thus hypothesized to have direct effects on the animal's arousal physiology (e.g. reduction of size and function of the adrenal glands and sympathetic nervous system), along with pleiotropic knockdown effects on pigmentation and skull shape. Changes in vocal behaviour are also known in domesticated animals, but we know very little about pleiotropic effects in the vocal or acoustic domain, due to a current lack of model organisms.

In the current study, we investigate potential pleiotropic effects of artificial selection on one vocal trait on other vocal traits across development. In particular, we examine how selective breeding on rat vocal output in an early developmental stage impacts vocal acoustics of later developmental stages.

Rats are an excellent model species to investigate pleiotropy in communication due to their rapid development and well-understood vocal repertoire. Rats produce a wide range of vocalizations and call types, and most of which are above the human hearing range, at over 20 kHz (Brudzynski, 2018). Calls above this threshold are called ultrasonic vocalizations (USVs). Young pups mostly produce USVs in the range of 30–65 kHz to maintain contact with their mother, who is crucial to their survival (Brunelli, Shair, & Hofer, 1994). Adult rats USVs split into two separate USV ranges to convey appetitive and aversive motivation. Appetitive calls centre around 50 kHz and aversive USVs at 22 kHz (Portfors, 2007). A large body of literature describes and uses these USVS as indicators of emotional states in rats (Brudzynski, 2015; Burgdorf et al., 2009; Simola & Brudzynski, 2018). However, both adults and pups also produce low-frequency calls (1–6 kHz) within the hearing range of humans. These calls serve to fend off predators and warn conspecifics, when the animal is in pain, and during rough-and-tumble play (Brudzynski, 2010, 2018).

The production mechanism of low-frequency calls is generally well understood in many mammals and results from vocal fold vibration in the larynx (Herbst et al., 2012) and is presumed to function similarly in rodents (Riede, York, Furst, Müller, & Seelecke, 2011; Roberts, 1975), and the mechanism of rodent USV production is debated. There is wide agreement that USVs are the product of a whistle mechanism (Johnson, Ciucci, Russell, Hammer, & Connor, 2010; Mahrt, Agarwal, Perkel, Portfors, & Elemans, 2016; Riede, Borgard, & Pasch, 2017; Roberts, 1975; Sanders, Weisz, Yang, Fung, & Amirali, 2001), but disagreement exists concerning the exact underlying production mechanism. Mahrt et al. (2016) suggests an intra-laryngeal planar impinging jet (Mahrt et al., 2016) to be responsible for USV production, while Riede et al. (2017) describe an edge-tone whistle mechanism as the source (Riede et al., 2017).

The present study investigates a selected line of rats, whose breeding was based on the production of infant USVs during maternal separation. This selection has resulted in two unique, stable lines, where pups emit USVs at high and low rates (Brunelli & Hofer, 2007). Infantile USVs are regarded as an indicator of the negative affective state of the pup (hence the term "distress calls"; Allin & Banks, 1972; Shair, 2007) since they decrease after the administration of anti-anxiety agents such as the benzodiazepines and neurosteroids (Carden & Hofer, 1990; Winslow & Insel, 1991; Zimmerberg, Brunelli, & Hofer, 1994). In earlier work, variations in acoustical features other than call rate were also found to be by-products of the selective breeding on these two lines. These unselected, pleiotropic by-products include longer call duration, louder relative amplitude and broader frequency bandwidth in the high-production line (Spence, Aslam, Hofer, Brunelli, & Shair, 2016). Studies of the behaviour of these high and low lines at later ages have documented the concurrent divergence of the lines into two “affective temperaments”. High line rats tend to be more anxious, depressed and less playful, with sympathetic nervous system over-activity, while low line rats tend to be more active and aggressive, with parasympathetic nervous system underactivity (Brunelli, 2005; Brunelli, Zimmerberg, & Hofer, 2010; Zimmerberg, Brunelli, Fluty, & Frye, 2005). Low line adult rats also perform better in spatial learning and object recognition tasks as compared to high line rats.

Burgdorf et al. (2009) independently produced two similar rat lines based on a different selection process. Their selection pressure was based on adult 50kHz USV responsiveness to positive social interaction (“tickling”). Adults of the responsive line produced a higher number of 50kHz calls as adolescents and adults, compared with the less responsive line. This selection for tickling responsiveness to positive social interaction not only led to the formation of two distinct lines, but it also led to the emergence of pleiotropic affective traits. Responsive lines with a high rate of 50kHz calls proved to be more stress resilient and had a more positive affectivity, compared with rats of the unresponsive line.

Both Brunelli-Hofer's and Burgdorf's rat lines have provided insight into pleiotropic changes affecting behaviour and physiology. To date however, we know little about how selection for vocal output at one stage might affect the vocal communication output at a different developmental stage. Our study bridges this gap by investigating the pleiotropic changes early selection pressure has on acoustic output later in development.

Based on the results presented by Burgdorf et al. (2009), and the selection pressure our rat lines were subjected to (i.e. to produce more pup USVs in the high line), we hypothesized that our adult rats would produce USVs in accordance with their behaviour as pups. One consideration supporting this hypothesis concerns the costliness of USV production: Compared with low-frequency squeaks which are very energy efficient, USV production has a higher metabolic cost (Kelm-Nelson, Lenell, Johnson, & Ciucci, 2018). Therefore, we hypothesized that selection pressure for increased pup USV production would also select for ability to sustain a higher level of communicative effort in adults. Second, in an earlier study, pup line USVs were reported to differ in call duration and range (Spence et al., 2016); our second hypothesis predicts that this will remain true for the adult rat line USVs. Finally, selection for USV rate in pups potentially influences the development and structure of the larynx, which in turn could impact the production

of other calls. However, due to their different production mechanisms, low-frequency (human-audible) vocalizations produced by vocal fold vibration may be unaffected relative to high frequency whistles. Our third hypothesis thus predicts that USV-focussed selection will have little measurable impact on the low-frequency (audible) calls between lines.

2 | METHODS

2.1 | Ethical approvals

All housing and testing procedures were approved by the Williams College Animal Use and Care Committee (ethical approval number ZB-B-17).

2.2 | Line breeding

Subjects for this experiment were offspring from the 57th (adults) and 58th generation (pups) of N:NIH rats that were selectively bred for high and low ultrasonic vocalization rates as induced by brief (20 min) maternal separation at 10 days of age, with selection methods described in detail in Brunelli, Vinocur, Soo-Hoo, and Hofer (1997).

2.3 | Housing

Mating was conducted within each line. Pregnant females, determined by the presence of a vaginal plug, were individually housed in plastic cages (45 × 25 × 15 cm) in an isolated nursery and had continuous access to standard laboratory chow and water throughout their pregnancies. The day of birth was denoted post-natal day (PN) 0. Distinct litters were used for pup and adult tests. Each litter was represented by no more than one male or one female in any behavioural test. For adult subjects, the pups stayed in the cage with their mother until weaning at PN 25. At weaning, the animals were housed in same sex pairs until 3 days prior to the start of habituation, when they were housed individually.

All rats were housed in standard rodent hanging cages and were given access to food and water ad libitum. All animals were kept on a 12/12 cycle of dark and light with the lights turning on at 06:00. Colony temperatures were maintained between 21.7 and 22.8°C with humidity levels between 44% and 55%. Both pups and adult rats were weighed after testing with a standard laboratory scale in grams with a two decimal place accuracy.

2.4 | Pup test paradigm

Pup ultrasonic vocalizations were measured following the brief maternal separation paradigm (Brunelli et al., 1997). On PN day 10, a total of 50 individuals with a sex:line ratio of 11 high line males, 14 low line males, 12 high line females and 13 low line females were tested in this experiment. Each litter was represented by at most only one male and female pup from a total of 12 high line litters and 13 low line litters. Each litter was removed from the home cage by hand and placed in a transport cage with bedding. The transport cage was then taken to the dimly lit adjacent test room. The cage was placed on a heating pad set at medium (slightly below body temperature) for 20 min. After this maternal separation period, we randomly selected one male and one female subject for testing. Subjects were placed individually in a circular glass dish (10 cm tall and 20 cm in diameter) and taken to an adjacent testing room. We recorded ultrasonic vocalizations (USVs) for 2 min with an ultrasonic recording system Model 3EM+ from Wildlife Acoustics (https://www.wildlifeacoustics.com/) held approximately 20 cm over the container (Figure 1a).

2.5 | Adult test paradigm

Adult rat vocalizations of both lines were measured using a tickling paradigm designed to elicit play behaviour and affiliative vocalizations in rats. This paradigm was developed by Panksepp and Burgdorf (2000) and since then has been adopted by other groups (LaFollette, O'Haire, Cloutier, Blankenberger, & Gaskill, 2017). We tested 51 adult rats recording 14 high line males, 13 low line males, 12 high line females and 12 low line females (not the same individuals tested as pups). One low line male rat was excluded on the first day of handling because it refused to interact with the experimenters. For the habituation and the experiment, the 50 remaining individuals were randomly split into three groups (Ns = 17, 17, 16). We staggered the starting day of each group in one day intervals and habituated and tested each group over four consecutive days. The whole adult elicitation ("tickle") experiment and habituation procedure were completed over six consecutive days.

2.5.1 | Handling during habituation and testing

For the first two days of habituation, the rats' cages were taken out of the holding shelf and placed on a cart. On the cart, the rats were carefully removed from their home cage by picking them up with one hand placed under the thorax and supporting them with the second hand under the hind legs. For habituation, they were placed in and habituated to a test cage (30 × 30 × 15 cm) for a duration of two minutes. After habituation, the rat was carefully picked up again and placed back into its home cage, which was returned to the holding shelf. Experimenters always wore the same reusable cotton gloves while handling the animals to ensure the same smell and texture for the rats. This same protocol was used on the third day of habituation and the testing day with the addition that the test cage was covered with a metal mesh to transfer the animal to the testing room. After removing the metal lid the animals were habituated/tested in the testing room for two minutes. After habituation/testing, the rat was

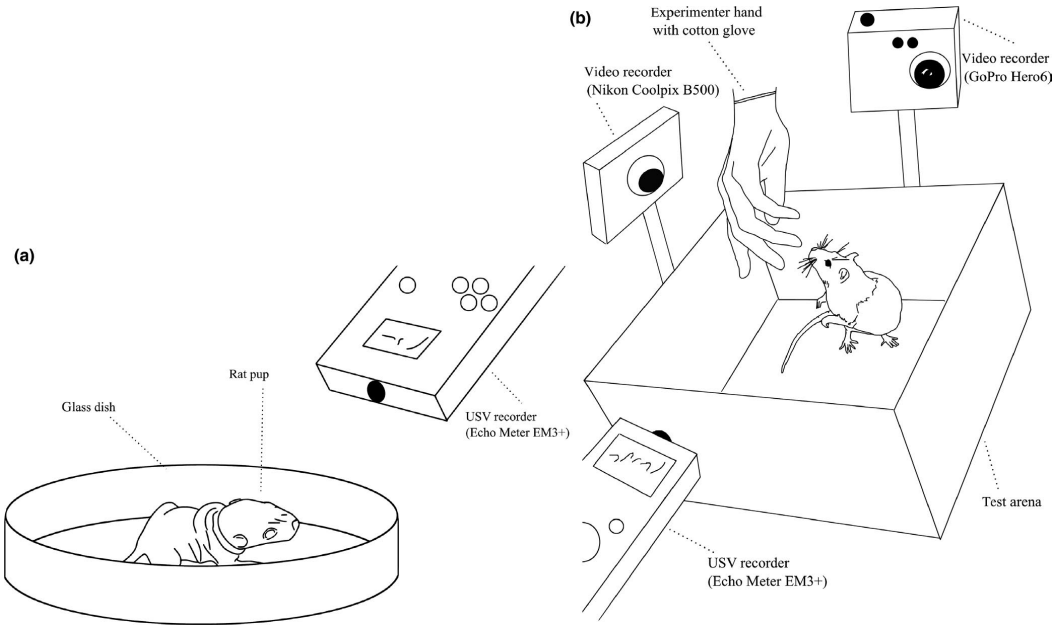

FIGURE 1 Schematic depiction of the two-minute recording period test set-ups for the elicitation paradigms. (a) Test set-up for the pup separation paradigm. The rat pup is isolated on a glass dish and recorded with a USV recorder. (b) Test set-up for the adult rat elicitation ("tickle") paradigm. The experimenter is wearing a cotton glove and interacts with the rat in the test cage. The whole procedure is recorded with two video cameras and one USV audio recorder

carefully carried back in the secured test cage and placed back into its home cage.

2.5.2 | Habituation protocol

Following Panksepp and Burgdorf (Panksepp & Burgdorf, 2000), we adapted the habituation process as follows. Each day the habituation lasted two minutes.

Day 1: The experimenters slowly introduced each rat to human contact and interaction. We encouraged the rats to sniff the experimenter's hand, and once the rat was comfortable with the experimenter, we softly stroked the animal's head and back. The habituation was completed in a test cage in the same room in which the animals were housed.

Day 2: We introduced the rat to more contact with the human hand and tried to elicit a play response. Once they were comfortable with the protocol, we picked the rats up and tickled them on the belly. The habituation was again performed in a test cage in the housing room.

Day 3: We invited the rat to play with the human's hand. All of the rats were comfortable being touched by the human experimenter within the first minute. Some rats started to chase the experimenter's hand as a playful response. In the second minute, we picked up the rats and tickled their belly. The habituation was done in a test cage in the testing room.

2.5.3 | Test protocol

The test protocol started with tickling the rat on the back, neck, and head region. These short interactions were interspersed with play invitations to chase the experimenter's hand (Figure 1b). The tickling and hand chasing alternated for the first minute. In the second minute, we picked up the rat and tickled it on the belly. This again was alternated with periods of inviting the rat to chase the experimenter's hand. The whole test was recorded with the recording equipment specified below. We cleaned the plastic cages with the odour eliminating animal-safe cleaner "Odor Mute" (Hueter Toledo Inc, Bellevue, Ohio) prior to testing each individual.

2.6 | Recording and analysis

2.6.1 | Recording equipment

We video recorded the affiliative/play task from two different angles using a Nikon Coolpix B500 (HD 1,920 × 1,080, mp4 format) and a GoPro Hero6 (HD 1,920 × 1,080, mp4 format). We audio recorded both the adult elicitation and pup separation experiment with a handheld bat detector (Echo Meter EM3+ from Wildlife Acoustics; https://www.wildlifeacoustics.com/). The sampling rate was set to 256 kHz for the tickle paradigm and 384 kHz for

the separation task. The recordings were saved in wav format with 16-bit quantization. The audio trigger mode (real time expansion) was set to 18 kHz, 0 dB SPL and 3.0 s to record continuously. The audio recordings gave us a total of 102 min of adult and 100 min of pup recordings.

2.6.2 | Acoustic definitions

In this study, we defined calls based on physical frequency range. Below, we present commonly used definitions for USVs and audible vocalizations:

"Audible vocalizations": Vocalizations with a fundamental frequency below 20 kHz and within the typical human hearing range.

"USVs": Vocalizations with a fundamental frequency above the human hearing range; in rats vocalizations ranging from 20 to 80 kHz.

2.6.3 | Acoustic analysis

We performed the acoustic analysis with four custom semi-automated scripts. All pup and adult vocalization recordings were trimmed to the two-minute test length (using Praat (Version 6.0.23; www.fon.hum.uva.nl/praat/)) prior to running the scripts.

2.7 | Adult analysis

The varying signal to noise ratio and diverse background noises (e.g. rat nails on the arena floor, sniffing the microphone, rustling of the fur) required hand labelling of all USVs and audible calls. After labelling an automated script exported the calls into individual wav files. In preparation for further analysis, all calls of most individuals were merged into two recoverable wav chains (audible and USV), but to avoid errors we created a separate USV chain for individual 38 due to his high number of 22-kHz calls. All chains were then processed by a semi-automated pitch tracking script that provided a manually adjustable visual representation of the proposed pitch tracking. For USV calls, the Praat internal function for the autocorrelation algorithm was set to: Advanced pitch settings: 0, 130000, "no," 10, 0.0003, 0.00045, 0.3, 0.35, 0.002 with Pitch settings: 25000, 90000, "Hertz," "autocorrelation," and "automatic," The same functions with the following setting changes were used for the audible vocalization chain: Advanced pitch settings: 0, 9000, "no," 15, 0.03, 0.45, 0.01, 0.35, 0.14 and Pitch settings: 1400, 6000, "Hertz," "autocorrelation," "automatic." Then, the pitch contour was extracted automatically with the Praat internal command "Extract visible pitch contour" for every call and saved as a pitch contour. A final automated script measured the acoustic parameters duration, $\bar{x}$ mean f0, max f0 and min f0 using the following Praat internal commands: Get total duration, Get mean(0, 0, "Hertz"), Get maximum (0, 0, "Hertz," "Parabolic") and Get minimum (0, 0, "Hertz," "Parabolic"), respectively. These values were saved to a text file. F0 range was then calculated by subtracting each minimum f0 from the maximum f0.

2.8 | Pup analysis

Because pups were generally immobile, there was less extraneous noise in their recordings, and this more consistent signal to noise ratio allowed us to use a completely automated system for the labelling and extraction of USV calls. We used the algorithm USVSEG (usvseg08r6; version 8, revision 6; 9.12.2018) by Tachibana, Kanno, Okabe, Kobayasi, and Okanoya (2020; running in MATLAB (version: R2018a)) for both labelling and the extraction. The USVSEG settings were set at time step (ms): 0.5, freq min (kHz): 20, freq max (kHz): 120, threshold (*SD*): 4.5, dur min (ms): 5, dur max (ms): 300, gap min (ms): 30, margin (ms): 15, wavfile output: on, image output: on, image type: orig, trace output: off, read size (s): 130 and map (kHz): 1,6. The algorithm saved all calls as individual wav files. They were manually concatenated into recoverable chains of calls for each individual using Praat. Just as with the adult analysis, all chains were put through a semi-automated script that tracked and extracted the pitch contour. The overall pitch tracking settings were pre-set (Advanced pitch settings: 0, 130000, "no," 10, 0.0003, 0.00045, 0.35, 0.4, 0.002; Pitch settings: 30000, 73000, "Hertz," "autocorrelation," "automatic") and could be adjusted and changed manually before the script ran through all calls and extracted each individual contour with the Praat internal command "Extract visible pitch contour." The last fully automated script took acoustic measurements from the previously extracted pitch contours. Duration, minimum, maximum and mean fundamental frequency were extracted with the same Praat commands as before and f0 range was again calculated by subtracting each minimum f0 from the maximum f0.

2.9 | Statistical analysis

Statistical analyses were performed in R (Version 3.6.1; www.r-project.org/) and RStudio (Version 1.2.1335; www.rstudio.com/) with the packages nlme, lme4, MASS, car, ggplot2, ggthemes, cowplot, multcomp, MuMIn, piecewiseSEM, r2glmm, visreg, dplyr, doBy, data.table, xlsx, GGally, openxlsx, car, readxl, plyr, stats, mclust, doBy, cowplot, gridExtra, gtable, grid, influence.ME, factoextra, olsrr and parallel.

We analysed calls collected from an N of 50 pups and 50 adults. We first used the package mclust to cluster all adult rat vocalizations and assign them to three categories: audible calls (<20 kHz), 22 kHz calls and 50 kHz calls (Portfors, 2007). The cluster analysis revealed seven categories of which four were in the 50 kHz range, two in the audible range and another one the 22 kHz call range. The four 50 kHz clusters were assigned to the 50 kHz category and the two audible call clusters to the audible call category. The 22 kHz range was only represented by 18 calls produced by one individual and therefore excluded

from a separate analysis due to the small sample size. The 92 audible calls were analysed separately from the 390 50 kHz USVs. The different call contribution was taken into account for both the audible and 50 kHz data. This was done by including the individuals ID as a random effect in all models; allowing us to control for individuals of both lines contributing varying numbers of calls. Clustering prior to statistical analysis was not necessary for the 7,895 pup USV calls since pups only produce one range of pup specific USV calls.

We used generalized linear models (GLMs) of the lme4 package to fit all measured f_0 parameters mean, maximum, minimum, range and duration, for all 3 categories: pup USVs, adult rat USVs and adult rat audible calls. For the pup data, no good model fit could be achieved while running GLMs on the complete data set due to singularity issues. Therefore, we collapsed all measurements down to the mean of the individual to achieve a distribution that could be analysed in our models. We then ran linear models for all acoustic measurements of pup USVs except for maximum f0 which required a generalized linear model with Gamma (log link) distribution. For adult rats, audible calls were analysed with linear mixed models, and for adult USVs we used generalized linear mixed models with Gamma (log link) distributions, but duration was fitted with a Gaussian (log link) distribution. We applied random effect intercepts for each individual in all adult rat models to correct for individual variation and call contribution. We had to refrain from creating random slopes for the individuals to avoid convergence issues. We tested all models for their stability, checked for collinearity issues and visualized their residuals to check for possible violations of distribution assumptions. Due to the sexual dimorphism in adult rats, we included a dummy-coded factor of rat line and sex resulting in four categories (high line male, low line male, high line female, low line female), dubbed line combinations. This line combination, bodyweight and their interaction were input as fixed factors to the full models and compared with null models lacking these fixed factors. To disentangle the involvement of sex and line, a third model including sex, bodyweight and their interaction were run and included as an additional control in the comparison of full-null model testing. The factors bodyweight, sex and line combination indicated possible collinearity issues with a VIF (variance inflation factor) value between 3 and 4. We plotted the affected factors and upon visual inspection we could confirm that there was considerable variation among these factors, and therefore, the VIF value was no problem for the model fit (Mundry, 2014).

3 | RESULTS

As described under "Statistical Analysis," we created a dummy-coded factor for the combination of sex and line. For concision this factor will be referred to as "line combination" below.

3.1 | Number of vocalizations in rat pups and adults

For the maternal separation paradigm, we found the full model for the number of rat pup calls to be significantly better than the null model (Pr(>Chi) < 2.2e-16). High line pups produced calls numbering in the hundreds, whereas low line pups barely produced any calls and never exceeded one hundred calls (Figure 2a; $\bar{x}$ ± *SD*: high ♂: 331 ± 123, low ♂: 16 ± 26, high ♀: 332 ± 125, low ♀: 3 ± 3). Thus, the two lines show a distinct difference in the selected trait.

In contrast, in adults we did not find such a distinct difference in the number of calls, either for adult rat USVs or adult audible calls. The full model for adult USVs, including line combination, bodyweight and their interaction, was significantly better than the null model and the reduced model (Pr(>Chi) = 1.176e-10) in explaining our data's distribution (Figure 2b; $\bar{x}$ ± *SD*: high ♂: 5 ± 7, low ♂: 16 ± 24, high ♀: 8 ± 15, low ♀: 3 ± 5). The full model for adult audible calls was not significantly better than the reduced model just including sex, bodyweight and their interaction (Figure 2c; Pr(>Chi) = 0.372; $\bar{x}$ ± *SD*: high ♂: 3 ± 4, low ♂: 1 ± 1, high ♀: 2 ± 6, low ♀: 2 ± 4). Thus, while pup USVs were much more frequent in the line selected for high vocal production, adult lines did not show such a clear distinction in the number of calls produced. Whereas the number of the adult audible calls did not differ across lines the significant difference found in the adult USV calls seems to be driven by the high number of USVs produced by low line males.

3.2 | Pup USV acoustics

Pup USV measurements mean f0, maximum f0, minimum f0 and duration, recorded during the separation paradigm, were most accurately predicted by the full models including fixed factors line combination, bodyweight and their interaction (mean: Pr(>Chi) = 0.0165, max: Pr(>Chi) = 0.004972, min: Pr(>Chi) = 0.0124, dur: Pr(>Chi) < 0.000025). Pups at the age of testing did not differ significantly in bodyweight (Figure 3f; Pr(>Chi) = 0.6502). In both the mean f0 ($\bar{x}$ ± *SD*: high ♂: 42,250 ± 1,670, low ♂:45,870 ± 6,302, high ♀: 45,181 ± 1,623, low ♀: 52,965 ± 9,861), maximum f0 ($\bar{x}$ ± *SD*: high ♂: 45,381 ± 2,612, low ♂:47,951 ± 7,491, high ♀: 47,622 ± 1,877, low ♀:57,513 ± 10,715) and minimum f0 ($\bar{x}$ ± *SD*: high ♂: 39,785 ± 1,407, low ♂: 44,603 ± 6,257, high ♀: 42,615 ± 1,172, low ♀:48,856 ± 8,995), high line males on average produced the lowest frequencies and low line females the highest (Figure 3a,b,c). Based on visual inspection, across these three f0 measures low line female and male pups produced USVs with a higher degree of variability in comparison with their high line counterparts. Average USV call duration was longest in males of the high line and lowest in females of the low line (Figure 3e; $\bar{x}$ ± *SD*: high ♂: 0.15 ± 0.03, low ♂:0.11 ± 0.04, high ♀:0.13 ± 0.02, low ♀:0.08 ± 0.03). High line individuals of both sexes produced, on average, longer calls than low line pups. We did not find the f0 range model to be significantly better than the null model (Figure 3d).

3.3 | Adult USV acoustics

In contrast to the pup results, adult rat USVs did not differ across line combinations; mean f0 ($\bar{x}$ ± *SD*: high ♂: 56,696 ± 8,690, low ♂:

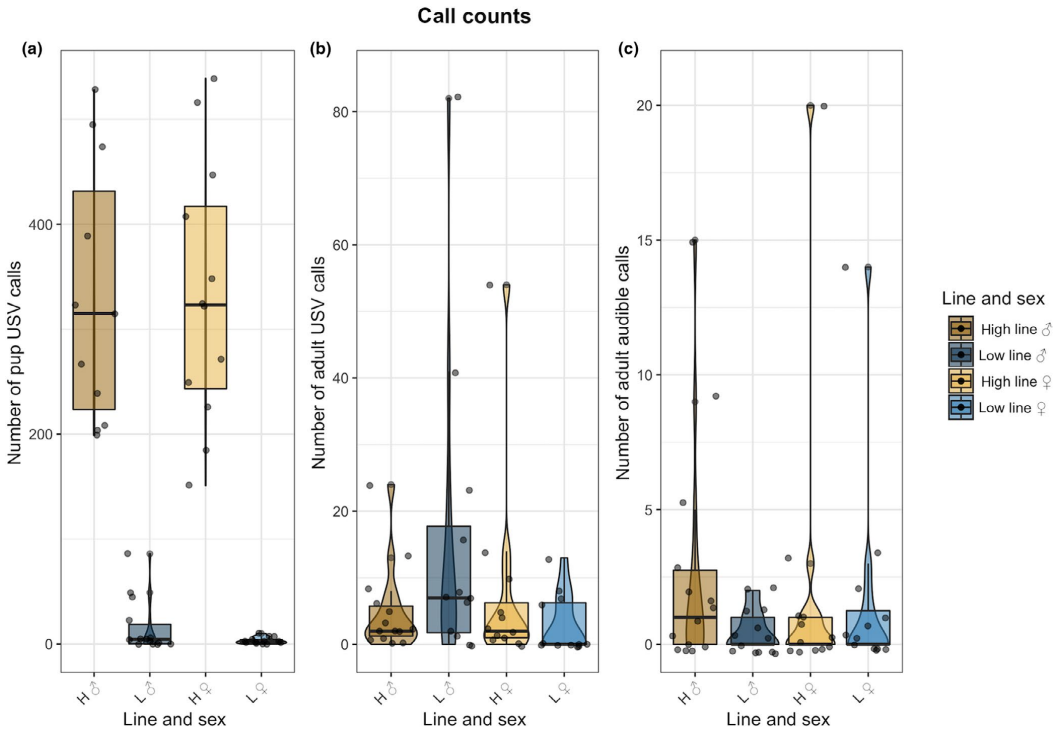

FIGURE 2 Total counts of pup and adult vocalizations per individual. The different lines are indicated by letters (H = high line, L = low line), and sex is indicated by symbols (♂ = male, ♀ = female). (a) Total number of USVs produced by pups plotted over the line and sex combinations. (b) Total number of USVs produced by adult rats and plotted over the line ad sex combinations. (c) Total number of audible calls produced by adult rats plotted over the line and sex combinations

57,381 ± 9,435, high ♀: 61,122 ± 9,741, low ♀: 61,096 ± 12,487), maximum f0 ($\bar{x}$ ± *SD*: high ♂: 60,515 ± 11,234, low ♂:63,007 ± 13,271, high ♀:65,121 ± 11,059, low ♀:65,170 ± 14,205), minimum f0 ($\bar{x}$ ± *SD*: high ♂: 51,173 ± 12,258, low ♂: 50,917 ± 11,930, high ♀: 55,512 ± 12,764, low ♀: 57,915 ± 14,115) and USV duration ($\bar{x}$ ± *SD*: high ♂: 0.03 ± 0.04, low ♂: 0.03 ± 0.01, high ♀: 0.03 ± 0.02, low ♀: 0.02 ± 0.02). For all acoustic parameters, full generalized linear mixed models were not significantly better than the null model (Figure 4). Due to persistent overdispersion in the f0 range (1.6), the technically significant difference of Pr(>Chisq) = 0.0195 over the null model cannot be interpreted as reliable. Only the full model for bodyweight was significantly better than the null model, showing a distinct difference in bodyweight explainable by both sex dimorphism and line combination (Pr(>Chisq) < 2.2e-16). Thus, selection focused on pup USVs did not have a corresponding effect on adult USV acoustics.

3.4 | Adult audible (low-frequency) acoustics

Finally, we come to low-frequency (audible) vocalizations. Unlike the models for adult USVs, the full models for audible vocalizations with the fixed factors line combination, bodyweight, and their interaction were a better fit than the null model for both $\bar{x}$ mean f0 (Pr(>Chisq) = 0.01803; Figure 5a; $\bar{x}$ ± *SD*: high ♂: 2,450 ± 235, low ♂:2,359 ± 339, high ♀:2,710 ± 108, low ♀:2,962 ± 335) and maximum f0 (Pr(>Chisq) = 0.01864; Figure 5b; $\bar{x}$ ± *SD*: high ♂: 2,330 ± 239, low ♂:2,210 ± 323, high ♀: 2,584 ± 102, low ♀:2,826 ± 310), in adult audible calls. The full model for minimum f0, f0 range and call duration were not significantly better than the null model (Figure 5c,d,e). Thus, to our surprise, selection on pup USV calls led to complex changes in adult audible calls, but not in adult USVs. Low line females produced the highest mean and maximum f0, and low line males produced both the lowest mean and maximum f0; high line males had a higher average f0 compared with the low line males.

Again (since the same individuals provided data), the full model for bodyweight was significantly better than the null model (Pr(>Chisq) < 2.2e-16; Figure 5f). However, body size was not consistently related to f0. Although the males of the high line were heavier than their low line counterparts, they produced on average a higher mean and maximum fundamental frequency. This was not true for the high line females, although they too were heavier than their low line counterparts.

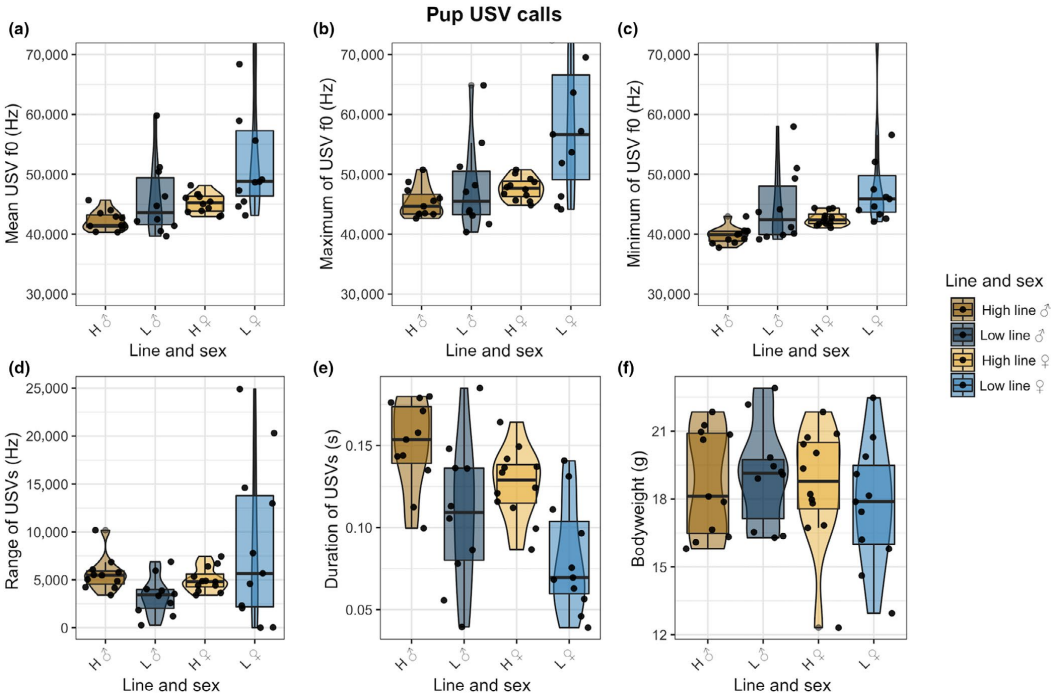

FIGURE 3 Pup USV acoustic measurements. The different lines are indicated by letters (H = high line, L = low line) and sex is indicated by symbols (♂ = male, ♀ = female). (a) Mean f0 of pup USVs plotted over the line and sex combinations. (b) Maximum f0 of pup USVs plotted over the line and sex combinations. (c) Minimum f0 of pup USVs plotted over the line and sex combinations. (d) f0 range of pup USVs plotted over the line and sex combinations. (e) Call duration of pup USVs over the line and sex combination. (f) Bodyweight in grams over the line and sex combination

4 | DISCUSSION

Our results indicate an unexpected pattern of changes in the low-frequency vocalizations of adult rats after selection on the USVs of rat pups. Despite our hypothesis that selection for pup USV rate should impact adult USV production without affecting the lower frequency calls, we found the opposite to be true. While adult USV call metrics did not differ, we found low-frequency (human-audible) calls to be affected by the pup USV selection procedure. Male adult rats that were bred to produce many USVs as pups (high line) had a higher fundamental frequency in audible calls than low line males. The opposite was true for female adult rats; high line females produced audible calls lower in f0 than the low line females. Apart from the findings in the adult animals, we also found interesting changes in the pup USVs. High line pups had lower f0s than their respective low line counterparts. To sum up, we analysed acoustic parameters in calls produced by rat lines selected for high or low ultrasound production in both pups and adults of both lines. We found selection pressures on neonate acoustic output to further impact the animals' vocal communication system across development.

Our first hypothesis predicted that adult rats produce USVs in accordance with their line-specific behaviour as pups, for example low line individuals should produce few USVs as adults and high line individuals should produce many. Contrary to our prediction, we found that adult low line males produced, on average, slightly more USVs than the high line. This difference could potentially be explained by an inherently different stress response to the tickle paradigm within the two lines. High line rats have a more anxious and depressed stress response compared with low line individuals (Brunelli, 2005; Brunelli et al., 2010; Zimmerberg et al., 2005). Taking line-specific stress responses into consideration, one might reasonably expect this precise pattern of less stressed (low line) rats vocalizing more during the human interaction (tickling).

Our second hypothesis predicted that the previously observed longer and broader-band calls (Spence et al., 2016) of high line pups would also be found in our population of pups. We did not find a broader f0 range in the high line pups, but we did find a longer call duration. Furthermore, we predicted longer and broader-band calls in adult high-line USVs, but neither USV range nor duration differed between lines in adults.

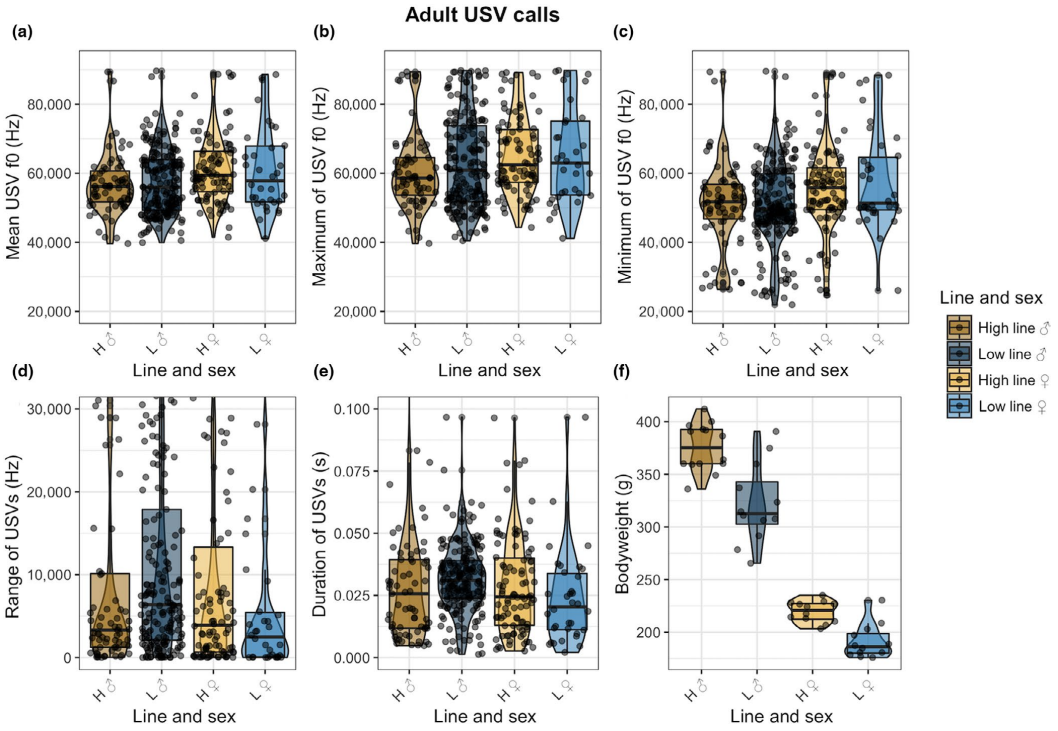

FIGURE 4 Adult USV acoustic measurements. The different lines are indicated by letters (H = high line, L = low line), and sex is indicated by symbols (♂ = male, ♀ = female). (a) Mean f0 of adult USVs plotted over the line and sex combinations. (b) Maximum f0 of adult USVs plotted over the line and sex combinations. (c) Minimum f0 of adult USVs plotted over the line and sex combinations. (d) f0 range of adult USVs plotted over the line and sex combinations. (e) Call duration of adult USVs over the line and sex combination. (f) Bodyweight in grams over the line and sex combination

Our third hypothesis predicted that USV-focussed breeding would have little measurable impact on low-frequency (audible) calls. Again, our data did not support this hypothesis. While rat line was irrelevant in predicting mean f0 of adult USV calls, rat line was highly important in predicting the data distribution of adult audible f0 call parameters.

All of our results are related to different measurements of fundamental frequency, suggesting a potential link between line-specific stress responses and its possible influence on f0 measurements (Briefer, 2012). So far, three independent rat breeding programs (Brunelli, 2005; Burgdorf, Panksepp, Brudzynski, Kroes, & Moskal, 2005; Frank et al., 2006), with different selection procedures, have linked selection on pup USV production with differences in adult anxiety. This may suggest that differences found in the acoustic parameters measured here were due to line-specific stress responses impacting f0 measurements. Schehka and Zimmermann (Schehka & Zimmermann, 2009) found an increased f0 to be an indication of emotional arousal, which again directly links to stress coping mechanisms (cf., Briefer, 2012). Consistent with this idea, we found that high line males, which are more responsive to stress and more easily aroused than their low line counterparts, produced higher frequencies than males of the low line. But the opposite is true for the females: high line females produced on average lower mean f0s in their audible calls than did low line females.

We argue that this finding, and the fact that adult rats of both lines produced 50kHz USVs (indicators of positive affective states) that did not differ in their call metrics, leads to the conclusion that acoustic line differences are not simply a reflection of different stress coping. Rather we suggest, since animals of both lines playfully engaged in social interaction with the human experimenter, the audible calls produced in this context do not reflect differing stress levels. If correct, this argument suggests that the different frequencies of audible calls may be a product of sex-dependent laryngeal differences (e.g. larynx size or mineralization, or vocal fold length).

Another peculiar finding was the distinct difference of f0 measurements in pup USVs. We found pup males to produce lower f0s than females of the same line, despite no significant difference in bodyweight. Aside from this sex dimorphism in pup USV f0, we also found high line individuals to produce lower f0s than their respective low line counterparts. Bowers and colleagues (Bowers,

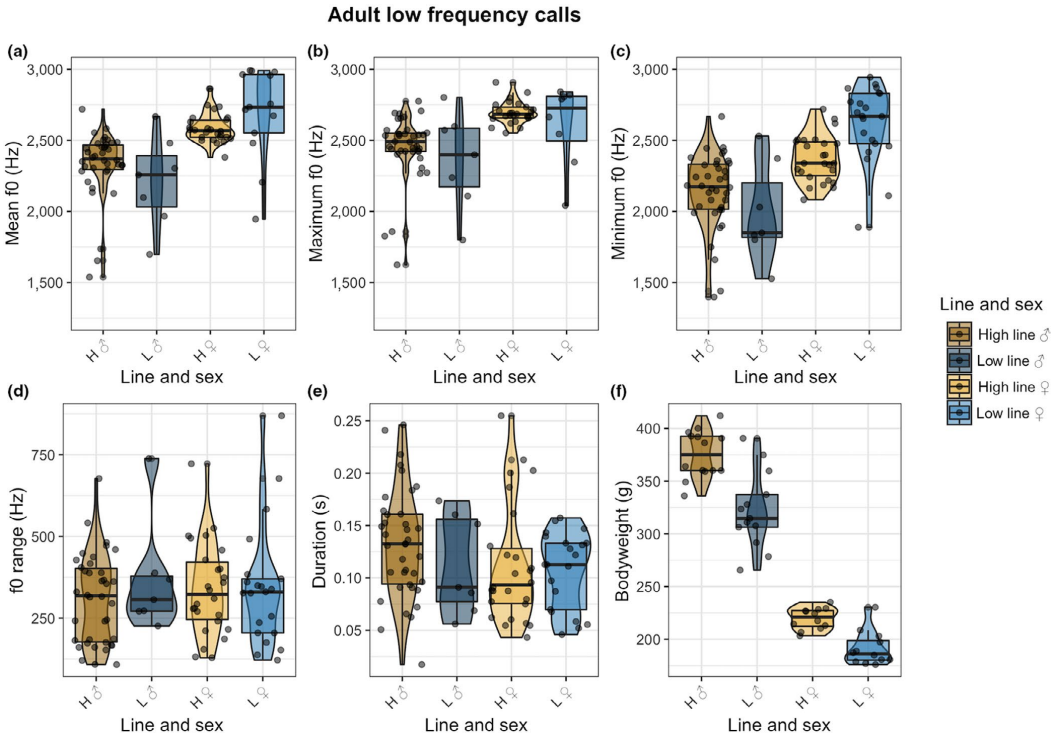

FIGURE 5 Adult audible call acoustic measurements. The different lines are indicated by letters (H = high line, L = low line) and sex is indicated by symbols (♂ = male, ♀ = female). (a) Mean f0 of adult audible calls plotted over the line and sex combinations. (b) Maximum f0 of adult audible calls plotted over the line and sex combinations. (c) Minimum f0 of adult audible calls plotted over the line and sex combinations. (d) f0 range of adult audible calls plotted over the line and sex combinations. (e) Call duration of adult audible calls over the line and sex combination. (f) Bodyweight in grams over the line and sex combination

Perez-Pouchoulen, Edwards, & McCarthy, 2013) reported lower USV f0 in male pups and a higher number of USVs produced during maternal separation. While we did not find sex-specific differences in the number of pup USVs produced, we did find males of both lines to produce lower f0s compared with their respective lines' females. Bower's and colleagues' findings help explain the sex dimorphism in pups USV f0, but not this line-specific difference. Male high line pups produced a lower f0 than low line males; the same pattern applied to females.

Our results only allow us to speculate whether these line-specific, selection-induced acoustic differences occur due to neuro-behavioural or physiological changes; they might also be found in their interplay. The multiple effects of artificial selection on one specific type of vocal output on other vocalizations (or behaviours) that we document here in rats have interesting potential implications in the context of vocal changes in domestication. Some domestic animals show somewhat different acoustic behaviours than their wild counterparts. For example, adult dogs bark more than wolves (Feddersen-Petersen, 2000) and adult domestic cats meow, but in wildcats this vocal type is common only juveniles. Physiologically, adults in both sub-species are able to produce these vocalizations, but nevertheless they produce them in different proportions, and the reasons for these vocal differences remain unclear.

More generally, our results suggest that selection on a particular vocalization type, at a particular developmental stage, may have surprising and unpredictable effects on other vocalization types and/or at other developmental stages. This has interesting implications for trade-offs in the evolution of communication (cf. Dunn et al., 2015), suggesting that (as in the evolution of body form) selection on a single trait may have a host of unselected by-product effects on other traits (Kozłowski & Weiner, 1997). The example we have uncovered in the current study is experimentally tractable, insofar as the specific selection history of the animals is known, and the physiological basis for the production of vocalizations is well-understood. The results of further investigations in our example will have implications for the evolution of communication in other species, where selection history (and perhaps details of vocal production) is not as well understood.

In summary, artificially selected line-specific differences found in early developmental stages were lost in later developmental

stages, but had unpredicted effects on the acoustics of different vocalization types. The selection pressure, focused entirely on the number of USVs produced by pups, influenced the production of adult audible calls (which are based on a different acoustic production mechanism), but did not affect adult USV calls. Our results indicate a surprising form of pleiotropy that violated most of our a priori predictions. Our evaluation of body size effects demonstrates that this is not a simple result of body size changes, and also shows that vocal production characteristics can readily change under selection, independent of changes in body size. Despite difference in temperament between the two lines, we do not think that these results affect different anxiety or arousal levels either, though this should be controlled for in future research. This leaves changes in vocal anatomy between the lines as the most likely explanation of our acoustic results. Anatomical investigations into possible changes in larynx morphology are in progress to evaluate and investigate the precise nature of these effects, and thus to better understand the effects that selection for acoustic output may have on vocal organs.

ACKNOWLEDGEMENTS

We thank the animal care staff at the Williams College for taking excellent care of the rats. The authors declare no conflict of interests.

ORCID

Raffaela Lesch https://orcid.org/0000-0001-7151-252X
W. Tecumseh Fitch https://orcid.org/0000-0003-1830-0928

REFERENCES

Allin, B. Y. J. T., & Banks, E. M. (1972). Functional aspects of ultrasound production by infant albino rats (*Rattus norvegicus*). *Animal Behaviour*, *20*, 175–185. https://doi.org/10.1016/S0003-3472(72)80189-1

Bowers, J. M., Perez-Pouchoulen, M., Edwards, N. S., & McCarthy, M. M. (2013). Foxp2 mediates sex differences in ultrasonic vocalization by rat pups and directs order of maternal retrieval. *Journal of Neuroscience*, *33*(8), 3276–3283. https://doi.org/10.1523/JNEUROSCI.0425-12.2013

Briefer, E. F. (2012). Vocal expression of emotions in mammals: Mechanisms of production and evidence. *Journal of Zoology*, *288*(1), 1–20. https://doi.org/10.1111/j.1469-7998.2012.00920.x

Brudzynski, S. M. (2010). *Handbook of mammalian vocalization: An integrative neuroscience approach*, 1st ed. Amsterdam, The Netherlands: Elsevier. https://doi.org/10.1016/s1569-7339(10)70001-1

Brudzynski, S. M. (2015). Pharmacology of ultrasonic vocalizations in adult rats: Significance, call classification and neural substrate. *Current Neuropharmacology*, *13*(2), 180–192. https://doi.org/10.2174/1570159X13999150210141444

Brudzynski, S. M. (2018). *Handbook of ultrasonic vocalization: A handbook into the emotional brain*, 1st ed. London, UK: Levy, Niki.

Brunelli, S. A. (2005). Selective breeding for an infant phenotype: Rat pup ultrasonic vocalization (USV). *Behavior Genetics*, *35*(1), 53–65. https://doi.org/10.1007/s10519-004-0855-6

Brunelli, S. A., & Hofer, M. A. (2007). Selective breeding for infant rat separation-induced ultrasonic vocalizations: Developmental precursors of passive and active coping styles. *Behavioural Brain Research*, *182*(2), 193–207. https://doi.org/10.1016/j.bbr.2007.04.014

Brunelli, S. A., Shair, H. N., & Hofer, M. A. (1994). Hypothermic vocalizations of rat pups (*Rattus norvegicus*) elicit and direct maternal search behavior. *Journal of Comparative Psychology*, *108*(3), 298–303. https://doi.org/10.1037/0735-7036.108.3.298

Brunelli, S. A., Vinocur, D. D., Soo-Hoo, D., & Hofer, M. A. (1997). Five generations of selective breeding for ultrasonic vocalization (USV) responses in N:NIH strain rats. *Developmental Psychobiology*, *31*(4), 255–265. https://doi.org/10.1002/(SICI)1098-2302(199712)31:4<255:AID-DEV3>3.0.CO;2-Q

Brunelli, S. A., Zimmerberg, B., & Hofer, M. A. (2010). Developmental effects of selective breeding for an infant trait. In K. E. Hood, C. T. Halpern, G. Greenberg, & R. M. Lerner (Eds.), *Handbook of developmental science, behavior, and genetics* (pp. 285–322). Malden, MA: Wiley-Blackwell.

Burgdorf, J., Panksepp, J., Brudzynski, S. M., Beinfeld, M. C., Cromwell, H. C., Kroes, R. A., & Moskal, J. R. (2009). The effects of selective breeding for differential rates of 50-kHz ultrasonic vocalizations on emotional behavior in rats. *Developmental Psychobiology*, *51*(1), 34–46. https://doi.org/10.1002/dev.20343

Burgdorf, J., Panksepp, J., Brudzynski, S. M., Kroes, R., & Moskal, J. R. (2005). Breeding for 50-kHz positive affective vocalization in rats. *Behavior Genetics*, *35*(1), 67–72. https://doi.org/10.1007/s10519-004-0856-5

Carden, S. E., & Hofer, M. A. (1990). Independence of benzodiazepine and opiate action in the suppression of isolation distress in rat pups. *Behavioral Neuroscience*, *104*(1), 160–166. https://doi.org/10.1037/0735-7044.104.1.160

Cheverud, J. M. (1996). Developmental integration and the evolution of pleiotropy. *American Zoologist*, *36*(1), 44–50. https://doi.org/10.1093/icb/36.1.44

Dunn, J. C., Halenar, L. B., Davies, T. G., Cristobal-Azkarate, J., Reby, D., Sykes, D., … Knapp, L. A. (2015). Evolutionary trade-off between vocal tract and testes dimensions in howler monkeys. *Current Biology*, *25*(21), 2839–2844. https://doi.org/10.1016/j.cub.2015.09.029

Feddersen-Petersen, D. U. (2000). Vocalization of European wolves (*Canis lupus lupus L.*) and various dog breeds (*Canis lupus f. fam.*). *Archiv Für Tierzucht*, *43*(4), 387–397.

Frank, E., Salchner, P., Aldag, J. M., Salomé, N., Singewald, N., Landgraf, R., & Wigger, A. (2006). Genetic predisposition to anxiety-related behavior determines coping style, neuroendocrine responses, and neuronal activation during social defeat. *Behavioral Neuroscience*, *120*(1), 60–71. https://doi.org/10.1037/0735-7044.120.1.60

Herbst, C. T., Stoeger, A. S., Frey, R., Lohscheller, J., Titze, I. R., Gumpenberger, M., & Fitch, W. T. (2012). How low can you go? Physical production mechanism of elephant infrasonic vocalizations. *Science*, *337*(6094), 595–599. https://doi.org/10.1126/science.1219712

Johnson, A. M., Ciucci, M. R., Russell, J. A., Hammer, M. J., & Connor, N. P. (2010). Ultrasonic output from the excised rat larynx. *The Journal of the Acoustical Society of America*, *128*(2), EL75–EL79. https://doi.org/10.1121/1.3462234

Kelm-Nelson, C. A., Lenell, C., Johnson, A. M., & Ciucci, M. R. (2018). Laryngeal activity for production of ultrasonic vocalizations in rats. In S. M. Brudzynski (Ed.), *Handbook of behavioral neuroscience* (1st ed., Vol. *25*, pp. 37–43). Amsterdam, The Netherlands: Elsevier B.V.

Klingenberg, C. P. (2008). Morphological integration and developmental modularity. *Annual Review of Ecology, Evolution, and Systematics*, *39*(1), 115–132. https://doi.org/10.1146/annurev.ecolsys.37.091305.110054

Kozłowski, J., & Weiner, J. (1997). Interspecific allometries are by-products of body size optimization. *American Naturalist*, *149*(2), 352–380. https://doi.org/10.1086/285994

LaFollette, M. R., O'Haire, M. E., Cloutier, S., Blankenberger, W. B., & Gaskill, B. N. (2017). Rat tickling: A systematic review of applications,

outcomes, and moderators. *PLoS One*, *12*(4), 1–22. https://doi.org/10.1371/journal.pone.0175320

Mahrt, E., Agarwal, A., Perkel, D., Portfors, C., & Elemans, C. P. H. (2016). Mice produce ultrasonic vocalizations by intra-laryngeal planar impinging jets. *Current Biology*, *26*(19), R880–R881. https://doi.org/10.1016/j.cub.2016.08.032

Mundry, R. (2014). Statistical issues and assumptions of phylogenetic generalized least squares. In L. Z. Garamzegi (Ed.), *Modern phylogenetic comparative methods and their application in evolutionnary biology* (pp. 131–153). Berlin Heidelberg: Springer-Verlag.

Paaby, A. B., & Rockman, M. V. (2013). The many faces of pleiotropy. *Trends in Genetics: TIG*, *29*(2), 66–73. https://doi.org/10.1016/j.tig.2012.10.010

Panksepp, J., & Burgdorf, J. (2000). 50-kHz chirping (laughter?) in response to conditioned and unconditioned tickle-induced reward in rats: Effects of social housing and genetic variables. *Behavioural Brain Research*, *115*(1), 25–38. https://doi.org/10.1016/S0166-4328(00)00238-2

Portfors, C. V. (2007). Types and functions of ultrasonic vocalizations in laboratory rats and mice. *Journal of the American Association for Laboratory Animal Science : JAALAS*, *46*(1), 28–34.

Riede, T., Borgard, H. L., & Pasch, B. (2017). Laryngeal airway reconstruction indicates that rodent ultrasonic vocalizations are produced by an edge-tone mechanism. *Royal Society Open Science*, *4*(11), 170976. https://doi.org/10.1098/rsos.170976

Riede, T., York, A., Furst, S., Müller, R., & Seelecke, S. (2011). Elasticity and stress relaxation of a very small vocal fold. *Journal of Biomechanics*, *44*(10), 1936–1940. https://doi.org/10.1016/j.jbiomech.2011.04.024

Roberts, L. H. (1975). The rodent ultrasound production mechanism. *Ultrasonics*, *13*(2), 83–88. https://doi.org/10.1016/0041-624X(75)90052-9

Sanders, I., Weisz, D. J., Yang, B. Y., Fung, K., & Amirali, A. (2001). The mechanism of ultrasonic vocalization in the rat. *Society of Neuroscience Abstracts*, *27*(88.19).

Schehka, S., & Zimmermann, E. (2009). Acoustic features to arousal and identity in disturbance calls of tree shrews (*Tupaia belangeri*). *Behavioural Brain Research*, *203*(2), 223–231. https://doi.org/10.1016/j.bbr.2009.05.007

Shair, H. N. (2007). Acquisition and expression of a socially mediated separation response. *Behavioural Brain Research*, *182*(2), 180–192. https://doi.org/10.1016/j.bbr.2007.02.016

Simola, N., & Brudzynski, S. M. (2018). Rat 50-kHz ultrasonic vocalizations as a tool in studying neurochemical mechanisms that regulate positive emotional states. *Journal of Neuroscience Methods*, *310*, 33–44. https://doi.org/10.1016/j.jneumeth.2018.06.018

Spence, H. R., Aslam, A. M., Hofer, M. A., Brunelli, S. A., & Shair, H. N. (2016). Vocal coselection in rat pup ultrasonic vocalizations. *Ecology and Evolution*, *6*(7), 1922–1929. https://doi.org/10.1002/ece3.1907

Stearns, F. W. (2010). One hundred years of pleiotropy: A retrospective. *Genetics*, *186*(3), 767–773. https://doi.org/10.1534/genetics.110.122549

Tachibana, R. O., Kanno, K., Okabe, S., Kobayasi, K. I., & Okanoya, K. (2020). USVSEG: A robust method for segmentation of ultrasonic vocalizations in rodents. *PLoS ONE*, *15*(2), 1–16. https://10.1371/journal.pone.0228907

Wilkins, A. S., Wrangham, R. W., & Fitch, W. T. (2014). The "domestication syndrome" in mammals: A unified explanation based on neural crest cell behavior and genetics. *Genetics*, *197*(3), 795–808. https://doi.org/10.1534/genetics.114.165423

Winslow, J. T., & Insel, T. R. (1991). The infant rat separation paradigm: A novel test for novel anxiolytics. *Trends in Pharmacological Sciences*, *12*(C), 402–404. https://doi.org/10.1016/0165-6147(91)90616-Z

Zimmerberg, B., Brunelli, S. A., Fluty, A. J., & Frye, C. A. (2005). Differences in affective behaviors and hippocampal allopregnanolone levels in adult rats of lines selectively bred for infantile vocalizations. *Behavioural Brain Research*, *159*(2), 301–311. https://doi.org/10.1016/j.bbr.2004.11.009

Zimmerberg, B., Brunelli, S. A., & Hofer, M. A. (1994). Reduction of rat pup ultrasonic vocalizations by the neuroactive steroid allopregnanolone. *Pharmacology, Biochemistry and Behavior*, *47*(3), 735–738. https://doi.org/10.1016/0091-3057(94)90181-3

How to cite this article: Lesch R, Orozco A, Shilling M, Zimmerberg B, Fitch WT. Selection on ultrasonic call rate in neonatal rats affects low frequency, but not ultrasonic, vocalizations in adults. *Ethology*. 2020;00:1–12. https://doi.org/10.1111/eth.13075

Chapter 3|

Selection on vocal output affects laryngeal morphology in rats

This chapter is accepted as an article in a peer-reviewed journal.

Citation: **Lesch, R.**; Schwaha, T.; Orozco, A.; Shilling, M.; Brunelli, S.; Hofer, M.; Bowling, D.; Zimmerberg, B.; Fitch, W. T. Selection on vocal output affects laryngeal morphology in rats. Journal of Anatomy; accepted

Selection on vocal output affects laryngeal morphology in rats

Rat selective breeding and larynx morphology

Raffaela Lesch*[a], Thomas Schwaha[d], Andrea Orozco [b], Margaret Shilling [b], Susan Brunelli [c], Myron Hofer [c], Daniel L. Bowling[a,e], Betty Zimmerberg [b], W. Tecumseh Fitch*[a]

[a] *Department of Behavioral & Cognitive Biology, University of Vienna, Vienna, Austria*

[b] *Department of Psychology, Williams College, Williamstown, MA, USA*

[c] *Department of Psychiatry, Columbia University, New York, NY , USA*

[d] *Department of Evolutionary Biology, Integrative Zoology, University of Vienna, Vienna, Austria*

[e] *Department of Psychiatry and Behavioral Sciences, Stanford University School of Medicine*

** corresponding authors*

Abstract

Although laryngeal morphology often reflects adaptations for vocalization, the structural consequences of selection for particular aspects of vocal behavior remain poorly understood. In this study we investigated the effects of increased ultrasonic calling in pups on the adult larynx morphology in selectively bred rat lines. Laryngeal morphology was assessed using multiple techniques: mineralized cartilage volumes were compared in 3D-models derived from microCT scans, internal structure was compared using clearing and staining procedures combined with microscopy, cellular structure was compared using histology and microscopy, and element composition was assessed with scanning energy dispersive X-ray spectroscopy. Our results show that adult rats from lines bred to produce ultrasonic calls at higher rates as pups have shorter vocal folds and a more mineralized thyroid cartilage compared to rats bred to produce ultrasonic calls at lower rates. The change in vocal fold length appears to account for differences in low-frequency calls in these two rat lines. We suggest that the observed increases in mineralization of the thyroid cartilage in the high-ultrasound lineage provide increased reinforcement of the laryngeal structure during ultrasonic call production. Our findings therefore demonstrate an effect of selection for vocal behavior on laryngeal morphology, with acoustic consequences.

Keywords

vocal anatomy; larynx; development; rodent communication; ultrasonic vocalizations; morphology; selection.

Introduction

The mammalian larynx has dual functions of airway protection and acoustic signal production (Shiba, 2009). Satisfying both has produced an organ that is effective for vocal production, but not particularly energetically efficient (Titze, 2018). The functional morphology of the mammalian larynx is relatively well understood, mostly due to extensive research on its role in the production of human speech, and the realization that many of the essential principles (e.g., as described by source-filter theory and myoelastic-aerodynamic theory) are widely conserved across mammalian clades (Titze, 1994; Riede and Fitch, 1999; Fitch, 2016). In contrast, the avian syrinx, which functions more purely in sound production due to its location deep in the chest, exhibits a much higher degree of diversity across species (Fitch and Hauser, 2006). Thus, the fact that the larynx must satisfy requirements for at least two critical functions can be estimated to have constrained its evolution and development, particularly with respect to modifications made solely for the purpose of vocal communication.

The mammalian larynx consists of a cartilaginous thyroid, arytenoids, cricoid, epiglottis, and a supporting set of hyoid bones (Schneider, 1964; Harrison, 1995). Despite a high degree of interspecific conservation, a number of impressive modifications of the mammalian larynx have been documented (Fitch, 2016). Among such adaptations we find enlargements of the larynx itself in howler monkeys, pads on the vocal folds in lions, or the ability to produce high frequency whistles in rodents (Klemuk *et al.*, 2011; Dunn *et al.*, 2015; Dent and Fay, 2018). In some rodents, such as rats and mice, another structure, the alar cartilage is added to the "standard" structures of the mammalian larynx (Inagi, Schultz and Ford, 1998).

Murine rodents have two distinct vocalization ranges, defined by low-frequency calls and ultrasonic vocalizations (USVs) (Brudzynski, 2005, 2018). This provides a very interesting and unusual opportunity to analyze functional changes to larynx morphology. USVs in rats can further be categorized into two distinct ultrasonic vocalization ranges used to communicate different affective states of the caller: 22kHz vocalizations communicate negative affect whereas 50kHz calls communicate positive affect (Portfors, 2007). These two ranges are specific to adult rats; when isolated, rat pups produce USVs in a single range centered around 40kHz (Portfors, 2007). Apart from these well-studied ultrasonic vocalizations, rats also produce low-frequency calls within the human hearing range (Brudzynski, 2010, 2018). Extrapolating from what is known about vocalization in other

mammals, the production mechanism of low-frequency calls in rodents is predictable on the basis of "ordinary" laryngeal vocal fold vibration (Roberts, 1975b; Riede *et al.*, 2011; Herbst *et al.*, 2012). Previous studies agree that USVs are also produced in the larynx, but by a different "whistle" mechanism (Sanders *et al.*, 2001; Johnson *et al.*, 2010; Mahrt *et al.*, 2016; Riede, Borgard and Pasch, 2017). However, debate remains over the nature of this mechanism: Mahrt and colleagues suggest a planar impinging jet. In contrast, Riede and colleagues argue for an edge-tone whistle mechanism (Mahrt *et al.*, 2016; Riede, Borgard and Pasch, 2017). These two different mechanisms are associated with different types of morphological changes in the larynx, for example in the alar cartilage (thought to be essential in the production of USVs in rat communication (Inagi, Schultz and Ford, 1998; Riede, Borgard and Pasch, 2017)). This leads to the question addressed by our study: how does selection for USV production rate influence laryngeal anatomy, including both USV-related anatomy, and morphological characteristics associated with low-frequency vocal production?

We address this question here by analyzing vocal anatomy in two rat lines that have been selected for over 50 generations to produce low or high rates of USVs as pups, during a maternal separation paradigm (Brunelli *et al.*, 1997; Brunelli and Hofer, 2007). This sustained artificial selection upon USV call rate in pups yielded two distinct rat lines with both different vocal behavior and distinctive stress responses (Zimmerberg, Brunelli and Hofer, 1994; Brunelli and Hofer, 2007). An earlier study of rats from these two selected lines revealed clear effects of selection on vocal acoustics (Lesch *et al.*, 2020), but the morphological basis of the line-specific acoustic differences documented there remain unknown.

In particular, our previous study showed that selection on rat pup USV rate did not affect acoustic parameters of adult USVs, but rather affected acoustic aspects of adult low-frequency calls. Specifically, we found that "high line" individuals (i.e., pups selected for increased USV production) were heavier in body weight than "low line" rats (i.e., pups selected for decreased USV production), but did not produce lower frequencies in their calls. This apparent exception to a general correlation (allometry) between body mass and fundamental frequency of low-frequency calls led us to formulate the hypothesis that selection for acoustic output in pups will lead to changes in adult laryngeal morphology. In the present study, we used multiple visualization and measurement techniques to quantitatively demonstrate an effect of artificial selection for a specific aspect of vocal behavior on the laryngeal morphology of rats. We found that larynges of high line adults have shorter vocal folds and a more mineralized thyroid cartilage compared to low line adults.

These results support our hypothesis that selection on pup calling will lead to differential changes in adult laryngeal morphology, depending on rat line.

Methods

Rat lines

The rat lines studied here were initially described in Brunelli *et al.*, (1997) and have been bred since the 1990s (57-58 generations) on the basis of differences in the rate of USV calls produced by pups in response to a maternal separation paradigm. Both lines were developed from the same founding population of N:NIH *Rattus norvegicus domestica*. The N:NIH strain was developed in the 1980s to provide an outbred and heterogeneous strain from eight inbred strains (including the Wistar lineage; Hansen and Spuhler, 1984). From a founding population of these N:NIH rats one line was bred to produce high rates of pup USV calls during a maternal separation paradigm ("high line"), while the "low line" was bred to produce few calls during the same paradigm. In the maternal separation paradigm, the dam was separated from the litter 20 minutes prior to testing, to elicit separation induced calls from the pups. During a two minute test phase each pup's USV call rate was measured. This rate was used as the basis from which the high- and low-lines were bred. Within five generations the high line pups produced up to 300 USVs within two minutes whereas the low line pups produced less than 50 calls. In the 58th generation high line individuals produced on average over 300 calls with some individuals reaching over 400 USVs; low line individuals produced less than 50 USVs with the exception of one individual that produced 86 USVs (Lesch *et al.*, 2020). The breeding was conducted at Williams College Animal Facility and all procedures were approved by the Williams College Animal Care and Use Committee.

Larynx extraction

We dissected larynges from 51 adult rats (between 133 and 135 days old; high line: 14♂, 13♀; low line: 12♂, 12♀) that were culled from the breeding population of the two rat lines maintained at Betty Zimmerberg's laboratory at Williams College in Williamstown, MA, USA. Each larynx was extracted with the hyoid, tongue, and 0.5cm of trachea still attached. After dissection, the larynges were stored in individual plastic bags and frozen at -25° C. These larynges were double bagged, placed in a cardboard box, surrounded by ice packs, and shipped in an insulated cooler to W. Tecumseh Fitch's lab at the University of

Vienna in Vienna, AT. Upon arrival all larynges remained frozen, and were transferred to -21° C freezers.

Overview

Out of the 51 larynges we randomly chose 8 larynges of each group (high male, high female, low male, low female), adding up to a total of 32 larynges. All 32 larynges were used in the microCT analysis and after the scan four of these larynges were stained, three were used for the energy dispersive X-ray (EDX) and one underwent sectioning.

microCT scans

Our primary method for comparing larynges between lines was based on the volumes of key cartilages. Each laryngeal specimen was scanned using microCT (μCT) to obtain a 3-dimensional computer model that allowed us to quantitatively determine the volume of mineralized tissue in the hyoid bone, and each of the main laryngeal cartilages (cricoid, arytenoids and thyroid).

Specimen preparation

32 larynges (8 per sex and line combination) were prepared for microCT scanning at the University of Vienna. The individual larynges were thawed and further dissected for the scanning procedure. The majority of the tongue was cut off and other surplus tissues were removed. We flushed the larynx with 0.9% saline solution to remove any tissue or fluid from within the larynx and trachea. Afterwards we placed the larynx in a plastic tube, stabilized its position with plastic straws and added a drop of saline at the bottom to prevent dessication. The tubes were sealed with parafilm and mounted on the scanner platform for scanning.

Scanning process and visualization

We scanned all larynges with an XRadia MicroXCT-400 (Carl Zeiss X-ray Microscopy, Pleasanton, CA, USA) using the 0.4x detector assembly. Scanning parameters were set to a source voltage of 40 kVp and 200 μA beam intensity. Projection images were recorded with 1s exposure time (detector binning = 4) per projection and an angular increment of 0.250° between projections. Tomographic sections were reconstructed using the XMReconstructor software supplied with the scanner. Isotropic voxel size in the

reconstructed volumes was 45.1 µm. Reconstructed volumes were exported in Digital Imaging and Communications in Medicine (DICOM) format.

DICOM image stacks were loaded in the Amira software package (version 6.4.0) and the following structures were segmented manually: thyroid, arytenoids, cricoid, tracheal rings, and the hyoid apparatus. Since the microCT scans only reliably capture mineralized structures, these reconstructions only show the mineralized parts of the cartilages and hyoid apparatus. We used the threshold tool in the Amira segmentation editor to aid manual segmentation of the cartilages and hyoid apparatus (basihyal, ceratohyal, chondrohyal, hypohyal and thyrohyal; Sharma and Sivaram, 1967). The threshold tool was adjusted for each individual to perfectly capture the hyoid apparatus and these same settings were then used for the rest of the (mineralized) larynx. We measured the mineralized volume of the hyoid, cricoid, arytenoids and thyroid and vocal fold length on the reconstructed 3D models of all larynges. Vocal fold length was approximated based on the mineralization patterns of the 3D reconstructions. We placed landmarks on the dorsal border of each arytenoid cartilage, and in the area of vocal fold attachment on the thyroid cartilage; we then measured the distance between the arytenoid and thyroid landmarks, resulting in two vocal fold measurements per individual (right and left; Bowling *et al.*, 2020). We recorded the average value as "mean vocal fold length" for each individual to be used for all further statistical analysis.

Clearing and staining

To visualize the entire (and not just mineralized) laryngeal anatomy in our specimens, we selected four larynges (high male: #18, high female: #42, low male: #34, low female: #2; microCT scans and 3D reconstructions were done for all specimens) to create cleared and stained specimens, according to an adapted staining protocol based on Rigueur and Lyons (Rigueur and Lyons, 2014). Excess tissue was mechanically removed for faster preparation. The main steps in the preparation were fixation of frozen larynges in 4% formaldehyde, followed by the first staining step in an ethanolic Alcian blue solution for staining cartilage. Afterwards, soft tissue was preincubated and macerated in potassium hydroxide (KOH) solution, followed by staining in aqueous Alizarin Red solution in KOH. Additional soft tissue removal and clearing was then accomplished using KOH. Whole specimens were finally transferred into glycerol for documentation and analysis with a Nikon SMZ25

stereomicroscope equipped with a Nikon DsRi-2 camera (Nikon Instruments, Tokyo, Japan) or a Hirox RH-2000 digital microscope system (Hirox, Limonest, France).

Electron microscopy and energy dispersive X-ray spectroscopy (EDX)

To assess the elemental composition of the mineralization we used three larynges (high line male #19, low line male #26, high line female #15) for elemental analysis. The distribution of elements was identified using EDX throughout cartilages of the larynx and the hyoid bone. Both the cricoid and thyroid cartilage were cut in half and attached to carbon adhesive discs on aluminum stubs with their cut surfaces facing upward. The hyoid bones were attached to the carbon disks as a whole. Additional conductive carbon cement (Leit-C) was applied to enable better contact to the stubs and prevent excessive charging. Prior to analysis, specimens were carbon coated with a Leica EM MED20 (Leica Microsystems, Wetzlar, Germany). Analysis was conducted with a Jeol-IT3000 scanning electron microscope with the following parameters: BED-C, 20kV, WD 11mm, STd.P.C 63.7-67.7, 100 Pa and 350-600 times magnification.

Histological analysis

To distinguish between calcification and ossification of the mineralized parts, one larynx (high line male #25) was embedded in epoxy resin for sectioning and histological analysis. The specimen was first fixed as described in the clearing and staining section. It was then decalcified with 20% EDTA and dehydrated with acidified dimethoxypropane prior to embedding into Agar LVR resin (Agar Scientific, Stansted, UK) using acetone as an intermediate. Sections of cured resin blocks were sliced at 1µm section thickness with a Leica UC6 ultramicrotome equipped with a diamond knife (Diatome, Nidau, Switzerland) and analyzed with a Nikon NiU compound microscope.

Statistical analysis

To determine the effect of line breeding on vocal fold length and mineralized cartilage volumes we constructed separate generalized linear models. The models compare each of the following measurements between lines: cricoid mineralized volume, thyroid mineralized volume, arytenoid mineralized volume and vocal fold length. A Gamma log distribution was used for all models. The significance of line specific differences was assessed by comparing the predictive value of full models against null models lacking the relevant predictor. In the full models we included the covariate bodyweight and a dummy coded variable representing

line and the respective sex within line. For all model comparisons, the null model only included the covariate ‘body weight’ due to our default allometric expectation that larger individuals should have larger vocal structures, and accounting for the fact that the different selected rat lines showed significant differences in body weight. The covariate bodyweight was scaled and centered before running any analysis. All models were inspected and plotted to determine if model assumptions were satisfied. All variance inflation factors were <4 and overdispersion was <0.4.

Statistical analyses were performed in R (Version 3.6.1; www.r-project.org/) and R-Studio (Version 1.2.1335; www.rstudio.com/) using the following packages and versions: performance_0.4.4, boot_1.3-22, plyr_1.8.4, readxl_1.3.1, openxlsx_4.1.0.1, forcats_0.4.0, MuMIn_1.43.6, ggthemes_4.2.0, car_3.0-3, carData_3.0-2, MASS_7.3-51.4, lme4_1.1-21, Matrix_1.2-17, stringr_1.4.0, dplyr_0.8.3, purrr_0.3.2, readr_1.3.1, tidyr_0.8.3, tibble_2.1.3, ggplot2_3.2.0, and NCmisc_1.1.6.

Results

Cartilage volumes, vocal fold lengths and bodyweight allometry

The reconstructed larynx models allowed us to measure a proxy of vocal fold length and quantify the mineralized volumes of each cartilage and the hyoid apparatus (Fig. 1). The full models for vocal fold length as well as arytenoid, thyroid and cricoid volume were all significantly superior to the null models (Tab. 1-4); only the full model for the hyoid was not significantly better than the null model (Tab. 5). Therefore differences between lines were identified for all major laryngeal cartilages, independent of body size, suggesting deviations for expected allometry (Fig. 2D-F). The hyoid is the only vocal structure whose volume follows the expected size allometry: larger animals have a larger hyoid bone and therefore a greater mineralized volume (Fig. 2C). The three measured cartilages deviate from this simple bodyweight-volume allometry indicating that selection on the different rat lines significantly affects the degree of mineralization in laryngeal cartilages. (Tab. 1-4).

The mineralized portions of the cartilages were as follows (see Fig. 1): In the thyroid a shield-like portion centered on the insertion points of the vocal folds was mineralized; the arytenoids were mineralized on the dorsal transitions to the cricoid; the cricoid ring itself and the dorsal "shield" were mineralized in the shape of an "M" from the arytenoids to the center of the cartilage (e.g. Fig. 1, right panel; Fig. 3D). For the thyroid cartilage, the mineralization difference is clear with high line males and females having a more mineralized thyroid than the low line (Fig. 2E). In the arytenoids and cricoid the difference in mineralization is less clear and may just be caused by one group (low line females; Fig. 2D,F).
A deviation from simple bodyweight allometry also applies to vocal fold length (Fig. 2A); although high line individuals are on average larger in size, they have *shorter* vocal folds compared to the smaller low line individuals, which have *longer* vocal folds (Fig. 2B). Thus, selection for vocalization during infancy had clear and statistically significant effects on adult larynx morphology, and led to a deviation from simple allometric predictions.

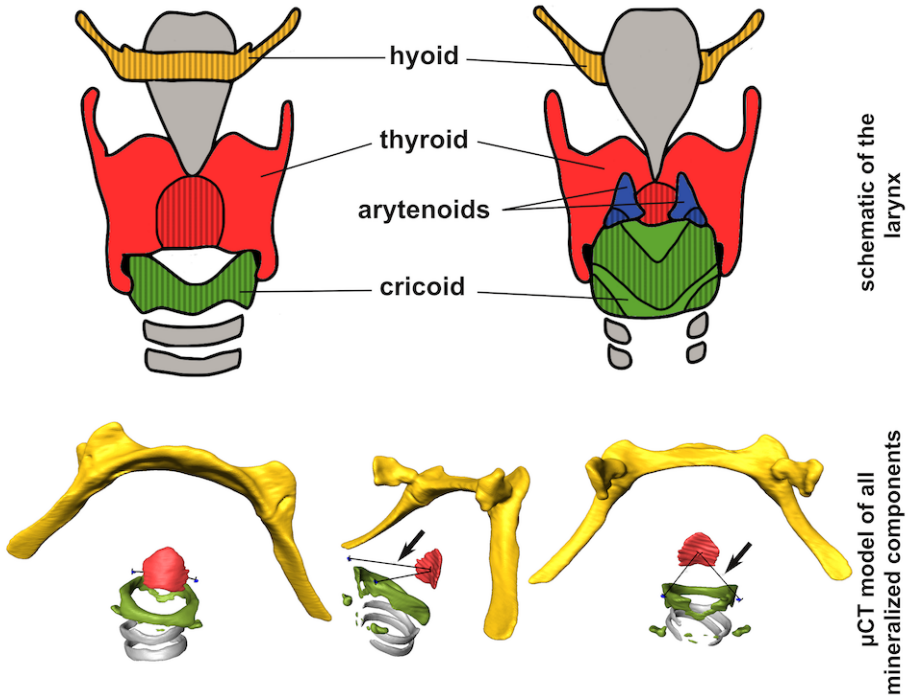

Figure 1: Schematic drawing of a mammalian larynx and 3D reconstructions of the mineralized larynx components of a high line male rat (#H16). The hyoid is figured in yellow, the thyroid in red, the arytenoids in blue and the cricoid in green. The trachea and epiglottis are shown in grey. The vertical-line shading in the schematic drawing indicates the mineralization visible in the microCT scans. An alar cartilage is not represented in the schematic and was not visible in our microCT scans. The epiglottis also was not visible in the scans. Vocal fold length measurements are indicated in the 3D reconstructions with thin black lines between the thyroid and arytenoid cartilages and pointed out by black arrows.

Table 1: Null/full model comparison and summary of the model for vocal fold length. High line males are included in the intercept.

vocal fold length				
	Pr(>Chi)			
full/null model comparison	$5.38 * 10^{-6}$			
	estimate	std. error	t value	Pr(>|t|)
intercept ♢	1.18827	0.02541	46.755	$< 2 * 10^{-16}$
bodyweight g	0.05633	0.01844	3.054	0.00503
low line male	0.07383	0.02031	3.636	0.00115
high line female	0.03663	0.03954	0.926	0.36252
low line female	0.09932	0.04533	2.191	0.03727

♢ intercept includes high line males

Table 2: Null/full model comparison and summary of the model for cricoid volume. High line males are included in the intercept.

cricoid volume				
	Pr(>Chi)			
full/null model comparison	0.002512			
	estimate	std. error	t value	Pr(>|t|)
intercept ♢	1.0912	0.1807	6.04	$1.9 * 10^{-6}$
low line male	-0.2339	0.1443	-1.62	0.11678
high line female	-0.605	0.2811	-2.152	0.04048
low line female	-1.0136	0.3222	-3.145	0.00401
bodyweight g	-0.2749	0.1311	-2.097	0.04549

♢ intercept includes high line males

Table 3: Null/full model comparison and summary of the model for arytenoid volume. High line males are included in the intercept.

arytenoid volume				
	Pr(>Chi)			
full/null model comparison	0.002138			
	estimate	std. error	t value	Pr(>|t|)
intercept ♢	-2.8381	0.4713	-6.022	$2 * 10^{-6}$
low line male	-0.6067	0.3766	-1.611	0.1188
high line female	-0.9778	0.7333	-1.333	0.1935
low line female	-2.1325	0.8407	-2.537	0.0173
bodyweight g	-0.5337	0.342	-1.56	0.1303

♢ intercept includes high line males

Table 4: Null/full model comparison and summary of the model for thyroid volume. High line males are included in the intercept.

thyroid volume				
	Pr(>Chi)			
full/null model comparison	$6.1 * 10^{-9}$			
	estimate	std. error	t value	Pr(>|t|)
intercept ♢	0.07738	0.18877	0.41	0.685101
low line male	-0.57998	0.15082	-3.846	0.000665
high line female	-0.24695	0.2937	-0.841	0.40783
low line female	-0.88356	0.33671	-2.624	0.01412
bodyweight g	-0.12008	0.13699	-0.877	0.388478

♢ intercept includes high line males

Table 5: Null/full model comparison and summary of the model for hyoid volume. Since the full model was not significantly better than the null model analysis was stopped here.

hyoid volume	
	Pr(>Chi)
full/null model comparison	0.7564

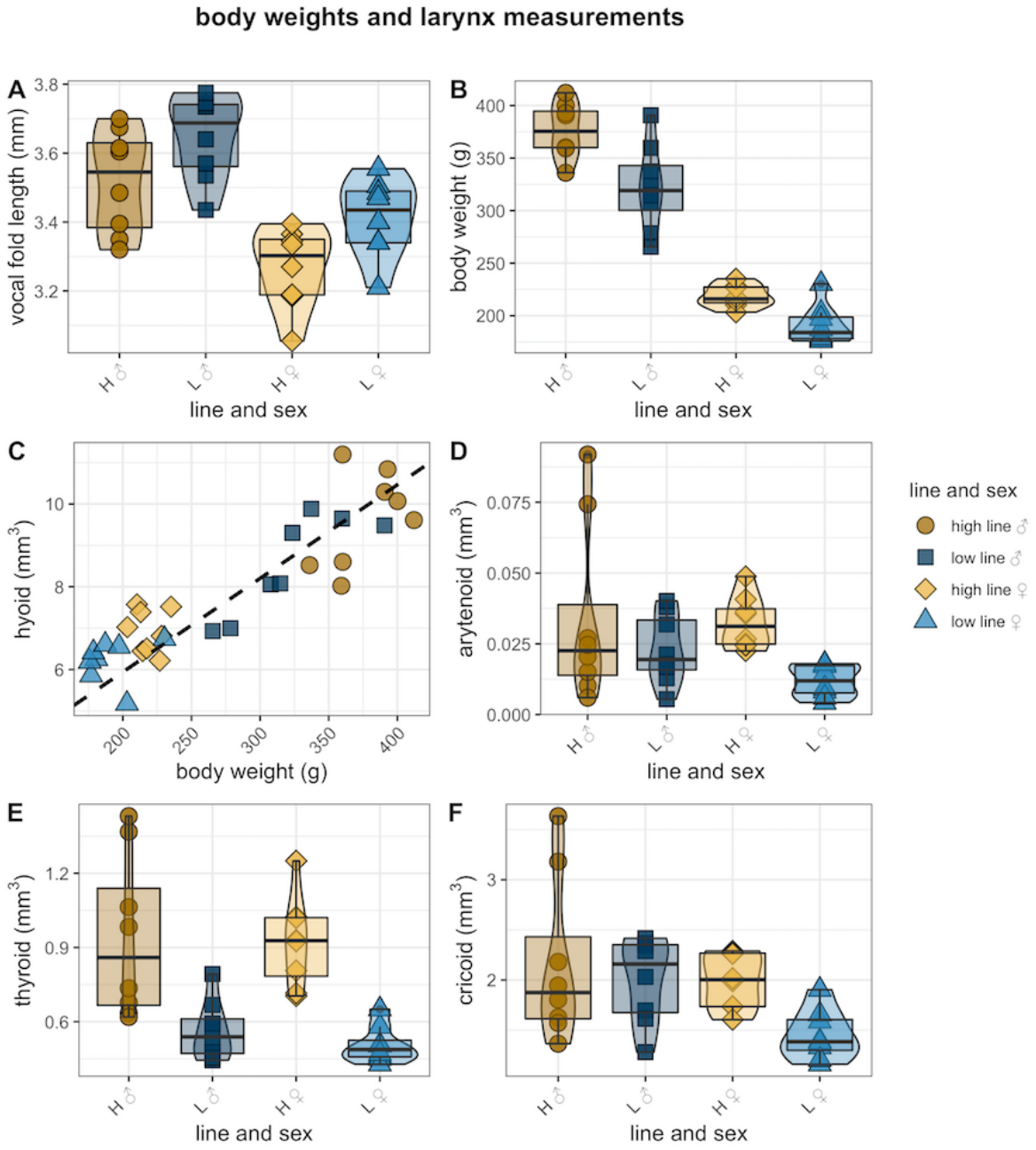

Figure 2: Overview of bodyweight, vocal fold length and volume of mineralized parts of the cartilages and the hyoid bone for the two selected lines, by sex. The different lines are indicated by letters (H=high line, L=low line), and sex is indicated by symbols (♂=male, ♀=female). Circles indicate high line males, squares indicate low line males, diamonds indicate high line females and triangles indicate low line females. A: Mean vocal fold length of each individual in mm plotted in groups of line and sex. B: Body weight in grams of all individuals plotted in groups of line and sex. C: Reconstructed hyoid volume of all 32 specimen plotted relative to their body weight. D-F: Volumes of specific laryngeal cartilages for all 32 CT specimens, plotted in groups of line and sex.

Composition and nature of mineralized areas

Clearing and staining of larynges was used to determine the nature of mineralized structures; with our staining methods calcified structures are stained purple and cartilage stains blue (Fig. 3). These specimens confirmed that the mineralized cartilage volumes indicated in microCT scans are calcifications (Fig. 1). Additional elemental analysis using EDX of the cross sections of the mineralized regions in laryngeal cartilages show the same elemental components as the hyoid bone (Fig. 4A-C). Both show a high degree of phosphate and calcium which is typical for hydroxyapatite (the mineralization of bone and calcified cartilage). Histological sections of the cartilage, including mineralized areas, show typical hyaline cartilage with chondrocytes clustering to form chondrons in a large structural matrix (Fig. 5A, B). Mineralized areas stain more intensely than the remaining cartilaginous matrix (Fig. 5) and located in various patches of the territorial and interterritorial matrix between chondrons. This distribution around chondrocytes indicates that the mineralized areas represent calcified cartilage rather than true bone tissue.

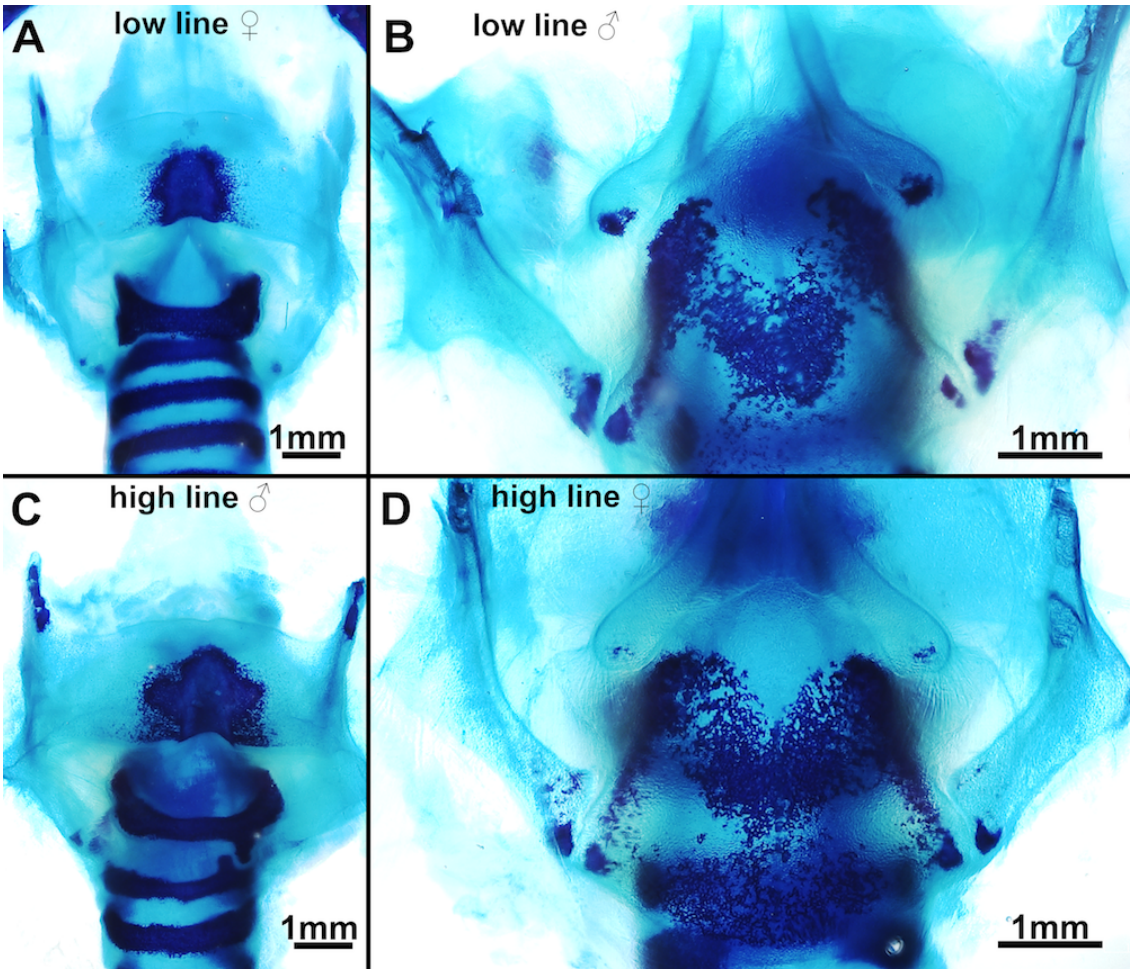

Figure 3: Selection of photographs of the 4 specimens whole mount stains. Calcified material is stained in purple and cartilage in blue. The high line individual shows a much more calcified thyroid compared to the low line. A: Ventral view of the larynx and hyoid of a low line female (#2). B: Dorsal view of the cricoid and arytenoids of a low line male (#34). C: Ventral view a larynx (lacking the hyoid) of a high line male (#18). D: Dorsal view of the thyroid and cricoid of a high line female (#42).

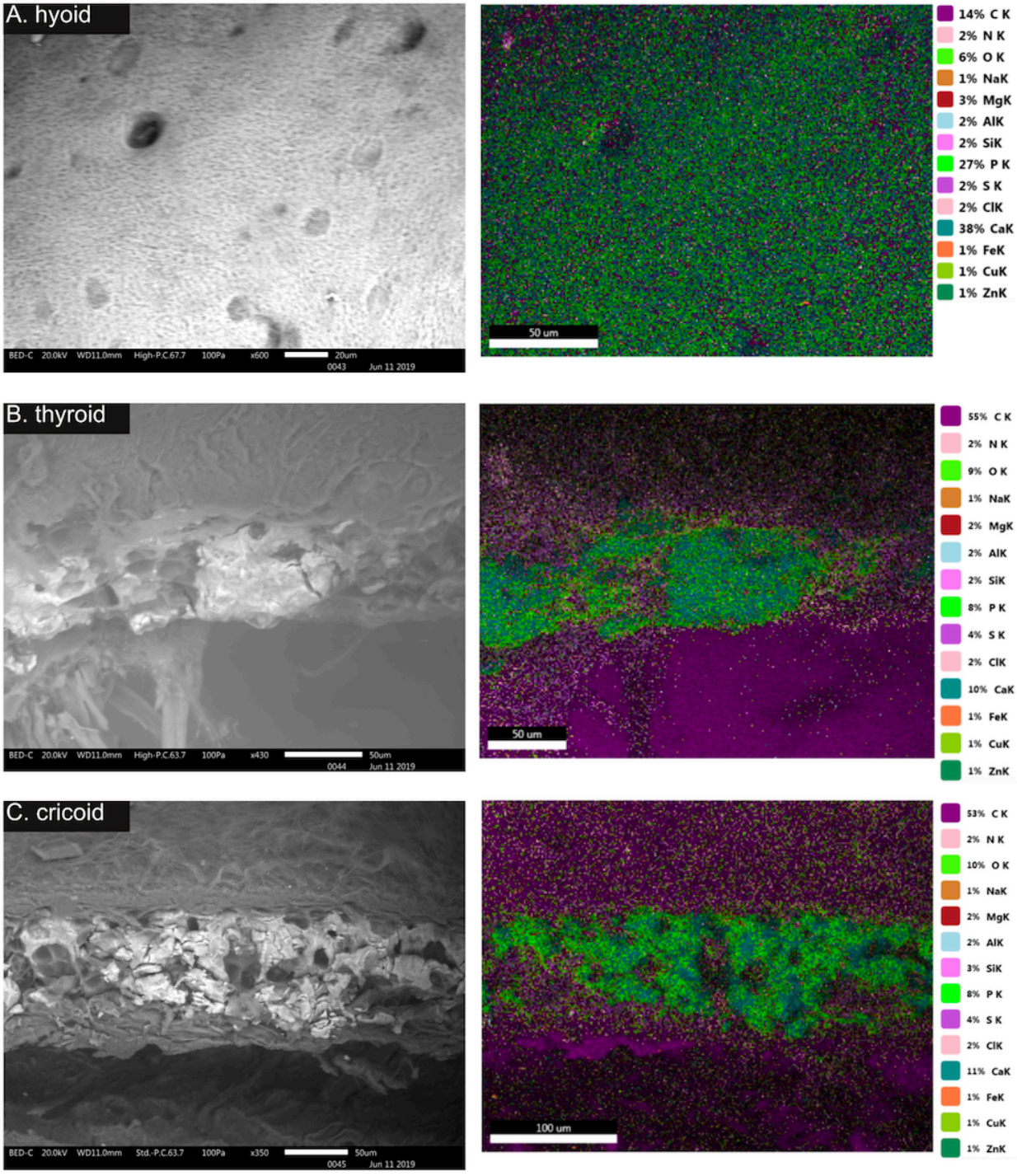

Figure 4: Photographs and elemental analysis (element map) from one individual (#19, high line male) showing the element distribution in the cricoid and thyroid in comparison to the hyoid. The cross sections of the mineralized regions in the laryngeal cartilages show a similar composition to the hyoid. A: Photograph and element map of the hyoid surface. B: Photograph and element map of the cross section of the thyroid. C: Photograph and element map of a cross section of the cricoid.

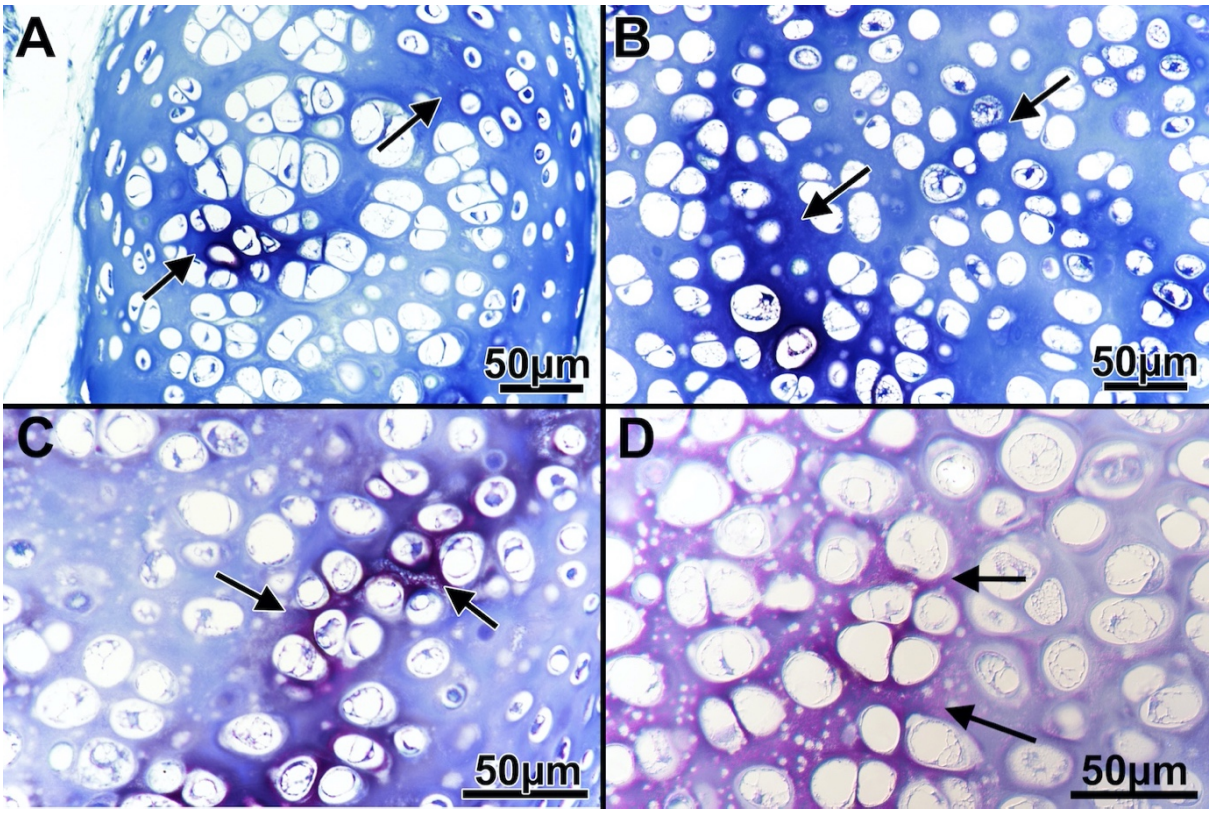

Figure 5: Cross-sections of a mineralized thyroid cartilage of a high line male (#25). Semi-thin sections, stained with toluidine blue. Mineralized areas are shown by intense staining indicated by arrows in all images. A, B: Overview of two sections showing hyaline cartilage with several chondrons. C, D: Details of mineralized areas located mainly in the inter-territorial matrix between chondrons.

Discussion

We investigated the impact of selective breeding for increased or decreased infant USVs on adult rat larynx morphology, finding that adult rats from lines bred to produce more USVs during maternal separation as pups had substantially more calcified thyroid cartilages, and shorter vocal folds. The opposite was true for rats bred to produce few USVs during maternal separation as pups, which had longer vocal folds and less-calcified thyroid cartilages as adults. This confirms our hypothesis that selective breeding can lead to changes in laryngeal morphology. Our anatomical findings are consistent with previous evaluations of acoustic measures in the same rat lines (Lesch et al., 2020).

USVs are at the center of research associated with emotional states, and are often used as an easily measured proxy for stress response styles in rats (Branchi, Santucci and Alleva, 2001; Brunelli, 2005; Simola and Brudzynski, 2018). Therefore it is important to document and understand possible changes to the vocal production mechanisms that may arise as a result of selective breeding (and, by extension, natural selection). In Lesch et al. (2020), analyses of vocal outputs from rat lines selectively-bred for USVs as pups showed that line breeding affected the fundamental frequency of low-frequency calls but not USVs in adults. The likely mechanism by which these low-frequency calls are produced is standard vocal fold vibration in the larynx, as detailed in the myo-elastic aerodynamic theory (Titze, 2006; Elemans *et al.*, 2015), implicating potential differences in vocal fold length as the main cause of these differing fundamental frequencies (Roberts, 1975a; Riede *et al.*, 2011; Herbst *et al.*, 2012). Our approximate measurements of vocal fold length from microCT reconstructions showed that high line individuals indeed have shorter vocal folds, which is consistent with the higher fundamental frequency of their low-frequency calls. In contrast, low line individuals have longer vocal folds and a correspondingly lower fundamental frequency. The differences in vocal fold length found in the current study thus explain the line difference we observed in the fundamental frequency of low-frequency calls in our previous study (i.e. high line males producing higher frequencies than low line males; Lesch *et al.*, 2020).

Furthermore, our microCT reconstructions revealed significant differences in mineralization of the thyroid cartilage in the two rat lines. While the significant difference in mineralization was clearly line specific in the thyroid cartilage, the significant differences in mineralization in the arytenoids and cricoid might just be driven by females of the low line. Histological analysis of stained larynges suggested that the mineralized components observed in the reconstructed microCT scans are indeed calcifications. Further EDX analyses

confirmed that these calcifications in the laryngeal cartilages have the elemental components of hydroxyapatite [$Ca_5(PO_4)_3(OH)$], present in both bone and calcified cartilage (Boskey, 1988). The histological structure, however, shows patchy distribution of the mineralized areas between chondrocytes, indicating calcified cartilage rather than bone. Definitive distinction of calcified cartilage vs bone would require specific staining against collagen I for bone and collagen II for cartilage (Fratzl, 2008; Hall, 2015).

Based on a comprehensive review of mammalian laryngeal anatomy, Schneider (1964) concluded that there is no generalizable rule for the process of calcification in the mammalian larynx, and suggested that the thyroid cartilage in eutherians tends to first calcify in areas that are under less deformation pressure e.g. inferior and superior thyroid horn (Schneider, 1964). This prediction does not match the pattern of calcification we found in our rat larynges, which were mainly calcified in areas that can be under significant deformation pressure, specifically the specific areas of vocal fold attachment. Carter (2020) compared calcifications in the trachea and larynx across laryngeally echolocating bats and found that higher-intensity vocalizing species tend to have a more mineralized thyroid cartilages than lower-intensity species. Thyroid mineralization in the high line rats might help to facilitate and sustain USV production, similar to that observed in laryngeally echolocating bats. However, it remains unclear whether this mineralization is genetically determined irrespective of vocalization, or whether increased rates of vocalization in young animals might have led to the increased mineralization of thyroid cartilages that we observe in adults. Detailed anatomical investigations through development, e.g. using microCT, would be necessary to test this prediction.

A limitation of this study is the fact that we can't determine whether the line specific differences that we have documented would develop reliably based purely on genetics, whether they result from behavioral differences (e.g., high vocalization rates) having a developmental effect on morphology, or a combination of the two. More precisely, the observed changes to laryngeal morphology might be caused by differences in usage during early developmental stages and/or genetically determined developmental differences. For example, low line individuals might have a less calcified thyroid simply due to lower rates of USV production ("use it or lose it"); while high line individuals, due to their frequent vocalizations, provide more frequent stress that provides the stimulus for increased mineralization. Addressing this question would require detailed individual-specific lifelong documentation of vocalization rates, and or molecular-genetic and developmental investigations. However, this question of developmental causation is secondary to the

primary questions addressed here, i.e., does selection on vocalization affect laryngeal morphology, and if so how.

Another limitation is that, because this is the first investigation of laryngeal anatomy in these rat lines, we cannot determine when the anatomical changes we have documented have occurred during the 50+ generations of ongoing selection. Behaviorally, changes in pup vocalization occurred quite rapidly, in the first five generations of selection (Brunelli *et al.*, 1997). But this does not necessarily indicate that the anatomical changes occurred equally rapidly. Unfortunately, addressing this question would entail starting the selection experiment afresh from unselected lines, and acquiring anatomical specimens at each generation, a research program far beyond the scope of our study."

It is well known that, despite its relatively conservative structure, the form of the mammalian larynx can be modified to suit its particular functions (Charlton and Reby, 2016; Fitch, 2016). For example, the larynx and hyoid of howler monkeys are enormously enlarged to support their extremely loud, low-frequency vocalizations, and these changes reflect specific selection in different species of howler monkeys (Dunn *et al.*, 2015). The large "roaring cats" (lions, tigers, jaguars and leopards) have several modifications of vocal fold structure and hyoid morphology that again support the production of loud, low-frequency vocalizations (Titze *et al.*, 2010; Klemuk *et al.*, 2011). Many similar modifications for loud low-frequency vocalizations have been documented in a range of ungulates (Frey and Hofmann, 2000; Frey and Riede, 2003; Frey *et al.*, 2007, 2011). Nonhuman primates show a suite of specific vocal modifications, including many types and sizes of laryngeal air sac (Schön Ybarra, 1995; Fitch, 2016), and large-scale comparisons of laryngeal morphology in primates and carnivores suggest that the primate larynx has evolved more rapidly than that of carnivores (Bowling *et al.*, 2020).

Given these well-known modifications on an evolutionary time-scale, it is clearly important to better understand how selection for specific vocal traits (whether lower- or higher-frequency calls, louder calls, or more frequent calling) affects the morphology and development of the main sound producing organ, the larynx (that is, to examine both the ontogeny and phylogeny of changes in vocal anatomy). Our study takes a first step in this direction and reveals that two specific aspects of laryngeal structure - vocal fold length and thyroid mineralization – can be modified by artifical selection on rate of vocalization alone, and in a relatively short time span. Furthermore, it should be noted that this targeted selection produced a host of other "side-effects," including e.g., on voice fundamental frequency, body

size, and temperament. This implies that selection on the voice can have a considerable diversity of effects that are of clear relevance for an animal's behavior and fitness.

Finally, our results also show that the expected allometric correlation between overall body size and the size of specific organs can easily be overridden by selection, at least with respsect to laryngeal size. Although our high line rats were larger in overall body size, they had shorter vocal folds and produced vocalizations with higher fundamental frequencies. Thus, our results are consistent with the hypothesis that laryngeal morphology, and the vocal acoustic parameters that depend on it such as fundamental frequency, is relatively unconstrained by overall body size in mammals (Fitch and Hauser, 2006; Garcia *et al.*, 2017). Put differently, under strong selection for vocal output, laryngeal size can change independent of body size. This offers comparative insight into the peculiar fact that human men, due to laryngeal hypertrophy, have a much lower fundamental frequency than women (about 50%) despite being on average only 20% larger (Pisanski *et al.*, 2014), and belies the common assumption that fundamental frequency automatically reflects body size as "a law of physics" (Morton, 1977). The presence or absence of acoustic allometry – the scaling of bioacoustic parameters with body size (e.g.,Bowling *et al.*, 2017) – additionally depends strongly on biological factors, specifically whether laryngeal morphology correlates with overall body size. This is a matter of anatomy and physiology, not of physics.

To sum up, our data support the hypothesis that rats selectively bred for USV production as pups show changes in larynx morphology in adulthood, which in turn have clear and predictable results on vocal production. This finding is clearly relevant to researchers using USVs to gain insight into the emotional state and stress response, as well as to broader questions in the evolution of communication (cf. Dunn *et al.*, 2015; Fitch, 2016; Bowling *et al.*, 2020).

Acknowledgements

We thank Stephan Handschuh for scanning the specimens used in the microCT reconstruction and Daniela Gruber for helping us collect EDX data. Scanning electron microscopy was performed at the Core Facility Cell Imaging and Ultrastructure Research, University of Vienna - member of the Vienna Life-Science Instruments (VLSI). Preparation of this paper was supported by Austrian Science Fund (FWF) DK Grant “Cognition & Communication 2” (#W1262-B29) to WTF. Ethical approval was received by the University of Vienna Faculty for Life Science Tierschutzgremium #2018-003. The data used here is made available as supplementary material to this manuscript. The authors state no conflict of interest.

Author Contributions

Conceptualization, R.L., D.B., B.Z. and W.T.F; Data curation, R.L.; Formal analysis, R.L., D.B. and T.S.; Funding acquisition, W.T.F., B.Z. and M.H.; Investigation, R.L., T.S., B.Z., A.O. and M.S.; Methodology, R.L., W.T.F and T.S.; Project administration,. R.L.; Resources, T.S., W.T.F., B.Z., M.H. and S.B.; Software, R.L., T.S., D.B.; Supervision, W.T.F.; Validation, R.L. and T.S.; Visualization, R.L. and T.S.; Writing—first draft, R.L., W.T.F. and T.S.; Writing—review and editing, R.L., T.S., D.B., W.T.F. and B.Z.

References

Boskey, A. L. (1988) ‘Calcified Tissues: Chemistry and Biochemistry’, in Calcium in Human Biology, pp. 171–186. doi: 10.1007/978-1-4471-1437-6_7.

Bowling, D. L. et al. (2017) ‘Body size and vocalization in primates and carnivores’, Scientific Reports. Nature Publishing Group, 7, p. 41070. doi: 10.1038/srep41070.

Bowling, D. L. et al. (2020) ‘Rapid evolution of the primate larynx?’, PLoS Biology, pp. 1–21. doi: 10.1371/journal.pbio.3000764.

Branchi, I., Santucci, D. and Alleva, E. (2001) ‘Ultrasonic vocalisation emitted by infant rodents: A tool for assessment of neurobehavioural development’, Behavioural Brain Research, 125(1–2), pp. 49–56. doi: 10.1016/S0166-4328(01)00277-7.

Brudzynski, S. M. (2005) ‘Principles of rat communication: Quantitative parameters of ultrasonic calls in rats’, in Behavior Genetics, pp. 85–92. doi: 10.1007/s10519-004-0858-3.

Brudzynski, S. M. (2010) Handbook of Mammalian Vocalization: An Integrative Neuroscience Approach. 1st edn. Edited by S. M. Brudzynski. Elsevier. doi: 10.1016/s1569-7339(10)70001-1.

Brudzynski, S. M. (2018) Handbook of Ultrasonic Vocalization: A Handbook into the Emotional Brain. 1st edn. London: Levy, Niki.

Brunelli, S. A. et al. (1997) ‘Five generations of selective breeding for ultrasonic vocalization (USV) responses in N:NIH strain rats.’, Developmental psychobiology, 31(4), pp. 255–65. doi: 10.1002/(SICI)1098-2302(199712)31:4<255::AID-DEV3>3.0.CO;2-Q.

Brunelli, S. A. (2005) ‘Selective breeding for an infant phenotype: Rat pup ultrasonic vocalization (USV)’, Behavior Genetics, 35(1), pp. 53–65. doi: 10.1007/s10519-004-0855-6.

Brunelli, S. A. and Hofer, M. A. (2007) ‘Selective breeding for infant rat separation-induced ultrasonic vocalizations: Developmental precursors of passive and active coping styles’, Behavioural Brain Research, 182(2), pp. 193–207. doi: 10.1016/j.bbr.2007.04.014.

Carter, R. T. (2020) ‘Reinforcement of the larynx and trachea in echolocating and non-echolocating bats’, Journal of Anatomy, (October 2019), pp. 1–9. doi: 10.1111/joa.13204.

Charlton, B. D. and Reby, D. (2016) ‘The evolution of acoustic size exaggeration in terrestrial mammals’, Nature Communications. Nature Publishing Group, 7, p. 12739. doi: 10.1038/ncomms12739.

Dent, M. L. and Fay, R. R. (2018) Rodent Bioacoustics. Edited by R. R. Fay, A. N. Popper, and M. L. Dent. Cham: Springer.

Dunn, J. C. et al. (2015) ‘Evolutionary trade-off between vocal tract and testes dimensions in howler monkeys’, Current Biology, 25(21), pp. 2839–2844. doi: 10.1016/j.cub.2015.09.029.

Elemans, C. P. H. et al. (2015) ‘Universal mechanisms of sound production and control in birds and mammals’, Nature Communications, 6, p. 8978. doi: 10.1038/ncomms9978.

Fitch, W. T. (2016) ‘Vertebrate bioacoustics: Prospects and open problems’, in Suthers, R. A. et al. (eds) Vertebrate Sound Production and Acoustic Communication. New York: Springer, pp. 297–328.

Fitch, W. T. and Hauser, M. D. (2006) ‘Unpacking “Honesty”: Vertebrate Vocal Production and the Evolution of Acoustic Signals’, Acoustic Communication, pp. 65–137. doi: 10.1007/0-387-22762-8_3.

Fratzl, P. (2008) Collagen: Structure and Mechanics. Edited by P. Fratzl. New York: Springer.

Frey, R. et al. (2007) ‘Nordic rattle: The hoarse vocalization and the inflatable laryngeal air sac of reindeer (*Rangifer tarandus*)’, Journal of Anatomy, 210(2), pp. 131–159. doi: 10.1111/j.1469-7580.2006.00684.x.

Frey, R. et al. (2011) ‘Descended and mobile larynx, vocal tract elongation and rutting roars in male goitred gazelles (*Gazella subgutturosa* Güldenstaedt, 1780)’, Journal of Anatomy, 218(5), pp. 566–585. doi: 10.1111/j.1469-7580.2011.01361.x.

Frey, R. F. and Riede, T. R. (2003) ‘Sexual Dimorphism of the Larynx of the Mongolian Gazelle (*Procapra gutturosa* Pallas , 1777)’, Zoologischer Anzeiger, 242, pp. 33–62.

Frey, R. and Hofmann, R. R. (2000) ‘Larynx and vocalization of the Takin (*Budorcas taxicolor* Hodgson, 1850 - Mammalia, Bovidae)’, Zoologischer Anzeiger, 239, pp. 197–214.

Garcia, M. et al. (2017) ‘Acoustic allometry revisited: morphological determinants of fundamental frequency in primate vocal production’, Scientific Reports. Springer US, 7(1), pp. 1–11. doi: 10.1038/s41598-017-11000-x.

Hall, B. K. (2015) Bones and Cartilage: Developmental and Evolutionary Skeletal Biology. 2nd edn.

Hansen, C. and Spuhler, K. (1984) ‘Development of the National Institutes of Health Genetically Heterogeneous Rat Stock’, Alcoholism: Clinical and Experimental Research, 8(5), pp. 477–479. doi: 10.1111/j.1530-0277.1984.tb05706.x.

Harrison, D. F. N. (1995) The anatomy and physiology of the mammalian larynx. Cambridge: Cambridge University Press.

Herbst, C. T. et al. (2012) ‘How low can you go? Physical production mechanism of elephant infrasonic vocalizations’, Science, 337(6094), pp. 595–599. doi: 10.1126/science.1219712.

Inagi, K., Schultz, E. and Ford, C. N. (1998) ‘An anatomic study of the rat larynx: Establishing the rat model for neuromuscular function’, Otolaryngology - Head and Neck Surgery, 118(1), pp. 74–81. doi: 10.1016/S0194-5998(98)70378-X.

Johnson, A. M. et al. (2010) ‘Ultrasonic output from the excised rat larynx’, The Journal of the Acoustical Society of America, 128(2), pp. EL75–EL79. doi: 10.1121/1.3462234.

Klemuk, S. A. et al. (2011) ‘Adapted to roar: Functional morphology of tiger and lion vocal folds’, PLoS ONE, 6(11), pp. 1–12. doi: 10.1371/journal.pone.0027029.

Lesch, R. et al. (2020) ‘Selection on ultrasonic call rate in neonatal rats affects low frequency, but not ultrasonic, vocalizations in adults’, Ethology, 126(11), pp. 1007–1018. doi: 10.1111/eth.13075.

Mahrt, E. et al. (2016) ‘Mice produce ultrasonic vocalizations by intra-laryngeal planar impinging jets’, Current Biology. Elsevier, 26(19), pp. R880–R881. doi: 10.1016/j.cub.2016.08.032.

Morton, E. S. (1977) ‘On the Occurrence and Significance of Motivation-Structural Rules in Some Bird and Mammal Sounds’, The American Naturalist, 111(981), pp. 855–869. doi: 10.1086/283219.

Pisanski, K. et al. (2014) ‘Vocal indicators of body size in men and women: A meta-analysis’, Animal Behaviour, 95, pp. 89–99. doi: 10.1016/j.anbehav.2014.06.011.

Portfors, C. V (2007) ‘Types and functions of ultrasonic vocalizations in laboratory rats and mice.’, Journal of the American Association for Laboratory Animal Science : JAALAS, 46(1), pp. 28–34.

Riede, T. et al. (2011) ‘Elasticity and stress relaxation of a very small vocal fold’, Journal of Biomechanics, 44(10), pp. 1936–1940. doi: 10.1016/j.jbiomech.2011.04.024.

Riede, T., Borgard, H. L. and Pasch, B. (2017) ‘Laryngeal airway reconstruction indicates that rodent ultrasonic

vocalizations are produced by an edge-tone mechanism', Royal Society Open Science, 4(11). doi: 10.1098/rsos.170976.

Riede, T. and Fitch, T. (1999) 'Vocal tract length and acoustics of vocalization in the domestic dog (*Canis familiaris*).', The Journal of experimental biology, 202, pp. 2859–2867.

Rigueur, D. and Lyons, K. M. (2014) 'Skeletal Development and Repair', Methods Molecular Biology, 1130, pp. 113–121. doi: 10.1007/978-1-62703-989-5.

Roberts, L. H. (1975a) 'The functional anatomy of the rodent larynx in relation to audible and ultrasonic cry production', Zoological Journal of the Linnean Society, 56(3), pp. 255–264. doi: 10.1111/j.1096-3642.1975.tb00268.x.

Roberts, L. H. (1975b) 'The rodent ultrasound production mechanism', Ultrasonics, 13(2), pp. 83–88. doi: 10.1016/0041-624X(75)90052-9.

Sanders, I. et al. (2001) 'The mechanism of ultrasonic vocalization in the rat', Society of Neuroscience Abstracts, 27(88.19).

Schneider, R. (1964) 'Der Larynx der Säugetiere', Handbuch der Zoologie. Berlin, 5(7), pp. 1–128.

Schön Ybarra, M. A. (1995) 'A comparative approach to the non-human primate vocal tract: implications for sound production', Current Topics in Primate Vocal Communication, pp. 185–198. doi: 10.1007/978-1-4757-9930-9.

Sharma, D. R. and Sivaram, S. (1967) 'The hyoid complex of *Rattus rattus rufescens*', J. Zool., Lond., 153, pp. 369–379. doi: 10.1017/CBO9781107415324.004.

Shiba, K. (2009) 'Functions of larynx in breathing, vocalization and airway protective reflexes', in Brudzynski, S. M. (ed.) Handbook of Mammalian Vocalization: An Integrative Neurosciennce Approach. 1st edn. New York: Elsevier, pp. 373–381.

Simola, N. and Brudzynski, S. M. (2018) 'Rat 50-kHz ultrasonic vocalizations as a tool in studying neurochemical mechanisms that regulate positive emotional states', Journal of Neuroscience Methods. Elsevier, 310(February), pp. 33–44. doi: 10.1016/j.jneumeth.2018.06.018.

Titze, I. R. (1994) Principles of Voice Production. Prentice Hall.

Titze, I. R. (2006) 'The myoelastic aerodynamic theory of phonation', in National Center for Voice & Speech.

Titze, I. R. et al. (2010) 'Vocal power and pressure-flow relationships in excised tiger larynges', Journal of Experimental Biology, 213(22), pp. 3866–3873. doi: 10.1242/jeb.044982.

Titze, I. R. (2018) 'Where has all the power gone? Energy production and loss in vocalization', Speech Communication. Elsevier B.V., 101, pp. 26–33. doi: 10.1016/j.specom.2018.05.003.

Zimmerberg, B., Brunelli, S. A. and Hofer, M. A. (1994) 'Reduction of rat pup ultrasonic vocalizations by the neuroactive steroid allopregnanolone', Pharmacology, Biochemistry and Behavior, 47(3), pp. 735–738. doi: 10.1016/0091-3057(94)90181-3.

Concluding discussion

Thesis overview

In this thesis I provided insight into three questions at the intersection of bioacoustics and domestication: I discussed 1) how stress and human personality might affect companion animal-directed speech, 2) how artificial selection pressures can affect vocal behaviour, and 3) how artificial selection pressures can change the laryngeal structures crucial for sound production. I applied methods ranging from acoustic analysis and μCT scans to histology and electron spectroscopy to access these two different aspects of my thesis. My findings were twofold: Companion animal-directed speech is modulated differently depending on the owner's gender and personality and my data supports the idea of a differential use of voice pitch and pitch range. I further found support for the idea that artificial selection pressures for infant ultrasonic call rate in rats can change both the adult rat's larynx morphology and vocal output.

Contributions to human-animal communication

One core aspect of domestication is the closeness between domesticated species and humans. Especially in dogs this closeness manifests in their ability to closely monitor and asses human gestures and facial expressions (Hare *et al.*, 2002; Hare and Tomasello, 2005; Virányi *et al.*, 2008). A main focus of research, so far, investigated how human attempts at communication are interpreted and understood by our domesticated animals. In this thesis, I strived to understand how humans adapt their acoustic communication system when interacting with their domesticated animals.

There is only limited research on companion animal-directed speech, but comparisons of infant-directed speech and companion animal-directed speech have shown that companion animal-directed speech shares a lot of similarities with the speech that humans use to address young children (Burnham and Francis, 1998; Burnham, Kitamura and Vollmer-Conna, 2002). Both infant-directed speech and companion animal-directed speech share higher fundamental frequencies, higher pitch ranges and a high rate of repetition, differing only in the lack of

hyper-articulation in companion animal directed speech (Hoff, 2009; Xu *et al.*, 2013; Ben-Aderet *et al.*, 2017). I investigated companion animal-directed speech during a (stress-inducing) strange situation procedure in human-dog dyads and found that companion animal-directed speech is modulated by the owners' gender and personality. The reliable production of companion animal-directed speech by all dog owners supports the idea that companion animals, in general, are able to elicit this vocal response in humans; a response that evolved to provide comfort and reassurance to human children.

Not only us humans communicate vocally with our domesticated animals but so do domesticated mammals with us. Next to domesticated foxes there are further well documented instances of domesticated animals vocally communicating with humans, but these signals origins are often unclear and not well documented. Cats seem to use specific solicitation purrs to solicit food from their human caregivers (McComb *et al.*, 2009); dogs use barks to draw their humans attention and generally bark in a much wider array of contexts than wolves (Hare, Call and Tomasello, 1998; Feddersen-Petersen, 2000). The work on domesticated foxes hints towards domestication being the unifying factor driving those adaptations and changes in vocalizations (Hare and Tomasello, 2005).

Contributions to domestication and artificial selection

Although the process of domestication has long been viewed as a classic example of pure artificial selection, reality is more complicated; while pure bred lines of domesticated species clearly underwent an artificial selection process, the creation of domesticated species was a slow process of animals adapting to a human environment and is, therefore, more of a mixture of both artificial and natural selection (Kohane and Parsons, 1988; Francis, 2015). Humans actively and passively shaped both their environment and their domesticated species (Hemmer, 1990). The close contact of humans with their domesticated animals not only led to changes in the domesticated species but also in how humans interact with them, which in turn could result in further changes in domesticated species. In this thesis, I approached both the aspect of how we can shape domesticated animals through clear artificial selection pressures and how the human companion-animal relationship shapes our way of interacting with them.

Artificial selection and domesticated animal breeds were essential in Darwin's description of natural selection; his view of domesticated animal breeds as a window into natural selection processes were a core principle to this thesis (Darwin, 1868; Evans, 1984; Price, 2002). In my work with the rat lines, I add to the knowledge of how strong artificial selection pressures can affect acoustic behaviour and sound producing structures in domesticated mammals. I found artificial selection pressures on vocal call rate in infant rats change both vocal behaviour and larynx morphology in adults. Güttinger suggested in his work on canaries that traits can only be artificially selected for if these traits are already present to certain degree in the originally domesticated group (Güttinger, 1985). Both Güttinger's suggestion and Darwin's view on artificial selection and domesticated animal breeds supports my idea of investigating the impact of artificial selection pressures on the vocal production structures in domesticated mammals to further our understanding of similar processes found in nature (Güttinger, 1985; Price, 2002). My findings along these lines provide groundwork for future studies comparing wild and domesticated mammals larynx and acoustic behaviour.

Domesticated animals in current bioacoustic research

A big part of current research combining bioacoustics with domesticated animals investigates how domesticated animals perceive human language or how human speech changes domesticated animals' behaviour.

One such example for an investigation into the perception of human language by domesticated animals is the consonant bias. To use an example presented by Mallikarjun et al. (Mallikarjun, Shroads and Newman, 2020): if an adult human would hear the non-existant word "dunkey" one could assume that there are equal chances that the listener would hear it either as the word "monkey" or "donkey". They both differ in pronunciation by one sound, yet adult humans would identify "dunkey" as donkey due to a tendency to rely more on consonant rather than vowel information in identifying words (Cutler *et al.*, 2000; Mallikarjun, Shroads and Newman, 2020). Research on rats initially indicated that they perceived a change in the vowels as more disruptive than a change to the consonant, therefore showing a clear vowel bias (Bouchon and Toro, 2019). A similar study investigating this bias in dogs argued that dogs have a lot more experience with human language than rats do and

therefore would be optimal candidates to rerun the study, yet they found the same results of a vowel bias rather than a consonant bias in dogs (Mallikarjun, Shroads and Newman, 2020).

Three recent studies on domestic piglets and cows combined research on how human speech is perceived by animals with the effects speech has on the animal's behaviour. In the first study, piglets were presented with different playbacks in a T-maze and their response behaviour was measured. The piglets were more attentive to the playback of human voices compared to background noise and spent more time close to the high-pitched, slow voice compared to the low-pitched, fast voice (Bensoussan *et al.*, 2019). In a second study, Bensoussan et al. (2020) investigated how the broadcast of human voices influences the behaviour of the pigs. The study found that pigs used to a vocal human experimenter showed signs of stress and increased interactive behaviour once they were presented with the same experimenter lacking vocal communication. In a study by Lange et al. (2020), researchers combined stroking cows with either playback or live acoustic stimuli consisting of speech with low-pitched, long vowels. They found a stronger decrease in heart rate after the stroking with live speech compared to the playback stimuli, indicating that there might be a preference for live speech over pre-recorded stimuli. All these studies suggest that animals pay attention to human voices and it seems that under the right circumstances the human voice is a very relevant part of human-animal interactions.

Finally a recent study by Ghazanfar et al. (2020) on marmoset monkeys (a non-domesticated mammal) found another possible link between the neural crest cells and acoustic behaviour. The authors of the study suggest that wild species can be put under selection pressures for domesticated phenotypes and hypothesise that there is a link between vocal cooperation and domestication; more specifically, they predict the size of the white patch on the forehead to correlate with the probability of the individual responding to contact calls (Ghazanfar *et al.*, 2020). The studies data supported this prediction and found a positive correlation between patch size and call response probability. While this study focusses more on possible results of self-domestication, which I haven't discussed in this thesis, it still underlines the relevance of investigating the impact of domestication on animals acoustic behaviour.

Future research directions: domestication and bioacoustics

Domesticated animals are so engrained in our everyday life that even amidst very human centric settings we often don't notice them or we forget that they are there. An excellent example to illustrate this is the following: while there is no record on any particular horse drawn carriage in London in the 19th century, we have plenty of information on Walter Rothschild's zebra drawn carriage in front of Buckingham palace in 1898. While most of us wouldn't be surprised to see a horse drawn carriage in front of the Buckingham palace, seeing a carriage pulled by zebras sure does catch the attention of onlookers. The domestication process and domesticated animals themselves might sometimes be overlooked similar to the horse drawn carriage in comparison to the zebras, yet domesticated animals provide a fascinating window into both artificial and natural selection processes which carry a lot of opportunities and potential for not only bioacoustic research.

As indicated in the discussion of current research in bioacoustics and domestication we have little knowledge of how the process of domestication shaped and formed the vocal production mechanisms of domesticated animals. Research comparing laryngeal structures between domesticated species, their wild cousins/ancestors and possible hybrids has the potential to offer crucial insight into this barely researched field.

Another indispensable factor in understanding and researching domestication is the impact domesticated animals (and their housing) have on nature and wildlife. A classic example for a domestic species impacting wild species are our domestic cats. Hybridisation events between domestic cats and European wildcats, as well as habitat destruction by humans almost wiped out the entire European wildcat population (Senn *et al.*, 2019; Quilodrán *et al.*, 2020). Another example of domestic cats impacting wild species is a topic discussed passionately both within and outside the scientific community: the influence of free ranging domestic cats on the numbers of birds and reptiles. These two examples listed here might be cat specific, but the general issue of domesticated animals impacting wildlife and nature isn't. Humans introduce domesticated species wherever they go and it is indispensable to understand and manage the impact our domesticated animals have on our surroundings.

To tackle these more general issues in often bigger areas, acoustic monitoring coupled with ever evolving technologies of machine learning might offer semi-automated opportunities for tracking movement, population size and density of free roaming domestic animals and their possible impact on the surroundings.

References

Ben-Aderet, T. et al. (2017) ‘Dog-directed speech: Why do we use it and do dogs pay attention to it?’, Proceedings of the Royal Society B: Biological Sciences, 284(1846), p. 20162429. doi: 10.1098/rspb.2016.2429.

Bensoussan, S. et al. (2019) ‘Domestic piglets (*Sus scrofa domestica*) are attentive to human voice and able to discriminate some prosodic features’, Applied Animal Behaviour Science. Elsevier B.V., 210, pp. 38–45. doi: 10.1016/j.applanim.2018.10.009.

Bensoussan, S. et al. (2020) ‘Broadcasting human voice to piglets (*Sus scrofa domestica*) modifies their behavioural reaction to human presence in the home pen and in arena tests’, Applied Animal Behaviour Science. Elsevier, 225, p. 104965. doi: 10.1016/j.applanim.2020.104965.

Bouchon, C. and Toro, J. M. (2019) ‘Is the consonant bias specifically human? Long–Evans rats encode vowels better than consonants in words’, Animal Cognition. Springer Berlin Heidelberg, (0123456789). doi: 10.1007/s10071-019-01280-3.

Burnham, D. and Francis, E. (1998) ‘Are you my little pussy-cat? Acoustic, phonetic and affective qualities of infant-and pet-directed speech’, in Proc. 5th International Conference on Spoken Language Processing (ICSLP 98).

Burnham, D., Kitamura, C. and Vollmer-Conna, U. (2002) ‘What’s new, pussycat? On talking to babies and animals’, Science, 296(5572), p. 1435. doi: 10.1126/science.1069587.

Cutler, A. et al. (2000) ‘Constraints of vowels and consonants on lexical selection: Cross-linguistic comparisons’, Memory and Cognition, 28(5), pp. 746–755. doi: 10.3758/BF03198409.

Darwin, C. (1868) The variation of animals and plants under domestication.

Evans, L. T. (1984) ‘Darwin’s use of the analogy between artificial and natural selection’, Journal of the History of Biology, 17(1), pp. 113–140. doi: 10.1007/BF00397504.

Feddersen-Petersen, D. U. (2000) ‘Vocalization of European wolves (*Canis lupus lupus L.*) and various dog breeds (*Canis lupus f. fam.*)’, Archiv für Tierzucht, 43(4), pp. 387–397.

Francis, R. (2015) ‘Preface’, in Domesticated: Evolution in a Man-Made World, pp. 1–6.

Ghazanfar, A. A. et al. (2020) ‘Domestication Phenotype Linked to Vocal Behavior in Marmoset Monkeys’, Current Biology. Elsevier Ltd., pp. 1–7. doi: 10.1016/j.cub.2020.09.049.

Güttinger, H. R. (1985) ‘Consequences of domestication on the song structures in the canary’, Behaviour, 95(3/4), pp. 254–278.

Hare, B. et al. (2002) ‘The domestication of social cognition in dogs’, Science, 298(5598), pp. 1634–1636. doi: 10.1126/science.1072702.

Hare, B., Call, J. and Tomasello, M. (1998) ‘ Communication of Food Location Between Human and Dog (*Canis Familiaris*) ’, Evolution of CommunicationEvolution of Communication. An international multidisciplinary journal, 2(1), pp. 137–159. doi: 10.1075/eoc.2.1.06har.

Hare, B. and Tomasello, M. (2005) ‘Human-like social skills in dogs?’, Trends in Cognitive Sciences, 9(9), pp. 439–444. doi: 10.1016/j.tics.2005.07.003.

Hemmer, H. (1990) ‘Changes in behaviour’, in Domestication: The decline of environmental appreciation, pp. 81–92.

Hoff, E. (2009) Language Development. 5th edn. Wadsworth: Jon-David Hague.

Kohane, M. J. and Parsons, P. A. (1988) ‘Domestication: Evolutionary Change under Stress’, Evolutionary Biology, 23, pp. 31–48.

Lange, A. et al. (2020) ‘Talking to Cows: Reactions to Different Auditory Stimuli During Gentle Human-Animal Interactions’, Frontiers in Psychology, 11(October). doi: 10.3389/fpsyg.2020.579346.

Mallikarjun, A., Shroads, E. and Newman, R. S. (2020) ‘The role of linguistic experience in the development of the consonant bias’, Animal Cognition. Springer Berlin Heidelberg, (0123456789). doi: 10.1007/s10071-020-01436-6.

McComb, K. et al. (2009) ‘The cry embedded within the purr’, Current Biology, 19(13), pp. 507–508. doi: 10.1016/j.cub.2009.05.033.

Price, E. O. (2002) ‘Artificial selection’, in Animal domestication and behavior, pp. 43–51.

Quilodrán, C. S. et al. (2020) ‘Projecting introgression from domestic cats into European wildcats in the Swiss Jura’, Evolutionary Applications, 13(8), pp. 2101–2112. doi: 10.1111/eva.12968.

Senn, H. V. et al. (2019) ‘Distinguishing the victim from the threat: SNP-based methods reveal the extent of introgressive hybridization between wildcats and domestic cats in Scotland and inform future in situ and ex situ management options for species restoration’, Evolutionary Applications, 12(3), pp. 399–414. doi: 10.1111/eva.12720.

Virányi, Z. et al. (2008) ‘Comprehension of human pointing gestures in young human-reared wolves (*Canis lupus*) and dogs (*Canis familiaris*)’, Animal Cognition, 11(3), pp. 373–387. doi: 10.1007/s10071-007-0127-y.

Xu, N. et al. (2013) ‘Vowel hyperarticulation in parrot-, dog- and infant- directed speech’, Anthrozoos, 26(3), pp. 373–380. doi: 10.2752/175303713X13697429463592.

Zusammenfassung

Menschen leben seit Jahrtausenden mit domestizierten Tieren zusammen und im Laufe dieser Zeit beeinflussten sie sowohl die anatomische Form als auch das Verhalten domestizierter Tiere in subtiler bis auffälliger Weise. Hängeohren, Ringelruten, weiße Flecken und eine verkürzte Schnauze sind allgegenwärtig in domestizierten Säugetieren, aber erst kürzlich konnte ein grundlegender Faktor identifiziert werden der diese Ansammlung an Eigenschaften erklären könnte: Während des Domestikationsprozesses haben Menschen aktiv und/oder passiv Tiere künstlich für ihre Zahmheit selektiert. Diese künstliche Selektion führte zu einem (minimalen) Defizit in den Neuralleistenzellen. Dieses Defizit hat das Potenzial all die Merkmale, die typisch für domestizierte Säugetiere sind zu erklären. Neuralleistenzellen sind auch ein Schlüsselkomponent in der Entwicklung der Kehlkopfstrukturen und stellen somit eine direkte Verbindung zwischen dem Domestikationsprozess und stimmproduzierender Strukturen dar.

An dieser Schnittstelle von Stimmproduktion und Domestikation findet sich eine Vielzahl an Facetten und offenen Fragestellungen, die von Signalproduktion und Signalwahrnehmung in domestizierten Tieren bis hin zu zwischenartlicher Kommunikation reicht. In den Kapiteln dieser Arbeit diskutiere ich Ergebnisse zu zwei Fragestellungen: 1) wie verwenden Menschen Sprache um mit ihren domestizierten Tieren zu kommunizieren und 2) wie beeinflussen künstliche Selektionsdrucke Stimmproduktionsmechanismen und Vokalisation?

In Kapitel 1 untersuche ich die differenzierte Verwendung von Tonhöhe und Variationsbreite der menschlichen Stimme in hundegerichteter Sprache. Außerdem zeige ich auf, dass hundegerichtete Sprache sowohl von der Persönlichkeitsachse Offenheit sowie von Umweltstressoren beeinflusst wird. In Kapitel 2 widme ich mich dem vokalen Verhalten von Ratten und zeige, dass starke künstliche Selektionsdrucke für niedrige/hohe Anzahl von Ultraschallrufen in Jungtieren zu Veränderungen im akustischen Verhalten von adulten Tieren führen. In Kapitel 3 untersuche ich wie sich künstliche Selektionsdrucke auf die Kehlkopfanatomie auswirken. Ich beschreibe Veränderungen in der Mineralisation des Schildknorpels und Änderungen in der Länge der Stimmfalten. Die Kapitel dieser Dissertation sollen helfen weitreichendere Fragen der Mensch-Tierbeziehung sowie der akustischen Kommunikation zu beantworten.

Abstract

Humans have been living with domesticated animals for up to 30,000 years. During this prolonged period, humans have influenced both the anatomical form and behaviour in domesticated mammals, in ways both obvious and subtle. Floppy ears, curly tails, white patches and a reduced muzzle are ubiquitous across domesticated mammals. Recent research has identified a unifying factor potentially able to explain this collection of traits: during the process of domestication, active and/or passive artificial selection for increased tameness in animals resulted in a mild deficiency in neural crest cells. This deficiency could explain these morphological changes. Apart from the neural crest cell's role in the domestication process, these cells are also a key component in the formation of laryngeal structures, linking domestication and voice producing structures.

At the intersection of voice production and domestication, there are a multitude of facets and smaller questions to study, ranging from signal production and perception in domesticated species to interspecies communication. This thesis allows me to discuss findings on two fronts: 1) how humans engage with their domesticated animals on a vocal level and 2) how artificial selection pressures affect vocal production and output.

In chapter 1, I investigated the differential use of mean pitch and pitch range in dog-directed speech and suggest that companion animal-directed speech is affected by both the personality trait "openness" and environmental stressors. In chapter 2, I turn to vocal behaviour in rats, showing that strong artificial selection pressures for ultrasonic call rates in pups affect the adult rats' acoustic behaviour. In chapter 3, I turn to anatomy, investigating how this strong artificial selection pressure on rat pups affected the adult laryngeal anatomy, finding changes to the thyroid cartilage's mineralization and to vocal fold length. This thesis will help inform on broader questions regarding the human-animal relationship and acoustic communication.