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# Multilevel communication of static and dynamic cues in the calls of domestic dog puppies (*Canis familiaris*)

Mathilde Massenet

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## Multilevel communication of static and dynamic cues in the calls of domestic dog puppies (*Canis familiaris*)

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# Abstract

In vertebrates, in order to provide optimal care to their young, parents are expected to use their vocal signals to assess kin, individuality or condition as well as more dynamic emotions or motivations. Despite the fact that many highly accessible species of domesticated mammals, that produce large and highly vocal litters, provide an ideal model for studying the mechanisms underlying such multilevel communication, they remain largely under-investigated. In this thesis, I aim to fill this gap by investigating the production and the perception of such information encoded in the offspring calls of domestic dogs, a large litter-rearing mammal.

In the first chapter, I analyse whine acoustics to examine what vocal cues encodes for litter, individual and condition of puppies. At 3 weeks of age, when puppies heavily rely on their mother to receive care, I show that variation in whine  $f_0$  (fundamental frequency) between litter and between puppies within their litters are predicted by body variation. Playbacks of resynthesized whines also indicate that mothers use  $f_0$  for discrimination of kin, individuality and condition at both litter and individual levels. In the second chapter, I move on to the investigation of indicators of intra-individual variation in arousal. I analyse NLP (nonlinear phenomena) – a well-known cue to arousal in infant mammals- in whines given by puppies during a short separation from their mother. I show that the production of whines affected by NLP increases as time since separation increases, suggesting that NLP function as vocal cue to heightened arousal. These findings are supported by psychoacoustic experiments showing that NLP in whines increased perceived levels of distress in human listeners, including professional caregivers.

In sum, my thesis work provides evidence for the acoustic encoding of multilevel static and dynamic information in a single call type, that mediate interactions between offspring and their caregivers and may facilitate the provision of optimal care. More systematic studies should now describe parent-offspring multilevel communication in a broader range of species that rear multiple offspring to better understand the conditions under which these complex interactions have evolved.

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# General introduction

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In many vertebrate and invertebrate species, parental care is crucial for increasing the chances of offspring survival and thus improving reproductive success (Royle, Smiseth and Kölliker, 2012). Parental care can be provided via different forms ranging from the preparation of environments receiving offspring (e.g., nests, dens, cavities or burrows), offspring defence and protection, to offspring feeding (Balshine, 2012). Crucially, misdirected, insufficient or excessive parental care can be costly for both parents and offspring. In this context, many species have evolved signalling systems that support the simultaneous communication of offspring identity and needs to ensure adequate provision of care. Among vertebrates, parent-offspring interactions are mediated via different sensory modalities, including visual signals (e.g., sheep (*Ovis aries*): Kendrick et al., 1996; Australian sea lions (*Neophoca cinerea*): Charrier, Pitcher and Harcourt, 2022) or olfactory signals (blue petrels (*Halobaena caerulea*): Leclaire, Bourret and Bonadonna, 2017; zebra finches (*Taeniopygia guttata castanotis*): Golüke, Bischof and Caspers, 2021; Australian sea lions: Charrier et al 2022; pigs (*Sus domesticus*): Maletínská et al., 2002; cats (*Felis catus*): Bánszegi et al., 2017; sheep: Keller et al., 2003). However, a wide range of species also use acoustic signals, as reported in crocodylians (Vergne et al., 2011; Chabert et al., 2015), birds (Beecher, Beecher and Hahn, 1981; McArthur, 1982; Barg and Mumme, 1994; Aubin and Jouventin, 2002) or in mammals (Holekamp et al., 1999; Torriani, Vannoni and McElligott, 2006; Sèbe et al., 2007; Rendall, Notman and Owren, 2009; Briefer and McElligott, 2011b; Knörnschild, Feifel and Kalko, 2013; Padilla de la Torre et al., 2016; Bouchet et al., 2020; Charrier, 2020). Compared to visual and olfactory signals, acoustic signals are relatively directional, can propagate over large distances and also function when visibility is reduced (Bradbury and Vehrencamp, 2011). As such, acoustic signals are an ideal candidate for mediating parent-offspring interactions.

Below, I provide a comprehensive overview of the research focusing on vocal interactions between parents and offspring in vertebrates. Specifically, I review the nature of parent-offspring communication systems in species rearing a single offspring as well as in species producing large broods or litters. I highlight the importance of the acoustic encoding of multilevel information related to offspring identity, condition and short-term need.

## Parent-offspring communication systems

### *Kin recognition in species rearing single young*

The nature and complexity of offspring recognition systems can largely depend on the behavioural ecology of the considered species. It has indeed been suggested that selective pressures associated with different social organisations, anti-predator strategies and/or reproductive strategies (Aubin and Jouventin, 2002; Torriani, Vannoni and McElligott, 2006; Briefer and McElligott, 2011b; Caro *et al.*, 2016; Charrier, 2020) have shaped the mechanisms underlying offspring recognition by parents.

Specifically, offspring recognition via acoustic signals is likely to be absent or rudimentary in species producing immature offspring with limited mobility such as, e.g., in altricial species. Similarly, parents that spend most of their time with their young in a burrow or a den rely on spatial proximity for care provisioning, as individuals within a given location are likely to be genetically related to them (Nowak *et al.*, 2000). In contrast, it has been suggested that selective pressures should favour the emergence of bidirectional vocal recognition in group-living or colonial species, where offspring can be relatively mobile (e.g., precocial species) or separated from their parents over long periods (e.g., *Aptenodytes* penguins or pinnipeds). These species are indeed exposed to high risks of misallocating parental care or aggression from group members or predators. In these contexts, both parents and offspring must develop their own and sophisticated acoustic distinctiveness shortly after birth to find each other within a group of up to several hundred conspecifics. A classic example is found in penguins, where non-nestling species such as emperor penguins (*Aptenodytes forsteri*) use more complex calls (relying on a dual-source information) than nestling species such as Adélie penguins (*Pygoscelis adeliae*) (Aubin, Jouventin and Hildebrand, 2000; Aubin and Jouventin, 2002). Pinnipeds are also an excellent model for understanding mother-young vocal interactions in mammals. Indeed, as reviewed in Charrier (2020), mother-young vocal recognition in phocids appears to covary with the colony's density. For example, in the highly colonial Northern elephant seal (*Mirounga angustirostris*), playback experiments have revealed that mothers use vocalisations to identify pups as theirs (Linossier *et al.*, 2021), whereas in Weddell seals (*Leptonychotes weddellii*), a species forming relatively smaller colonies, mothers' behaviour does not significantly change in response to playbacks of their own *versus* stranger pup calls (Opzeeland *et al.*, 2012).

Studies focusing on ungulates have also illustrated how selective pressures related to predation risks can act on kin recognition systems. In species that mainly rely on fleeing or group defence to protect themselves against predators, the young closely follows its mother and both use a bidirectional recognition system to find each other within the group (sheep: Sèbe *et al.*, 2007; cattle (*Bos taurus*): Padilla de la Torre *et al.*, 2016). In contrast, species living in relatively small groups that cannot benefit from group defence against predators, hide their vulnerable and relatively immobile offspring in the vegetation during their first weeks of life (as observed fallow deer (*Dama dama*)) (Lent, 1974). In these species, a relatively simpler unidirectional recognition system has been identified, where only the mother produces individualised vocalisations that can be recognised by the hiding offspring (Torriani, Vannoni and McElligott, 2006).

In sum, these observations support the hypothesis that the social organisation of a species and its associated risks of predation can influence its kin recognition systems, ranging from relatively simple (e.g., unidirectional recognition in fallow deer) to more complex and sophisticated (e.g., bidirectional recognition in emperor penguins) forms. However, while most research focused on species that typically care for a single offspring, our understanding of kin recognition systems in litter- or brood- rearing species is very limited, despite strong predictions that number of offspring should shape the nature of parent-offspring vocal interactions.

#### *Kin and individual discrimination in species rearing multiple young*

Parents that simultaneously rear multiple offspring face a specific problem. Indeed, in such systems, selection pressures linked to kin recognition should favour the acoustic distinctiveness of calls between own *versus* stranger litters (or broods) by limiting inter-individual variation within the litter (figure 1). On the other hand, individual recognition subserving optimised individual care should push for interindividual call differentiation within the litter (figure 1). As such, parents producing multiple offspring are not only expected to perform kin recognition at group level (i.e., litter) in order to limit resource misallocation, but also to discriminate at individual level to detect dynamic variation in condition and/or motivation and to provide individualised care. Several studies have already demonstrated that parents are able to discriminate between their offspring and strangers' offspring in litter- or brood-rearing species (McArthur, 1982; Stoddard and Beecher, 1983; Holekamp *et al.*, 1999; Illmann *et al.*, 2002; Levréro *et al.*, 2009; Mogi *et al.*, 2017). For instance, cliff swallows (*Petrochelidon pyrrhonota*) and pinyon jays (*Gymnorhinus*

*cynocephalus*) visit their nest significantly more in response to natural begging calls of their chicks than to strangers' begging calls (McArthur, 1982; Stoddard and Beecher, 1983). Furthermore, in spotted hyenas (*Crocuta crocuta*), playback experiments of natural woop vocalisations given by kin cubs elicit stronger behavioural responses in mothers compared to non-kin woop (Holekamp *et al.*, 1999). However, because the playback experiments reported in these studies used natural sounds, they do not allow to determine whether the parents identified their offspring as kin at litter level ("group signature", Monk, Koenig and Koenig, 1997; Illmann *et al.*, 2002; Syrová *et al.*, 2017), at individual level (sum of "individual signatures", Monk, Koenig and Koenig, 1997; Levréro *et al.*, 2009; Briefer and McElligott, 2011; Scheumann *et al.*, 2012; Dreiss, Ruppli and Roulin, 2014; Syrová *et al.*, 2017), or both. It is thus now crucial to describe what vocal cues in offspring calls encode for kin information, whether these cues are present at the group and/or at the individual level, and to test how these levels contribute to their recognition by parents using methods of sound resynthesis that allow for controlled, independent manipulations.

#### *Condition and temperament discrimination in offspring calls*

Parents are not only expected to recognise their offspring as theirs, but they are also expected to identify those that require most care. Parental perception of condition in offspring calls has mainly been explored in birds (with few exceptions in crocodylians or mammals). A recent comparative study including more than 140 bird species has shown that low-condition chicks beg more and are preferentially fed by parents if food is relatively abundant in their environment (Caro *et al.*, 2016), suggesting that parents are able to detect individual variation in body condition in begging calls of their chicks within their brood. In crocodiles, there is also evidence for maternal perception of condition in the calls of their young: females are more often attracted by sound playbacks of high-pitched synthetic contact calls (simulating a small, young offspring) compared to low pitched calls (simulating a bigger, older offspring) (Chabert *et al.*, 2015). Finally, in another study conducted on pigs - a mammal species producing large litters - playback experiments have revealed that mothers exhibited stronger vocal responses to relatively high-pitched calls given by the smallest offspring their litter.

Because sibling competition for parental resources can critically affect offspring body condition and survival (where dominant offspring are typically in better condition than subordinates, Hofer *et al.*, 2016; López-Jiménez *et al.*, 2016; Bebbington *et al.*, 2017), parents are

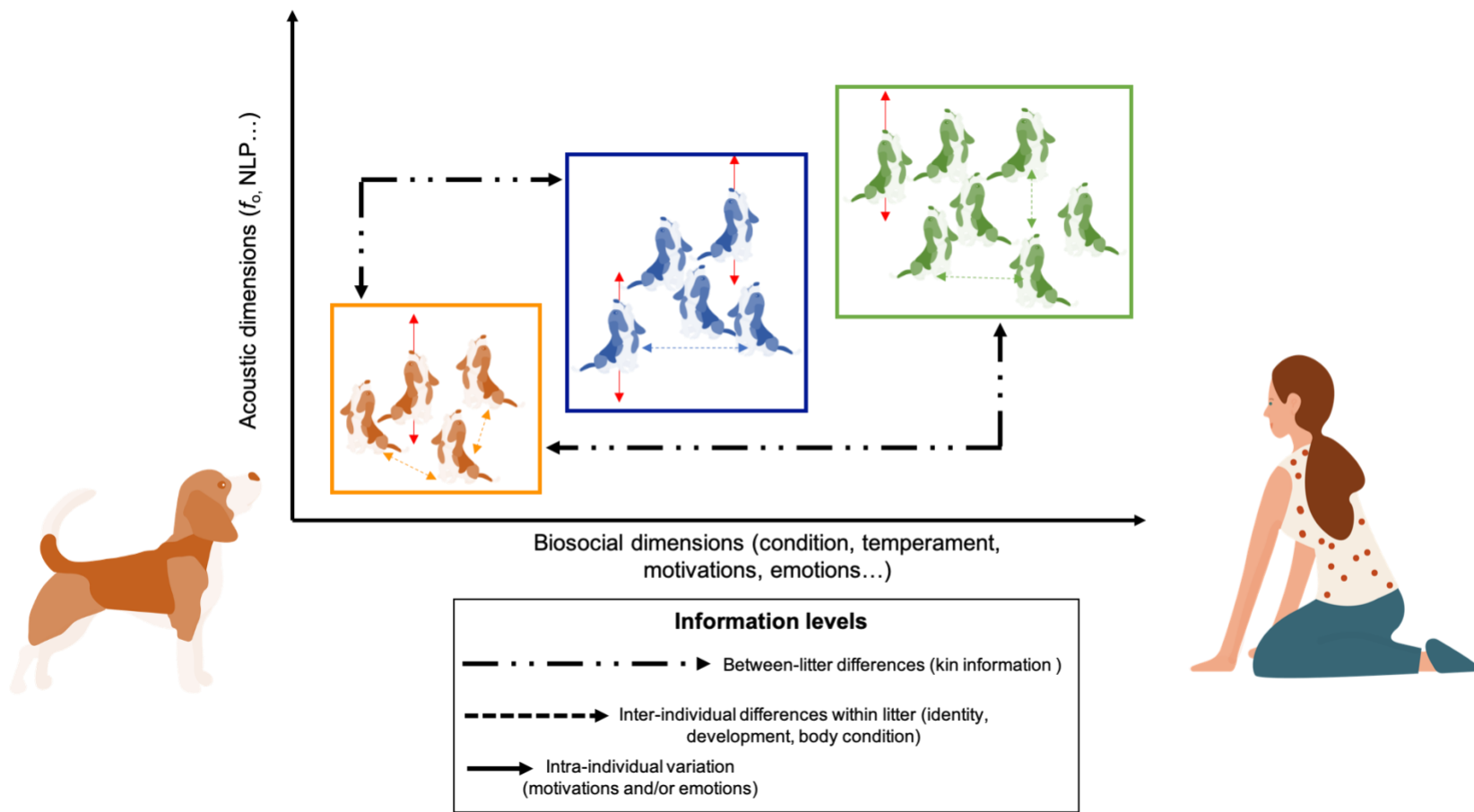
also predicted to perceive cues to temperament in their offspring calls to adjust parental care and minimize costs of sibling rivalry. For instance, observations in spotted hyenas report that mothers provide more care to subordinate than dominant cubs (White, 2008).

#### *Motivation and emotion discrimination in offspring calls.*

Finally, while kin, individual and/or condition discrimination are important in parent-offspring vocal interactions, parents should be able to detect intra-individual variation in arousal in order to adjust care accordingly to the level of short-term needs of their young. Indeed, parents are expected to perceive short-term variation in their offspring vocalisations that reflect motivation or emotions (e.g., hunger, aggression or distress) (figure 1). In this context, the familiarisation of parents with their offspring calls that is necessary for them to learn to discriminate between individuals can also support their detection of dynamic cues to emotions and/or motivations. Indeed, by learning the baseline vocal behaviour of each individual, parents can become experts of their individual offspring vocalisations and thus become better at detecting departures from baseline that indicate fluctuating need, emotions or motivations (Corvin *et al.*, 2022). Several studies provide evidence for parent's ability to detect of varying level of need or distress in their offspring calls. Indeed, this has been reported in a wide range of vertebrate including crocodylian, avian as well as mammalian species rearing a single or multiple offspring (Leonard and Horn, 2001; Smiseth and Lorentsen, 2001; Quillfeldt, 2002; Manser *et al.*, 2008; Vergne *et al.*, 2011; Lingle *et al.*, 2012; Corvin *et al.*, 2022). For instance, the communication of varying distress level has been described in humans (*Homo sapiens*), where caregivers are able to detect varying levels of pain in their baby cries after a short learning period (Corvin *et al.*, 2022). Another study also found that black caiman mothers (*Melanosuchus niger*) exhibit stronger behavioural responses to high-pitched distress calls given by juveniles compared to low-pitched contact calls (Vergne *et al.*, 2011), suggesting that females may use specific vocal cues in their offspring calls to assess level of distress.

Moreover, begging calls reflect varying level of hunger in offspring and usually elicit food provision by parents. For instance, in mammals, adult meerkat caregivers (*Suricata suricatta*) provide food to pups in response to playbacks of begging calls. Specifically, they increase food provisioning as the number of high-pitched begging calls increases, suggesting that caregivers are able to perceive intra-individual variation in the pups' level of hunger through the acoustics of begging calls (Manser *et al.*, 2008). Parental perception of short-term needs has also been

described in a large number of birds, and especially those producing large broods. Most studies report that parents systematically deliver larger meals to their chicks when offspring increase the production of begging calls (Clark and Lee, 1998; Leonard and Horn, 2001; Quillfeldt, 2002; Quillfeldt, Masello and Hamer, 2004). A recent study also found that parental investment was greater in response to higher-pitched calls produced by a brood that received corticosterone (a stress hormone) compared to control broods (without corticosterone administration) (Perez *et al.*, 2016). However, as most investigations of parents' perception of need/distress levels in their offspring calls reported field observations or tested this hypothesis using natural sound playbacks (black caimans: Vergne *et al.*, 2011; reed warbler (*Acrocephalus scirpaceus*): Kilner, Noble and Davies, 1999; trees swallows (*Tachycineta bicolor*): Leonard and Horn, 2001; Wilson's storm petrels (*Oceanites oceanicus*): Quillfeldt, 2002; Quillfeldt, Masello and Hamer, 2004; zebra finches: Perez *et al.*, 2016; grey seals (*Halichoerus grypus*): Smiseth and Lorentsen; meerkats: Manser *et al.*, 2008; humans: Corvin *et al.*, 2022), what vocal cues are actually used to assess intra-individual variation of need/distress in their offspring calls remains to be demonstrated.



**Figure 1:** Different levels of information potentially encoded in a single call type: litter-level information may enable mothers to discriminate between own and stranger, to assess litter condition, and to position individuals within litter (weaker or stronger than average). Within-litter, individual information may enable mothers and caregivers to discriminate between individuals, identify condition and allocate individualised care. Within-individual emotional and motivational dynamic information may enable mothers and caregivers to assess immediate needs. These communication levels are expected to be in conflict, as increasing distinctiveness of litters is likely to rely on within litter homogeneity and thus limit individual variation within litters, and as increasing intra-individual variation to efficiently express emotional or motivational states may be at the expense of individuality.

## **Overview and gaps in research on parent-offspring vocal communication**

Together, the literature reviewed above suggests that parent-offspring vocal communication systems rely on the production and perception of multilevel information related to identity (litter and/or individual), condition, and short-term varying need encoded in offspring calls. While these vocal interactions only occur at individual level in species rearing a single offspring, however, in species producing large litters, selective pressures must have favoured fine-tuned perception in parents to detect differences at the litter level as well as at individual level (within their litter) (figure 1). Parents are expected to perceive differences between litters to ensure kin and individual recognition, and to perceive inter and intra differences between individual offspring within the group to ensure the provision of adapted care according to their condition and short-term need (figure 1). Despite the fact that many mammal species, including domesticated ones, can produce large and highly vocal litters, our understanding of such complex multilevel communication remains surprisingly poor. It is thus crucial to uncover the mechanisms underlying vocal interactions between parents and their multiple offspring in litter-rearing mammalian species. A first step consists of investigating how multilevel information can be encoded in a single call type. To do this, we must consider how the mechanisms of sound production affect the encoding of information in their acoustic structure (Taylor and Reby, 2010)

## **Information encoded in offspring mammal calls**

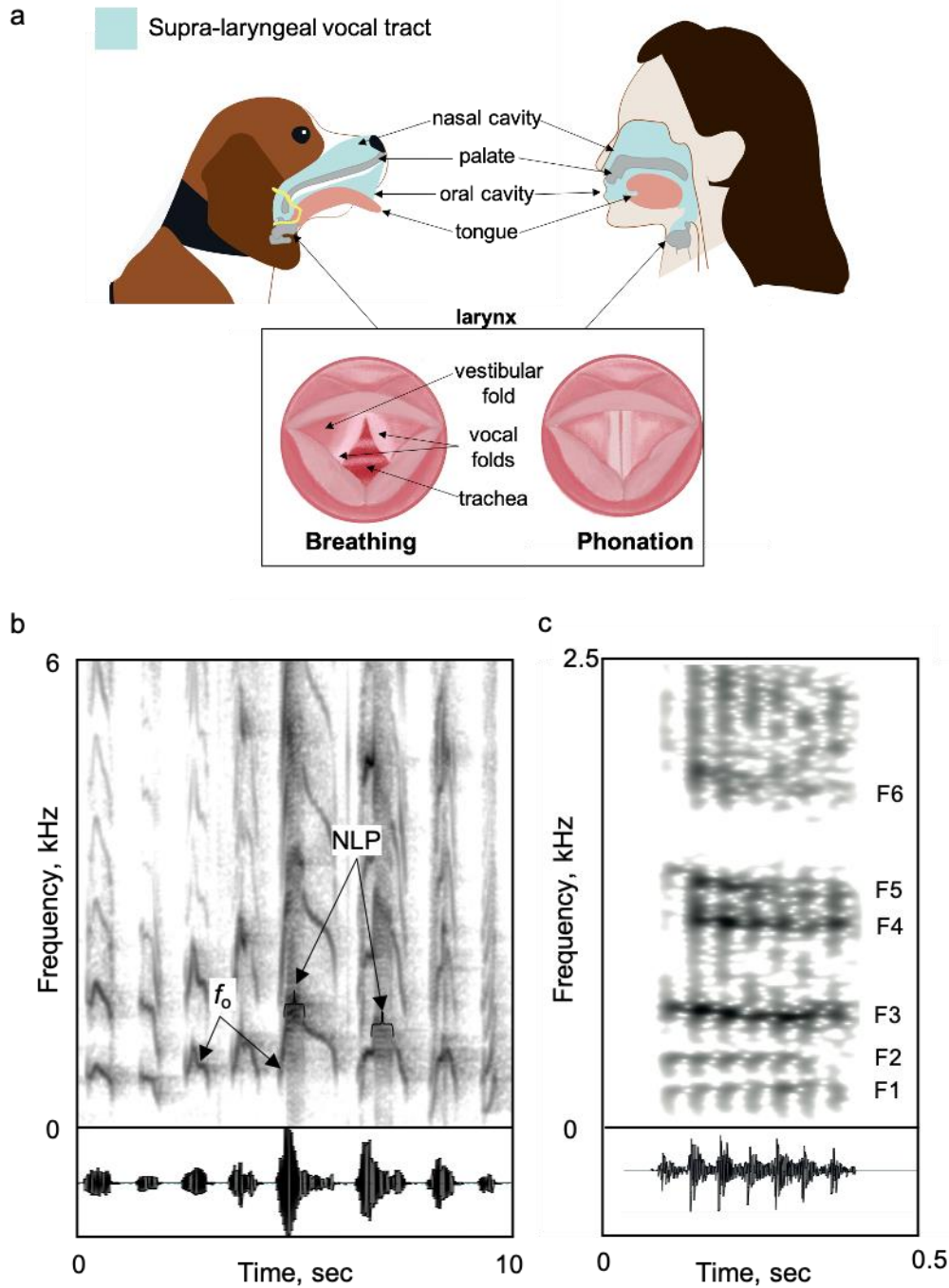
### *Mechanisms of sound production*

In terrestrial mammals, with a wide range of species presenting strong similarities in their vocal anatomy, shared mechanisms of sound production underlie a phylogenetically preserved system of vocal communication. Most species indeed possess a larynx (containing a pair of vocal folds) and a supra-laryngeal vocal tract (composed of the laryngeal, oral and/or nasal cavities) (figure 2a). The source-filter theory posits that sound production follows a two-step mechanism. Specifically, sounds are first generated in the larynx by the vibration of vocal folds (described as the “source”) and then are filtered in the vocal tract (described as the “filter”) (Titze, 1994) before its radiation through the mouth (or nostrils) into the external environment. The rate of vocal fold vibration determines the fundamental frequency  $f_0$  of vocalisations, which is perceived as pitch (Titze, 1994) (figure 2b). In addition, the shape and size of the supra-laryngeal vocal tract



determine the vocal tract resonances or formant frequencies, also perceived as timbre (Titze, 1994) (figure 2c).

While typically stable and periodic, vocal fold vibration can however become irregular and lead to the production of nonlinear phenomena (hereafter NLP, see figure 2b) (Titze 1994). Nonlinear phenomena are extremely common (e.g., koalas (*Phascolarctos cinereus*): Charlton, 2015; marmots (*Marmota marmota*): Blumstein and Récapet, 2009; rhesus macaques (*Macaca mulatta*): Fitch, Neubauer and Herzel, 2002; humans: Mende, Herzel and Wermke, 1990; Raine *et al.*, 2018; meerkats: Townsend and Manser, 2011; dogs (*Canis famialiris*): Marx *et al.*, 2021; North American elk (*Cervus canadensis*): Reby *et al.*, 2016; cattle: Green *et al.*, 2020; African elephants (*Loxodonta Africana*): Stoeger *et al.*, 2011; Fuchs *et al.*, 2021). As such, NLP are considered an important feature of vocal signals, and include four main types: frequency jumps, subharmonics, deterministic chaos and biphonation (Wilden *et al.*, 1998; Fitch, Neubauer and Herzel, 2002). Nonlinear phenomena are often responsible for the perceived harshness, roughness and vocal instabilities of vocalisations (Anikin *et al.*, 2021). Therefore,  $f_0$ , formants and NLP have been largely described as key vocal features in infant mammal calls encoding for both static and dynamic cues. Static cues are related to relatively long-term, stable information that typically characterises animal identity and biological traits including physical attributes such as individual's sex, age, body size, body condition, testosterone levels, as well as social attribute such as individual's temperament. Dynamic cues correspond to short term variation, for example in caller's emotional or motivational states (Briefer, 2020; Charlton *et al.*, 2020).



**Figure 2:** Comparative vocal anatomy between humans and domestic dogs (a) and key features of mammal calls (b-c). Panel (a) illustrates shared vocal anatomy among mammal species. Panel (b) shows a spectrogram of a sequence of puppy whines emitted during a short separation from their mother and littermates. Fundamental frequency ( $f_0$ ) of whines, as well as episodes of nonlinear phenomena (NLP) are annotated. Panel (c) presents a spectrogram of a fallow deer groan emitted during the breeding season. The first six formant frequencies are annotated. Panels (a) and (c) are adapted from Root-Gutteridge, Pisanski and Reby (2019) and Charlton and Reby (2011) with the authors' permission.

### *Vocal communication of litter and individual information*

In terrestrial mammals, fundamental and formant frequencies are usually individualized because they reflect the vocal anatomy of callers (as reviewed by Taylor and Reby, 2010; Taylor, Charlton and Reby, 2016; Charlton *et al.*, 2020). For instance, due to allometric scaling, formants can predict inter-individual differences in body size in most mammal species (Charlton *et al.*, 2020). Fundamental frequency can also depend on individual variation in the length or stiffness of vocal folds, where longer and laxer vocal folds vibrate at a lower rate and produce signals with a relatively low  $f_0$  (Titze, 1994). However, infant calls are typically emitted with a relatively high  $f_0$  and are thus characterized by relatively low spectral density (Lingle *et al.*, 2012). In this context, vocal tract resonances of distress calls are not clearly emphasized (Titze, 1994) and formant frequencies are unlikely to play a crucial role in signalling cues to offspring individuality to mothers.

Fundamental frequency has been demonstrated to strongly predict inter-individual differences in calls given by a wide range of infant mammal species (e.g., Australian sea lion pups: Charrier and Harcourt, 2006; kittens: Scheumann *et al.*, 2012; goat kids: Briefer and McElligott, 2011; calves: Padilla de la Torre *et al.*, 2015; piglets: Syrová *et al.*, 2017; infant chimpanzees (*Pan troglodytes*): Levréro and Mathevon, 2013; human babies: Gustafsson *et al.*, 2013). Moreover,  $f_0$  can vary between individuals within their litter and between litters (Illmann *et al.*, 2002; Briefer and McElligott, 2012; Syrová *et al.*, 2017). For instance, it has been demonstrated that  $f_0$  is a key contributor for vocal distinctiveness of piglet calls between individuals of a same litter and between different litters (Illmann *et al.*, 2002; Syrová *et al.*, 2017). Higher levels of acoustic similarities in  $f_0$  between full siblings than between half siblings also supports the hypothesis that litter-specific cues may have genetic component as suggested in goats (Briefer and McElligott, 2012). These differences can remain stable throughout development as demonstrated in humans, wherein  $f_0$  in infancy and childhood strongly predicts voice  $f_0$  into adulthood (Fouquet *et al.*, 2016; Levréro *et al.*, 2018).

According to the acoustic allometry principle, animals are expected to develop larger vocal folds and longer vocal tracts as they grow (Fitch, 1997; Reby and McComb, 2003; Taylor and Reby, 2010). As such, vocal features of offspring calls are expected to encode age information, as indeed demonstrated in several species, where older and larger individuals typically produce vocalisations with relatively lower  $f_0$  than do newborns (goats: Briefer and McElligott, 2011a;

humans: Titze, 1994; Corsican deer (*Cervus elaphus corsicanus*): Kidjo *et al.*, 2008). Furthermore, sexual dimorphism in caller's vocal anatomy typically emerges at puberty (e.g., as well-described in humans Fitch and Giedd, 1999). These anatomical changes are mediated by androgens (i.e., testosterone) that influence vocal fold size (Evans *et al.*, 2008). Indeed, adult males present higher levels of testosterone, longer vocal folds and produce vocalisation with lower  $f_0$  than females (goitred gazelles (*Gazella subgutturosa*): Efremova *et al.*, 2011; Corsican deer: Kidjo *et al.*, 2008; elephants: Baotic and Stoeger, 2017; baboons: Rendall *et al.*, 2005; humans: Puts *et al.*, 2016). Therefore, before puberty, offspring sex does not contribute to interindividual differences in the calls of several mammal species (e.g., human babies: Levréro *et al.*, 2018; kittens: Scheumann *et al.*, 2012; calves: Padilla de la Torre *et al.*, 2015; piglets: Garcia *et al.*, 2016)

There is also some evidence that  $f_0$  could signal the body condition of individuals as, e.g., a consequence of slower development. Indeed, lower-condition individuals are expected to have shorter vocal folds and produce vocalisations with relatively high  $f_0$ , compared to individuals benefiting from a better condition. In infant mammals, several studies support these predictions. For instance, neonate body weight is significantly correlated to their cry  $f_0$ , where relatively big babies cry with a lower  $f_0$  than do small babies (Wermke and Robb, 2010). Fundamental frequency is also higher in cries given by babies born at preterm compared to full-term (Shinya *et al.*, 2014). In addition, a study conducted in litter-rearing piglets has suggested a negative relationship between call  $f_0$  and body weight (an index of body condition in vertebrates, Ronget *et al.*, 2018) (Weary, Lawson and Thompson, 1996). In contrast, the acoustic allometry also suggests that weak newborns can present smaller lung capacities (Bartlett and Areson, 1977) and/or weaker muscles preventing them from increasing subglottal pressure at the needed levels to significantly increase the  $f_0$  of their calls (also characterised by high amplitude and duration), compared to fitter offspring (Titze, 1994; Fischer *et al.*, 2004). It has indeed been demonstrated that male quality/condition is positively correlated with call  $f_0$  and duration in adult baboons (Fischer *et al.*, 2004). Therefore, we can expect inter-individual differences in the call features of young mammals, particularly their  $f_0$ , to be explained by two potentially opposing mechanisms: vocal fold size and/or lung capacity.

Moreover,  $f_0$  can also explain differences in individual temperament and androgens levels, as demonstrated in several species (e.g., fallow deer: Vannoni and McElligott, 2008, pandas (*Ailuropoda melanoleuca*): Charlton *et al.*, 2011 and humans: Puts *et al.*, 2007; Puts *et al.*, 2016; Cartei *et al.*, 2020). Recent findings in children indeed report a negative relationship between voice

$f_0$ , salivary testosterone levels and perceive masculinity (index of dominance) (Levréro *et al.*, 2018; Cartei *et al.*, 2020). In species producing multiple offspring, where sibling competition is common, temperament and androgen level of young can considerably affect their body condition (Frank, Glickman and Licht, 1991; Hofer *et al.*, 2016; López-Jiménez *et al.*, 2016; Bebbington *et al.*, 2017). As such, within a litter, relatively larger, more dominant littermates with higher levels of testosterone are thus expected to produce vocalisations characterised by lower  $f_0$  (and vice versa).

#### *Vocal encoding of dynamic information related to need or distress*

Besides the communication of individual information, acoustic features of offspring calls have been described as vocal indicators of intra-individual varying levels of need (e.g., hunger or distress) (Lingle *et al.*, 2012; Briefer, 2020). Indeed, changes in callers' emotional states affect the somatic and autonomic nervous system, which in turns affect the configuration of the vocal apparatus (Scherer, 2003). Specifically, increase in arousal is typically associated with a high subglottal pressure (resulting from an intense respiration), which ultimately leads the production of vocalisations characterized by a longer duration, a higher amplitude and a higher  $f_0$  (Briefer, 2012). Some of these vocal changes have been observed in the infant vocalisations of several mammal species including infant elephants (Stoeger *et al.*, 2011), panda cubs (Stoeger *et al.*, 2012) or piglets (Syrová *et al.*, 2017). For example, infant elephant roars produced during separation from their mother (high arousal context), had a significantly higher  $f_0$  and duration than those produced during suckling (low arousal context) (Stoeger *et al.*, 2011).

Heightened levels of the caller's need or distress can also affect the typically regular pattern of vocal fold vibration and thus lead to the production of NLP (Titze, 1994; Berry *et al.*, 1996; Briefer, 2012; Herbst, 2014). As such, NLP have been described as important acoustic features in the communication of need or distress (Wilden *et al.*, 1998; Fitch, Neubauer and Herzel, 2002). Nonlinear vocal phenomena are indeed commonly found in offspring distress vocalisations (Stoeger *et al.*, 2011, 2012; Scheumann *et al.*, 2012; Koutseff *et al.*, 2018). Because of their perceptual unpredictability, another potential and non-mutually exclusive communicative function of NLP is to reduce habituation in receivers by making calls difficult to ignore (Fitch, Neubauer and Herzel, 2002; Reby and Charlton, 2012; Karp *et al.*, 2014; Charlton, Watchorn and Whisson, 2017). For instance, NLP that naturally occur in alarm calls of meerkats induce escaping

behavioural responses and reduce habituation by increasing individual's vigilance after sound exposures (Townsend and Manser, 2011).

Finally, as mentioned above, because high emotional state typically predicts a high vocal effort by the caller (Briefer, 2012), the associated physiological changes in the vocal apparatus of highly aroused animals may in turn affect key vocal cues to individuality. While some findings indeed demonstrate that individual encoding decreases with increasing levels of arousal (Rendall, Notman and Owren, 2009), vocalisations can remain individualized in highly-aroused vocalisers (Scheumann *et al.*, 2012; Green *et al.*, 2019; Pisanski, Raine and Reby, 2020), suggesting that parents may recognise their offspring from distress vocalisations while simultaneously assessing their level of need or distress.

### **The domestic dog: an excellent model to study multilevel parent-offspring communication**

Together with wolves (*Canis lupus*), coyotes (*Canis latrans*), jackals (*Canis aureus*), dingoes (*Canis dingo*), domestic dogs form the *Canis* genus. Dogs diverged from their wild ancestor, the wolf due its domestication by humans between 14,000 and 40,000 years ago (Wang *et al.*, 2013; Skoglund *et al.*, 2015; Botigué *et al.*, 2017; Perri *et al.*, 2021). Although selective breeding has favoured phenotypical and behavioural divergences that now characterize more than 300 different dog breeds (recognized by the International Canine Federation), domestic dogs produce large and highly vocal litters and provide maternal care, as also observed in other canids species.

Shortly after birth, puppies are able produce vocalisations such as groans, grunt, and whines (Bleicher, 1963) in contexts typically associated with discomfort or distress (e.g., when puppies are cold, hungry, or seeking for physical contact with their parents or littermates: Bleicher, 1963; Cohen and Fox, 1976). Specifically, the relatively high frequency whines appear to be particularly evocative for mothers who typically respond by providing care to puppies (Bleicher, 1963; Cohen and Fox, 1976; personal observations). Indeed, since puppies' vision, audition and thermoregulation are very limited during their first weeks of life (Lord, 2013), their survival mainly relies on their mothers' care. During this period, females intensively feed, groom, warm and also protect their puppies (Lezama-García *et al.*, 2019; Santos, Beck and Fontbonne, 2020).

Yet, while in this context puppy whines are expected to convey multilevel information including their litter's identity, individual identity, condition and short-term needs to support the

optimal provision of this essential care, our understanding of their communication function remains surprisingly poor.

## **This thesis**

The aim of this thesis is thus to fill a gap in the scientific understanding of parent-offspring vocal communication systems of species producing multiple offspring. Specifically, I investigate the production and perception of multilevel information in the offspring calls of the domestic dog, a large litter-rearing mammal. To do so, I analyse key vocal features of puppy whines, including  $f_0$  and NLP, and perform playback experiments using methods of sound (re)-synthesis to test how these key vocal features inform caregivers. This thesis is divided into two main strands. The first focuses on the acoustic encoding and decoding of information to litter and individuality in puppy whines, and the second focuses on the acoustic encoding and decoding of intra- individual variation in need/distress. More precisely, in the first chapter I investigate how vocal cues to litter and individuality are encoded in puppy whines and their perception by mothers. I examine whether whine acoustics are different between litters and between littermates (within their litter) and whether these differences are predicted by individual variation in body weight, temperament and testosterone levels. Then, I use methods of sound resynthesis and playbacks to test whether mothers use specific vocal features of puppy whines to recognize their offspring at both litter and individual levels while also assessing their condition. In the second chapter, I move on to the encoding of dynamic information and I investigate how specific vocal features of puppy whine (NLP and  $f_0$ ) can encode arousal in the context of a short separation of the puppies from their mothers and littermates. Finally, given the possible effects of human intervention in the context of dog breeding, I also examined human perceptions of puppy whines. Specifically, in the third chapter, I test the perceptual effects of the presence and extent of NLP in whines, as well as effects of varying whine  $f_0$  in human listeners, including participants with extensive experience in puppy caregiving.

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# Chapter 1: Simultaneous communication of individual and litter identity in domestic dog puppies (*Canis familiaris*)



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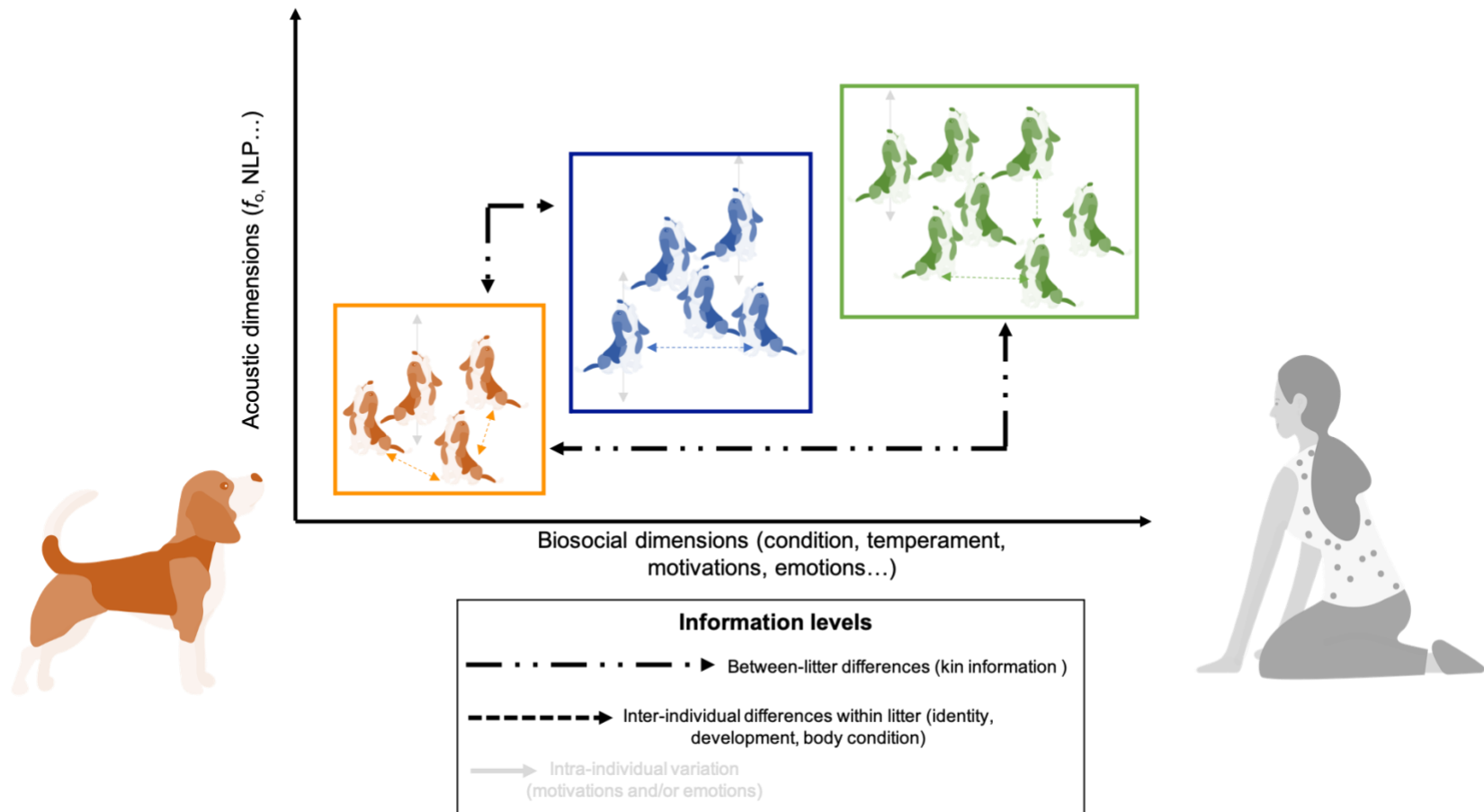
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## Aim



In this first chapter, I analyse key features in puppy whines ( $f_0$ , harmonicity, and duration) and perform playbacks to investigate: (1) what vocal cues encode litter and individuality in calls and (2) whether mothers use them to assess kin and condition.

## Abstract

In vertebrates, and more specifically mammals, it is well established that caregiving parents can recognise their offspring and assess their condition through their calls. However, the case of litter-producing mammals, where parents must recognise a large number of offspring as kin, whilst discriminating among them, has received little scrutiny. Indeed, in such systems, kin recognition is expected to favour acoustic differences of calls between own and stranger litters by limiting inter-individual differences within the litter. At the same time, individual recognition within litters can optimise individual care, and thus selection is also expected for interindividual call differentiation. Here, we disentangle this multilevel mother-young communication system in a mammal species - the domestic dog (*Canis familiaris*) - by investigating how vocal cues to litter and individuality are encoded in puppy whines and their perception by mothers. We found differences in whine  $f_0$  between litters and between puppies within litters that were driven by different relatively stable attributes, such as puppies body weight or temperament (but not testosterone or sex). Specifically, at 3-weeks of age, the relationship between  $f_0$  and weight was negative within litters, whereas this relationship was, surprisingly, positive across litters. These findings indicate that two opposing mechanisms facilitate simultaneous acoustic encoding of litter identity and puppy identity within a single call type. Crucially, to test whether mothers actually use whine  $f_0$  to assess kin at the litter and/or individual levels, we exposed 16 mothers to synthetic whines of their own or stranger 3-week-old puppies. We manipulated whine  $f_0$  to fall inside or outside of the tested female's litter range, where each condition had an associated low and high frequency variant. This allowed us to simulate whines given by relatively small/weak *versus* large/fit puppies inside or outside of the litter range. We found that mothers exhibited stronger stress-related and maternal behaviours when the frequency of playback whines from their kins was litter-typical. When  $f_0$  fell within litter range, females provided even more care in response to relatively high  $f_0$  whines indicating weaker individuals. We suggest that the integration of kin information at litter and individual levels may support the provision of optimal care to puppies. Our study fills a gap in the scientific understanding of the function and evolution of parent-offspring interactions in vertebrates by revealing a dual-encoding mechanism to support offspring recognition in mammalian species that produce litters.

## Introduction

Because misdirected, insufficient or excessive parental care can be costly for both parent and offspring, theory predicts that parents should be able to recognise their offspring and to assess their condition. While parent-offspring communication systems have been extensively described among vertebrates, most studies have focused on species where parents need to recognise a single offspring (Aubin and Jouventin, 2002; Torriani, Vannoni and McElligott, 2006; Padilla de la Torre *et al.*, 2016; Charrier, 2020). Yet, in species producing large litters (or broods), parents are faced with a more complex problem. Indeed, in such systems, while selective pressures related to kin recognition should favour acoustic differentiation of calls between litter (by limiting within-litter interindividual variation), within litter, individual recognition subserving optimised individual care should push for interindividual call differentiation.

This multi-level system of recognition raises two related questions. How do parents recognise their entire litter, and how do they also recognise each offspring within that litter? One hypothesis is that parents may have developed a recognition system relying on kin detection at both litter and offspring individual levels. While there is evidence for such dual-level recognition in birds (Stoddard and Beecher, 1983; Barg and Mumme, 1994; Draganoiu *et al.*, 2006; Caro *et al.*, 2016), very little is known in mammals, despite the fact that they provide an excellent study model with many species producing a large number of very vocal littermates (e.g., pigs (*Sus domesticus*): Illmann *et al.*, 2002; dogs (*Canis familiaris*): Bleicher, 1963; cats (*Felis catus*): Scheumann *et al.*, 2012). Here, to uncover the mechanisms underlying mother-young vocal interactions in a mammalian species producing large litters, we report an extensive investigation of the communicative function of distress whines given by domestic dog puppies. Specifically, we investigate which vocal cues encode litter and individual information (e.g., age, sex, condition, temperament) in whines and how mothers use them to recognise their litter and/or puppies.

Among vertebrates, parent-offspring communication is often achieved through various modalities including visual cues (e.g., sheep (*Ovis aries*): Kendrick *et al.*, 1996; Australian sea lions (*Neophoca cinerea*): Charrier, Pitcher and Harcourt, 2022) and olfactory cues (blue petrels (*Halobaena caerulea*): Leclaire, Bourret and Bonadonna, 2017; zebra finches (*Taeniopygia guttata castanotis*): Golüke, Bischof and Caspers, 2021; Australian sea lions: Charrier, Pitcher and Harcourt, 2022; pigs: Maletínská *et al.*, 2002, cats: Bánszegi *et al.*, 2017; sheep: Keller *et al.*, 2003). However, kin recognition is often mediated by vocal signals, as observed in a broad range

of vertebrate species (cliff swallows (*Petrochelidon pyrrhonota*): Beecher, Beecher and Hahn, 1981; pinyon jays (*Gymnorhinus cyanocephalus*): McArthur, 1982; Florida scrub jays (*Aphelocoma coerulescens*): Barg and Mumme, 1994; *Aptenodytes* penguins: Aubin and Jouventin, 2002; pinnipeds: Charrier, 2020; spotted hyenas (*Crocuta crocuta*): Holekamp *et al.*, 1999; fallow deer (*Dama dama*): Torriani, Vannoni and McElligott, 2006; sheep: Sèbe *et al.*, 2007 ; goats (*Capra hircus*): Briefer and McElligott, 2011; cattle (*Bos taurus*): Padilla de la Torre *et al.*, 2016; pigs: Illmann *et al.*, 2002; bats (*Saccopteryx bilineata*): Knörnschild, Feifel and Kalko, 2013, baboons: Rendall, Notman and Owren, 2009; humans (*Homo sapiens*): Bouchet *et al.*, 2020). Compared to visual and olfactory signals, such acoustic signals are relatively directional, propagate over longer distances, and function when visibility is impaired (Bradbury and Vehrencamp, 2011), thus making the vocal modality an ideal candidate for selection for kin recognition to operate on.

Offspring vocal recognition can rely on complex acoustic encoding (e.g., dual source in emperor penguins Aubin, Jouventin and Hildebrand, 2000) – and has been shown to vary along with the ecological constraints of the species. Therefore, it has been suggested that systems for parent-offspring recognition likely reflect different selective pressures as consequences of different social organisations, anti-predator strategies and/or reproductive strategies (Aubin and Jouventin, 2002; Torriani, Vannoni and McElligott, 2006; Briefer and McElligott, 2011b; Caro *et al.*, 2016; Charrier, 2020). Specifically, kin recognition is likely to be absent or rudimentary in species where parents spend most of their time with their young (e.g., in a nest or burrow) (Nowak *et al.*, 2000). In contrast, selective pressures should favour the emergence of bidirectional recognition systems in group-living or colonial species that are indeed exposed to relatively heightened risks of misallocating parental care, and also aggression from group members or predators. There, both parents and offspring are thus expected to produce individualized calls shortly after birth or hatching, to be able to locate each other within groups of up to several hundred conspecifics. This hypothesis of parent-offspring mutual recognition and its rapid onset has now been verified in a wide range of group-living or colonial species, including penguins (Aubin and Jouventin, 2002), pinnipeds (Charrier, 2020), sheep (Sèbe *et al.*, 2007), and cattle (Padilla de la Torre *et al.*, 2016).

Studies focusing on ungulates have also illustrated how selective pressures related to predation risks can act on kin recognition systems. Specifically, in ungulates with short or no

hiding phase, the young closely follows their mother as they rely on fleeing, or maternal and/or group defence to protect themselves against predators. In these species mother-young recognition consists of a bidirectional system (Sèbe *et al.*, 2007; Padilla de la Torre *et al.*, 2016). In contrast, species living in relatively small groups, and thus benefiting from weak group defence against predators, hide their vulnerable offspring in vegetation during their first week of life (as observed in fallow deer) (Lent, 1974). Because mothers use the location of their offspring to find them, it has been suggested that females have lost the ability to recognise them from their calls. As such, in these species, anti-predator strategies appear to have favoured “simpler”, unidirectional mother-young recognition systems, where only the mother produces individualized calls that are subsequently recognised by the hidden offspring (Torriani, Vannoni and McElligott, 2006).

Besides the social organisation of species and their associated anti-predation strategies, the number of offspring within a litter (or brood) can also influence the nature of vocal interactions between parents and their offspring. Indeed, to reduce misallocation of care, parents are able to recognise their offspring (zebra finches: Levréro *et al.*, 2009; pinon jays: McArthur, 1982; cliff swallows: Stoddard and Beecher, 1983; spotted hyenas: Holekamp *et al.*, 1999) suggesting that they use cues to kin at the litter level (“group signature”, Monk, Koenig and Koenig, 1997; Illmann *et al.*, 2002; Syrová *et al.*, 2017) or at the level of each individual offspring (sum of “individual signatures”, Monk, Koenig and Koenig, 1997; Levréro *et al.*, 2009; Briefer and McElligott, 2011; Scheumann *et al.*, 2012; Dreiss, Ruppli and Roulin, 2014; Syrová *et al.*, 2017). However, because these studies have examined kin recognition using the playback of natural calls, they could not disentangle whether parents identified their offspring at the group and/or at the individual level. It is thus now crucial to determine whether kin information is acoustically encoded at group and/or individual levels and to experimentally test how these levels contribute to offspring recognition occurs using methods of sound resynthesis that allow for controlled independent manipulations.

To provide adapted care to each individual offspring within the brood or litter, parents are expected to develop a fine-tuned perception of intra- and inter-individual variation in their offspring calls (Weary, Lawson and Thompson, 1996; Draganoiu *et al.*, 2006; Caro *et al.*, 2016) to support the assessment of their individual condition (Weary, Lawson and Thompson, 1996). A recent comparative study including more than 140 bird species has indeed shown that chicks with a low body condition beg more and are preferentially fed by parents if food abundance is relatively high in their environment (Caro *et al.*, 2016). In litter-rearing mammalian species, the potential for

such multilevel kin recognition at both litter and individual levels remains poorly investigated, except in pigs. Indeed, playback experiments have revealed that mothers exhibit stronger vocal responses to natural calls from their own piglets than to strangers (Illmann *et al.*, 2002), while a different study has shown that vocal responses of mothers were stronger to higher frequency calls of their smallest piglet compared to the lower frequency ones given by their bigger offspring (Weary, Lawson and Thompson, 1996). While these two studies suggest that pig mothers are able to recognise their offspring at litter and individual levels, future studies should investigate which vocal cues provide mothers with reliable information about their kin.

Finally, because sibling competition for parental resources can critically affect offspring body condition and survival (where dominant offspring is typically in better condition than subordinates, Hofer *et al.*, 2016; López-Jiménez *et al.*, 2016; Bebbington *et al.*, 2017), parents are also predicted to perceive cues to temperament in their offspring calls to adjust parental care and minimize costs of sibling rivalry. For instance, observations in spotted hyenas indicate that mothers provide more care to subordinate than dominant cubs (White, 2008).

Together, these observations indicate that offspring calls communicate information on kin, identity, condition and temperament. In order to investigate how such information may be encoded in a single call type, it is necessary to consider how mechanism of sound production affects the encoding of information in their acoustic structure of mammal calls (Taylor and Reby, 2010). In terrestrial mammals, the source-filter theory posits that sound production follows a two-step mechanism. Specifically, sounds are first generated in the larynx by the vibration of vocal folds (described as the “source”) and then are filtered in the vocal tract (described as the “filter”) (Titze, 1994). The rate at which vocal folds vibrate determines the fundamental frequency  $f_0$  of the produced signal, while the shape and size of the supra-laryngeal vocal tract determine the vocal tract resonances or formant frequencies (Titze, 1994). Fundamental and formant frequencies are key features of vocal signals and are usually individualized because they reflect the vocal anatomy of callers (as reviewed by Taylor and Reby, 2010; Taylor, Charlton and Reby, 2016; Charlton *et al.*, 2020). Due to allometric scaling, formants can predict inter-individual differences in body size in most mammal species (Charlton *et al.*, 2020). However, spectral density is relatively low in high frequency distress calls including puppy whines (Lingle *et al.*, 2012). As such, vocal tract resonances of distress calls are not clearly emphasized (Titze, 1994) and formant frequencies are unlikely to play a crucial role in signalling cues to offspring individuality to mothers.



In contrast,  $f_0$  is highly perceptually salient while also depending on individual variation in the length or stiffness of vocal folds, with longer and laxer vocal folds vibrating at a lower rate and produce signals with a relatively low  $f_0$  (Titze, 1994; Charlton *et al.*, 2020). Therefore,  $f_0$  has been demonstrated to strongly predict inter-individual differences in calls given by a wide range of infant mammal species (e.g., Australian sea lion pups: Charrier and Harcourt, 2006; kittens: Scheumann *et al.*, 2012; goats: Briefer and McElligott, 2011; calves: Padilla de la Torre *et al.*, 2015; piglets : Syrová *et al.*, 2017; infant chimpanzees (*Pan troglodytes*): Levréro and Mathevon, 2013; human babies: Gustafsson *et al.*, 2013). Moreover,  $f_0$  can vary between individuals within their litter and between litters (Illmann *et al.*, 2002; Syrová *et al.*, 2017). For instance, it has been demonstrated that  $f_0$  is a key contributor for vocal distinctiveness of piglet calls between individuals of a same litter and between different litters (Illmann *et al.*, 2002; Syrová *et al.*, 2017). Higher levels of acoustic similarities in  $f_0$  between full siblings than between half or non-siblings also supports the hypothesis of litter-specific cues in calls species such as piglets (Syrová *et al.*, 2017) or goats (Briefer and McElligott, 2012). These differences can remain stable throughout development as demonstrated in humans, wherein  $f_0$  in infancy and childhood strongly predicts voice  $f_0$  into adulthood (Fouquet *et al.*, 2016; Levréro *et al.*, 2018).

Inter-individual differences in the vocal parameters of offspring calls are likely to arise from variations in relatively stable physical (e.g., age, sex, body size and condition, testosterone levels) or social attributes (e.g., temperament) (Taylor and Reby, 2010; Charlton *et al.*, 2020). According to the principle of acoustic allometry, animals are expected to have larger vocal folds and longer vocal tracts as they grow (Fitch, 1997; Reby and McComb, 2003; Taylor and Reby, 2010). Consequently, older and larger individuals typically produce vocalisations with relatively lower  $f_0$  than do newborns (goats: Briefer and McElligott, 2011a; Corsican deer (*Cervus elaphus corsicanus*): Kidjo *et al.*, 2008; humans: Titze, 1994). Furthermore, sexual dimorphism in caller's vocal anatomy typically emerges at puberty (e.g., as well-described in humans Fitch and Giedd, 1999). These anatomical changes are mediated by androgens (i.e., testosterone) that influence vocal fold size (Evans *et al.*, 2008). Indeed, male present higher levels of testosterone, longer vocal folds and produce vocalisations with lower  $f_0$  than females (goitred gazelles (*Gazella subgutturosa*): Efremova *et al.*, 2011; Corsican deer: Kidjo *et al.*, 2008; elephants (*Loxodonta africana*): Baotic and Stoeger, 2017; baboons: Rendall *et al.*, 2005; humans: Puts *et al.*, 2016). Therefore, before puberty, offspring sex does not contribute to interindividual differences in the

calls of several mammal species (e.g., human babies: Levréro *et al.*, 2018; kittens: Scheumann *et al.*, 2012; cattle: Padilla de la Torre *et al.*, 2015; piglets: Garcia *et al.*, 2016).

There is also some evidence that  $f_0$  could function as an indicator of body condition, where relative low body weight constitutes an index of poor condition (Ronget *et al.*, 2018) and lower-condition individuals are expected to produce vocalisations with relatively high  $f_0$ . Within the same litter, piglet body weight is indeed negatively correlated to call  $f_0$  (Weary, Lawson and Thompson, 1996). In contrast, weak newborns can also present smaller lung capacities (Bartlett and Areson, 1977) and/or weaker muscles preventing them from increasing subglottal pressure at the levels needed to significantly increase the  $f_0$  of their calls (also characterised by high amplitude and duration), compared to fitter offspring (Titze, 1994; Fischer *et al.*, 2004). It has indeed been demonstrated that male quality/condition is positively correlated with call  $f_0$  and duration in adult baboons (Fischer *et al.*, 2004). Therefore, we can expect inter-individual differences in the call features of young mammals, particularly their  $f_0$ , to be explained by two potentially opposing mechanisms: the vocal fold size and/or the lung capacity.

Moreover,  $f_0$  can also explain differences in individual temperament and androgens levels, as demonstrated in several species (e.g., fallow deer: Vannoni and McElligott, 2008, pandas (*Ailuropoda melanoleuca*): Charlton *et al.*, 2011 and humans: Puts *et al.*, 2007; Puts David A. *et al.*, 2016; Cartei *et al.*, 2020). Recent findings in children indeed report a negative relationship between voice  $f_0$ , salivary testosterone levels and perceive masculinity (index of dominance) (Levréro *et al.*, 2018; Cartei *et al.*, 2020). Likewise, we recently showed that low  $f_0$  puppy whines are also perceived as sounding more dominant by human caregivers than are high frequency whines (Massenet *et al.*, 2022, see Chapter 3 of this thesis). In species producing multiple offspring, where sibling competition is common, temperament and androgen level of young can considerably affect their body condition (Hofer *et al.*, 2016; López-Jiménez *et al.*, 2016; Bebbington *et al.*, 2017). Within a litter, relatively larger, more dominant littermates with higher levels of testosterone are thus expected to produce vocalisations characterised by lower  $f_0$  (and vice versa).

The domestic dog is an excellent mammalian model to study the mechanisms underlying mother-young vocal interactions in species producing multiple offspring. Puppies produce vocalisations shortly after birth (Bleicher, 1963), typically to signal discomfort or distress in contexts associated with e.g., separation from their mother or littermates (Bleicher, 1963; Cohen

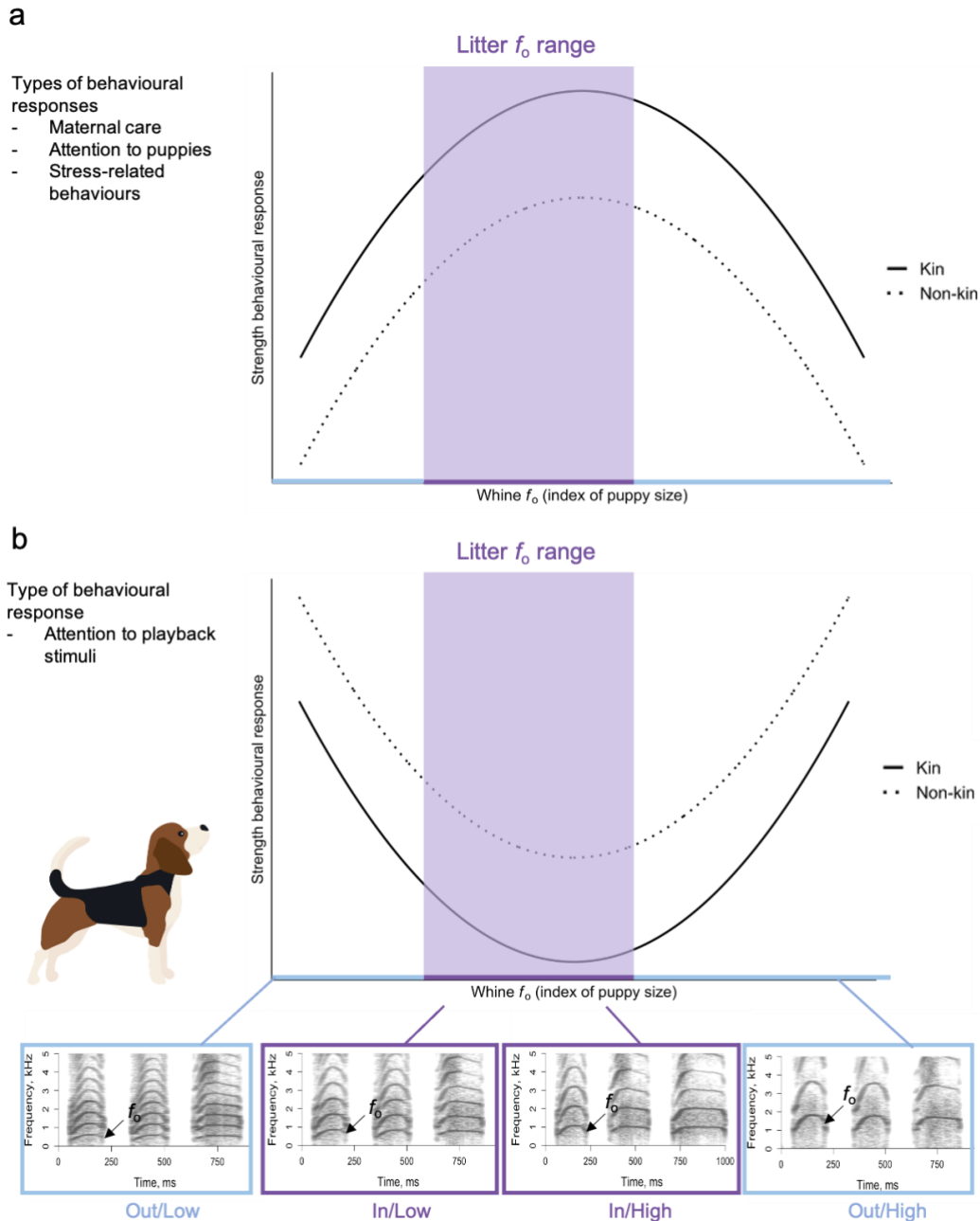
and Fox, 1976). As in human caregivers (Massenet *et al.*, 2022), mothers are sensitive to puppy whines and often provide care in response to these calls (Bleicher, 1963; Cohen and Fox, 1976; personal observations). Indeed, since puppies' vision, audition and thermoregulation are very limited during their first week of life (Lord, 2013), their survival mainly relies on mothers. During this period, females intensively feed, groom, warm and also protect their puppies (Lezama-García *et al.*, 2019; Santos, Beck and Fontbonne, 2020). Observations also report mothers gently grabbing puppies with their muzzle to put them back within the nest (Lezama-García *et al.*, 2019).

In this current study, we report an extensive investigation of the communicative function of distress puppy whines in the context of mother-young interactions. Specifically, to unravel mechanisms underlying “multi-level” interactions in litter-rearing species, we examined which vocal cues encode litter's identity or individuality in puppy whines and then tested whether mothers use these vocal cues to assess kin and condition among multiple offspring. Therefore, we determined when and how litter or individual differences in whines arise during the two first months of puppies' life. To address this question, we recorded 220 Beagle puppies from 40 litters at 3-, 6- or 9-weeks of age during a 5-minute separation from their mother and littermates (table 1). We performed acoustic analyses on collected whines and measured vocal parameters related to call  $f_0$ , harmonicity and duration. Because distress puppy whines are characterised by a relatively high  $f_0$  compared to other call types (such as grunts, groans, barks or growls, Bleicher, 1963), formant frequencies should not be highly excited. Therefore, based on previous findings in mammal infants (Charrier and Harcourt, 2006; Briefer and McElligott, 2011b; Scheumann *et al.*, 2012; Gustafsson *et al.*, 2013; Levréro and Mathevon, 2013; Padilla de la Torre *et al.*, 2015; Syrová *et al.*, 2017), we predicted that whine  $f_0$  would be a key parameter contributing to acoustic differences in whines between litters, and between puppies within their litter. We also predicted that harmonicity or duration of whines may also contribute to interindividual differences by covarying with  $f_0$ . In addition, to better understand the nature of inter-litter and inter-individual differences in whines, we also identified puppies' sex, body weight (proxy for puppy body condition and size: Ronget *et al.* 2018; Dikmen *et al.* 2008) and temperament, and collected blood samples to evaluate testosterone levels. While we did not expect sexual dimorphism in puppy whines, we predicted that weight-related variation would explain inter-litter and inter-individual differences in whines. More specifically, we expected puppies from relatively heavy litters to produce relatively lower pitched whines than puppies from lighter litters. Similarly, we also

predicted that whines given by big/large puppies would be lower-pitched than those given by a smaller littermate. In addition, we predicted that whine  $f_0$  would be negatively correlated to temperament, such as puppies' dominance, and to testosterone levels, as verified in several mammal species (Vannoni and McElligott, 2008; Charlton *et al.*, 2011; Cartei *et al.*, 2020; Stern *et al.*, 2021). Finally, we predicted that  $f_0$  would also decrease as puppies grow but that inter-individual differences in whines would remain stable until weaning.

In a second part, we tested whether mothers actually use whine  $f_0$  as cues to litter or individuality to assess kin and condition of offspring. To do so, we exposed 16 females in the presence of their puppies to natural and resynthesized whines of their own or stranger 3-week-old puppies (thereafter kin *versus* non-kin). Here, we applied methods of sound resynthesis to disentangle mechanisms of kin recognition at litter and individual levels. Specifically, we manipulated whine  $f_0$  to fall inside or outside of the tested mother's litter range. In addition, each condition had an associated low and high frequency variant, allowing us to simulate whines given by relatively small/weak *versus* large/fit puppies inside or outside of the litter range. We analysed female behavioural responses to each playback and quantified level of maternal care, stress-related behaviours, attention directed to playbacks and to their puppies.

If mothers use whine  $f_0$  as a cue to litter, we expected that they should respond with stronger attention, maternal care, and stress-related behaviour when exposed to whines with a manipulated  $f_0$  falling within their litter's frequency range, regardless of their origin (kin or non-kin). Yet, if mothers use individual cues to recognise kin, they should exhibit stronger responses to kin whines compared to non-kin, even when the  $f_0$  of the whine is re-synthesised to fall outside the litter range. Finally, if whine  $f_0$  provides mothers with reliable information about their puppies' condition, we predicted that they should react more to their puppy whines whose  $f_0$  has been raised while remaining within their litter frequency range (figure 1).



**Figure 1:** Predicted strength of mother’s behavioural responses to experimental conditions tested with playbacks of resynthesized whines of kin and non-kin puppies. We predicted that females would exhibit stronger maternal care, attention directed to puppies, stress-related behaviours in response to kin whines whose  $f_0$  was manipulated to fall inside their litter range (a). These responses were expected to be even stronger to kin whines with increased  $f_0$  (while remaining within litter range of the tested mother). We also predicted same pattern but weaker responses to non-kin whines. Finally, female’s attention to playback stimuli was expected to be stronger in response to non-kin compared to kin whines (b), and in response to whines characterized by a  $f_0$  resynthesized outside of litter range compared to resyntheses inside of the litter range.

## Material and methods

To disentangle the mechanisms underlying a possible multilevel mother-young vocal communication system, we investigated how vocal cues to litter and individuality are encoded in puppy whines and their perception by mothers. We collected data in Beagle puppies as follows: (1) recording whines at approximately 3, 6, and/or 9 weeks of age (table 1); (2) measuring body weight at each recording trial (proxy for body size and condition: Dikmen, Elmaz and Askoy, 2008; Ronget *et al.*, 2018); (3) collecting blood samples to measure testosterone; (4) performing behavioural tests to assess puppies' temperament within their litter; (5) conducting playback experiments on mothers.

**Table 1:** Overview of collected data on Beagle puppies. Sample sizes are given for each metric across the three age classes.

Age class	Acoustic recordings	Body weight measurements	Temperament behavioural tests	Testosterone measurements
<b>3-week-old</b>	40 litters 220 puppies	40 litters 220 puppies		
<b>6-week-old</b>	12 litters 62 puppies	12 litters 62 puppies		
<b>9-week-old</b>	10 litters 51 puppies	10 litters 51 puppies	4 litters 20 puppies	5 litters 26 puppies

At 3 weeks, 36 litters shared one parent at 3 weeks, while 8 litters had a common parent at 6 and 9 weeks. Our sample includes 12 litters that could be recorded in at least two different age classes. We did not measure temperament and testosterone levels in 3- and 6-week-old puppies.

### *Puppy whines*

#### Acoustic recordings

We recorded whines produced by puppies at four different breeding facilities in France. Females and their litter were kept in the same enclosure during the first two months of puppies' lives, until weaning. Puppy whines were collected in a context of separation from the individual's mother and littermates. During recordings, each puppy was carried to a separate room and placed alone in a pen (roughly 100 x 50 cm) without any visual and vocal contacts with its mother and littermates. Individuals were recorded for approximately 5 minutes once they produced a first whine. At the end of each recording session, puppies were immediately reunited with their mother. All puppies

immediately displayed normal behaviours (including suckling or interacting with their mother and littermates) indicating that any stress experienced during the recording had been low and temporary. No puppies showed signs of distress such as excessive panting, yawning or lip licking (Beerda *et al.*, 1998; Godbout *et al.*, 2007), which otherwise would have led to the immediate interruption of the experiment. Mothers, which were habituated to being temporarily separated from their litter (e.g., during daily walks) and accustomed to breeders manipulating puppies during daily care (e.g., weight measurements evaluating physical condition of puppies), displayed no sign of stress during the separations for their puppies' recordings.

All whine recordings were performed with a Sennheiser MKH70 directional microphone connected to an audio recorder Zoom H4n (44.1kHz, 24bit). The microphone was positioned at approximately 30 cm from the animal's mouth. Whines of each individual were recorded during two sessions, that were at least separated by four hours. This allowed us to increase the repeatability and thus, the biological relevance of our results, while also limiting pseudo replication.

We collected whines in a total of 220 puppies from 40 litters at approximately 3 weeks of age (table 1). Out of these 220 puppies, 62 of them (N=12 litters) were also recorded at 6 and/or 9 weeks, allowing us to investigate whine ontogeny during their two first months of life. Specifically, one litter of 6 puppies were recorded at 6 and 9 weeks, one litter of 5 puppies were recorded at 3 and 6 weeks, and finally the ten remaining litters (n= 52 puppies) were recorded across the three age classes.

### Acoustic analyses

We analysed 10 whines produced in the first minute of puppies' separation from their mother and littermates in order to create a balance dataset of whines emitted in relatively low arousal levels. Whines were selected in the absence of marked background noise or co-vocalising other dogs and if separated by at least three other whines to reduce homogeneity associated with the production of consecutive calls. We used a custom-based script in Praat version 6.0.33 (Boersma and Weenink, 2022) to measure 10 vocal parameters (table 2). Contours of the whines'  $f_0$  were extracted and verified using a cross-correlation algorithm with a detection range of 250–2500 Hz, a window length 0.05sec and a time step of 0.01sec (command *To Pitch (cc)*). We measured the duration of each whine and to their associated mean  $f_0$ , max  $f_0$  and min  $f_0$ . Our acoustic analyses

also included a coefficient of variation ( $f_0$  CV) as well as measurements related to the harmonicity of whines such as harmonic-to-noise ratio (hereafter HNR), jitter and shimmer. To characterise frequency modulations in whines, we also applied two different algorithms of vocal smoothing on each verified  $f_0$  contour. The first smoothing algorithm used a broad bandwidth (*Smooth* command with a parameter of 25) to suppress very short-term frequency variation while preserving minor modulation events. This allowed for quantifications of small frequency modulations ( $FM_{\text{minor}}$ ). The second smoothing algorithm used a narrower bandwidth (*Smooth* command with a parameter of 2) to characterise strong frequency modulations ( $FM_{\text{major}}$ ).

**Table 2:** List and definitions vocal parameters measured in puppy whines.

<b>Vocal parameter</b>	<b>Definition</b>
Duration (ms)	Duration of the whine
mean $f_0$ (Hz)	Mean $f_0$ calculated over the whine duration
max $f_0$ (Hz)	Maximum $f_0$ value calculated over the whine duration
min $f_0$ (Hz)	Minimum $f_0$ value calculated over the whine duration
$f_0$ CV (Hz)	Coefficient of variation expressed as the ratio between standard deviation and mean $f_0$ . High values of $f_0$ CV are associated with highly variable $f_0$
$FM_{\text{minor}}$	Coefficient characterising small frequency modulations calculated after a broadband smoothing. $FM_{\text{minor}}$ is expressed as the sum of inflection points (change in $f_0$ contour's derivative sign) divided by the whine duration. High values of $FM_{\text{minor}}$ are associated with whines containing high level of small frequency modulations.
$FM_{\text{major}}$	Coefficient characterising small frequency modulations calculated after a broadband smoothing ( <i>Smooth</i> command, 2). $FM_{\text{major}}$ is expressed as the sum of inflection points (change in $f_0$ contour's derivative sign) divided by the whine duration. High values of $FM_{\text{major}}$ are associated with whines containing high level of strong frequency modulations.
HNR (dB)	Harmonic-to-noise ratio of the whine. Low values of HNR indicate a rough, harsh vocal quality whereas high values characterise high tonality in whines.
Jitter (%)	Coefficient characterising small variation between consecutive periods of a whine. It is expressed as the average absolute difference between consecutive periods divided by the average period. High values of jitter are typically associated with a harsh, rough vocal quality.
Shimmer (%)	Coefficient characterising small variation in the peak amplitude between consecutive periods of a whine. It is expressed as the average absolute difference between the amplitude of consecutive periods divided by the average whine amplitude. High values of shimmer are typically associated with a harsh, rough vocal quality.



## *Individual temperament in 9-week-old puppies*

### Behavioural tests

We assessed temperament in 9-week-old puppies (N= 20 individuals from 4 litters) using four standardized behavioural tests (McGarrity *et al.*, 2015). Specifically, we performed a sequence of 3 consecutive tests in a fixed order as follows. We presented a puppy to (1) a novel environment (i.e., unfamiliar and empty testing room), (2) a human stranger and (3) a novel object (i.e., a moving and noisy item) (table 3). These tests aimed to assess temperament traits related to puppies' activity, sociability and fearfulness respectively. We also measured individual dominance using a behavioural test adapted from Wright 1980 that consisted in presenting a food treat (i.e., a bone) to two littermates for 10 minutes. Experiments were conducted in a familiar room where puppies usually played and interacted with littermates. We positioned the bone at the room centre, and both individuals were released simultaneously when the experiment started. All possible dyads of littermates were tested within a litter. A same puppy was not tested more than three times successively and each experiment was separated by at least 5 min (range= 5-43 min; mean $\pm$  SD = 10.62  $\pm$  7.33min).

During each temperament test, the experimenter stepped out from the experimental arena, did not interact with puppies and avoided direct visual contact. This allowed us to reduce puppies' response to separation from their mother and littermates during experiments (personal observations). We video recorded puppies' behaviour in each temperament test.

**Table 3:** Description of behavioural tests assessing temperament in 9-week-old puppies (N=4 litters; n= 20 puppies).

Tested temperament trait	Order	Description of the temperament test	Test duration	Reference
Activity	1	The tested puppy was placed in a novel and empty testing arena.	2 min	Hennessy <i>et al.</i> , 2001
Sociability	2	The human stranger was an adult woman crouching at approximately 2m away from the puppy. She was allowed to call the puppy in a friendly manner once the experimenter had released the tested individual. If the puppy came towards the unfamiliar person, she will pet it on its back for 20sec. Otherwise, the test stopped after 45 sec.	20 – 45 sec	Riemer <i>et al.</i> , 2014
Fearfulness	3	The novel object was positioned at the room centre. Using a remote-controlled system, the experimenter triggered the novel object when the tested puppy was at approximately 1m from it.	1 min 30 sec	Kopechek 2010 Riemer <i>et al.</i> , 2014
Dominance	-	Two littermates competed for a single bone positioned at centre of the familiar testing room.	10 min	Wight 1980

#### Behavioural analyses of puppies' temperament

To quantify puppies' response to each temperament test, we measured a total of 22 behavioural metrics using Boris open-source software (table S1 for details) (Friard and Gamba, 2016). Specifically, we assessed puppies' activity with measures of time spent at exploring, moving and whining. For instance, we expected active puppies to highly explore a new environment without intense whining. Furthermore, we assessed puppies' sociability with human strangers by encoding tail wagging, approaches and interactions. In tests exposing puppies to a new - and possibly scary - object, we also measured 7 behavioural parameters including escaping attempts, approaches, and vocal activity (barking, growling and whining rates). Finally, we quantified 9 behavioural parameters in tests evaluating puppies' relative dominance. For instance, we measured time spent at sharing the bone with a littermate or keeping it (Wright 1980). In addition, by encoding playful postures (playbows) and tail wagging, we were able to discriminate between playful and competitive/dominant individuals (Beerda *et al.*, 1999; Hasegawa, Ohtani and Ohta, 2014). We also reported submissive postures and bites as behaviours reflecting possible submissiveness or

dominance (Van Der Borg *et al.*, 2015). We never observed bites that could have injured a littermate, which otherwise would have led to the interruption of the experiment.

### *Testosterone measurements in 9-week-old puppies*

We collected 5mL blood samples at the jugular vein of 9-week-old puppies (N= 6 litters; n=26 individuals). Blood sampling involved two experimenters with extensive experience in animal medical care such as veterinarians or animal physiologists. Specifically, a first experimenter was responsible for restraining and raising the jugular vein while also reassuring the puppy. The second experimenter was responsible for taking the blood in heparin tubes (preventing blood coagulation). No signs of distress were observed (as listed earlier, see section *Acoustic recordings*). In addition, after each sample, puppies exhibited low stress-level behaviours such as eating or playing with littermates and experimenters.

Each sample was centrifuged and plasmas were frozen until testosterone measurements. Testosterone assays were performed in duplicate after steroids extraction from 200 $\mu$ L of plasma, using a competitive ELISA immunoassay. The detection threshold was 0.15 ng/mL and the intra-assay coefficient of variation was 6.1%.

### *Playback experiments of 3-week-old puppy whines*

#### Whine stimuli

We predicted that mothers would use whine  $f_0$  to recognise their puppies and assess their condition, where small/weak puppies are expected to produce higher  $f_0$  whines than bigger littermate (according to principle of size allometry). As such, to prepare acoustic stimuli, we first characterized litter  $f_0$  range of the tested mother using her puppies' whine recordings (see above). Mean and median  $f_0$  of whines given during the first minute of each recording were measured using the Voice Report function in Praat (Boersma and Weenink, 2022). The most extremes  $f_0$  values were used as upper and lower boundaries characterising the litter frequency range of the tested mother. Then, we selected two exemplars of whine sequences emitted by puppies of the tested mother (hereafter kin whines) and two others produced by stranger puppies (hereafter non-kin whines) to prepare one-minute stimuli. From each exemplar, we removed whines that overlapped with other vocalisations emitted by surrounding conspecifics, while also preserving temporal

dynamics in the whine production. In addition, low frequency background noise was filtered or attenuated. Using kin and non-kin exemplars, we applied methods of sound resyntheses (PSOLA algorithm, Praat) to prepare different variants. Specifically, we manipulated whine  $f_0$  to fall inside or outside of the tested female's litter range. Each condition had an associated low and high frequency variant to simulate whines given by a relatively small/weak *versus* large/fit puppies inside or outside of the litter range.

Together, we prepared two sets containing six different whine stimuli. The first set included natural exemplar of whines given by kin puppies and its four associated synthetic variants (figure 1). An exemplar of natural non-kin whines was also broadcasted to contrast behavioural responses to natural kin versus non kin whines. Similarly, the second set included an exemplar of natural whines produced by non-kin puppies, the synthetic variants, as well as natural whines from a kin puppy. Natural whine playbacks also ensured that females did not simply react to the synthetic component of variants (as statistically verified, see table S11). Sound pressure levels of each stimulus were adjusted to match the average natural level of 3-week-old puppy whines (see below for details).

#### Playback procedure

We conducted playback experiments from  $22 \pm 1$  days postpartum when puppies' hearing, vision, locomotion and thermoregulation are still limited (Lord, 2013). Provision of care such grooming, feeding, warming puppies are commonly observed, indicating that maternal is high after 3 postpartum weeks (Lezama-García *et al.*, 2019; Santos, Beck and Fontbonne, 2020).

A total of 192 playback trials were performed. Each mother (N=16) was exposed to sets of six stimuli over two days of experiments. Stimuli were randomly broadcasted from a Sound Bose Mini Link II ( $\pm 3$  dB in 0.2-20kHz frequency range) at an average sound pressure level of 67dB (1 meter from the sound source). Sound source levels were preliminary measured in 3-week-old whining puppies using a sonometer Rion NL-52 set for Z-weighted fast responses. The loudspeaker was covered by a padded cover to mimicry tactile sensations when mothers grabbed or gently pushed puppies (Lezama-García *et al.*, 2019, personal observations). Because kin recognition via olfaction has been described in several mammal species (Lévy, Keller and Poindron, 2004; Bánszegi *et al.*, 2017), including dogs (Hepper, 1994), the padded cover was replaced between successive experiments.

We played back whines to mothers with their puppies present in the enclosure (hosting them until weaning). Enclosures contained a parturition box, characterizing the nest, in which puppies spend most of their time during their first weeks (Santos, Beck and Fontbonne, 2020). Most experiments were conducted while the tested female was resting outside of the nest (except for 20/192 experiments, N= 2 females), with her puppies sleeping. The speaker was also placed behind a wooden screen to avoid direct visual sight with the loudspeaker. We observed real-time females' behavioural responses to playbacks and recorded them with a remote-controlled GoPro camera. Thus, we were able to stop a playback if the tested mother retrieved the loudspeaker in the nest. As such, we could increase the biological relevance of our experiment, as a puppy is supposed to relax when cared by its mother. Between two successive experiments, a recovery period ranging from approximately 20 minutes to 4 hours (58min 20sec  $\pm$  41min 44sec) allowed mothers to return to baseline, calm behaviour (e.g., resting). Finally, because time between nursing sessions can affect mother responses to offspring calls (Sèbe *et al.*, 2008), we also delayed the next experiment by 25 minutes if we observed maternal care (i.e., grooming, feeding) during the recovery period.

#### Behavioural analyses of mother's responses

To characterise female behavioural responses to playbacks, we measured a total of 12 metrics from video recordings collected during each experiment (table 6). For each tested female, behavioural analyses were performed during the playback and two minutes following the sound exposure. Depending on the metric, we counted behaviours or measured their duration using Boris open-source software (table S2) (Friard and Gamba, 2016).

We created indexes of reactions by dividing each playback experiment period into bins. We then encoded with a 0-1 variable whether each behaviour was displayed. When two behaviours happened simultaneously, e.g., mother grooming and feeding her puppies, the associated score to the bin was 2. We multiplied each score per bin by a latency weight (i.e., 1-1/number of bins) to correct for relative contribution of behaviours during the playback experiment. All score per bins were summed to calculate our index of reaction.

Using this procedure, we calculated four indexes of reactions that respectively characterised mother's attention to playback whine stimuli, attention to their puppies, care and stress-related behaviours. Index related to attention to playbacks included female's time spent at

looking, inspecting the loudspeaker and head-cocking, while time spent at looking and inspecting puppies were used to quantify mother's attention to their puppies. The association of whining and locomotor activity were used to calculate index of stress-related behaviours. Finally, we measured time spent at grooming, feeding and we counted feeding postures to quantify the index of *maternal care*. If the mother retrieved the loudspeaker into the nest, we encoded this behaviour until the end of the experiment as another form of maternal care.

### *Statistical analyses*

#### Vocal distinctiveness between whines litters and littermates

At 3, 6, and 9 weeks, we investigated whether whines were different between litters and, within litters, between puppies. To address this hypothesis, we performed two types of Discriminant Function Analyses (hereafter DFA) to classify, at each age class, puppy whines between litters or individuals (within litters) (MASS R package, Venables and Ripley, 2002). Our DFA models included litter's or puppy's identity as grouping variables and vocal parameters measured on whines as predictors (Table 2).

We used a forced entry of acoustic predictors because this method is less vulnerable to collinear variables, random effects or type I errors than a stepwise entry (Mundry and Sommer, 2007). Moreover, as DFAs are sensitive to unbalanced datasets, we fed our models with 20 whines per puppy. Specifically, we used 10 whines per puppy extracted from the first recording session to train the model to attribute calls to the correct litter or individual. Then, we used 10 other whines per puppy that were emitted during the second recording session as "untrained" data for cross-validation. This "testing" dataset examined the accuracy of the model to classify whines.

To test for vocal distinctiveness of whines between litters, we performed 100 DFAs (or iterations) while randomly selecting 3 puppies per litter at each iteration (minimum litter size). This prevented litter size to influence correct classifications of whines to litters. Randomly selected puppies were the same between both training and testing datasets.

In addition, when investigating whether whines were different between littermates, we randomly selected 2 males and 2 females within each litter to create our training and testing (or cross-validated) datasets. This allowed us to control for same call contribution between the two sexes and to ensure that individual's sex did not influence correct attributions to puppies' identity.

We ran a DFA on each possible combination of puppies for each litter, which led to the exclusion of litters containing less than two males and two females.

In sum, we thus performed a total of 6 types of DFA (i.e., 3 classifying whines to litters and 3 classifying whines to puppies within their litter at respectively 3, 6 and 9 weeks). Correct classification derived from each DFA was compared to chance levels (i.e., 1/ total number of groups) and average classifications across all tested iterations are reported in tables S3 and S4.

#### Predictors of differences in whines between litters or littermates

We investigated whether physical attributes such as puppy age, sex, body weight and testosterone levels, or social attributes, such as puppy temperament, contributed to differences in whines between litters and littermates. We tested these hypotheses using linear mixed models performed in lme4 R package (Bates *et al.*, 2015). Because some vocal parameters of whines were highly correlated, we selected four independent acoustic variables, i.e., whine duration, mean  $f_o$ ,  $FM_{\text{minor}}$  and HNR, as predictors in our models. Puppy age, sex, body weight, testosterone levels or temperament were modelled as fixed effects in separated models. In addition, each model also included litter, parents and puppy identity as well as recording session (i.e., recording sessions n°1 or n°2) as random effects. We used a likelihood ratio test to evaluate the significance of each predictor (Dobson and Barnett, 2018). All model residuals were normally and homogeneously distributed, except for models fitting whine duration that we subsequently log-transformed. For all models, effects with p value  $\leq 0.05$  were considered as statistically significant.

When testing for the relationship between body weight and acoustic parameters, we fitted a model with the average litter weight and with individual puppy weight to examine weight-effects across and within litters. In addition, because puppies were mainly interacting with their mother and littermates (e.g., playing) at 9 weeks old, we evaluated the effects of temperament traits (previously characterized with a principal component analysis, see electronic supplementary material) on whine acoustics within litters. To do so, we normalized the temperament traits (i.e., *activity*, *sociability with humans*, *boldness*, *wariness*, *possessive-like dominance*, *aggressive tendency*, *submissiveness* and *sociability with littermates*) of individuals relative to their littermates.

Finally, due to genetic relatedness between puppies and their parents, we also investigated whether parents contributed to litter-differences in whines.

### Mothers' responses to 3-week-old puppy whines

We examined whether mothers use  $f_0$  as cue to recognise their puppies (at both litter or individual levels) and to assess their condition. To address this question, we ran linear mixed models on each behavioural metrics (i.e., attention to sounds, attention to puppies, maternal care, stress-related behaviours) (lme4 R package, Bates *et al.*, 2015). Models included female's identity and playback order as random effects to account for repeated observations and possible habituation. Before running any models, we searched for outliers that may have influenced model predictions (using a method calculating Cook distance). All analyses were performed with and without outliers to ensure that overall conclusions were similar. Presented results correspond to analyses excluding identified outliers.

First, we investigated whether mothers were able to discriminate between whines given by kin *versus* non-kin puppies. We thus fitted a model testing the independent effect of whines' kinship on each behavioural response to both natural and resynthesized whine playbacks.

We then examined whether mother use whine  $f_0$  as a litter-specific cue. We tested two models examining effects of  $f_0$  manipulations (falling inside versus outside of litter frequency range of the tested mother) on behavioural responses to kin and non-kin whines respectively. Specifically, we modelled the effect of the absolute  $f_0$  distance of a tested stimulus as derived from litter frequency centre. A value close to 0 meant that  $f_0$  resynthesis was close to litter frequency centre and thus litter-typical for the tested mother.

Finally, we tested whether mothers use  $f_0$  to assess condition of puppies within their litter. To do so, we fitted two models examining effects of  $f_0$  manipulations within litter range (relatively high  $f_0$  versus relatively low  $f_0$ ) on behavioural responses to kin and non-kin whines respectively. Using a similar method as described above, we calculated to frequency distance between the tested stimulus and the centre of the litter range. As such, positive values corresponded to relatively high  $f_0$  stimulating a relatively small/weak puppy (and vice versa).



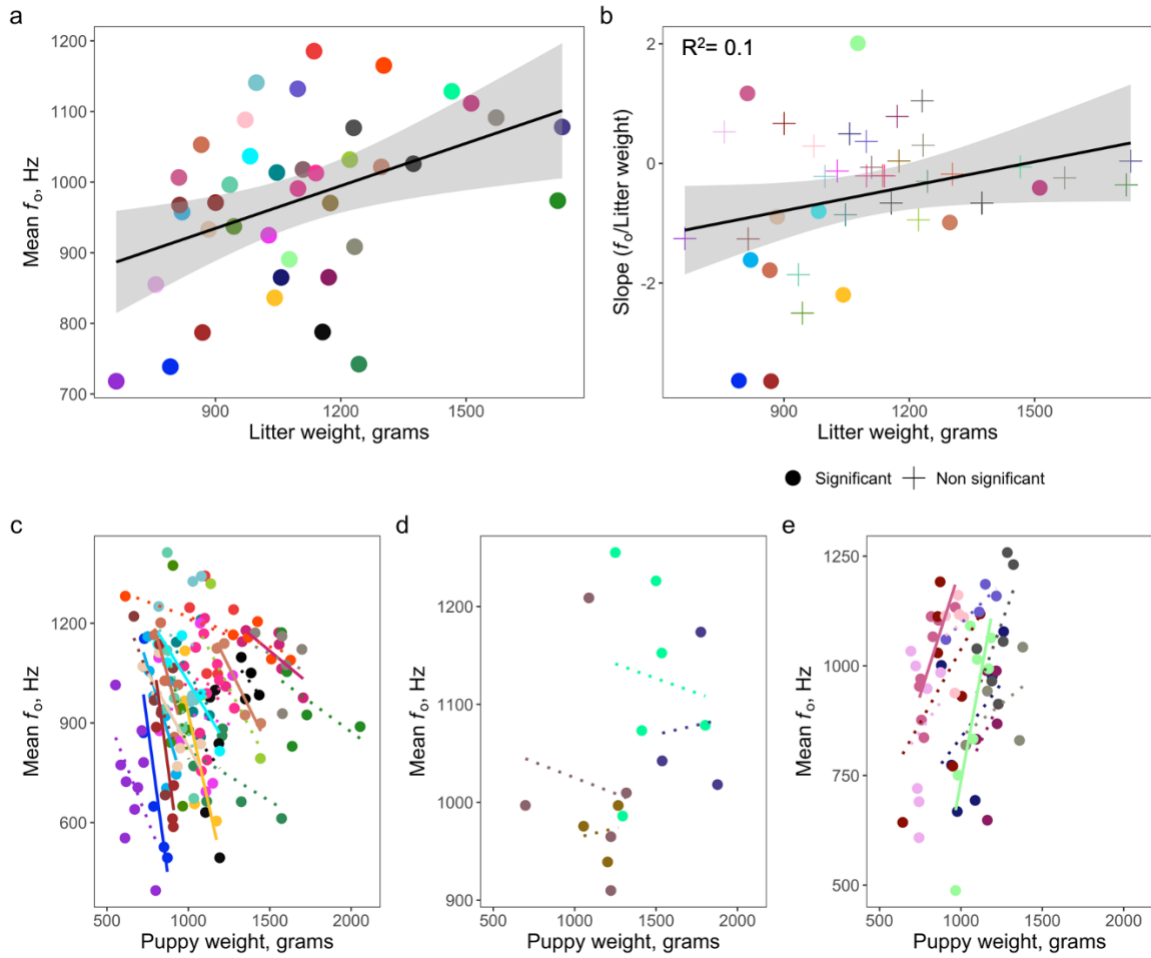
## Results

### *Are puppy whines different between litters?*

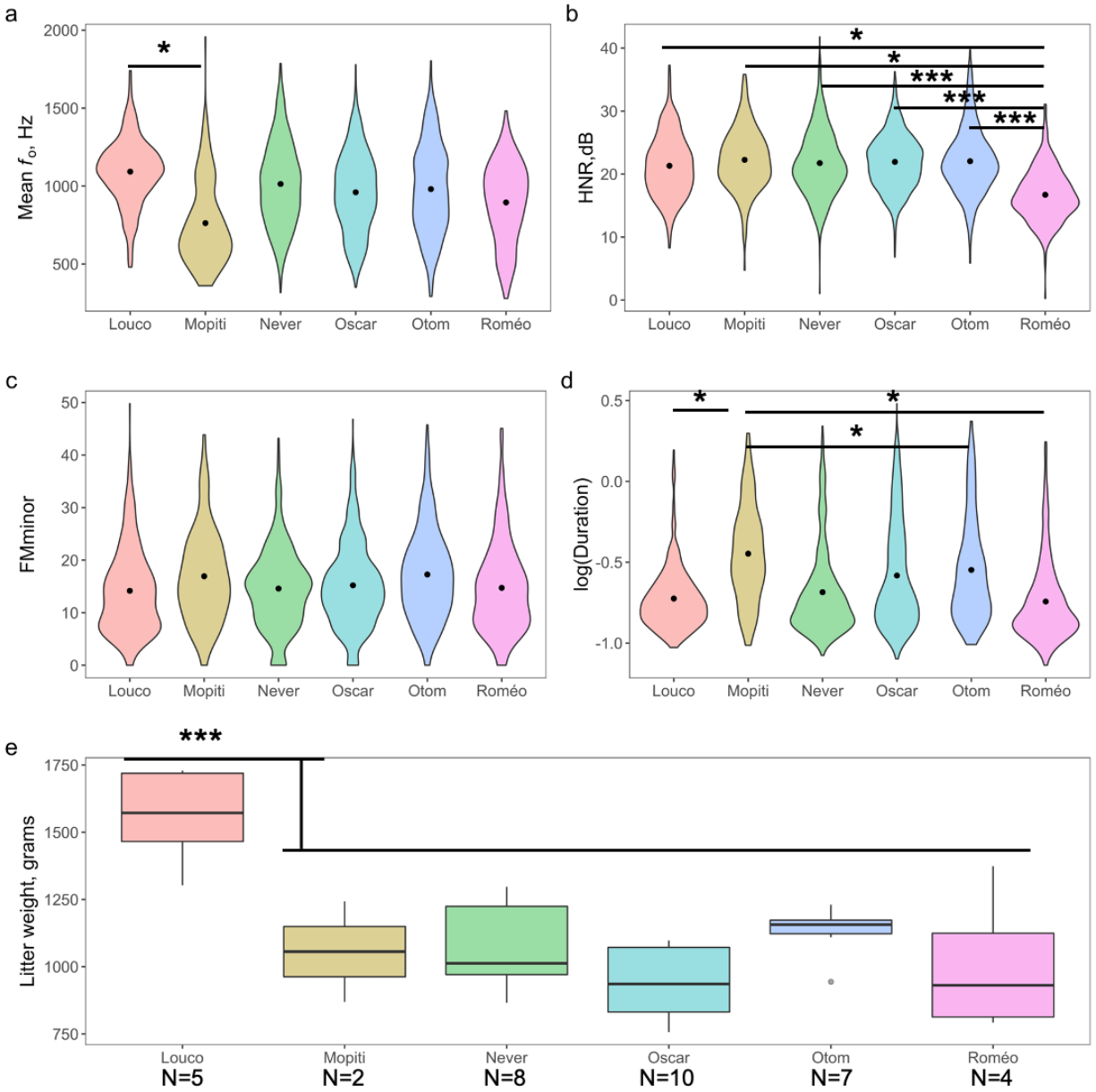
We first examined whether whines given by 3-, 6- and 9-week-old puppies were acoustically different between litters using discriminant function analyses (DFA). While controlling for litter size, we found that whines were on average attributed to correct litters at 3, 6, and 9 weeks above chance levels (table S3 and figure S1). Fundamental frequency (e.g., mean  $f_0$ ,  $f_0$  min,  $f_0$  max) and harmonicity (e.g., HNR, jitter, shimmer) of whines were key contributors to these acoustic differences at each age class (figure S1d-f).

Because puppy body weight can considerably vary across litters, we then investigated whether whine acoustics was predicted puppies' body weight. Specifically, we tested the effects of the litter weight (i.e., average weight of puppies within their litter) on four independent acoustic variables of whines (i.e., mean  $f_0$ , HNR,  $FM_{\text{minor}}$ ,  $\log(\text{duration})$ ) using linear mixed models. At 3 weeks, we found a positive relationship between whine  $f_0$  and weight (estimate  $\pm$  standard error =  $0.39 \pm 0.13$ ;  $p = 0.002$ ) (figure 2a). Specifically, puppies from heavy litters produced higher  $f_0$  whines than puppies from lighter litters. Our models did not reveal any other significant correlations between the litter weight and vocal parameters of whines at 3 weeks (table S5). No significant were also found at 6 and 9 weeks (table S5). Therefore, the acoustic distinctiveness in whines between litters that we found in older puppies did not appear to be mediated by a weight-effect across litters.

In our study, several litters also shared one and two parents. This provided us with an opportunity to examine whether differences in whines may be somewhat inherited. We tested this hypothesis in 3-week-old litters that shared a father as our sample was too small to perform reliable statistics in older litters or in 3-week-old litters that shared a same mother. We found that father's identity predicted between-litter variation in whine  $f_0$  ( $p= 0.002$ ), HNR ( $p<0.001$ ), duration ( $p<0.001$ ), but not frequency modulations ( $p=0.13$ ). Litter weight was also strongly predicted by father's identity ( $p<0.001$ ). Pairwise comparisons further revealed that males producing relatively heavy litter tend to correspond to litters emitting relatively high  $f_0$ , tonal and short whines (figure 3).



**Figure 2:** Across-litter (a-b) and within-litter (c-e) relationships between weight and whine  $f_0$  in 3-week-old puppies (N=40 litters, 220 individuals). In (a) and (b), each coloured dot corresponds to a litter and shaded areas to confidence intervals. (a) shows a significant correlation between average litter weight and  $f_0$ , whereas (b) represents a non-significant correlation between average litter weight and linear slope (calculated with whine  $f_0$  / puppy weight relationships within litters). In (b) markers indicate also whether within-litter correlations were significant. In (c), (d), (e), each dot corresponds to an individual puppy and its colour indicates the litter. Each dot represents average values per individual. For readability, (c), (d), (e) represent respectively negative, null or positive weight-effects on whine  $f_0$  within each litter, where significant relationships are represented with solid lines and non-significant relationships with dashed lines. Negative weight-effect: slope  $\leq -0.1$ , null:  $-0.1 < \text{slope} \leq 0.1$ , positive: slope  $> 0.1$ .



**Figure 3:** Paternal effects on whine acoustics (a-d) and litter weight (e). N corresponds to the total number of litters sharing a same father. Black dots correspond to average values in (a), (b), (c), (d) and blacks indicate median values in (d). Significant pairwise comparisons (following Tukey correction for multiple comparisons) are indicated ( $p < 0.001$ \*\*\*,  $p < 0.05$ \*).

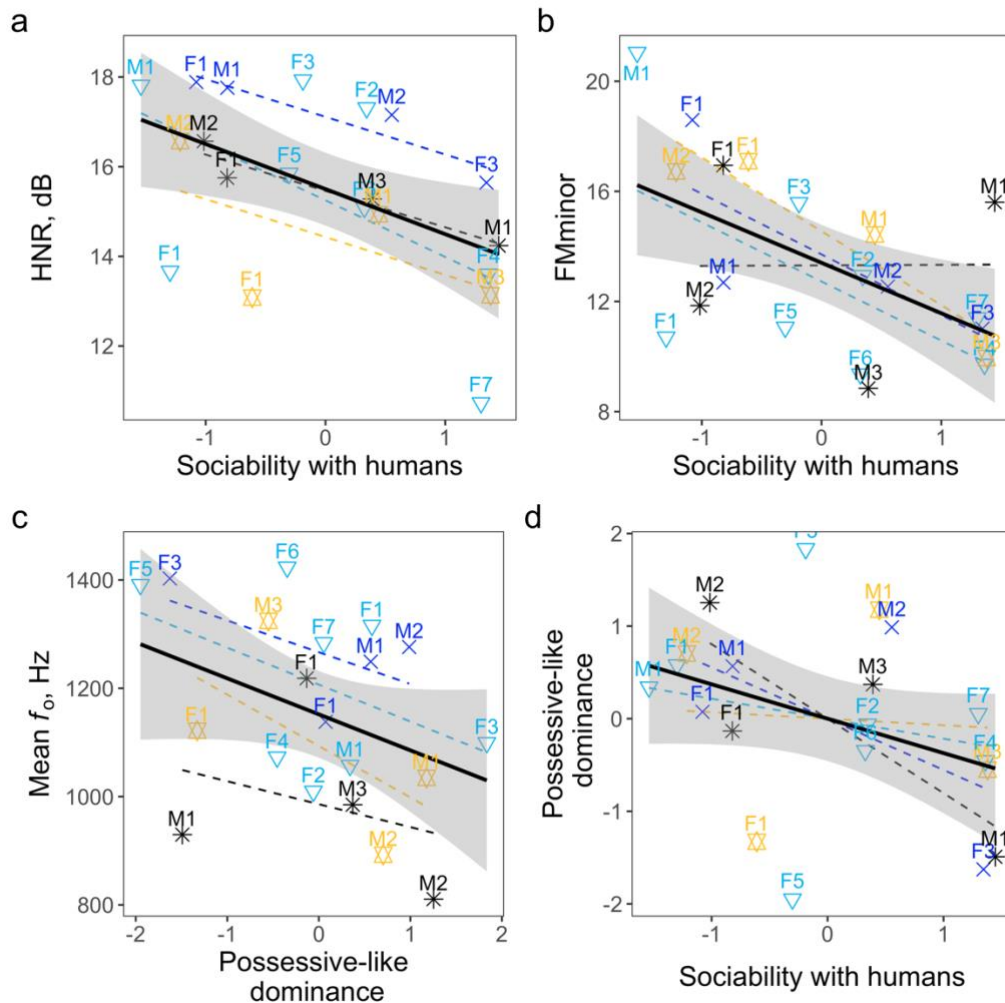
### *Are puppy whines different among littermates?*

Within each litter, we tested whether whines were different among 3-, 6- and 9-week-old puppies using discriminant function analyses. While controlling for puppies' sex, whines were correctly attributed to littermates above chance levels at each age class (table S4 and figure S2). Similar to their contribution to the acoustic distinctiveness of litters, whine  $f_0$  (i.e., mean  $f_0$ , min  $f_0$ , max  $f_0$ ) and harmonicity (i.e., HNR, jitter, shimmer) also predicted vocal differences between puppies within the same litter. Whine acoustics did not vary with puppy sexes (table S7) or testosterone levels (table S8) but rather with variation in body weight and temperament traits within litters (tables S5 and S8).

We found that body weight significantly predicted whine acoustics at 3, but not 6 and 9 weeks. Specifically, at 3 weeks, our model revealed a negative correlation between whine  $f_0$  and weight of puppies ( $-0.23 \pm 0.09$ ;  $p=0.01$ ) (figure 2c-e). When considering a negative relationship (significant or non-significant, slope  $< -0.1$ ), we found that the probability of finding a negative relationship between body weight and whine  $f_0$  within litters was also significantly higher than the probability of finding no correlation or a positive correlation (binomial test:  $p<0.001$ ). Body weight was indeed negatively correlated to whine  $f_0$  in 26 out 40 litters, where 9 litters presented a significant relationship (table S6). Interestingly, we found a non-significant trend for linear slopes between puppy's weight and  $f_0$  to become more negative as the overall litter weight decreased ( $-63.63 \pm 33.46$ ;  $p=0.06$ ) (figure 2b). Finally, interindividual differences in  $FM_{\text{minor}}$ , but not HNR or duration, appear to also be driven by variation in puppy' weight body within their litter (table S5). Together, our results thus show that relatively big puppies produced whines characterized significantly lower  $f_0$  and frequency modulations than smaller littermates at 3 weeks old.

While interindividual differences in whine acoustics were not mediated by body weight variation in older puppies, these differences were related to variation in puppies' temperament at 9 weeks (table S6). Although, there were no significant relationships between whine acoustics and 6 temperament traits measured in individuals (i.e., activity, bold, wary, possessive-like dominance, aggressivity, submissiveness and sociability with littermates) (table S6), we found that puppies' dominance and sociability with humans contributed to inter-individual differences in their whines. Because neither testosterone nor body weight were significantly correlated to puppies' temperament of dominance or sociability (table S8), relationships between individual temperament and whine acoustics were independent from variation in testosterone or body weight. Within litters,

our results show that sociable puppies with humans emitted significantly harsher and less frequency-modulated whines (HNR:  $-1.01 \pm 0.36$ ,  $p = 0.01$ ;  $FM_{\text{minor}}$ :  $-1.83 \pm 0.66$ ,  $p = 0.01$ ) than less sociable littermates (figure 4c-d) and that relatively dominant puppies produced whines with a significantly lower  $f_0$  ( $-66.45 \pm 33$ ,  $p = 0.05$ ) (figure 3a-c) in separation from their mother and littermates. Moreover, although dominant puppies tended to be relatively less sociable with humans (and vice versa), the relationship between these temperament traits was not significant ( $-0.37 \pm 0.21$ ,  $p=0.08$ ) (figure 3d).

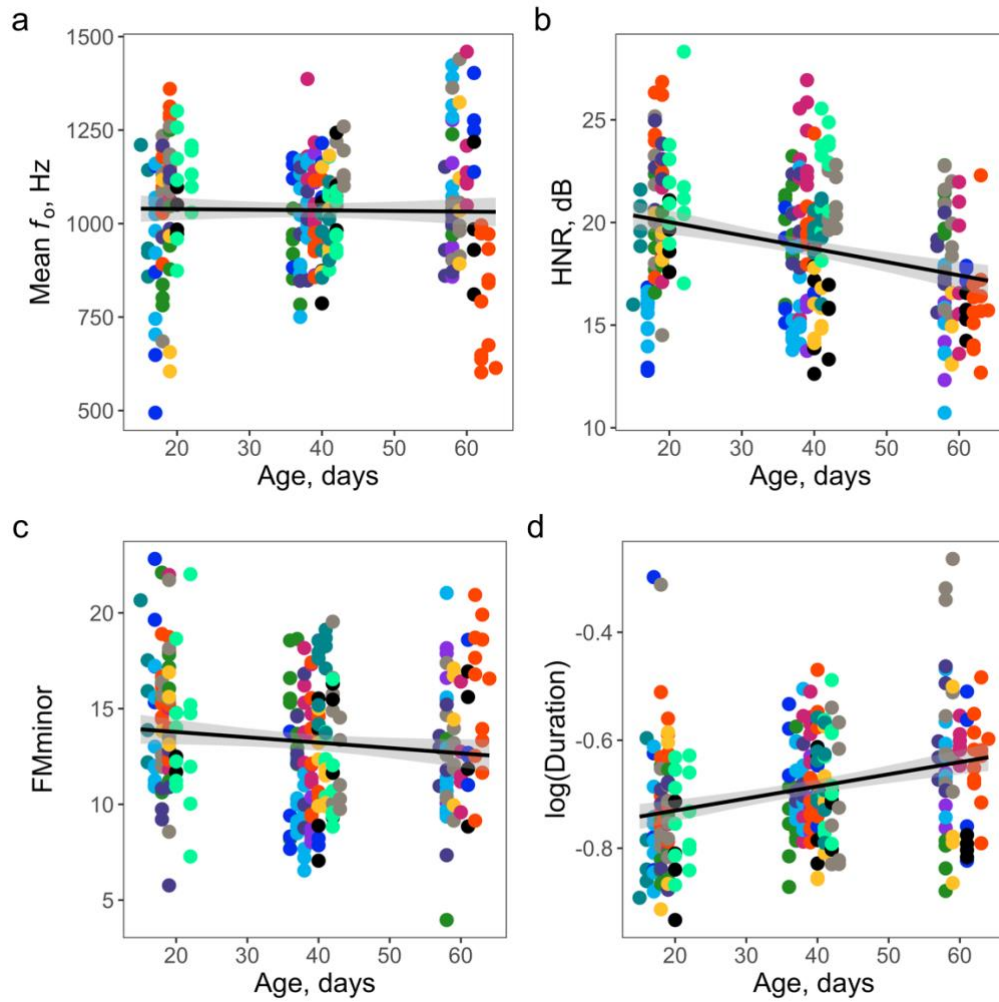


**Figure 4:** Within-litter effects of individual sociability (a-b) and dominance (c) on whine acoustics in 9-week-old puppies (N=4 litters, 20 puppies). Each marker corresponds to average values per individual, while labelled with sex (F, M) and identity (number) of the puppy. Markers' colour corresponds to the litter. Solid black lines show overall relationship in our population of puppies, whereas coloured, dashed lines show the within-litters effects.

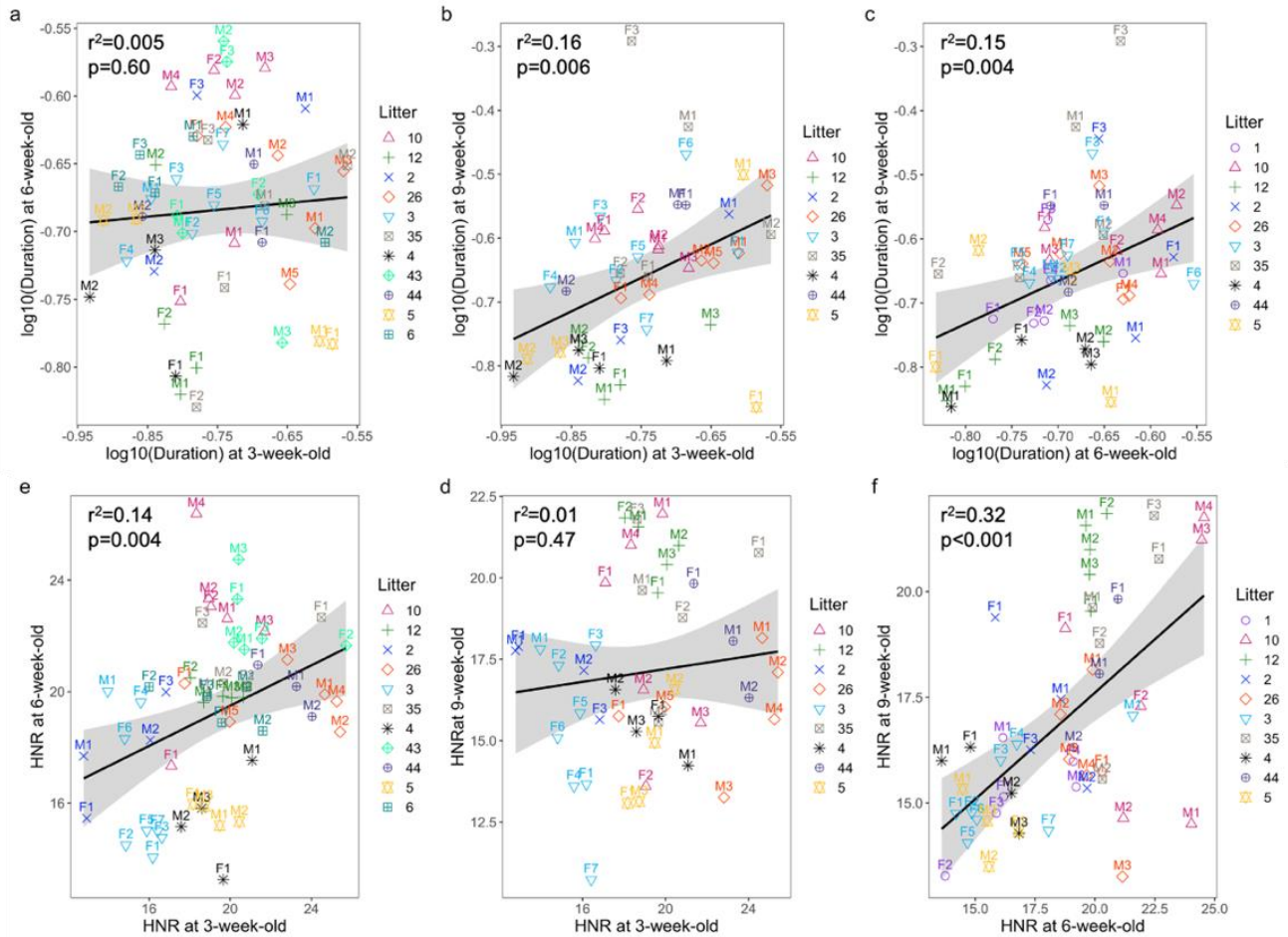
### *How do puppy whines vary with age?*

We investigated the ontogeny of whines from 3 to 9 weeks of age by examining whether whine acoustics changed with puppies' age. Because we did not find any significant differences in whine between males and females (Table S7), we tested independent effects of age on  $f_0$ , HNR,  $FM_{\text{minor}}$  and duration of their calls. In contrast to our prediction, we did not find that whine  $f_0$  significantly changed with puppies' development ( $62.86 \pm 154.57$ ,  $p = 0.67$ ) (figure 5a). There were however significant relationships between age and whine HNR ( $-4.28 \pm 2.03$ ,  $p=0.04$ ) and duration ( $0.21 \pm 0.05$ ,  $p= 0.01$ ) (but not  $FM_{\text{minor}}$ ,  $-1.22 \pm 3.15$ ,  $p= 0.66$ ). Specifically, whine harmonicity and frequency modulations significantly decreased as puppies grew (figure 5b and d).

Furthermore, we performed simple linear regression to test whether inter-individual differences in whine acoustics were stable during puppies' development (figure 6 and figure S3). Specifically, we examined the relationships between whine acoustics of puppies at 3 and 6 weeks and whines acoustic of those same individuals at 6 and/or 9 weeks. We found that the duration of whines given by puppies at 3 weeks ( $0.52 \pm 0.18$ ,  $p = 0.006$ ) and 6 weeks ( $0.67 \pm 0.22$ ,  $p= 0.004$ ) significantly predicted the whine duration of individuals at 9-weeks of age. We also found a positive correlation between HNR of whines emitted at 3 and 6 weeks of age ( $0.36 \pm 0.12$ ,  $p = 0.004$ ), as well as those produced at 6 and 9 weeks ( $0.50 \pm 0.10$ ;  $p < 0.001$ ). These findings indicate that whine duration and harmonicity are fairly stable acoustic markers within puppies, even as they get older. Inter-individual differences in whine  $f_0$  and frequency modulations were however not relatively stable during the two first months of puppies' life (figure S3).



**Figure 5:** Age effects on whine acoustics. Each dot represents a recording session, and their colour indicates the litter. Back solid lines show the overall relationship between age and whine acoustics and grey shaded ribbon correspond to confidence interval of that relationship. We collected individual whines in 51 puppies from 10 litters at 3,6 and 9 weeks but 1 litter of 6 puppies could be recorded at only 3 and 6 weeks



**Figure 6:** Within-individual relationship between whine duration (a-c) or HNR (e-f) of whines throughout the two first months of puppies’ lives. Each marker corresponds to average values per individual, while labelled with sex (F, M) and identity (number) of the puppy. Markers’ colour correspond to the litter. p values and  $R^2$  extracted from linear regressions are annotated. Interindividual differences in whine  $f_0$  and frequency modulations were not significantly stable before weaning (see figure S3). Significant effects correspond to  $p \leq 0.05$ .

*Do mothers use whine  $f_0$  to assess kin and condition?*

We have demonstrated that  $f_0$  in whines encodes rich information and characterises frequency ranges of litters at 3 weeks of age. Specifically,  $f_0$  contributes to acoustic differences in whines between litters and between littermates that reflect variation in body weight (between and within litters). Therefore, we predicted that mothers may use whine  $f_0$  to assess kin and condition by detecting specific cues to litter and puppies’ individuality. To address this hypothesis, we exposed mothers, in presence of their puppies, to playbacks of resynthesized whines given by their own puppies or by strangers. Whine  $f_0$  was manipulated to fall inside or outside of the tested female’s litter range. Each of these two experimental conditions had an associated low and high frequency



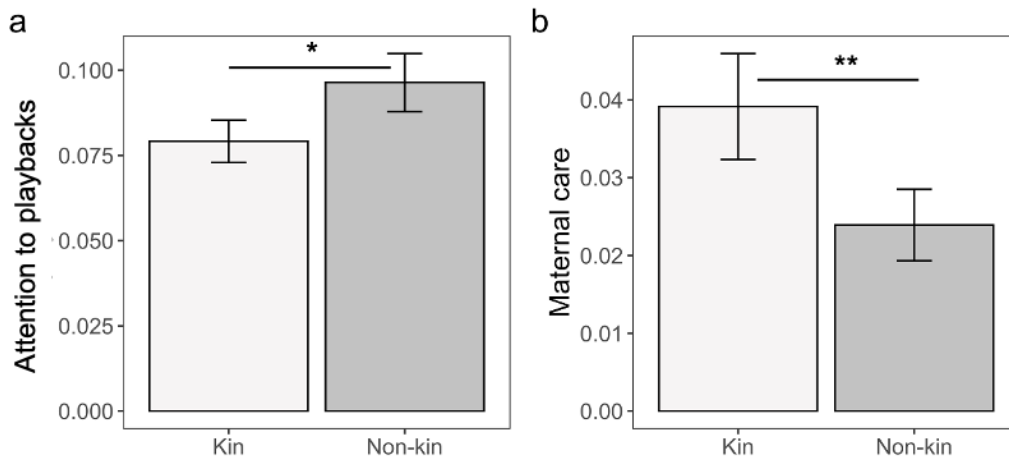
variant, allowing us simulate whines given by a relatively small/weak *versus* large/fit puppies inside or outside of the litter range (figure 1).

We first tested whether mothers discriminate between kin and non-kin whines, independently from whine  $f_0$ . To test this hypothesis, we fitted a model evaluating independent effects of whine kinship on the four behavioural metrics (i.e., mother's attention to playbacks, attention to puppies present her during sound exposure, stress-related behaviours, provision of care such as grooming or feeding behaviours). While we did not find any significant difference in female response to natural whines given by kin or non-kin, most non-significant effects were consistent with our results obtained with our sample of responses to resynthesized whines (see below) (table S10 and Figure S4). We suggest that the absence of kinship effects on females' responses to natural whines was due to a relatively smaller sample size, compared to our sample of responses to synthetic whines (N=63 playbacks of natural whine; N= 123 playbacks of resynthesized whines).

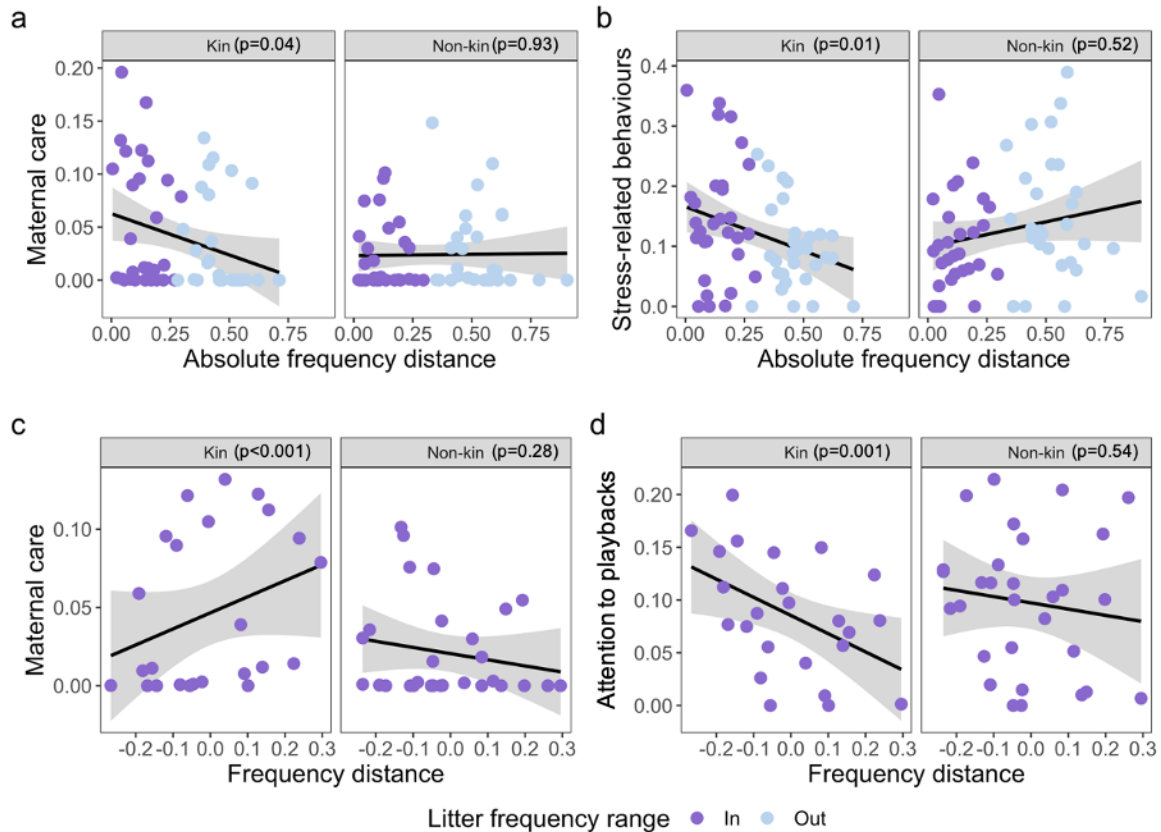
In response to synthetic whines, we found that kinship had a significant effect on females' attention to playbacks ( $0.02 \pm 0.01$ ,  $p=0.02$ ) and provision of care ( $-0.02 \pm 0.01$ ,  $p=0.01$ ), but not on their attention to puppies ( $-0.01 \pm 0.01$ ,  $p=0.25$ ) or stress-like behaviours ( $0.004 \pm 0.01$ ,  $p=0.71$ ). Specifically, females' attention to playbacks was significantly greater in response to non-kin whines compared to kin whines (figure 7). They indeed spent more time looking in the direction of non-kin sounds, inspecting the sound source while also exhibiting headcockings, relative to exposures of kin whines. Furthermore, the provision of maternal care to puppies was significantly higher during playbacks of kin *versus* non-kin whines (figure 7).

Then, we examined whether mothers used whine  $f_0$  as a specific cue to their litter. To do so, we also ran models testing for independent effects of the absolute acoustic distance between a tested stimulus as derived from the centre frequency of the litter (table S12a). These models were tested on females' responses to both kin and non-kin whines. When we broadcasted kin whines, mothers exhibited stronger stress-related behaviours ( $-0.06 \pm 0.03$ ;  $p=0.04$ ) and provided more care to puppies ( $-0.12 \pm 0.05$ ;  $p=0.01$ ) in response to more whines with a litter-typical  $f_0$  (distance close to 0) (figure 8a-b). Females' attention to playbacks and to their puppies in the enclosure did not significantly change in response to kin whines resynthesized with a  $f_0$  falling inside or outside of their litter range. Moreover, our results indicate that behavioural response to non-kin whines did not significantly differ between experimental conditions ( $f_0$  inside or outside litter range) (table S12a).

Finally, we investigated whether mothers used whine  $f_0$  to discriminate individual puppies and assess their condition within their litter. Using behavioural data collected during playbacks of whines whose  $f_0$  remained within litter range of tested mother, we examined independent effect of the frequency distance (between the stimulus and litter range centre) on females' responses to both kin and non-kin whines (table S12b). We found that level of maternal care significantly increased as kin  $f_0$  increased within the litter range (positive values) (figure 8c). In contrast, female's attention to the loudspeaker decreased significantly as kin  $f_0$  increased (figure 8d). There was no significant relationship between these two behavioural responses and  $f_0$  of non-kin whines. Attention directed to puppies and stress-like behaviour did not change as  $f_0$  of kin or non-kin varied within the litter range of the tested female (table S12b).



**Figure 7:** Average females' attention to broadcasted sounds and provision of care to their puppies in response to kin and non-kin whines. Significant differences are indicated ( $p < 0.01$ \*\*,  $p < 0.05$ \*).



**Figure 8:** Maternal responses to kin and non-kin whines according to  $f_0$  manipulations. In (a) and (b), we report effects of the absolute frequency distance of tested stimulus as derived from litter frequency centre on females' care and stress-related behaviour. Values of absolute frequency distance close to 0 means that  $f_0$  of the tested stimulus is close are litter-typical for the tested mother. In (c) and (d), we report effects of the frequency distance of tested stimulus as derived from litter centre (while remaining within the litter range of the tested female) on maternal care and attention to playback. Negative frequency distance simulates low-pitched whines given a relatively big/fit puppy of the litter, whereas positive frequency distance simulates high pitched whines produced by a relatively small/weak puppy of the litter. In all panels, each dot corresponds to a playback and its colour indicates whether  $f_0$  the tested stimulus was falling inside or outside litter range. Statistical significance between behavioural responses and frequency distances are indicated ( $p < 0.05$ ).

## Discussion

To explore the vocal communication of multilevel information in litter-rearing dogs, we first performed acoustic analyses to investigate whether vocal cues in puppy whines convey litter- and individual-specific information and then, conducted playback experiments to test whether mothers use these cues to assess kin and condition. We found that the  $f_0$  of a single call type - the puppy whine - encodes extremely rich information that is likely to be crucial for puppy's survival,

especially during their first week of life. Indeed, whine  $f_0$  mainly contributed to differences in whines between litters and littermates, as a consequence of inter-individual variation in puppies' body condition (i.e., weight) and/or temperament traits related to their dominance with littermates and sociability with humans. More specifically, at 3 weeks of age, puppy weight was positively correlated with whine  $f_0$  across litters, but this correlation was negative within litters. This result indicates that body weight has opposing allometric effects on  $f_0$  across and within litters. We thus suggest that these opposing mechanisms facilitate the acoustic encoding of litter and individual identity within a single call type to enable mothers to assess kin and condition at both litter and individual levels. Indeed, mothers exhibited stronger stress-related behaviours and provided more care as  $f_0$  of playback stimuli from their kins were litter-typical. When whine  $f_0$  was manipulated to fall inside the tested mother's litter range, females also provided more maternal care in response to higher-pitched whines compared to lower-pitched (simulating a relatively small/weak puppy versus a relatively large/fit puppy of her litter). Together, our results show that mothers strongly depend on whine  $f_0$  to identify kin and condition at both litter and individual levels. Below we discuss the possible mechanisms underlying the production and perception of multilevel information in puppy whines.

### *Whine acoustics during development*

#### Litter differences

We identified between-litter differences in puppy whines at 3-, 6- and 9-weeks after birth and found that  $f_0$  and harmonicity, but not duration, were key contributors to these acoustic differences. These results are consistent with previous findings in piglets or goat kids showing higher levels of acoustic similarities in  $f_0$  between full siblings calls than half or non-siblings calls (Illmann *et al.*, 2002; Briefer and McElligott, 2012; Syrová *et al.*, 2017), which suggests that  $f_0$  reliably conveys litter-specific cues across several mammal species.

Because puppy weight can vary considerably across litters, we expected that between-litter variation in whines should reflect body weight variation. We found that at 3 weeks, (but not 6 and 9 weeks), puppies from relatively heavy litters emitted whines with a significantly higher  $f_0$  relative to puppies from lighter litters. This result contrasts with our prediction expecting puppies from relatively heavy litter to have longer vocal folds and to produce lower  $f_0$  whines compared lighter

litters, as earlier verified in wide range of mammals (Charlton *et al.*, 2020). On the other hand, according to acoustic allometry principles predict that large and fit puppies are expected to have larger lung capacities and stronger muscles, enabling them to increase subglottal pressures at needed level for the production of and higher  $f_0$  calls compared to small and weak individuals (Bartlett and Areson, 1977; Titze, 1994). Therefore, we suggest that inter-litter differences in 3-week-old puppy whines are likely mediated by differences in lung capacities rather than vocal fold size. At 6 and 9 weeks, litter differences in whines were not mediated by body weight variation. We suggest that these differences may reflect the contribution of other attributes, such as litter-specific temperament variation arising from variation in provision of maternal care (Foyer *et al.*, 2016; Guardini *et al.*, 2017).

Furthermore, at 3 weeks, we also found that whine acoustics differed between parents. Puppies from father parenting heavier litters produced whines characterized by high  $f_0$  and harmonicity as well as by a short duration. Although these preliminary results suggest that litter “signatures” may have a genetic component (as previously suggested in goat kids: Briefer and McElligott, 2012), further analyses are needed to confirm these observations, while also investigating the effect of both parents’ body weight on litter weight and acoustics.

In sum, independent of the origins of acoustic differences in whines between litters, our results indicate that whines contain litter-specific cues, mainly related to  $f_0$ , that may enable kin recognition. At 3 weeks of age, these differences were driven by between-litter body weight variation which may support litter condition assessment and thus overall care provision by mothers.

#### Individual differences within litter

We found that, beyond contributing to the acoustic distinctiveness of litters, whine  $f_0$  and harmonicity also predicted vocal differences between puppies within the same litter. This suggests that whines convey cues to individuality, as has already been demonstrated in a wide range of young mammals (Charrier and Harcourt, 2006; Briefer and McElligott, 2011b; Scheumann *et al.*, 2012; Gustafsson *et al.*, 2013; Levréro and Mathevon, 2013; Padilla de la Torre *et al.*, 2015; Syrová *et al.*, 2017). We did not find differences in puppy whine acoustics between sexes, as also verified in the offspring calls of other mammalian species, including e.g., human babies (Levréro *et al.*, 2018), kittens (Scheumann *et al.*, 2012), or calves (Padilla de la Torre *et al.*, 2015). This absence

of sexual dimorphism in whines is consistent with the putative lack of size and shape differences in the vocal apparatus of prepubertal mammals, like humans (Titze, 1994; Fitch and Giedd, 1999). Instead, we found that individual variation in whine acoustics correlated with body weight (development/condition) and/or temperament.

More specifically, within-litter, there a significant relationship between puppies' body weight and whine  $f_0$  at 3 weeks but not 6 and 9 weeks. Relatively small puppies produced whines characterized by a significantly higher  $f_0$  compared to bigger littermates. This is consistent with the negative correlations between offspring weight and  $f_0$  observed in species producing large litters (e.g., piglets: Weary, Lawson and Thompson, 1996) or a single offspring (e.g., human babies: Wermke and Robb, 2010; Shinya *et al.*, 2014). In contrast to the positive weight-effect across litters, body weight is negatively correlated to  $f_0$  within litters. Here, our result is consistent with the acoustic allometry principle predicting that larger animals have longer vocal folds that produce lower-pitched calls. We suggest that individual differences in whine  $f_0$  are likely to be mediated by variation in vocal fold size (rather than lung capacities and muscle strength) within litters. Together, our results indicate that vocal differences between litters, and between puppies within their litter, are encoded within a same call type and by the same acoustic parameter. We thus suggest that two opposing mechanisms mediate the differences in whines between litter and littermates may enable mothers to simultaneously assess kin and condition in  $f_0$  characterising 3-week-old puppy whines.

While body weight did not explain interindividual differences in older puppy whines, we found significant relationships between whines and temperament traits, and not testosterone. Indeed, 9-week-old puppies characterized by a relatively high dominance and low sociability tended to produce lower  $f_0$  and more tonal whines than subordinate and sociable littermates. Our results are consistent with studies demonstrating that low  $f_0$  vocalisations are typically associated with dominance at both production and perceptual levels in terrestrial mammals (Vannoni and McElligott, 2008; Pisanski and Bryant, 2016; Puts David A. *et al.*, 2016; Cartei *et al.*, 2020; Stern *et al.*, 2021). In an earlier study, we also found that puppy whines synthesized at low  $f_0$  levels sounded more dominant to human listeners, including professional human caregivers, than higher  $f_0$  vocalisations (Massenet *et al.*, 2022). In species rearing multiple offspring, where offspring can compete for parental resources, communication of cues to dominance (themselves predicted by testosterone) are expected to play a crucial role in predicting outcome of sibling competition (as

reviewed by Roulin and Dreiss, 2002). However, sibling rivalry is relatively low in dogs (Arteaga *et al.*, 2013) as puppies do not display specific nipple preference and aggressive-related behaviours (e.g., puppies dislodging a suckling littermate) when suckling. As such, we expect vocal signalling of dominance via whine  $f_0$  to play a rudimentary role in supporting mothers' care adjustment (e.g., in contrast to spotted hyenas: White, 2008). On the other hand, communication of dominance cues could be involved in vocal interactions between littermates during play-fighting where puppies usually produce growls and barks that have the potential to convey such information (Yin and McCowan, 2004).

Finally, our results also indicate that individual differences in temperament could reflect varying levels of arousal in stressful contexts (e.g., separation from their mother or later from their owner). Indeed, in terrestrial mammals, low harmonicity and high  $f_0$  are considered as possible indicators of heightened vocaliser arousal in acoustic signals (Briefer, 2012, 2020). As such, we suggest that low tonality in whines given by relatively sociable puppies and high  $f_0$  in whines of relatively subordinate may reflect higher level of arousal experienced by individual during a separation from their mother and littermates.

#### Puppy whine ontogeny

We found that whines became longer and less tonal with age and that interindividual differences in whine HNR and duration were stable during the first two months of puppies' lives. Overall, the increase in whine duration in older individuals probably reflects their bigger lung capacities, allowing them to produce longer whines (Briefer and McElligott, 2011a). Yet, in contrast to our predictions, we did not find that whine  $f_0$  decreased with age. After one month of life, puppies usually start to actively interact with littermates and produce barks and growls (Bleicher, 1963). We suggest that the absence of age-related changes in whine  $f_0$  may be caused by the specialisation of signalling function in puppy vocalisations. Specifically, the expected vocal allometry in whines might be offset by the whines becoming specialised as a high  $f_0$  distress call, whereas barks and growls might take over whines in the communication of size (Riede and Fitch, 1999; Taylor, Reby and McComb, 2010) and motivations (Yin and McCowan, 2004; Taylor, Reby and McComb, 2008; Faragó *et al.*, 2014); a hypothesis that could be tested in future studies.

In addition, we also observed an overall decrease in whine harmonicity with age, suggesting stronger  $f_0$  instabilities that possibly reflect heightened level of distress conveyed by

whines in older puppies than younger. Indeed, socialisation corresponds to a developmental period in dogs (from approximately 1 to 6 months of age), where puppies are usually more sensitive to new stimuli and may experience higher levels of emotional arousal than when they were younger.

### *Mothers' behavioural responses to puppy whines*

Whine  $f_0$  appears to be an important vocal cue to litter and individual identity. The communication of such information can be crucial for puppies' survival, especially during their first week of life when they heavily rely on their mother to receive care (Lezama-García *et al.*, 2019; Santos, Beck and Fontbonne, 2020). As such, we predicted that mothers may actually use  $f_0$  of 3-week-old puppy whines to recognise a litter as their own, while also discriminating each individual offspring.

Independent from  $f_0$  manipulations, we found that mother's attention directed to the playback was significantly higher in response to non-kin whines compared kin whines. Indeed, relative to kin whines, mothers spent more time looking in the direction of sounds, inspecting the loudspeaker while also exhibiting headcockings - a common behaviour typically reflecting high attention to sounds in dogs (Sommese *et al.*, 2021). In addition, provision of care (i.e., grooming or feeding puppies) significantly decreased as kinship of whines decreased. Our findings thus indicate that females are able to discriminate between kin *versus* non-kin whines (by either using litter and/or individual cues), as similarly demonstrated in several species producing large litters (or brood) (e.g., spotted hyenas: Holekamp *et al.*, 1999; pigs: Illmann *et al.*, 2002; zebra finches: Levréro *et al.*, 2009, pinyon jays : McArthur, 1982; cliff swallows : Stoddard and Beecher, 1983). However, to understand whether mother use  $f_0$  as vocal cues to litter and/or puppies' individuality, we manipulated  $f_0$  to fall inside or outside litter's frequency range of the tested mother. We found that females exhibited stronger stress-related behaviours (i.e., moving and whining) and provided more care to their puppies as the frequency of whine stimuli more closely matched that of their litter. In contrast, the intensity of these two behaviours did not significantly differ in response to non-kin whines whose  $f_0$  was either falling inside or outside of litter's frequency range. Therefore, our results indicate that females can use whine  $f_0$  as a specific cue to litter, but also detect other litter- or individual related parameters (e.g., harmonicity) to assess kin.

Furthermore, when the  $f_0$  of their own puppy whines was falling within the tested mother's litter range, the level of provided care was significantly higher in response to high  $f_0$  whines compared to low  $f_0$  whines (respectively simulating a relatively small *versus* relatively big whining



puppy). Their attention directed to the playback also significantly decreased as whine  $f_0$  increased (while remaining in the female's litter range). We thus suggest that maternal discrimination of individual whine  $f_0$  may support the provision of optimal care to puppies within the litter. This is consistent with observations of brood-rearing bird parents detecting individual differences in begging calls (Draganoiu *et al.*, 2006) and adjusting provided food according to their chick's body condition (Caro *et al.*, 2016). Therefore, our study provides an experimental demonstration in a litter-rearing mammal of multilevel communication system relying on the detection of litter and individual information encoded within a unique call type that reflect variation in offspring condition. We suggest that perception of such dual-encoded information may facilitate provision of optimal care by females.

In the context of dog breeding, it has been suggested that the level of maternal care is lower in domesticated dogs than wild canids (Lord *et al.*, 2013; Macdonald *et al.*, 2019). Human caregivers can indeed provide care to puppies and increase their survival in cases of mothers neglect or rejection (Czerwinski *et al.*, 2016). As such, one could have expected a relatively rudimentary form of kin recognition by domesticated dog mothers. Yet, we found little support for this hypothesis. Indeed, because whines are different between littermates and litters, mothers that usually stay in the same enclosure with their puppies until weaning may have learned to recognise them. For example, human non-parents can quickly learn to recognise individuals after a short exposure to human babies' cries (Bouchet *et al.*, 2020). Therefore, we suggest that mothers' abilities to recognise their litter and individual puppies (within the litter) can reflect consequences of extensive learning opportunities and/or prior experience of caring for puppies. Furthermore, being able to perceive individuality in their puppy whines through familiarisation may allow mothers to better detect intra-individual variation in short-term need (i.e., hunger) through their calls and thus provide adapted care. Individual information is indeed usually stable across call types (Keenan *et al.*, 2020; Pisanski, Raine and Reby, 2020) and contexts (Scheumann *et al.*, 2012; Green *et al.*, 2019) in a range of terrestrial mammals including humans, allowing caregivers to recognise their offspring regardless of their emotional state.

## **Conclusion**

We report an experimental investigation of a “multi-level” kin recognition system in a litter-rearing mammalian species by disentangling maternal kin recognition at both individual puppy and litter levels. Specifically, we show that the  $f_0$  of the whine encodes for litter and individual information in 3, 6 and 9-week-old puppies. At 3 weeks, we propose that differences in whines between litters and between puppies within the litter are driven by two opposing weight-related rules at each level:  $f_0$  positively correlates with the average litter weight, but within litter it negatively correlates with individual littermate weight, following classic acoustic allometry. Crucially, we also show that mothers use whine  $f_0$  to recognise their litter and discriminate individuals within the litter. The integration of such kin information at litter and individual levels may support the provision of optimal care to puppies. Our study thus fills a gap in our understanding of the function and evolution of parent-offspring interactions in vertebrates by uncovering a mechanism for dealing with the complexity of offspring recognition in a mammalian species producing large litters.

## **Ethics**

Data collection performed under the ethical approval no. E-42-218-0901 (ENES lab agreement, Direction Départementale de la Protection des Populations, Préfecture du Rhône).

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Electronic Supplementary Materials

Simultaneous communication of individual and  
litter identity in domestic dog puppies (*Canis  
familiaris*)

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## Principal component analyses

To investigate whether vocal parameters predict interindividual differences in puppies' temperament, we first performed 4 principal component analyses (hereafter PCA) on behaviours measured during each temperament test using *prcomp* R package.

The first PCA on behaviours measured in tests assessing puppies' activity produced one principal component (hereafter PC) with eigenvalue  $>1$  (Kaiser's criterion) explaining 77% of the variance identified in our behavioural data. Loadings of this PC showed that puppies that spent more time exploring and moving were also the ones that produced whines later during the experiment. We interpreted this PC as an index of puppies' temperament of *activity*.

In addition, we found that the second PCA fitted with behaviours displayed during the test assessing puppies' sociability with humans resulted in one PC with eigenvalue  $> 1$  (predicted variance = 62%). This PC characterised puppies that wagged their tail, interacted with human strangers and also approached them quickly. We interpreted this PC as an index *sociability with humans*.

The third PCA on behaviours in response to a novel object (test assessing puppies' fearfulness) produced two PCs with eigenvalues  $> 1$ . Both PCs captured 63% of the variance identified in our dataset. The first PC characterised puppies that highly moved and that rapidly approached the novel object (after it was triggered) whereas the second PC characterised individuals that looked towards the object but that did not try to escape from the testing arena. These PCs were thus interpreted as indexes of puppies' *boldness* and *wariness* respectively.

We also found that the fourth PCA on behaviours observed in a context of competitive dominance produced four PCs which explained 79% of the variance in our data. Variables loadings of the first PC indicated that the possession of the bone was negatively associated with the production of whines and tail wagging behaviour. We thus interpreted this PC as an index of *possessive-like dominance* in puppies. The second PC was interpreted as an index of *aggressive tendency*, as bites were negatively associated with barking and playing postures. The third PC was characterised by puppies that displayed submissive postures and did not bark. This PC could thus be interpreted as an index of *submissiveness*. Finally, we found that the fourth PC described puppies' *sociability with littermates* as bites, submissive and playing postures were negatively correlated to the amount of time possessing the bone.

Together, we identified 8 temperament traits in puppies related to their *activity, sociability with humans, boldness, wariness, possessive-like dominance, aggressive-like behaviours, submissiveness* and *sociability with littermates*.

**Table S1:** Behavioural parameters analysed in each test assessing the temperament of activity, sociability, fearfulness and dominance in puppies.

Temperament test	Behaviour	Description	Variable	References
Activity	Exploration	The puppy is touching and/or sniffing the floor or walls	% time	Riemer et al., 2014 Friel et al. 2016
	Move	The puppy is moving forward or backwards or tuning on itself with its 4 paws	% time	Riemer et al., 2014
	Whining behaviour	The puppy is producing whines	Latency before whining (sec)	
Whines per minute				
Sociability	Active interaction	The puppy is in physical contact with the novel person while also looking at her to attract her attention	% time	Hennessy et al., 2001
	Tail wagging	The puppy is wagging its tail	% time	Riemer et al., 2014
	Approach	The puppy is moving towards the novel person	Latency before approaching (sec)	Hennessy et al., 2001
Fearfulness	Escape	The puppy has its two fore paws on a wall, eventually jumping, trying to leave the testing arena	% time	Hennessy et al., 2001
	Move	The puppy is moving forward or backwards or tuning on itself with its 4 paws	% time	Riemer et al., 2014
	Approach	The puppy is moving towards the novel object	% time	Riemer et al., 2014
			Latency before approaching	Hennessy et al., 2001
	Observation	The puppy is looking in the direction of novel object	% time	
	Barking behaviour	The puppy is producing barks	Barks per minute	Riemer et al., 2014
	Growling behaviour	The puppy is producing growls	Growls per minute	Riemer et al., 2014
Whining behaviour	The puppy is producing whines	Whines per minute		

Dominance	Alone	The puppy keeps the bone to itself	% time	Wright 1980
	Sharing	Two littermates have the bone in their mouths, are sniffing or licking it	% time	Wright 1980
	Tail wagging	The puppy is wagging its tail	% time	Pluijmakers et <i>al.</i> , 2010
	Barking behaviour	The puppy is producing barks	Barks per minute	Riemer and <i>al.</i> , 2014
	Growling behaviour	The puppy is producing growls	Growls per minute	Riemer and <i>al.</i> , 2014
	Whining behaviour	The puppy is producing whines	Whines per minute	
	Play bow	The puppy is laying down on its fore paws, typically to incite a littermate to play	Count	Swanepoel 1996
	Submissive posture	The puppy is laying down, exposing its belly and anogenital parts	Count	Swanepoel 1996
	Bite	The puppy is biting the muzzle or neck of its littermate	Count	Van der Borg et <i>al.</i> , 2015

**Table S2:** Behavioural parameters analysed in response to playbacks of 3-week-old puppy whines.

<b>Reaction score</b>	<b>Behaviour</b>	<b>Description</b>	<b>Variable</b>	<b>References</b>
Attention to the playback	Orientation towards sounds	The female is looking towards loudspeaker	Duration	Lehoscki et al., 2020
	Inspection of the loudspeaker	The female is inspecting the loudspeaker, i.e., sniffing or pushing it	Duration	
	Head-cocking	The female is titling her head, which indicates high attention directed to the playback	Duration	Cantalupo et al., 2002 Sommesse et al., 2021
Stress-related behaviours	Whining	The female is producing whines	Count	Grigg et al., 2021 Stellato et al., 2017
	Movement	The female is moving in her enclosure	Duration	Grigg et al., 2021
Attention to puppies	Orientation towards puppies	The female is looking toward her puppies	Duration	
	Inspection of puppies	The female is inspecting her puppies, sniffing or pushing them. She is in or close to the nest	Duration	Lezama-Garcia et al., 2019 Santos et al., 2020
Maternal care	Grooming	The female is licking her puppies, mainly their anogenital parts to stimulate urination and defecation. She is in the nest or close to it	Duration	Lezama-Garcia et al., 2019 Santos et al., 2020
	Breastfeeding	The female is sitting or lying in the nest with at least one puppy suckling	Duration	Lezama-Garcia et al., 2019 Santos et al., 2020
	Breastfeeding postures	The female is facilitating puppies' access to her nipples by sitting or lying	Count	personal observations
	Retrieval attempts	The female is grabbing the loudspeaker	Count	personal observations
	Retrieval	The female is retrieving the loudspeaker in the nest	Duration	Lezama-Garcia et al., 2019



**Table S3:** Results of DFA models classifying whines produced by 3-, 6-, 9-week-old puppies between litters.

Puppy age	Total number of litters	Correct classification (Mean $\pm$ SD %; N whines)		Chance level (%)
		Training dataset	Testing dataset (cross-validation)	
3 weeks	40	22.35 $\pm$ 1.20; N= 1200	13.65 $\pm$ 1.12; N= 1200	2.5
6 weeks	12	36.52 $\pm$ 2.46; N= 360	24.05 $\pm$ 2.04; N= 360	8.3
9 weeks	10	44.72 $\pm$ 2.09; N=300	27.06 $\pm$ 2.67; N=300	10

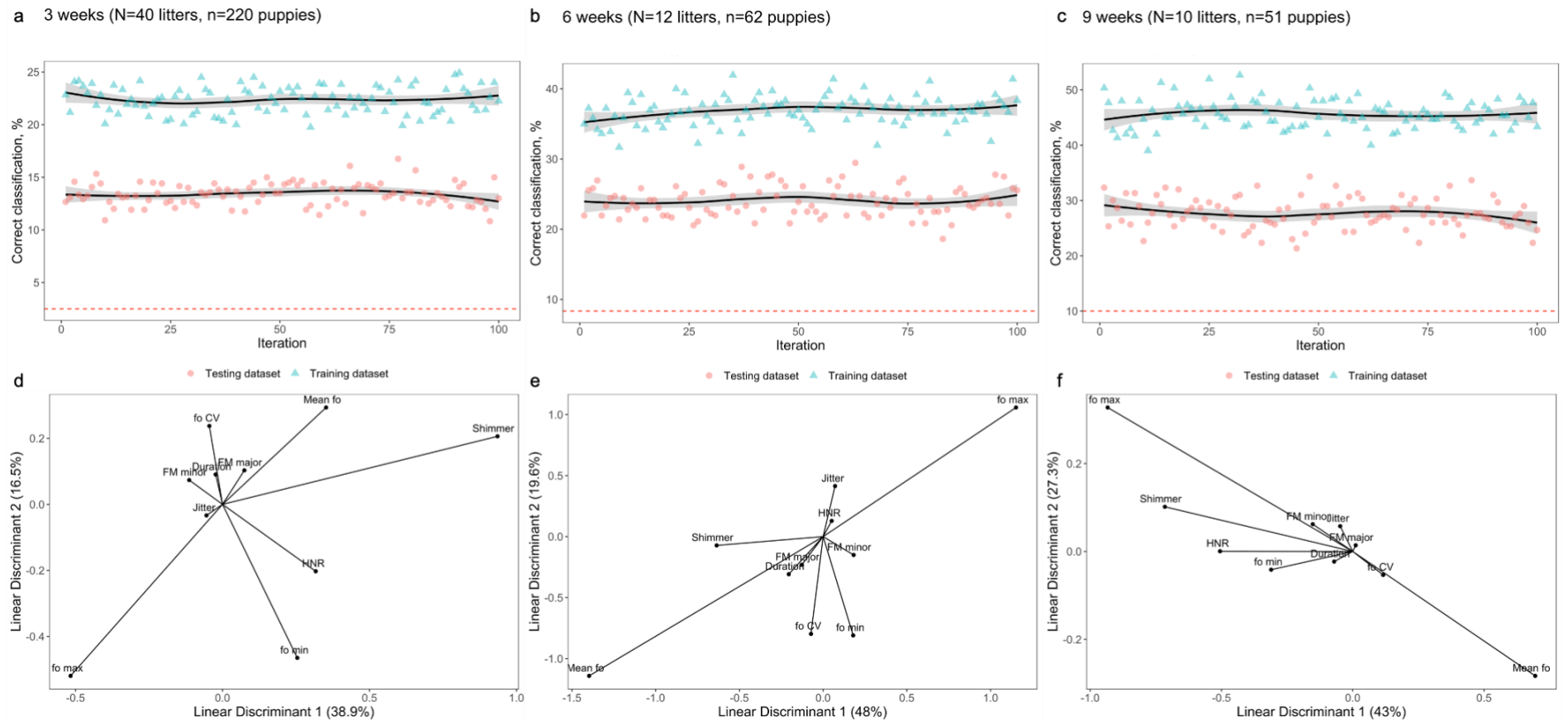
These analyses were performed using a training and testing dataset that included 3 randomly selected puppies from each tested litter. This allowed us to control for litter size effects on classifications. This procedure was applied 100 times, thus resulting in 100 DFAs at 3, 6, and 9 weeks. At each age class, chance level was set at 1/total number of litters.

**Table S4:** Results of DFA models attributing whines to individual puppies at each age class (3, 6, and 9 weeks of age).

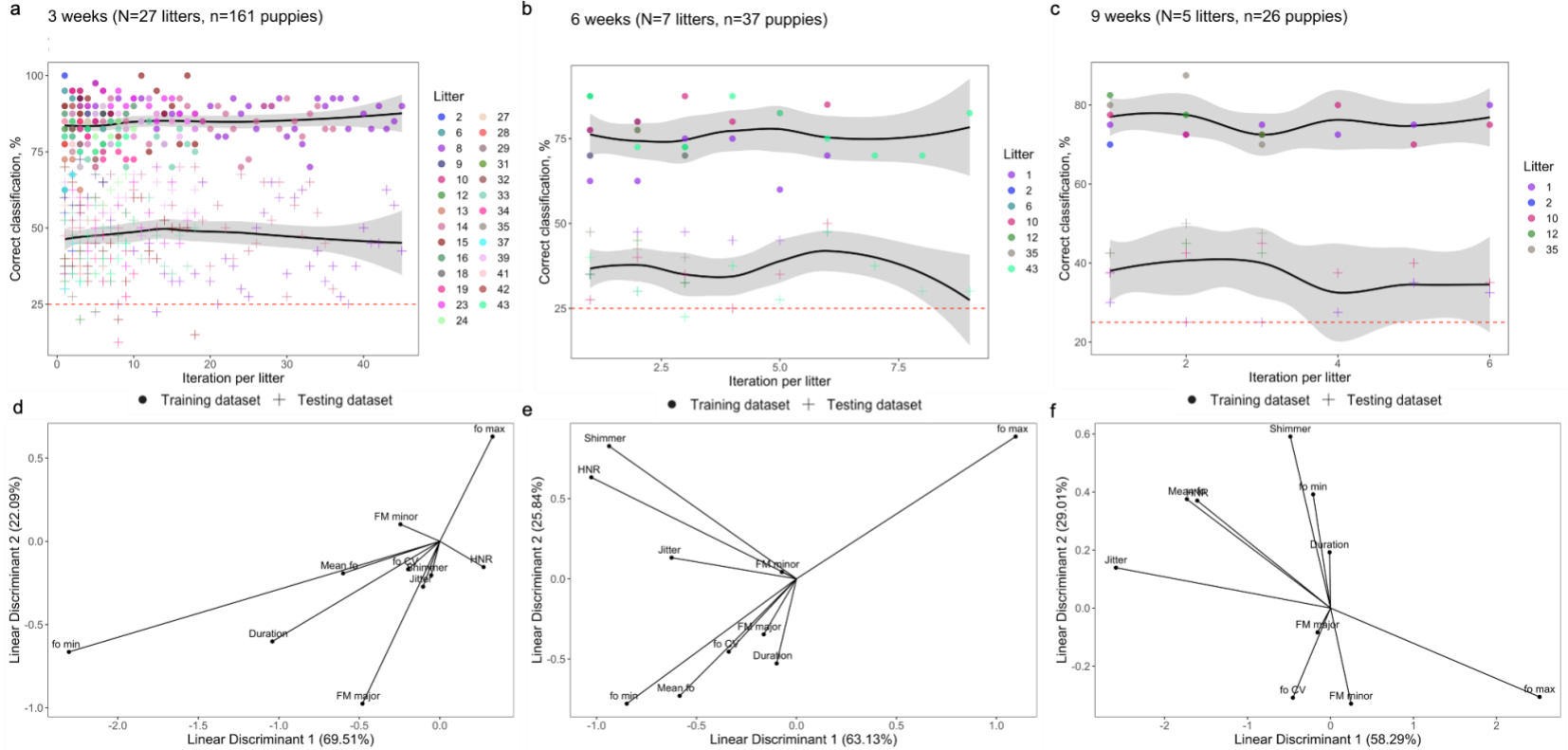
Litter's identity	Number of females (within litter)	Number of males (within litter)	Total number of tested male/female combinations	Correct classification (Mean $\pm$ SD %)	
				Training dataset	Testing dataset (cross-validation)
<i>3-week-old puppies</i>					
2	3	2	3	92.5 $\pm$ 7.5	51.67 $\pm$ 8.04
6	3	2	3	92.5 $\pm$ 2.5	52.5 $\pm$ 0
8	6	3	45	86.89 $\pm$ 5	43.5 $\pm$ 12.41
9	4	2	6	85 $\pm$ 5.92	63.75 $\pm$ 5.65
10	2	4	6	77.5 $\pm$ 4.74	43.33 $\pm$ 8.47
12	2	3	3	80 $\pm$ 4.33	34.17 $\pm$ 15.07
13	3	2	3	69.17 $\pm$ 5.77	40.83 $\pm$ 3.82
14	4	4	36	84.1 $\pm$ 5.71	52.08 $\pm$ 11.04
15	4	3	18	87.78 $\pm$ 8.22	42.36 $\pm$ 12.7
16	3	3	9	87.5 $\pm$ 4.68	47.78 $\pm$ 8.88
18	2	4	6	87.5 $\pm$ 4.47	35.42 $\pm$ 6.79
19	3	2	3	93.33 $\pm$ 2.89	45.83 $\pm$ 11.81
23	3	4	18	90.28 $\pm$ 3.92	52.64 $\pm$ 9.49
24	2	5	10	84.75 $\pm$ 3.62	63 $\pm$ 7.8
27	4	2	6	75.42 $\pm$ 6.21	44.17 $\pm$ 9.17
28	3	2	3	82.5 $\pm$ 4.33	35.83 $\pm$ 3.82
29	2	3	3	90.83 $\pm$ 3.82	34.17 $\pm$ 1.44

31	3	2	3	85±2.5	50±15.21
32	2	4	6	74.58±8.43	45±6.52
33	3	4	18	80.42±6.08	47.08±7.14
34	2	5	10	80.5±7.43	43.5±16.3
35	3	2	3	83.33±6.29	33.33±8.04
37	2	3	3	66.67±3.82	35.83±8.04
39	4	3	18	83.33±4.93	58.33±6.53
41	2	3	3	77.5±7.5	44.17±8.04
42	2	3	3	89.17±1.44	64.17±6.29
43	3	3	9	79.72±3.84	38.06±5.42
<b>6-week-old puppies</b>					
1	4	2	6	67.5±6.71	44.58±4.85
2	2	2	1	70	35
6	3	2	3	75.83±5.2	32.5±2.5
10	2	4	6	82.08±3.68	35.42±9
12	2	3	3	79.17±7.64	37.5±6.61
35	3	2	3	72.5±4.33	43.33±3.82
43	3	3	9	77.78±7.23	33.61±7.61
<b>9-week-old puppies</b>					
1	4	2	6	75±2.74	29.17±4.08
2	2	2	1	70	37.5
10	2	4	6	74.58±3.68	39.58±3.68
12	2	3	3	77.5±5	43.33±1.44
35	3	2	3	79.17±8.78	46.67±3.82

These analyses were performed using a training and testing dataset that included 4 puppies (2 males and 2 females) randomly selected within each tested litter. This allowed us to control for possible effects of puppies' sex on classifications. For each litter, we classified whines to individuals on every possible combination of males/females. As such, the number of DFA iterations per litter depended on the number of males/females combinations that we tested within each litter. Chance level was set at 25% when attributing whines to correct puppies within their litters.



**Figure S1:** Results of DFAs classifying whines between litters at 3 (a,d), 6 (b,e) and 9 (c,f) weeks of ages. In panels a-c, each marker corresponds to one DFA iteration, the solid black lines show to the average classification of all 100 DFA iterations (for both training and testing datasets) and the red dashed lines indicates to the chance level (i.e.,  $1/\text{total number of group}$ ). Panels a-c shows that whines were classified to litters above chance levels. Panels d-f show the average contribution of each vocal parameter to differences in whines between litters. The higher contributing, the more dispersed (from 0) a vocal parameter is. At each age class, fundamental frequency (hereafter  $f_0$ ) and harmonicity of whines were main parameters explaining acoustic distinctiveness between litters. These analyses were performed using a training and testing (cross-validated) dataset that included 3 randomly selected puppies from each tested litter. This allowed us to control for litter size effects on classifications. This procedure was applied 100 times, thus resulting in 100 DFAs at 3, 6, and 9 weeks. See table S1 for details about classifications scores.



**Figure S2:** Results of DFA attributing whines to individual puppies within their litter at 3 (a,d), 6 (b,e) and 9 (c,f) weeks. In panels a-c, each marker corresponds to one DFA iteration, the solid black lines show to the average classification all iterations per litter (for both training and testing datasets) and the red dashed lines indicates to the chance level set at 25%. Panels a-c shows that whines were on average attributed to the correct puppies at levels above chance. Panels d-f show the average contribution of each vocal parameter to differences in whines between puppies within their litter. The higher contributing, the more dispersed (from 0) a vocal parameter is. At each age class,  $f_0$  and harmonicity of whines were main parameters explaining acoustic distinctiveness between puppies within their litter. These analyses were performed using a training and testing dataset that included 4 puppies (2 males and 2 females) randomly selected within each tested litter. This allowed us to control for possible effects of puppies' sex on classifications. For each litter, we classified whines to individuals on every possible combination of males/females. As such, the number of DFA iterations per litter depended on the number of males/females combinations that we tested within each litter. See table S2 for details about classifications scores.

**Table S5:** Results of linear mixed models testing independent effects of litter weight and puppy weight on whine acoustics at 3, 6 and 9 weeks.

Vocal parameter	Litter weight (across-litter effect)		Puppy weight (within-litter effect)	
	Estimate $\pm$ Standard error	p value	Estimate $\pm$ Standard error	p value
<i>3-week-old puppies</i>				
$f_0$ mean	<b>0.39 <math>\pm</math> 0.13</b>	<b>0.002</b>	<b>-0.23 <math>\pm</math> 0.09</b>	<b>0.01</b>
HNR	0.003 $\pm$ 0.002	0.16	-0.001 $\pm$ 0.001	0.24
FM <sub>minor</sub>	0.004 $\pm$ 0.002	0.08	<b>-0.004 <math>\pm</math> 0.002</b>	<b>0.02</b>
log(Duration)	0.001 $\pm$ 0.001	0.50	0.0003 $\pm$ 0.0005	0.06
<i>6-week-old puppies</i>				
$f_0$ mean	0.01 $\pm$ 0.06	0.81	-0.02 $\pm$ 0.05	0.73
HNR	-0.001 $\pm$ 0.002	0.83	0.001 $\pm$ 0.001	0.46
FM <sub>minor</sub>	0.003 $\pm$ 0.00	0.07	0.0002 $\pm$ 0.001	0.82
log(Duration)	-0.002 $\pm$ 0.001	0.11	0.001 $\pm$ 0.0003	0.07
<i>9-week-old puppies</i>				
$f_0$ mean	-0.08 $\pm$ 0.07	0.24	-0.01 $\pm$ 0.04	0.89
HNR	0.002 $\pm$ 0.001	0.07	-0.001 $\pm$ 0.001	0.31
FM <sub>minor</sub>	0.00004 $\pm$ 0.001	0.96	-0.001 $\pm$ 0.001	0.37
log(Duration)	0.0004 $\pm$ 0.0002	0.37	-0.0002 $\pm$ 0.0001	0.57

The litter weight corresponds to averaged body weight of littermates. Significant effects ( $p \leq 0.05$ ) are indicated in bold.

**Table S6:** Results of linear mixed models testing independent effects of puppies' body weight on whine  $f_0$  within each litter included in our analyses at 3 weeks of age (N= 40 litters, 220 puppies).

Litter	Litter size	Estimate $\pm$ Standard error	p value
42	5	-3.64 $\pm$ 1.41	<b>0.02</b>
2	5	-3.63 $\pm$ 1.07	<b>0.005</b>
31	5	-2.5 $\pm$ 3.03	0.31
5	4	-2.2 $\pm$ 1.08	<b>0.03</b>
33	7	-1.86 $\pm$ 1	0.06
13	5	-1.79 $\pm$ 0.82	<b>0.03</b>
3	8	-1.61 $\pm$ 0.59	<b>0.01</b>
32	6	-1.26 $\pm$ 0.88	0.11
8	9	-1.26 $\pm$ 0.73	0.07
11	4	-0.99 $\pm$ 0.23	<b>0.003</b>
36	3	-0.94 $\pm$ 1.01	0.17
27	6	-0.89 $\pm$ 0.34	<b>0.01</b>
6	5	-0.86 $\pm$ 1.05	0.32
37	5	-0.8 $\pm$ 0.25	<b>0.01</b>
4	4	-0.66 $\pm$ 0.74	0.32
25	5	-0.66 $\pm$ 3.39	0.80

10	6	-0.41 ± 0.18	<b>0.03</b>
12	5	-0.36 ± 0.35	0.22
16	6	-0.3 ± 0.26	0.19
35	5	-0.24 ± 0.59	0.65
40	6	-0.22 ± 1	0.79
34	7	-0.21 ± 0.75	0.74
19	5	-0.21 ± 1.04	0.80
28	5	-0.2 ± 0.75	0.73
26	6	-0.18 ± 0.11	0.10
23	7	-0.13 ± 0.5	0.76
29	5	-0.06 ± 0.27	0.76
43	6	-0.06 ± 0.25	0.77
44	3	0.04 ± 0.48	0.89
30	3	0.04 ± 0.36	0.89
41	5	0.29 ± 1.18	0.79
17	4	0.3 ± 0.39	0.30
21	4	0.37 ± 0.21	<b>0.08</b>
9	6	0.5 ± 0.53	0.27
39	7	0.53 ± 1.29	0.63
15	7	0.67 ± 0.53	0.16
22	5	0.79 ± 1.49	0.50
18	6	1.05 ± 0.91	0.22
14	8	1.17 ± 0.4	<b>0.01</b>
24	7	2.01 ± 0.73	<b>0.01</b>

We considered a negative weight-effect when: slope  $\leq -0.1$ , null effects when  $-0.1 < \text{slope} \leq 0.1$ , and positive effects when slope  $> 0.1$ . Significant effects ( $p \leq 0.05$ ) are indicated in bold.

**Table S7:** Results of linear mixed models testing independent effects of puppy's sex on whine acoustics at 3, 6 and 9 weeks.

Vocal parameter	Estimate ± Standard error	p value
<i>3-week-old puppies</i>		
$f_0$ mean	-17.36 ± 23.34	0.47
HNR	0.3 ± 0.28	0.29
FM <sub>minor</sub>	-0.26 ± 0.43	0.53
log(Duration)	0.02 ± 0.02	0.44
<i>6-week-old puppies</i>		
$f_0$ mean	-19.71 ± 25.97	0.43
HNR	0.59 ± 0.44	0.19
FM <sub>minor</sub>	-0.12 ± 0.59	0.85
log(Duration)	0.02 ± 0.02	0.34
<i>9-week-old puppies</i>		
$f_0$ mean	-52.58 ± 47.28	0.27
HNR	-0.21 ± 0.65	0.77
FM <sub>minor</sub>	-0.67 ± 0.95	0.50

log(Duration)	0.01 ± 0.03	0.67
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This table shows that whines between males and females are not significantly different.

**Table S8:** Results of linear mixed models testing the independent effects of puppy's temperament (within their litter) and testosterone on whine acoustics at 9 weeks.

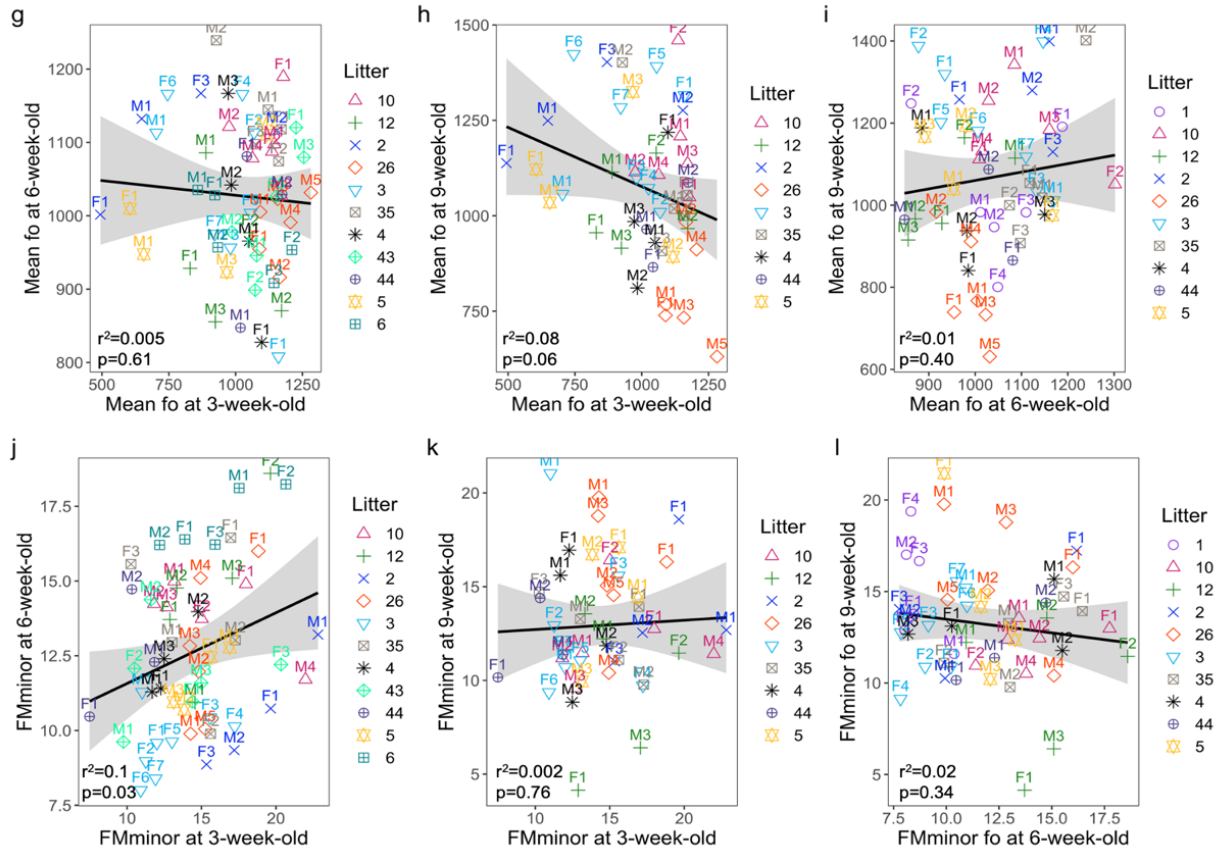
Predictor	Mean $f_0$		HNR		FM <sub>minor</sub>		log(Duration)	
	Estimate ± standard error	p value	Estimate ± standard error	p value	Estimate ± standard error	p value	Estimate ± standard error	p value
<i>Temperament traits</i>								
Activity	9.36 ± 37.24	0.80	0.1 ± 0.45	0.82	-0.68 ± 0.77	0.36	-0.01 ± 0.03	0.79
Sociability with humans	37.33 ± 36.0	0.29	<b>-1.01 ± 0.36</b>	<b>0.01</b>	<b>-1.83 ± 0.66</b>	<b>0.01</b>	-0.02 ± 0.02	0.33
Boldness	7.56 ± 37.27	0.83	-0.39 ± 0.44	0.37	-0.79 ± 0.77	0.29	-0.03 ± 0.02	0.20
Wariness	24.89 ± 36.7	0.49	-0.23 ± 0.45	0.60	-0.92 ± 0.76	0.21	0.01 ± 0.03	0.72
Possessive-like dominance	<b>-66.45 ± 33</b>	<b>0.05</b>	0.74 ± 0.4	0.07	0.44 ± 0.78	0.55	0.03 ± 0.02	0.23
Aggressive-like behaviours	-30.67 ± 36.46	0.39	-0.01 ± 0.45	0.98	-0.22 ± 0.79	0.77	0.01 ± 0.03	0.72
Submissiveness	20.88 ± 36.93	0.56	-0.12 ± 0.45	0.78	-0.2 ± 0.79	0.79	0.01 ± 0.03	0.55
Sociability with littermates	2.21 ± 37.32	0.95	-0.53 ± 0.43	0.21	0.42 ± 0.78	0.58	-0.04 ± 0.02	0.12
<i>Androgen levels</i>								
Testosterone	80.79 ± 214.83	0.79	-1.93 ± 2.28	0.56	-1.55 ± 4.04	0.94	-0.04 ± 0.13	0.72

Significant effects ( $p \leq 0.05$ ) are indicated in bold.

**Table S9:** Results of linear mixed models testing independent within-litter effects of puppies' body weight and testosterone on their temperament.

Temperament traits	Body weight		Testosterone	
	Estimate ± standard error	p value	Estimate ± standard error	p value
Activity	0.07 ± 0.23	0.73	0.28 ± 0.23	0.21
Sociability with humans	0.33 ± 0.23	0.13	0.15 ± 0.23	0.48
Boldness	-0.04 ± 0.241	0.85	-0.02 ± 0.24	0.93
Wariness	<b>-0.59 ± 0.20</b>	<b>0.003</b>	-0.04 ± 0.20	0.84
Possessive-like dominance	-0.01 ± 0.24	0.95	-0.12 ± 0.24	0.58
Aggressive-like behaviours	-0.08 ± 0.24	0.72	-0.21 ± 0.24	0.35
Submissiveness	-0.08 ± 0.24	0.7	-0.11 ± 0.43	0.61
Sociability with littermates	0.36 ± 0.22	0.08	0.26 ± 0.22	0.21

Significant effects ( $p \leq 0.05$ ) are indicated in bold. Although, there is a significant relationship between puppies' wariness and body weight, this temperament did not predict variation in whine acoustics.



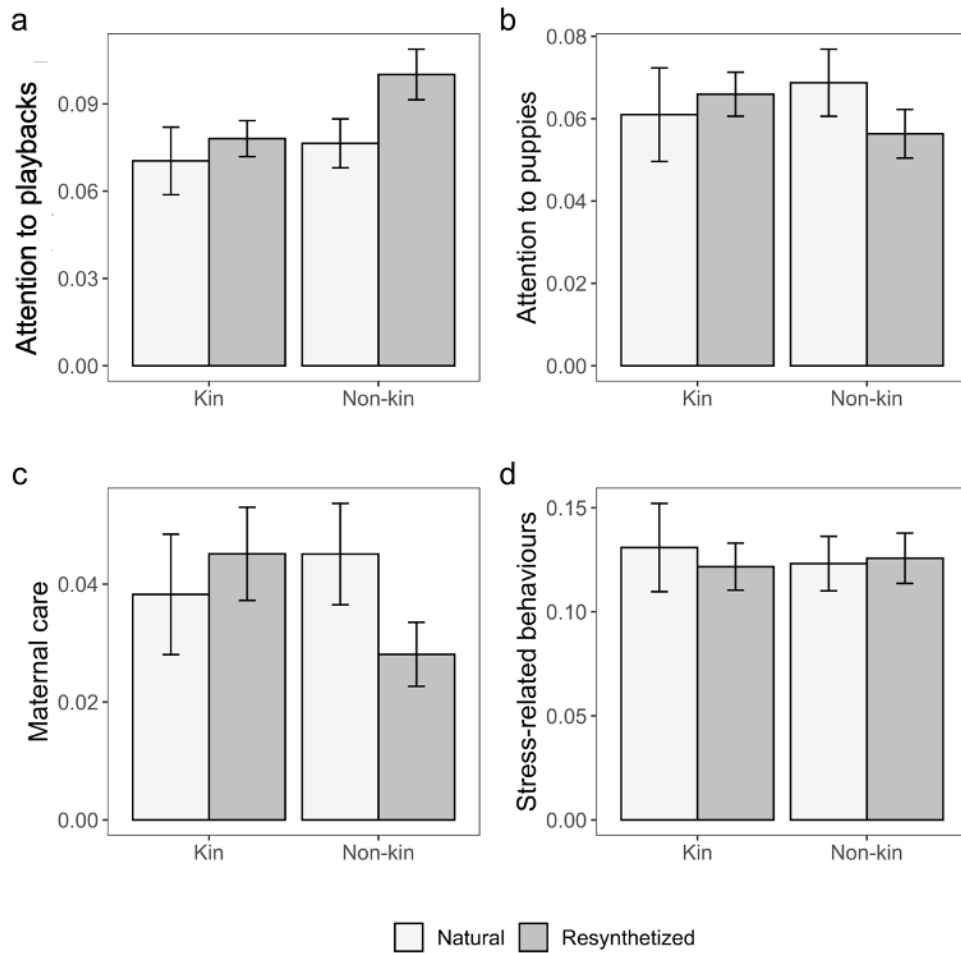
**Figure S3:** Within-individual relationship between whine  $f_0$  (g-i) and frequency modulations (j-i) of whines throughout the two first months of puppies' lives. Each marker corresponds to the average value per individual, while labelled with sex (F, M) and identity (number) of the puppy. The marker's colour corresponds to the litter. p values and  $R^2$  extracted from linear regressions are annotated. Significant effects correspond to  $p \leq 0.05$ .

**Table S10:** Results of linear mixed models testing effects of sound resynthesis (natural *versus* synthetic whines) on mother's behavioural responses.

Behavioural parameter	Estimate $\pm$ standard error	p value
Attention to the playback	0.012 $\pm$ 0.007	0.09
Attention to puppies	0.004 $\pm$ 0.006	0.52
Maternal care	0.006 $\pm$ 0.007	0.43
Stress-related behaviours	0.003 $\pm$ 0.012	0.83

This table shows that there are no significant differences in behavioural responses to natural and synthetic whines playbacks. N=63 natural whine playbacks and N=123 synthetic whine playbacks.





**Figure S4:** Mothers' responses to natural and resynthesis playbacks of whines given by kin or non-kin puppies. Note that most responses are consistent between playbacks of natural and resynthesized whines.

**Table S11:** Results of linear mixed models testing effects of kinship on mother's behavioural responses to natural whines.

Behavioural parameter	Estimate $\pm$ standard error	p value
Attention to the playback	0.01 $\pm$ 0.01	0.17
Attention to puppies	0.01 $\pm$ 0.01	0.13
Maternal care	0.01 $\pm$ 0.01	0.26
Stress-related behaviours	0.02 $\pm$ 0.04	0.66

This table shows that there are no significant differences in behavioural responses to kin *versus* non-kin natural whines. N=63 natural whine playbacks.

**Table S12:** Results of linear mixed models testing effect of  $f_0$  manipulations on mothers' behavioural responses.

Behavioural parameter	Interaction effect between kinship and $f_0$		Independent effect of kin whine $f_0$		Independent effect of non-kin whine $f_0$	
	Estimate $\pm$ Standard Error	p value	Estimate $\pm$ Standard Error	p value	Estimate $\pm$ Standard Error	p value
<i>Absolute frequency distance (a)</i>						
Attention to the playback	0.002 $\pm$ 0.04	0.14	0.004 $\pm$ 0.03	0.92	0.007 $\pm$ 0.027	0.77
Attention to puppies	-0.03 $\pm$ 0.03	0.53	0.001 $\pm$ 0.02	0.96	-0.01 $\pm$ 0.02	0.49
Maternal care	<b>0.05 <math>\pm</math> 0.03</b>	<b>0.01</b>	<b>-0.07 <math>\pm</math> 0.03</b>	<b>0.04</b>	-0.002 $\pm$ 0.02	0.93
Stress-related behaviours	0.12 $\pm$ 0.06	0.28	<b>-0.12 <math>\pm</math> 0.05</b>	<b>0.01</b>	0.02 $\pm$ 0.04	0.52
<i>Frequency distance (b)</i>						
Attention to the playback	<b>0.07 <math>\pm</math> 0.10</b>	<b>0.01</b>	<b>-0.19 <math>\pm</math> 0.06</b>	<b>0.001</b>	-0.07 $\pm$ 0.09	0.54
Attention to puppies	-0.16 $\pm$ 0.06	0.06	0.06 $\pm$ 0.05	0.23	-0.04 $\pm$ 0.04	0.3
Maternal care	<b>-0.16 <math>\pm</math> 0.07</b>	<b>0.01</b>	<b>0.18 <math>\pm</math> 0.03</b>	<b>&lt;0.001</b>	-0.04 $\pm$ 0.04	0.28
Stress-related behaviours	-0.07 $\pm$ 0.13	0.27	-0.05 $\pm$ 0.11	0.63	0.01 $\pm$ 0.07	0.91

(a) We tested effects of the absolute frequency distance of a tested stimulus as derived from litter frequency centre on females' behavioural response to investigate whether mothers use  $f_0$  as a specific cue to recognise their litter.

(b) We tested effects of the frequency distance of a tested stimulus as derived from litter frequency centre (while remaining within the litter range) on females' behavioural response to investigate whether mothers use  $f_0$  to assess puppies' body condition.

Note that these analyses were performed on responses to both kin and non-kin whines. We also tested the interaction to verify whether responses were significantly different according to  $f_0$  manipulation and whine kinship.

Significant effects ( $p \leq 0.05$ ) are indicated in bold.

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## Chapter 2: Nonlinear phenomena in puppy dog whines: a possible cue to arousal?



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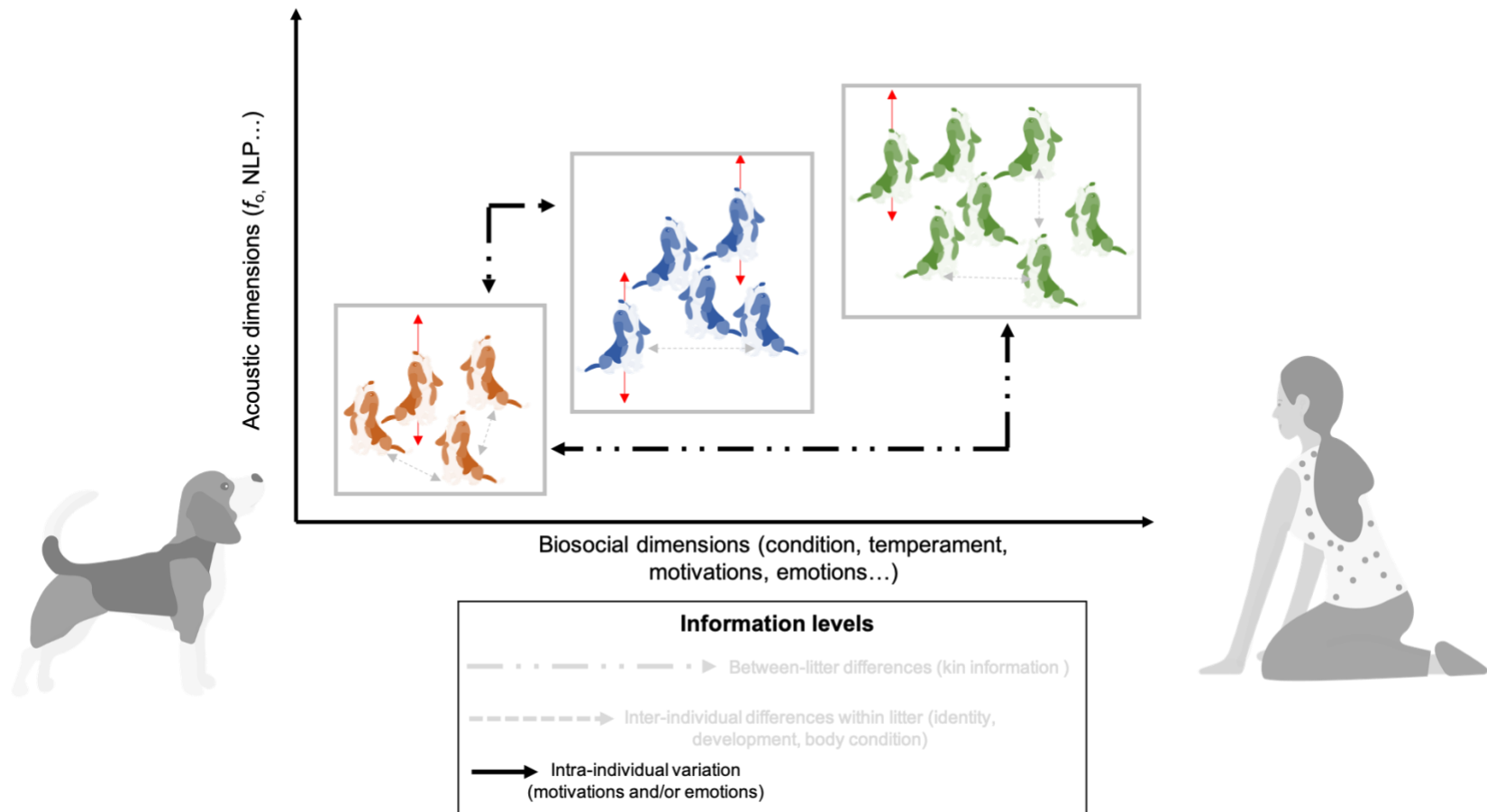
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## Aim



In this second chapter, I investigated cues to arousal in puppy whines emitted in the context of a short separation from their mother and littermates. Specifically, I provide an extensive description of NLP and their acoustic correlates such as whine  $f_0$ , amplitude, duration or harmonicity.

## Abstract

Animals often produce harsh-sounding vocalisations in high arousal distress or aggression contexts. These vocalisations typically contain nonlinear phenomena (NLP) including frequency jumps, subharmonics, chaos, and biphonation. While interest in the mechanistic origins and communicative functions of NLP is growing, how their production varies with caller's arousal state is poorly documented. Here, we describe NLP and their acoustic correlates in 13,346 natural whines given by puppies temporarily separated from their mother and littermates. Alongside an increase in whining, we show that the proportion of whines containing NLP, especially chaos, increases with time since separation. Within individual whines, all NLP types are usually produced during the first half of the whines' duration, typically corresponding to the steepest point of the rising phase in the average fundamental frequency ( $f_0$ ) and amplitude contours. Our results are consistent with the hypothesis that NLP arise in calls as a product of instabilities in the vocal system of mammals, possibly reflecting heightened arousal states. Our study also provides methodological tools to describe in details the production of nonlinear acoustics in natural mammal calls and thus explore their communicative functions in context of varying arousal state.

## Introduction

Commonalities in the vocal communication systems of terrestrial mammals are largely the consequence of selective pressures that shape the acoustic structure of vocalisations according to their adaptive functions (Taylor, Charlton and Reby, 2016). An important component of terrestrial mammal vocalisations is their fundamental frequency ( $f_0$ ), determined by the rate of vocal fold vibration, and influencing the perceived pitch of the call (Titze, 1994; Taylor and Reby, 2010). While typically stable and periodic, vocal fold vibration can become irregular and lead to the production of nonlinear phenomena (hereafter NLP) (Wilden *et al.*, 1998). These include four types of common acoustic perturbations: frequency jumps, subharmonics, deterministic chaos and biphonation. The presence of NLP in vocalisations is typically responsible for perceived vocal harshness, roughness, and instabilities (Bergan and Titze, 2001; Anikin *et al.*, 2021).

Despite their ubiquity in animal vocal communication, the production mechanisms driving NLP and their ostensible adaptive functions remain poorly understood (Wilden *et al.*, 1998; Fitch, Neubauer and Herzel, 2002). A key element that is missing, and that limits research progress in

this area, is a comprehensive understanding of the context NLP occurrence in a natural animal vocal communication system. Such an understanding would enable us to ground hypotheses regarding the communicative function of NLP in stronger ecological foundations. Here, to plug this gap, we provide an exhaustive description of the temporal distribution and acoustic correlates of NLP in puppy whines, a vocalisation type that naturally contains a high level of NLP and typically signals relatively transient distress experienced by individuals (e.g., during separation from mother and littermates, Bleicher, 1963).

NLP were initially described as nonadaptive vocal features or by-products (as reviewed by Fitch, Neubauer and Herzel, 2002), often characterising pathological human voices (Herzel *et al.*, 1994). Yet, because they are a common feature of mammal calls (e.g., koalas (*Phascolarctos cinereus*): Charlton, 2015; marmots (*Marmota marmota*): Blumstein *et al.*, 2008; rhesus macaque (*Macaca mulatta*): Fitch, Neubauer and Herzel, 2002; chimpanzees (*Pan troglodytes*): Riede, Arcadi and Owren, 2007; humans (*Homo sapiens*): Mende, Herzel and Wermke, 1990; Koutseff *et al.*, 2018; Raine *et al.*, 2018; meerkats (*Suricata suricatta*): Townsend and Manser, 2011; giant pandas (*Ailuropoda melanoleuca*): Stoeger *et al.*, 2012; dogs (*Canis familiaris*): Marx *et al.*, 2021; cats (*Felis catus*): Scheumann *et al.*, 2012; North American elk (*Cervus canadensis*): Reby *et al.*, 2016; cattle (*Bos taurus*): Green *et al.*, 2020; African elephants (*Loxodonta Africana*): Stoeger *et al.*, 2011; Fuchs *et al.*, 2021), recent studies have focused on their occurrence across different call types and behavioural contexts to uncover their ostensible communicative functions. Several hypotheses have since emerged regarding the potential signalling functions of NLP. In contexts of territorial defence or sexual competition, it has been suggested that NLP may signal or exaggerate physical traits (e.g., body size) or social attributes (e.g., the dominance status) of emitters (Fitch, Neubauer and Herzel, 2002; Anikin, 2020; Anikin *et al.*, 2021). Indeed,  $f_0$  and formant frequencies (vocal tract resonances influencing perceived timbre) represent key and well-described vocal indicators of size and dominance in terrestrial mammals, where relatively big and dominant animals produce low  $f_0$  and low formant frequencies (Charlton *et al.*, 2020). Crucially, NLP lower the perception of pitch (the acoustic correlate of  $f_0$ ) (Anikin *et al.*, 2021) and, can also emphasise the perception of formant frequencies by increasing spectral density. The hypothesis of NLP as vocal cues to body size and dominance is supported by recent studies showing that NLP experimentally added to synthetic mammal vocalisations increase the perceived size and

dominance of the vocaliser as judged by human listeners (Anikin, 2020; Anikin *et al.*, 2021; Massenet *et al.*, 2022, see Chapter 3 of this thesis)

Nonlinear phenomena have also been described as vocal indicators of arousal in behavioural contexts associated with aggression such as sexual competition (Reby and McComb, 2003), or those associated with distress such as during separation from other conspecifics (Wilden *et al.*, 1998; Stoeger *et al.*, 2011; Scheumann *et al.*, 2012), or an immediate risk of predation (Blumstein *et al.*, 2008). Nonlinear phenomena may thus inform receivers about the transient emotional or motivational states of vocalisers (Wilden *et al.*, 1998). This has further led to the suggestion that another potential function of NLP is to reduce habituation in receivers by making calls unpredictable and difficult to ignore (Fitch, Neubauer and Herzel, 2002; Reby and Charlton, 2012; Karp *et al.*, 2014; Charlton, Taylor and Reby, 2017). For example, harsh roars typically produced by highly motivated red deer stags during the breeding season (Reby and McComb, 2003) increase the level of attention in females (Reby and Charlton, 2012) but elicit fewer responses in other stags, suggesting that males may avoid potential conflicts when perceiving high levels of motivation in rivals (Garcia *et al.*, 2014). This is also in line with evidence that listeners associate NLP in human nonverbal vocalisations with aggression and intimidation (Anikin *et al.*, 2021). Finally, recent studies show that the intensity of perceived distress increases with the presence of chaos in human and dog vocalisations (Raine *et al.*, 2018; Anikin, Pisanski and Reby, 2020; Massenet *et al.*, 2022). Chaos is indeed considered as the most perceptually salient form of NLP and is commonly found in the distress vocalisations of several species including marmots (Blumstein *et al.*, 2008), African elephants (Stoeger *et al.*, 2011), Giant pandas (Stoeger *et al.*, 2012) and humans (Mende, Herzel and Wermke, 1990; Koutseff *et al.*, 2018; Raine *et al.*, 2018).

While it is now increasingly accepted that NLP likely convey biologically and socially relevant information, studies describing their mechanistic origins also support their role in the communication of arousal. Indeed, increases in the arousal state of the vocaliser are typically associated with a high subglottal pressure and/or strong muscle tension on the vocal folds (as reviewed by Briefer, 2012). As in pathological human voices (Herzel *et al.*, 1994), this configuration of the vocal apparatus may affect the vibratory regimes of vocal folds, which ultimately can lead to vocal instabilities (Berry *et al.*, 1994, 1996; Steinecke and Herzel, 1995; Herbst, 2014). Specifically, using *in vivo* or *in vitro* high-speed observations of phonating larynges (elephants: Herbst *et al.*, 2012; red deer: Herbst, 2014; dogs: Berry *et al.*, 1996; humans: Neubauer

*et al.*, 2001) and/or biomechanical simulations of vocal folds (e.g., a two-mass model: Berry *et al.*, 1994; Steinecke and Herzel, 1995; Jiang, Zhang and Stern, 2001), several studies have characterised regimes of vocal fold vibration that provide insight into the possible mechanisms underlying the production of NLP in vocalisations. For instance, it has been demonstrated that antero-posterior or left-right asymmetries in vocal folds elicit bifurcations from a regime of periodic vibrations (i.e., tonal segments of a call) to a regime characterised by aperiodic or period-doubled vibrations (i.e., segment of a call containing NLP) (Berry *et al.*, 1994, 1996; Steinecke and Herzel, 1995; Neubauer *et al.*, 2001). In highly emotionally aroused animals, these asymmetries may result from involuntary changes in muscle tensions applied on the vocal folds, thus likely causing a desynchronisation in their vibratory pattern and leading to vocal instabilities. Furthermore, it has been shown that increasing subglottal pressure is also responsible for bifurcations and complex vibration regimes typically leading to NLP (Berry *et al.*, 1996; Jiang, Zhang and Stern, 2001; Herbst, 2014).

The different types of NLP are based on the configuration and type of vocal fold vibration regimes. Frequency jumps are produced when the rate of vocal fold vibration suddenly changes (Riede, Wilden and Tembrock, 1997) and are characterised by upward or downward jumps of the call  $f_0$ , as typically observable on spectrograms (figure 1). Subharmonics appear when two or more independent modes compete for dominance in the pattern of vocal fold vibration (Titze, 1994). Indeed, because vocal folds form a highly coupled vibratory system, slight changes in their configuration (e.g., asymmetries between vocal folds) can drive them towards vibration at a frequency corresponding to an integer ratio of  $f_0$  (Fitch, Neubauer and Herzel, 2002). In mammals, subharmonics often result from a period doubling or tripling in the vocal folds vibration regimes (Steinecke and Herzel, 1995; Wilden *et al.*, 1998; Fitch, Neubauer and Herzel, 2002) and appear on spectrograms as additional frequency components parallel to the  $f_0$  occurring at an integer ratio of  $f_0$  (typically  $f_0/2$ ,  $f_0/3$ ; Fig. 1). Chaos is usually produced when vocal folds vibrate in asynchronous and nonperiodic regimes (Wilden *et al.*, 1998) and thus appears as a broadband “noisy” segment on spectrograms. Finally, biphonation involves two separate sound sources vibrating at two independent frequencies (Fitch, Neubauer and Herzel, 2002). For instance, the sound sources can correspond to air flowing through the glottis and a narrow supraglottic constriction (Frey *et al.*, 2016; Reby *et al.*, 2016). Biphonation can result from the amplitude modulation of a high supra-laryngeal frequency  $g_0$  by a relatively lower laryngeal frequency  $f_0$



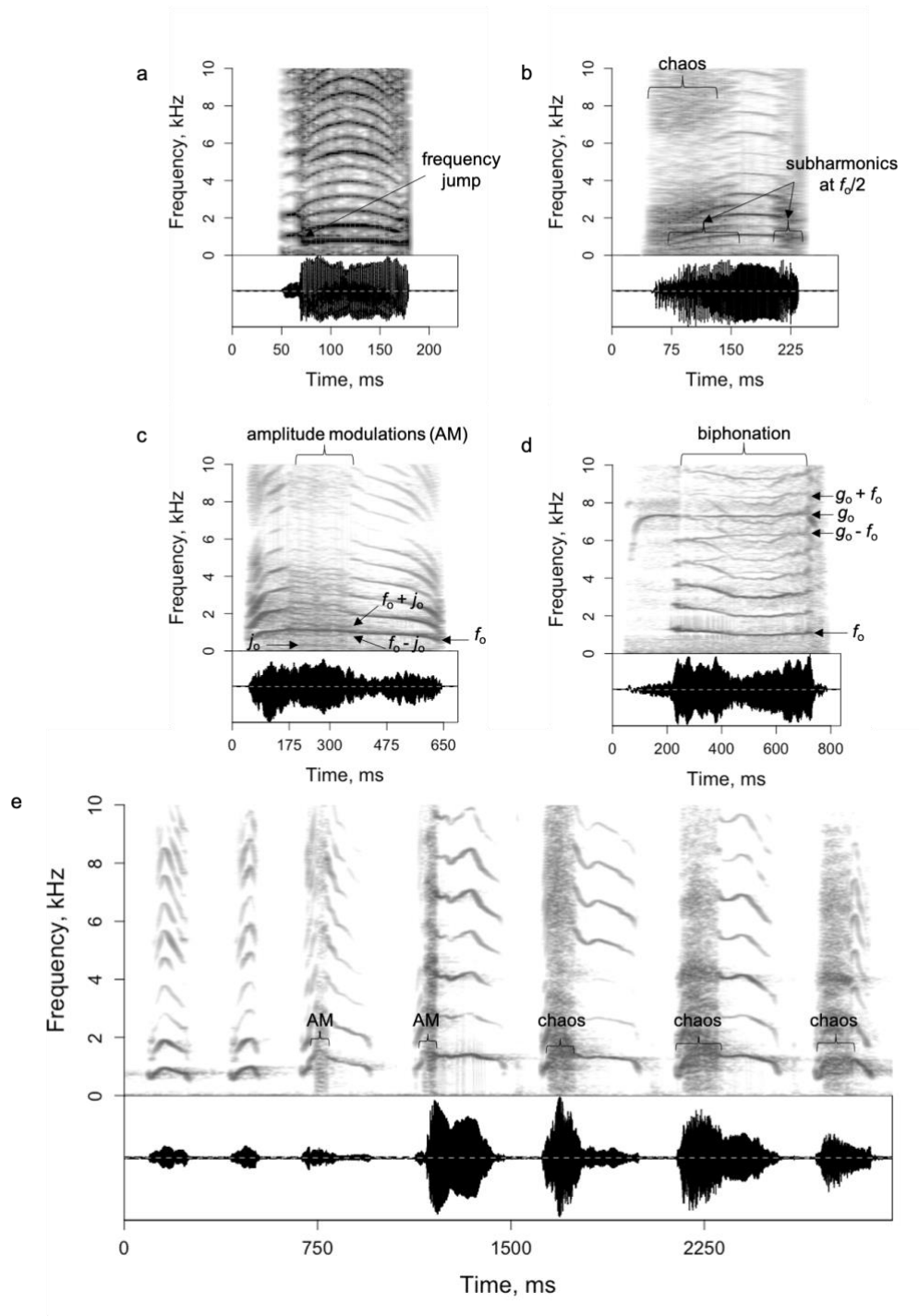
(Wilden *et al.*, 1998; Schneider and Anderson, 2011; Briefer *et al.*, 2015; Reby *et al.*, 2016). However, it is also possible that the laryngeal frequency  $f_0$  is amplitude-modulated by another lower supra-laryngeal frequency (here called  $j_0$ ). Segments of vocalisations presenting a frequency  $g_0$  higher than  $f_0$  will hereafter be characterised as containing *biphonation*, whereas segments presenting  $j_0$  lower than  $f_0$  will hereafter be characterised as containing *amplitude modulation*. The amplitude modulation of one frequency by a second lower frequency leads to visible sidebands as illustrated in figure 1.

Aside from inducing NLP, increases in subglottal pressure can also lead to a high vocal  $f_0$  and amplitude (Titze, 1994), as reported in the distress calls of several mammal species (Lingle *et al.*, 2012; Briefer, 2020). As such, NLP are expected to occur in relatively high-pitched vocalisations. For instance, the onset of NLP corresponds to  $f_0$  maxima in chimpanzee calls (Riede, Arcadi and Owren, 2007). This may result from nonlinear interactions between the larynx (“source”) and the vocal tract (“filter”), causing bifurcations in the dynamics of vocal fold vibration, particularly when  $f_0$  (or the first harmonic) crosses the first formant frequency (reviewed by Tokuda, 2018). Therefore, it is possible that the occurrence of NLP within calls or within vocal long sequences covaries with other acoustic features such as call  $f_0$ , amplitude, or duration.

While most studies only report the presence of NLP in vocalisations produced in contexts of high emotional state, the effect of arousal on NLP remains poorly documented. What is the effect of varying levels of arousal on the occurrence of different types of NLP within calls or within calling sequences? What is the acoustic context (e.g., amplitude,  $f_0$ , duration) of their occurrence? Such systematic descriptions in natural vocalisation models would inform both mechanistic and functional hypotheses that have emerged regarding the presence of NLP as possible vocal cues to signal heightened arousal in the communication systems of mammals. Here, we address these questions by providing detailed description of NLP and their acoustic correlates in a natural call system, the distress whines of domestic dog puppies (figure 1). Whine vocalisations, typically given in sequence (figure 1e), often signal distress, for example in contexts of separation from or aggression by the puppy’s mother or littermates (Bleicher, 1963; Lezama-García *et al.*, 2019). Similar to the distress vocalisations of several other mammal species (reviewed in Lingle *et al.*, 2012; Briefer, 2020), some acoustic features of puppy whines can vary dynamically with arousal, including variations in duration, amplitude and/or  $f_0$  (figure 1e). Like adult dog whines (Marx *et*

*al.*, 2021), puppy whines include NLP such as frequency jumps, subharmonics, chaos and biphonation.

To investigate the effect of a putative increase in puppies' arousal state on the occurrence of NLP in whines, we analysed whines produced in response to a short separation from their mother and littermates. For each whine, we annotated NLP episodes and measured key vocal parameters including  $f_0$ , duration, amplitude, and harmonicity (harmonic-to-noise ratio, HNR). This approach allowed us to describe the temporal distribution of NLP across whines during the separation period and within individual whines in relation to  $f_0$  and amplitude parameters. Based on behavioural observations showing that in mammals calling behaviour increases with arousal (Briefer, 2020), we predicted that whining activity in puppies would increase as the time since separation from their mother becomes longer. We also expected that heightened whining would be accompanied by an increase in  $f_0$  and duration of whines as well as an increase in the occurrence and duration of NLP. Furthermore, within whines, we predicted NLP to occur at the beginning of whines, rather than the end, as the rapid early increase in  $f_0$  and amplitude may reflect phonation instability. In order to investigate the stability of whine acoustic profiles during the puppies' development, these analyses were repeated longitudinally at approximately 1, 3, 6 and 9 weeks after birth.



**Figure 1:** Examples of NLP occurring in puppy whines. Panel (a) illustrates an example of whine containing a downward frequency jump. Panel (b) corresponds to whines characterised by a combination of two different types of NLP, i.e., chaos and subharmonics. Panels (c) and (d) show clear examples of amplitude modulation and biphonation. Sidebands are visible at a linear combination of  $m \cdot f_0 \pm n \cdot j_0$  ( $f_0$  higher than  $j_0$ ) or  $m \cdot g_0 \pm n \cdot f_0$  ( $g_0$  higher than  $f_0$ ) where  $m$  and  $n$  are integers. Note in (e) the increase of the whines' duration, amplitude, and  $f_0$  during the production of NLP.

## Material and methods

### *Recordings of puppy whines*

We recorded whines produced by Beagle puppies (N=32 individuals from 6 litters, table 1) in a context of separation from their mother and littermates. Puppy whines were collected at two different breeding facilities in France, where females and their litter are kept in the same enclosure during the first two months of puppies' lives. During recordings, each individual was carried to a separate room and placed alone in a pen (roughly 100 x 50 cm) without any visual and vocal contact with its mother and littermates. Puppies were recorded during their 1<sup>st</sup>, 3<sup>rd</sup>, 6<sup>th</sup> or 9<sup>th</sup> week after birth (table 1), using a Sennheiser MKH70 directional microphone connected to an audio recorder Zoom H4n (44.1kHz, 24bit). The microphone was positioned at approximately 30 cm from the animal's mouth. To avoid saturation of acoustic signals, when necessary, the input level was adjusted during the recording. While this prevented us to measure variation in whine amplitude as time since separation increased, we could however measure these variations within whines as these input level adjustments were instantaneous and thus did not affect intra-whine amplitude contours.

Each puppy was recorded during separations lasting  $3.5 \pm 2$  minutes (mean  $\pm$  SD). At the end of each recording trial, puppies were immediately reunited with their mother. All puppies displayed normal behaviours upon being reunited, including suckling or interacting with their mother and littermates, indicating that any stress experienced during the recording had been low and temporary. No puppies showed signs of distress (e.g., excessive panting, yawning, lip licking Beerda *et al.*, 1998; Godbout *et al.*, 2007) which otherwise would have led to the immediate interruption of the trial. The mothers in this study were habituated to being temporarily separated from their litter (e.g., during daily walks) and accustomed to breeders manipulating puppies during daily care (e.g., weight measurements evaluating physical condition of puppies). As a consequence, they did not display any sign of stress during the separations from their puppies for individual recordings.

**Table 1:** Total number of litters and individual puppies included in acoustic analyses for each age class (1, 3, 6 and 9 weeks after birth).

Litter's identity	Litter size	Age of puppies			
		1 week (6 ± 3 days)	3 weeks (18 ± 1 days)	6 weeks (39 ± 1 days)	9 weeks (59 ± 1 days)
3	8	8	6	3	4
6	5	5	3	3	
4	4	4	3	3	
2	5		3	2	3
5	4		2	2	4
1	6			4	6
<b>Total # of individuals</b>		<b>17</b>	<b>17</b>	<b>17</b>	<b>17</b>
<b>Total # of litters</b>		<b>3</b>	<b>5</b>	<b>6</b>	<b>6</b>

Within each age category column, cell values represent the number of individual puppies per litter whose whines were analysed. The age in days of puppies is given with the mean ± SD.

### *Acoustic analyses*

Our acoustic analysis of whines included the following steps: (1) extraction of individual whines from recordings, (2) measurement of several acoustic parameters such as whine  $f_0$ , amplitude, duration (table 2), and (3) manual annotations of NLP. Our original pool of recordings consisted of a total of 125 recordings. From our pool of recordings, we randomly selected recordings of 17 puppies from 3 to 6 different litters to create a balanced dataset across ages (table 1). As a same puppy was recorded several times during their two first months of life, whines from a given individual were analysed up to three times at different ages throughout their development (although we avoided this as much as possible to limit pseudo replication, and always controlled for the puppy's identity in statistical our models).

Of the 68 selected recordings, we visually inspected spectrograms of 13,346 whines in Praat (Boersma and Weenink, 2022) (Gaussian window, window length = 0.01 sec, dynamic range = 100 dB) and classified them into whines without NLP (i.e., tonal whines, N= 8330 calls) and whines with NLP (i.e., whines containing one or several episodes of one or several types of NLP, N= 5016 calls). While we manually extracted whines with NLP, the method applied for the

extraction of whines without NLP relied on their semi-automatic detection using the function *segment* (soundgen R package) (Anikin, 2019). Because the level of background noise can slightly change across whining sequences, we adjusted settings related to the proportion of noise or to the silence-to-noise ratio in order to improve the performance of the algorithm.

Our final dataset of whines thus included a total of 6995 whines without NLP and 5016 whines with NLP, from which duration and onset time during separation were also measured. In addition, we used the function *analyze* (soundgen R package) to extract the  $f_0$  and amplitude contours of every whine (divided into frames of 5% of the whine duration) and measured their associated harmonicity (HNR) (table 2). Because the presence of NLP in calls can lead to false automatic detection in  $f_0$  tracking, we supervised the extraction of  $f_0$  contours in whines that contained NLP by using the *pitch\_app* (soundgen R package). This was not necessary for whines without NLP from which  $f_0$  extraction was performed automatically. Indeed, when verifying the reliability of manual *versus* automatic extractions of  $f_0$  contours on a subset of 500 whines without NLP, we found a nearly perfect correlation between these two methods (Pearson's correlation  $r=0.99$ ).

Finally, we manually annotated the onset time and duration of each episode of NLP within each whine containing any NLP. Because the duration of whines with NLP varied widely (mean  $\pm$  SD =  $256 \pm 164$ ms, range [73ms, 1628ms]), we converted the absolute duration (ms) of chaos, subharmonics, and sidebands into a relative duration expressed as the proportion of each whine affected by NLP.

**Table 2:** Description of the 7 vocal parameters measured. With the exception of NLP duration and NLP onset time, all other parameters were measured in whines both with and without NLP (N=6995 and N=5016 respectively).

Vocal parameter	Definition
$f_0$ (Hz)	fundamental frequency of the whine. We extracted mean $f_0$ of whines as well as values from $f_0$ contour (see section Acoustic analyses)
HRN (dB)	harmonic-to-noise ratio of the whines. A low value of HNR indicates a rough, harsh voice quality whereas a high value characterises high tonality in calls.
Within-whine amplitude (RMS)	root mean square amplitude. We extracted values from amplitude contours (see section Acoustic analyses)
Duration (ms)	duration of the whine.
Whine onset time (ranging from 0 to 1)	relative onset time of the whine during separation from mother and littermates. For instance, a whine onset of 0.5 means that a whine occurred half-way through the separation period.
NLP duration (%)	proportion of the whine affected by subharmonics, chaos, amplitude modulation and/or biphonation
NLP onset time (ranging from 0 to 1)	relative position of frequency jump, subharmonics, chaos, amplitude modulation or biphonation within a whine containing NLP. For instance, an NLP onset of 0.5 means that the NLP occurs at half of the whine duration.

We investigated amplitude variation only within whines as measurements over the separation were not meaningful (see section *Recordings of puppy whines*)

### *Data analysis*

We used different statistical methods including Bayesian multilevel models fitted with the *brms* R package (Bürkner et al 2017) using default priors and generalized additive models fitted with *mgcv* R package, while controlling in both approaches for puppy's identity (to account for repeated measurements). Posterior distributions of model parameters were summarized by their median and 95% credible/confidence intervals.

The main aim of our analyses was to investigate how the vocal production of NLP and their acoustic correlates vary with increasing time since puppy's separation from their mother and littermates. To do so, we first tested a model testing the effects of separation on the production of whines (presence/absence) to evaluate whether whining activity of puppies increased as the period of separation became longer (model 1, table S2). Similar models were fitted to examine whether the production of whines containing NLP and their acoustics varied since separation (model 2-5, table S1).

Then, we ran models to investigate when NLP arise within whines by modelling their presence or extent in individual whines (models 6-8, table S1). In addition, to understand what acoustic change is associated with the onset of NLP within whines, we also fitted two additional models that examined the relationships between NLP onset and  $f_0$  and amplitude slopes (models 9 and 10, table S1).

Finally, we characterised acoustics of whines with NLP compared to whines without NLP by fitting a model testing the effects of the presence NLP on measured vocal features (i.e., mean  $f_0$ , duration and HNR) (models 11 and 12, table S1). We also examined the relationship between the production of NLP and puppy age (model 13, table S1) to describe NLP ontogeny from birth to two months of age. The syntax and data used for each model are detailed in table S1.

## Results

### *Acoustics of whines with NLP*

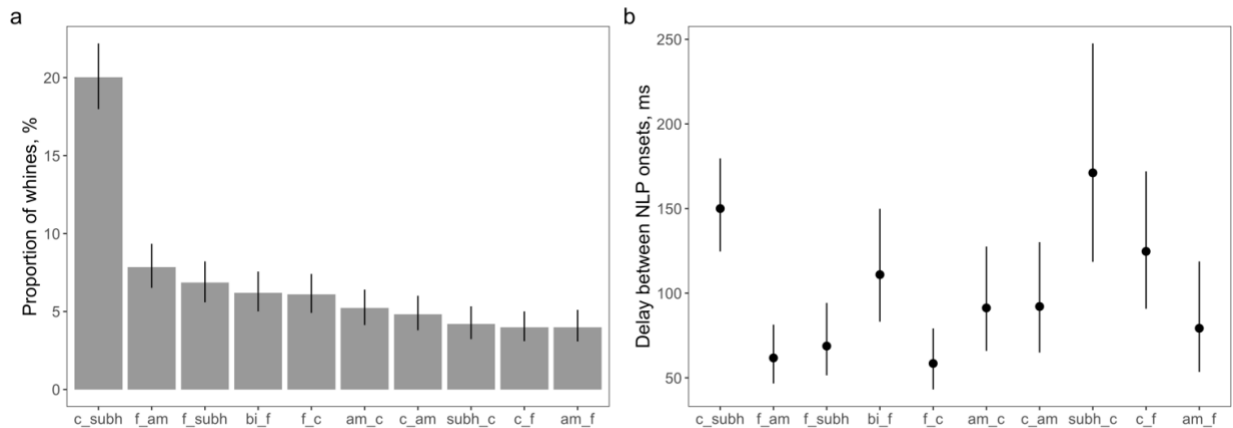
In the 5016 whines containing NLP, we identified a total of 7178 NLP episodes. Chaos and frequency jumps were the two most common NLP types found in puppy whines, occurring in 37% and 36.5% of calls, respectively. Subharmonics, amplitude modulation and biphonation occurred in 26.5%, 17.7% and 15.9% of whines with NLP, respectively. In addition, whines containing NLP were typically characterised by a single episode of NLP (N=3351/5016), although 1665 whines contained multiple NLP episodes, ranging from 2 to 6, with most such whines (1450) characterised by multiple episodes of several different types of NLP. Specifically, we identified 78 different NLP combinations in puppy whines, with chaos followed by subharmonics as the most common form of combination (N= 291/1450 whines) (see table S2 for details about the different types of NLP combinations). Within a whine, chaos and subharmonics were typically separated by a tonal section of 149.9ms [125.1, 181.9] (hereafter called *delay*). Furthermore, frequency jumps occurred in combination with any type of NLP (figure 2a). They were rapidly followed by other types of NLP, as we indeed found a short delay between frequency jumps and amplitude modulation (62ms [46.8, 82.5]), frequency jumps and subharmonics (69.1ms [52.3, 92.5]), or frequency jumps and chaos (58.1ms [42.6, 80.1]). However, this tonal section was slightly longer when frequency jumps followed biphonation (110.8ms [81.4, 153.2]), amplitude modulation (79.5ms [53.8, 118.4]) or chaos (125.1ms [89.8, 175.7]). Finally, whines containing amplitude modulation and chaos were



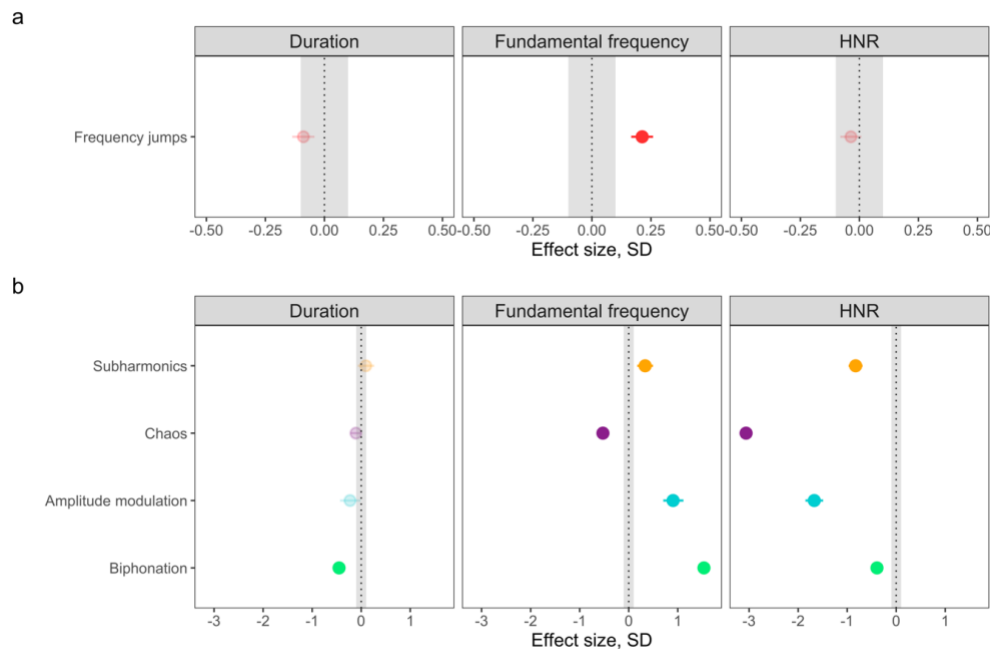
separated by a delay of roughly 100ms regardless of the order in which these two types of NLP occurred in the whine (figure 2b).

We also characterised the general acoustic structure of whines affected by each specific type of NLP. Whines containing any type of NLP (except frequency jumps) did present differences in their harmonicity (HNR) or  $f_0$ , but not in their duration (figure 3a) compared to whines without. Differences in HNR were the most noticeable in whines with chaos, as these whines were characterised by smaller values of HNR (3.1 SD [-3.2, -3]) relative to whines without chaos. We also found that whines containing subharmonics, amplitude modulation, biphonation and chaos slightly differed in their  $f_0$  (figure 3b). Indeed, relative to the  $f_0$  of other whines, episodes of subharmonics, amplitude modulation and biphonation, respectively, occurred in whines produced with a higher  $f_0$  (0.3 SD [0.2, 0.5], 0.9 SD [0.7, 1.1], 1.5 SD [1.4, 1.7] respectively). Chaos however occurred in whines characterised by a lower  $f_0$  (0.5 SD [-0.6, -0.4]) compared to whine without chaos.

Labels:  
 f= frequency jump; subh= subharmonics; c= chaos;  
 am= amplitude modulations; bi= biphonation



**Figure 2:** Description of the 10 most common types of NLP combinations occurring in puppy whines (N=1450). Panel (a) shows the proportion of whines containing these specific types of combinations. Note that the most common type of combination occurring in puppy whines corresponds to chaos followed by an episode of subharmonics. Panel (b) shows the delay (i.e., duration of a tonal section) between two consecutive NLP onsets associated with each type of combination. Note that the duration of the tonal segment between two consecutive NLP episodes varies depending on the type of combination, e.g., this delay is shorter when frequency jumps occur first in a combination than when frequency jump follow another other type of NLP. The order of NLP labels corresponds to their order of occurrence within a whine (e.g., c\_subh means that chaos occurs first and is then followed by subharmonics). Bars (panel a) or solid markers (panel b) correspond to medians of posterior distribution with their 95% credible intervals.



**Figure 3:** Differences in vocal parameters expressed in standard deviation (SD) as a function of (a) the presence of frequency jumps and (b) the proportion of whine affected by subharmonics, chaos, amplitude modulation and biphonation compared to whines without these NLP types. Gray rectangles indicate an interval of  $\pm 0.1$  SD in which we did not consider any changes in the measured vocal parameters. Circles correspond to medians of the posterior distribution with their 95% credible intervals. Note a lower HNR in whines containing chaos or amplitude modulation, or a higher  $f_0$  in whines containing biphonation, compared to whines not containing these NLP types (b).

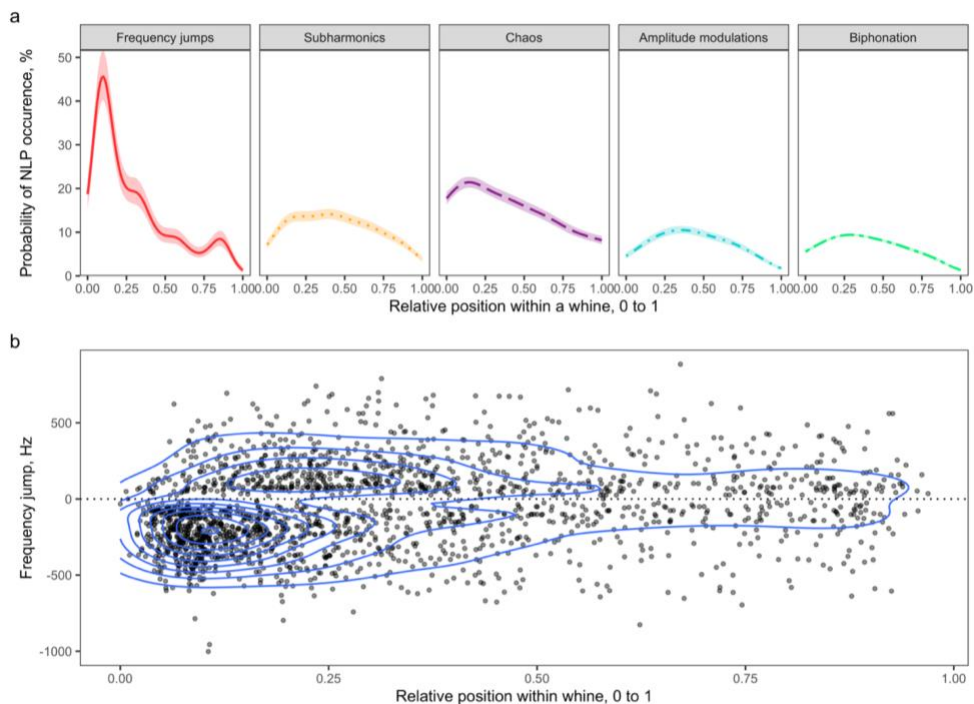
### *Nonlinear phenomena occur when $f_0$ and amplitude increase within whines*

We investigated the temporal dynamics of NLP within individual whine and found that NLP often occurred early in whines, typically during the first half (figure 4a). Indeed, peaks of NLP types occurred at 10% of the whine for frequency jumps, 14% for subharmonics, 29% for amplitude modulation, 35% for chaos, and 40% for biphonation.

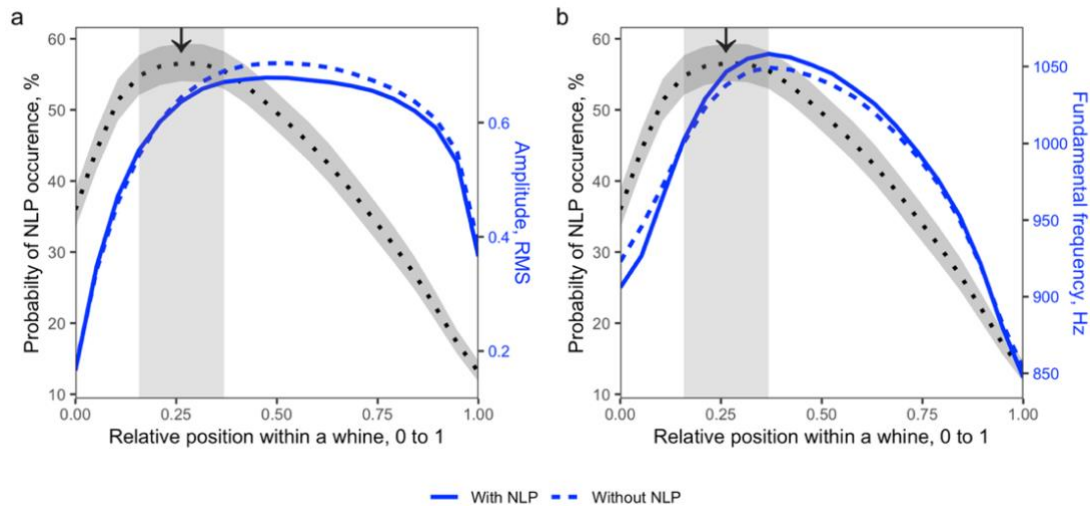
With the exception of frequency jumps, typically characterised by sudden and acute downward jumps in whine frequency (figure 4b), we found that all other forms of NLP showed variable durations within whines. Indeed, biphonation affected more than half of the whines' duration (57% [53, 62]), whereas subharmonics, amplitude modulations and chaos occurred in a smaller proportion of the whine (i.e., 26% [24, 28], 26% [24, 28], 34% [31, 37], respectively) (figure 5). Absolute durations of NLP within whines averaged 61ms [56, 65] for amplitude

modulations, 64ms [60, 69] for subharmonics, 82ms [76, 88] for chaos, and 114ms [105, 124] for biphonation.

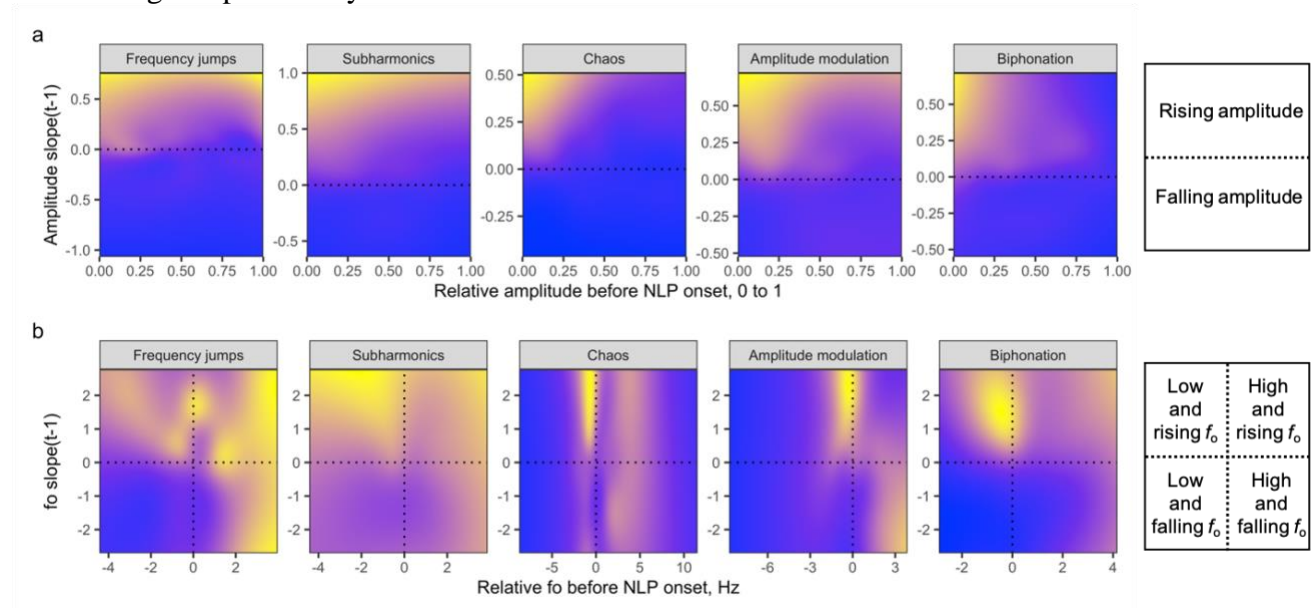
We also found that NLP occurred when the average amplitude and  $f_0$  of whines were typically rising, before reaching their maximum values (figure 5a and 5b), possibly reflecting instabilities in the puppies' vocal system. In line with this finding, when mapping whines' variation in amplitude (i.e., amplitude slope) before the onset of NLP, NLP were often preceded by a rising amplitude (figure 6a). As for  $f_0$  variations, we also found that NLP often occurred just after an episode of rising  $f_0$ , except for frequency jumps (figure 6b). This type of NLP could indeed appear in two different “scenarios”: frequency jumps were often preceded by a relatively high and falling  $f_0$ , or less commonly, by a low and rising  $f_0$ . Finally, while  $f_0$  preceding episodes of chaos or amplitude modulation did not differ from average  $f_0$ , it could be lower or higher when preceding subharmonics (figure 6b).



**Figure 4:** Temporal distribution of (a) NLP type and (b) the extent of  $f_0$  jumps within whines. In panel (a), lines correspond to fitted values extracted from the posterior distribution and associated ribbons to the 95% credible interval. In panel (b), each dot corresponds to a downward or upward frequency jump (change in Hz after compared to before the jump), and blue lines indicate the density. Panel (a) illustrates that all forms of NLP occurred during the first half of whines. Panel (b) shows that most  $f_0$  jumps correspond to acute downward frequency shifts occurring at the beginning of whines, and less commonly, an upward shift occurring slightly later in the whine.



**Figure 5:** Average (a) amplitude contours and (b)  $f_0$  contours in whines with and without NLP represented respectively with the probability of NLP occurrence. Note that NLP occur while amplitude and  $f_0$  slopes are at their steepest rising phase, but before they peak. Each line corresponds to fitted values extracted from the posterior distribution. The shaded rectangle shows the highest density interval of NLP posterior probability, corresponding to the whine region in which NLP most commonly occur. The arrow indicates where, in an average whine, NLP occur with the highest probability.



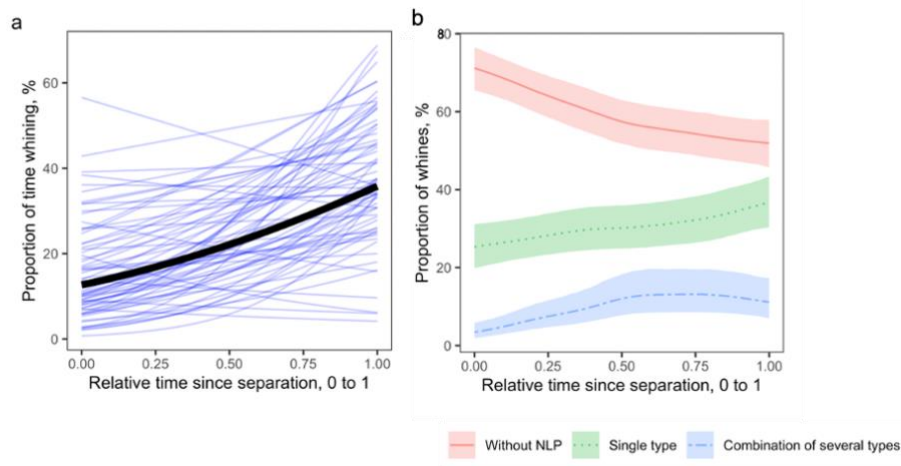
**Figure 6:** Probability of NLP occurrence within whines modelled as a smooth function of (a) amplitude or (b)  $f_0$ , and their slopes just before the onset of NLP. Yellow regions correspond to a high probability of NLP occurrence whereas blue regions correspond to a low probability of NLP occurrence. Note that NLP commonly occur just after an episode of rising amplitude and/or rising  $f_0$ .

### *Increasing production of NLP with time of separation*

We found that the puppies' vocal activity increased as separation from their mother and littermates became longer (figure 7a). Indeed, whines with NLP occurred more often towards the end of a separation period whereas whines without NLP (tonal whines) occurred more often towards the beginning (figure 7b). Specifically, while the proportion of whines without NLP decreased by an odds ratio of 1.2 [1.1, 1.4] during separation, the proportion of whines with a single type of NLP or a combination of several types of NLP increased towards the end of the separation period.

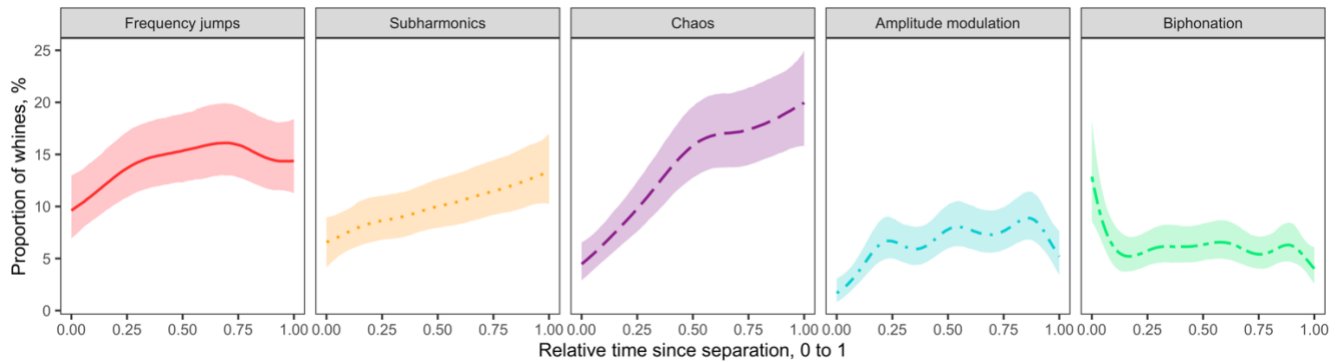
The increase in whines containing NLP was mainly driven by an increase in frequency jumps, subharmonics, chaos and amplitude modulation, wherein the predicted occurrence was respectively increased by an odds ratio of 1.4 [1.1, 1.9], 1.9 [1.5, 2.8], 3.7 [2.7, 5.5], 3 [1.6, 6] as time since separation became longer. In contrast, whines containing biphonation rapidly dropped at the beginning and then remained stable until the end of the separation period (figure 8). Furthermore, we predicted that the proportion (or duration) of NLP affecting whines would increase during separation. However, we did not find noticeable changes in NLP duration (figure 9).

While describing the occurrence of NLP, we also investigated whether the acoustics of whines varied as the separation period became longer. In this context, we predicted a higher  $f_0$ , a longer duration and a harsher, rougher voiced quality in whines produced towards the end of experiments than those produced at the beginning. Unexpectedly, we did not find such dynamics, as none of the measured vocal parameters noticeably varied with time since separation (figure 10).

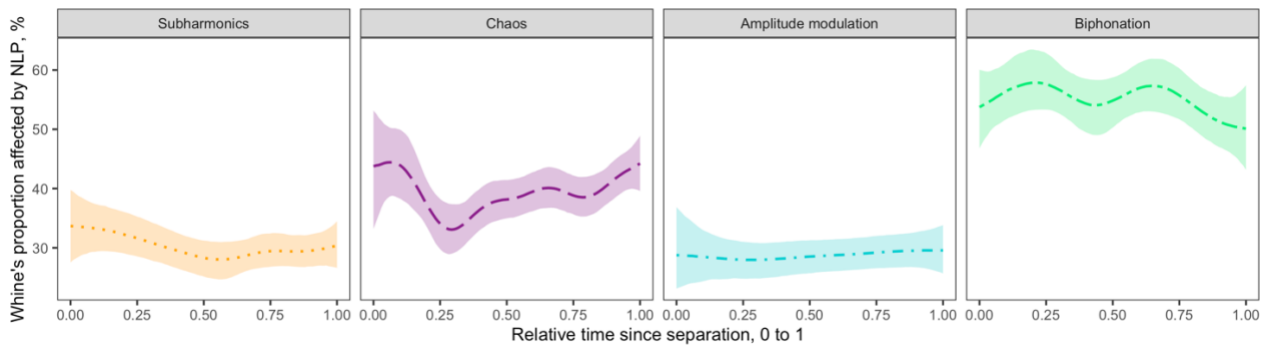


**Figure 7:** Whining behaviour of puppies in a context of separation from their mother and littermates. Because the duration of separation varied by  $\pm 2$  minutes between individuals, we

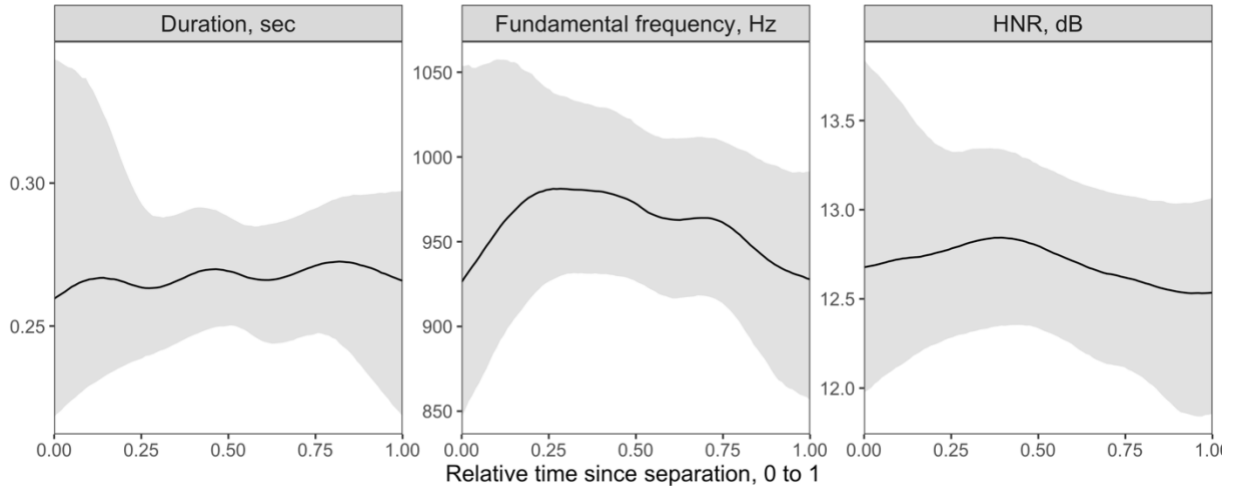
converted the absolute time since separation as a relative time ranging from 0 to 1. In panel (a), the thick black line represents the overall trend and thinner blue lines the trend for each whining sequence (N= 68) estimated by models. In panel (b), lines correspond to fitted values extracted from the posterior distribution of our model and ribbons correspond to 95% credible intervals. Panel (a) shows the increasing proportion of time spent whining as separation became longer. Panel (b) shows the proportion of whines without NLP, with a single NLP type and with a combination of NLP as a function of time since separation.



**Figure 8:** Temporal distribution of whines containing NLP, expressed in proportion of whines as a function of time since puppies’ separation from their mother and littermates. Because the duration of separation varied by  $\pm 2$  minutes between individuals, we converted the absolute time since separation to relative time ranging from 0 to 1. Lines correspond to fitted values extracted from the posterior distribution from our models and ribbons to the 95% credible interval. The proportion of whines containing frequency jumps, subharmonics, chaos, biphonation, but not amplitude modulation, varied during separation.



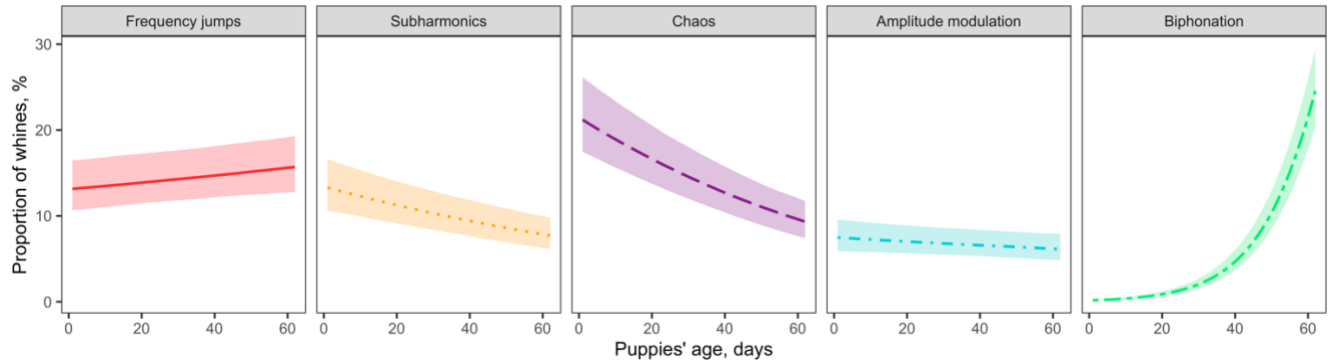
**Figure 9:** Proportion (or duration) of NLP affecting whines as a function of time since puppies’ separation from their mother and littermates. Because the duration of separation varied by  $\pm 2$  minutes between individuals, we converted the absolute time since separation as a relative time ranging from 0 to 1. Lines correspond to fitted values extracted from the posterior distribution of our model and ribbons to the 95% credible interval. The proportion of each NLP type remains relatively stable during separation. Also note that episodes of biphonation are longer than are episodes of subharmonics, chaos and amplitude modulation.



**Figure 10:** Variation of whine duration, pitch and harmonicity measured from whines as a function of time since puppies’ separation from their mother and littermates. Because the duration of separation varied by  $\pm 2$  minutes between individuals, we converted the absolute time since separation as a relative time ranging from 0 to 1. Lines correspond to fitted values extracted from the posterior distribution of our model and ribbons to the 95% credible interval. None of these vocal parameters changed significantly over the period of puppies’ separation from their mother and littermates.

*Production of NLP varies with puppy age*

Because our dataset includes whines produced by puppies at different ages (table 1), we investigated whether the production of NLP varies during the first months of puppies’ lives. We found a clear interaction effect between the proportion of whines containing NLP and the age of puppies. Indeed, from birth to two months of age, the predicted NLP occurrence decreased by an odds ratio of 0.6 [0.5, 0.7] and 0.5 [0.4, 0.6] for whines containing subharmonics or chaos, respectively. In contrast, the proportion of whines affected by biphonation noticeably increased after 6-weeks of age (figure 11). The occurrence of frequency jumps and amplitude modulation in whines, however, tended to be stable throughout the two first months of puppies’ development (figure 11).



**Figure 11:** Ontogeny of whines containing NLP, expressed in proportion of whines as a function of puppy age. This figure shows that the proportion of whines containing subharmonics and chaos decreased during puppies’ development whereas the occurrence of biphonation increased with age. Lines correspond to the fitted values extracted from the posterior distribution our model and ribbons to the 95% credible interval.

## Discussion

We investigated the temporal distribution and acoustic correlates of nonlinear phenomena (NLP) in whines produced by domestic dog puppies in a context of separation from their mother and littermates. We found that puppy whines include all key types of NLP, namely frequency jumps, subharmonics, chaos, amplitude modulation and biphonation, either as single episodes or as a rich but non-random variety of combinations. The production of NLP varied within individual whines, across whines throughout the separation period, but also throughout puppies’ development. Crucially, puppies produced more whines and specifically more whines with NLP as time since separation from their mother increased, suggesting that NLP in puppy whines may function as cues to signal distress to caregivers.

*Do the dynamics of  $f_0$  and amplitude predict the occurrence of NLP within whines?*

Yes. Extracted  $f_0$  and amplitude contours show that puppy distress whines are on average characterised by a typical chevron shape, as commonly observed in the distress vocalisations of a wide range of mammal species (reviewed in Lingle *et al.*, 2012). Specifically, whine amplitude and  $f_0$  rapidly increased at the beginning of calls and then dropped towards the end. According to mechanistic models predicting the production of NLP (Berry *et al.*, 1994; Titze, 1994; Jiang, Zhang and Stern, 2001; Herbst, 2014), we expected NLP to occur while the vocal system was unstable, possibly caused by rapid changes in both amplitude and  $f_0$ . Our results confirmed this



prediction as we found that all form of NLP occurred most often in the first half of whines. In addition, all NLP occurred after a rising  $f_0$  (and amplitude). This finding is consistent with research on chimpanzee pant-hoot calls showing that chaos, amplitude modulation or frequency jumps occur when  $f_0$  is rising to its maximum value (Riede, Arcadi and Owren, 2007). Pan-hoot calls are indeed given by individuals in sequences characterized by a climax phase, where calls are typically produced with a high  $f_0$ , amplitude and NLP. Fundamental frequency slopes in puppy whines containing NLP were steeper than in tonal whines. Therefore, the rapid increase in  $f_0$  (and amplitude), rather than the overall call  $f_0$ , may trigger the occurrence of NLP, possibility because of a specific configuration in the vocal anatomy that likely arises in aroused animals, which ultimately leads to nonlinear dynamics in the vibration of their vocal system.

*Does the proportion of whines containing NLP increase with time since separation?*

Yes. We found that the proportion of whines containing NLP increased towards the end of separation periods. This was mainly driven by an increase in the occurrence of frequency jumps, subharmonics, chaos and amplitude modulation. These types of NLP have been described as particularly evocative or attention-grabbing in distress contexts (Blumstein and Récapet, 2009; Townsend and Manser, 2011). For instance, playbacks of frequency jumps added to synthetic sounds (Slaughter *et al.*, 2013; Yan *et al.*, 2019) or subharmonics in natural alarming calls of meerkats (Townsend and Manser, 2011) induced potentially costly behavioural responses in animals such as an increased vigilance state or fewer foraging activities. In addition, chaos, the most perceptually salient type of NLP (Anikin *et al.*, 2021), increased perceived distress by human listeners when experimentally added to highly realistic synthetic vocalisations (Anikin, Pisanski and Reby, 2020; Massenet *et al.*, 2022). We also found that the production of whines containing biphonation quickly dropped and then, remained stable until the end of the separation period. In canids, biphonation often occurs in behavioural contexts associated with attention seeking or frustration (Schneider and Anderson, 2011; Taylor *et al.*, 2014). Thus, we suggest that biphonation may serve to grab receivers' attention at the beginning of a separation period, and that the later production of more perceptually salient types of NLP, such as chaos, may reinforce this attention-grabbing effect.

In addition, we showed that the duration of NLP events within whines did not increase as the proportion of whines containing NLP increased throughout the separation period. We thus

suggest that the presence of NLP in whines, rather than their duration, encode for heightened levels of arousal in puppies. Consistent with this, we recently found in psychoacoustic experiments involving human listeners that, while the presence of chaos in synthetic whines noticeably increased perceived distress, the amount of chaos did not influence the level of this perceived distress (Massenet *et al.*, 2022). In other words, the mere presence of chaos was enough to communicate distress to listeners.

High arousal in animals is typically associated with a high subglottal pressure (characterising a high  $f_0$ , amplitude, and duration in calls) and strong muscle tension on vocal folds (Briefer, 2012, 2020). This configuration of the vocal apparatus is often responsible for the production of NLP (Steinecke and Herzel, 1995; Berry *et al.*, 1996; Herbst, 2014). Thus, along with the higher occurrence of NLP towards the end of a separation period, we expected vocal parameters of these whines (i.e.,  $f_0$  or duration) to increase. For instance, it has been reported in several species that NLP occur in calls characterized by a relatively higher  $f_0$  and/or longer duration compared to tonal calls (Riede, Wilden and Tembrock, 1997; Peters *et al.*, 2004; Stoeger *et al.*, 2011; Digby, Bell and Teal, 2014). Yet, we found that neither whine  $f_0$  nor duration were higher in whines with NLP than in those without NLP, and that these parameters did not increase with time since separation. This absence of such dynamics may reflect limits of the vocal system of puppies for periodic/tonal phonation. Indeed, along with a possible increase in arousal since separation, we suggest that the vocal system may become unstable, ultimately leading to the production of NLP in whines without further increase of  $f_0$  and duration. Finally, we found that the HNR did not vary during the separation period. As we did not find that NLP duration changed within whine as time since separation increase, we suggest that the absence of variation in HNR during our experiments is due to the relatively short duration of NLP events that does not affect the overall whine HNR.

In addition to changes in vocal configuration associated with aroused physiological states in mammals, increases in cortisol levels, cardiac activity, locomotor and vocal activity have also been described as indicators of heightened arousal (as reviewed by Briefer, 2012). Here, we found that puppies spend more time whining towards the end of the separation period, indicating that arousal increases with prolonged separation. Therefore, we suggest that NLP may convey reliable information about the arousal level of puppies in a context of separation from their mother and

littermates. Additional correlations with variables quantifying stress-related behaviour (e.g., locomotion activity) are planned to confirm our findings.

### *Ontogenetic changes in the production of NLP*

We found that the production of NLP varied during the two first months of puppies' live, with the exception of frequency jumps and amplitude modulation. Specifically, the production of whines containing biphonation clearly increased after 6 weeks of age. In canids, biphonation is common and typically corresponds to the production of high frequency whistles, while simultaneously emitting whines (Wilden *et al.*, 1998; Frey *et al.*, 2016, 2016; Marx *et al.*, 2021). Several hypotheses have been put forward regarding mechanisms underlying the production of biphonation (Volodina *et al.*, 2006; Frey *et al.*, 2016). While several mammal species present specific adaptations in their vocal anatomy that are responsible for biphonation (e.g., vocal membranes in bats and some primate species) (Suthers, 1988; Fitch and Hauser, 1995), in canids, biphonation is more likely to result from the vibration of supra-laryngeal vocal structures (Frey *et al.*, 2016) occurring while the subglottal pressure (and  $f_0$ ) is relatively high (Riede *et al.*, 2000). Our result showing that biphonation occurs in high  $f_0$  whines supports this mechanistic hypothesis. We suggest that the absence of biphonation in whines produced by younger puppies may be due to their relatively small lung capacities. Indeed, new-born puppies may not be able to efficiently increase their subglottal pressure to produce vibration of the second supra-laryngeal sound source. In addition, we found that the probability of whines containing subharmonics and chaos noticeably decreased from birth to two months of age. We suggest that an incomplete maturation of neuro-motor control in new-born puppies may account for the high rate of nonlinear dynamics in the vocal fold vibrations (responsible for chaos and subharmonics) and for the absence of vibrations in the supra-laryngeal vocal structures (responsible for biphonation) (Mende, Herzel and Wermke, 1990).

During their first month of life, puppies have limited capacity for thermoregulation and they can hardly move, hear, or see their environment and are thus particularly vulnerable (Lord, 2013). Their survival therefore relies on their mothers who intensively breastfeed, groom and warm them (Lezama-García *et al.*, 2019). In this context, NLP in puppy whines may provide mothers with reliable information about their offspring's needs, and may thus have a critical survival function during early ontogeny. Because maternal investment often slowly decreases until weaning around 2-months of age (Santos, Beck and Fontbonne, 2020), we suggest that the age-

variation in NLP production observed throughout puppies' development may also reflect differential impacts of the separation from the mother, and thus different levels of associated arousal. However, while the occurrence of whines with chaos and subharmonics decreases with age, it is important to note that these whines remain common in the vocal repertoires of older puppies. While NLP may indicate age differences in callers (as shown in adult dogs: Marx *et al.*, 2021), NLP in mammal calls have also been described as indicators of arousal in contexts associated with distress (Blumstein *et al.*, 2008; Stoeger *et al.*, 2011; Lingle *et al.*, 2012; Koutseff *et al.*, 2018; Raine *et al.*, 2018).

## **Conclusion**

We found that the production of whines containing NLP increased towards the end of vocal sequences during a stressful separation event. Nonlinear vocal phenomena also typically occurred at the beginning of whines, concluding a rapid increase in  $f_0$  and amplitude. Our observations are thus consistent with experimental and theoretical studies that have linked the occurrence of NLP to rapid increases of subglottal glottal pressure and strong muscle tensions on vocal folds. Because this configuration of the vocal apparatus often arises in animals with aroused physiological states, we suggest that NLP in whines may reflect instabilities in the vocal system and thus heightened emotional states. This systematic description of the temporal distribution and acoustic correlates of NLP contributes to a much-needed ecological background for understanding the mechanisms and functions of NLP in mammal vocal communication systems.

## **Ethics**

Acoustic recordings of puppies were performed under the ethical approval no. E-42-218-0901 (ENES lab agreement, Direction Départementale de la Protection des Populations, Préfecture du Rhône).

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# Electronic Supplementary Materials

## Nonlinear phenomena in puppy dog whines: a possible cue to arousal?

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**Table S1:** Summary of table of statistical models performed to describe the temporal distribution of NLP and acoustic correlates in puppy whines.

Name	Model syntax	Data	Family	Model type	R package
Model 1	Presence/absence of whine ~ time + (time   recording name)	12011 whines with and without NLP	Binomial	Logistic regression	lme4
Model 2	Type of whine ~ s(time) + (1 puppy name)	12011 whines with and without NLP	Categorical	Bayesian Multilevel Model	brms
Model 3	Presence/absence of whines ~ NLP type + s(time, by = NLP type) + (1 puppy name)	12011 whines with and without NLP	Bernoulli	Bayesian Multilevel Model	brms
Model 4	Proportion of NLP affecting whines ~ NLP type + s(time, by = NLP type) + (1 puppy name)	5016 whines with NLP	Zero-one-inflated beta	Bayesian Multilevel Model	brms
Model 5	Vocal parameter ~ s(time, by = NLP type) + (1 puppy name)	12011 whines with and without NLP	Gaussian	Bayesian Multilevel Model	brms
Model 6	Presence or absence of NLP ~ NLP type + s(whine frame, by = NLP type) + (1 puppy name + whine name)	5139 NLP episodes	Bernoulli	Bayesian Multilevel Model	brms
Model 7	Relative duration of NLP ~ NLP + (1 puppy name)	5139 NLP episodes	Lognormal	Bayesian Multilevel Model	brms
Model 8	Absolute NLP duration of NLP ~ NLP + (1 puppy name)	5139 NLP episodes	Lognormal	Bayesian Multilevel Model	brms
Model 9	NLP onset ~ NLP type + s(amplitude, amplitude slope, by = NLP type)	5139 NLP episodes	Binomial	Generalized Additive Model	mgecv
Model 10	NLP onset ~ NLP type + s(fo, fo slope, by = NLP type)	5139 NLP episodes	Binomial	Generalized Additive Model	mgecv
Model 11	Vocal parameter ~ presence of frequency jumps (0/1) + (1 puppy name)	12011 whines with and without NLP	Gaussian	Bayesian Multilevel Model	brms
Model 12	Vocal parameter ~ proportion of NLP affecting whines + (1 puppy name)	12011 whines with and without NLP	Gaussian	Bayesian Multilevel Model	brms
Model 13	Presence/absence of whines ~ NLP type * age + (1 puppy name)	12011 whines with and without NLP	Bernoulli	Bayesian Multilevel Model	brms

**Table S2:** Relative occurrence of all possible combinations of NLP occurring in whines.

Type of NLP combination	Relative occurrence (median[95CI])	Type of NLP combination	Relative occurrence (median[95CI])
c_subh	20% [18.1, 22.2]	subh_c_am	0.2% [0.1, 0.6]
f_am	7.8% [6.5, 9.3]	subh_f_c	0.2% [0.1, 0.5]
f_subh	6.9% [5.6, 8.3]	am_f_subh	0.2% [0.1, 0.5]
bi_f	6.2% [5, 7.5]	am_f_subh_c	0.2% [0.1, 0.5]
f_c	6.1% [4.9, 7.4]	subh_f_am	0.2% [0.1, 0.6]
am_c	5.2% [4.2, 6.4]	f_bi_subh	0.2% [0.1, 0.5]
c_am	4.8% [3.8, 6]	f_am_bi	0.1% [0, 0.5]
subh_c	4.2% [3.2, 5.2]	subh_am_f	0.1% [0, 0.4]
c_f	4% [3.1, 5.1]	c_subh_am_f	0.1% [0, 0.4]
am_f	4% [3.1, 5]	c_f_am	0.1% [0, 0.4]
subh_f	3.6% [2.8, 4.6]	bi_c_f	0.1% [0, 0.4]
am_subh	2.7% [1.9, 3.6]	am_f_c	0.1% [0, 0.4]
bi_subh	1.5% [1, 2.2]	am_subh_f	0.1% [0, 0.4]
f_bi	1.3% [0.8, 2]	subh_bi_am	0.1% [0, 0.4]
subh_am	1.3% [0.8, 2]	bi_f_c_subh	0.1% [0, 0.4]
c_subh_f	1.2% [0.7, 1.9]	subh_am_c	0.1% [0, 0.4]
bi_am	1% [0.6, 1.5]	am_subh_c	0.1% [0, 0.4]
f_am_c	0.9% [0.5, 1.5]	f_am_subh_c	0.1% [0, 0.3]
bi_c	0.9% [0.5, 1.5]	bi_subh_f	0.1% [0, 0.3]
f_c_am	0.9% [0.5, 1.5]	f_subh_c_am	0.1% [0, 0.3]
f_subh_c	0.9% [0.5, 1.5]	am_bi_f	0.1% [0, 0.3]
c_f_subh	0.8% [0.4, 1.3]	am_bi_c	0.1% [0, 0.3]
f_c_subh	0.8% [0.4, 1.3]	am_f_bi	0.1% [0, 0.3]
am_bi	0.6% [0.3, 1.1]	bi_c_subh	0.1% [0, 0.3]
c_bi	0.6% [0.3, 1]	f_bi_am_c	0.1% [0, 0.3]
bi_am_f	0.6% [0.3, 1]	subh_c_am_f	0.1% [0, 0.3]
bi_f_am	0.5% [0.3, 1]	f_subh_c_bi	0.1% [0, 0.3]
f_subh_am	0.5% [0.2, 0.9]	subh_f_am_c	0.1% [0, 0.3]
bi_subh_c	0.5% [0.2, 0.9]	bi_am_c	0.1% [0, 0.3]
c_am_subh	0.4% [0.2, 0.9]	bi_f_subh_c	0.1% [0, 0.3]
subh_c_f	0.4% [0.2, 0.8]	c_am_bi	0.1% [0, 0.3]
bi_f_c	0.4% [0.2, 0.8]	bi_subh_am	0.1% [0, 0.3]
c_subh_am	0.4% [0.1, 0.7]	am_subh_f_c	0.1% [0, 0.3]
bi_f_subh	0.4% [0.1, 0.7]	subh_f_bi_c	0.1% [0, 0.3]
f_am_subh	0.3% [0.1, 0.7]	f_c_subh_am	0.1% [0, 0.3]
c_am_f	0.3% [0.1, 0.7]	f_bi_c	0.1% [0, 0.3]
am_c_subh	0.3% [0.1, 0.6]	bi_am_c_f	0.1% [0, 0.3]
subh_bi	0.3% [0.1, 0.6]	subh_f_c_am	0.1% [0, 0.3]
am_c_f	0.3% [0.1, 0.6]	c_am_bi_f	0.1% [0, 0.3]

Labels: f = frequency jumps; subh= subharmonics; c= chaos; am= amplitude modulation; bi= biphonation. Labels are given the order of NLP occurrence. For instance, c\_subh means that a combination is characterised by chaos followed by subharmonics.

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# **Chapter 3: Nonlinear vocal phenomena affect human perceptions of distress, size and dominance in puppy whines**



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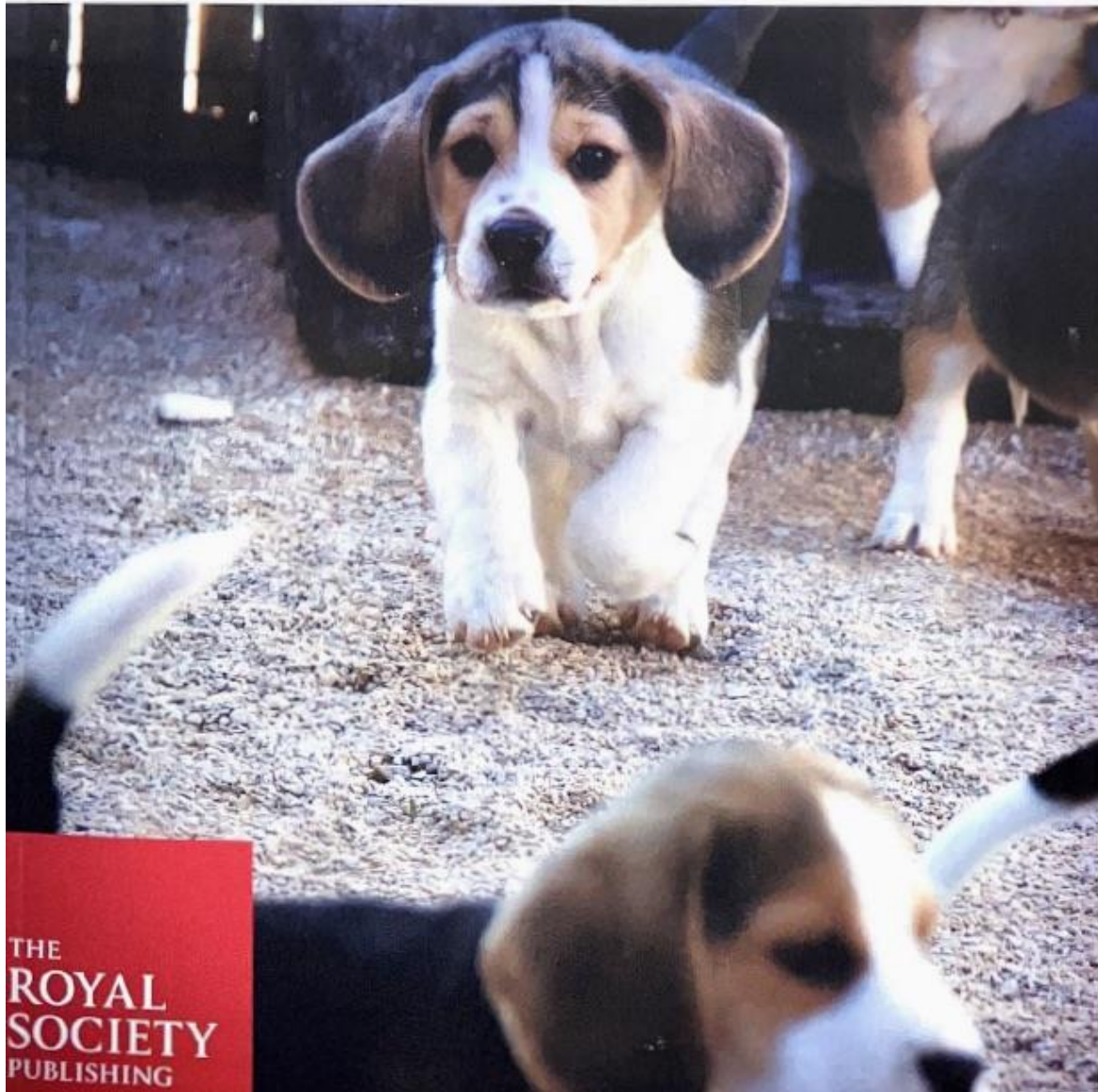
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# Nonlinear vocal phenomena affect human perceptions of distress, size and dominance in puppy whines

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While nonlinear phenomena (NLP) are widely reported in animal vocalizations, often causing perceptual harshness and roughness, their communicative function remains debated. Several hypotheses have been put forward: attention-grabbing, communication of distress, exaggeration of body size and dominance. Here, we use state-of-the-art sound synthesis to investigate how NLP affect the perception of puppy whines by human listeners. Listeners assessed the distress, size or dominance conveyed by synthetic puppy whines with manipulated NLP, including frequency jumps and varying proportions of subharmonics, sidebands and deterministic chaos. We found that the presence of chaos increased the puppy's perceived level of distress and that this effect held across a range of representative fundamental frequency ( $f_0$ ) levels. Adding sidebands and subharmonics also increased perceived distress among listeners who have extensive caregiving experience with pre-weaned puppies (e.g. breeders, veterinarians). Finally, we found that whines with added chaos, subharmonics or sidebands were associated with larger and more dominant puppies, although these biases were attenuated in experienced caregivers. Together, our results show that nonlinear phenomena in puppy whines can convey rich information to human listeners and therefore may be crucial for offspring survival during breeding of a domesticated species.

## 1. Introduction

There is now widespread evidence that vertebrate vocal signals communicate both static information (e.g. relatively stable physical and social attributes) and dynamic information (e.g. transient emotional and motivational states) about the animals that produce them [1,2]. Investigating how these signals are emitted, and how mechanisms of vocal production determine their acoustic structure, has enabled researchers to better understand their evolution and functions [2].

In mammals, the production of vocalizations is initiated in the larynx by vocal fold vibration [3]. The rate at which the vocal folds vibrate determines a key feature of vocal signals, their fundamental frequency ( $f_0$ ), which is largely responsible for their perceived pitch [3]. While vocal fold vibration is typically regular, perturbations can occur, causing abrupt changes in the tonal structure of acoustic signals [4,5]. This results in acoustic irregularities known as nonlinear phenomena (hereafter NLP), which are widely reported in the

vocalizations of terrestrial mammals including koalas (*Phascolarctos cinereus*) [6], marmots (*Marmota marmota*) [7], chimpanzees (*Pan troglodytes*) [8], meerkats (*Suricata suricatta*) [9], giant pandas (*Ailuropoda melanoleuca*) [10], dogs (*Canis lupus familiaris*) [11,12], North American elk (*Cervus canadensis*) [13], cows (*Bos taurus*) [14], African elephants (*Loxodonta Africana*) [15] and humans (*Homo sapiens*) [16,17]. Generally, the presence of NLP in vocalizations is responsible for perceived harshness, roughness and instability [18].

Four main types of NLP in animal vocalizations are frequency jumps, subharmonics, sidebands and deterministic chaos. Frequency jumps correspond to sudden changes in vocal fold vibration [19]. Subharmonics can appear when one vocal fold vibrates at a period corresponding to an integer fractional value of the second vocal fold (e.g. 1:2, 2:3) [20], while sidebands (resulting from biphonation) are produced by the vibration of two independent sound sources (e.g. vocal folds vibrating at two independent frequencies) [4]. Finally, deterministic chaos (hereafter chaos) usually occurs when vocal folds vibrate in asynchronous nonperiodic regimes [5]. Because of their ubiquity in mammal calls, a consensus is emerging that NLP are not merely by-products of vocal production but rather function to communicate information about the caller [4,5]. However, it remains unclear what kind of information NLP transmit.

It has been suggested that NLP are vocal indicators of high arousal [7,11,15,21] in contexts associated with distress (e.g. during separation from conspecifics or immediate risk of predation) [22]. Specifically, chaos represents the most severe and perceptually harsh form of vocal nonlinearity [18] characterizing the distress vocalizations of several mammal species [10,11,15,21–23], including humans [16,17]. For instance, perceptions of distress increase with the presence of NLP in nonverbal vocalisations of human infants [16] and adults [17]. This has led to the suggestion that a possible biological function of NLP may be to reduce habituation in receivers by making these calls unpredictable and thus difficult to ignore [9,24–26].

Aside from NLP,  $f_0$  (perceived as pitch) and formant frequencies (vocal tract resonances that influence perceived timbre) also affect the perceptual quality of vocalizations. In many terrestrial mammals, formants reliably predict inter-individual differences in body size, whereas  $f_0$  is usually a better predictor of dominance status [27]. Indeed, animals in aggressive motivational states commonly produce harsh low-pitched calls [28] to potentially signal and/or exaggerate their size and dominance [2]. These signals can therefore play a key role in determining the outcome of agonistic interactions, for instance during sexual competition or territorial defence [27]. In this context, another hypothesis regarding the potential functions of NLP may be to exaggerate perceived size and dominance [4,5]. Some NLP can indeed lower perceived pitch, enhancing the perceptibility of vocal tract resonances by increasing spectral density, and potentially, may affect perceptions of the vocalizer's size and dominance [4,18]. Together, several hypotheses have thus been put forward regarding the functions of NLP: attention grabbing, communication of distress, and/or size or dominance exaggeration.

Previous investigations of the perceptual effects of NLP in nonhuman animal vocalizations have focused on a single type (e.g. chaos in lizards, *Liolaemus chiliensis*: [29]; chaos in marmots [24]; subharmonics in meerkats [9]; chaos in red deer, *Cervus elaphus* [26]). Past studies also typically relied

on NLP variation in natural vocal sounds (e.g. frogs, *Odoranna tormota* [30]; meerkats [9,25]; marmots [24]; red deer [26]), and hence could not control for potentially covarying acoustic parameters (e.g.  $f_0$ , intensity). It thus remains crucial to understand the biological function(s) of each type of NLP by testing their independent perceptual effects across different species, call types, and behavioural contexts. Yet, until recently, a major limitation in the experimental study of NLP has been the absence of tools to synthesise naturalistic sounds with controlled levels of NLP.

Here, we take advantage of recent advances in parametric sound synthesis, thus far used only on human nonverbal vocalizations [18,31,32], to systematically add NLP to synthetic, yet highly realistic, nonhuman animal vocalizations. More specifically, we applied this method to investigate how the presence and duration of NLP in distress whines, produced by 1- to 9-week-old domestic dog puppies, affect their perception by humans. Whines are high-pitched tonal calls considered to signal distress, typically given by puppies during separation [33] or aggression by their mother [34]. Like adult dog whines [11,12], puppy whines also often contain NLP (see electronic supplementary material). Although puppies' survival mainly relies on mothers, who intensively breastfeed, groom and warm them [34], the intervention of human caregivers is sometimes required for breeding in this domesticated species [35]. As such, NLP in whines may be important to attract the attention of the mother, but also of humans, ensuring that puppies receive care. Indeed, the common presence of NLP across vertebrate infant distress calls suggests that these shared vocal features may support the interspecific vocal communication of need to human caregivers [22]. Therefore, to investigate the possible function of NLP as vocal indicators of distress in puppy-human interactions, we performed playback experiments using synthetic puppy whines in which we manipulated the presence, and duration, of NLP (frequency jumps, subharmonics, sidebands, chaos) across  $f_0$  levels (low, medium, high). We predicted that NLP added to whines, particularly chaos, would increase human perceptions of the puppy's distress, and that higher proportions of NLP would enhance these perceptual effects. Moreover, because NLP affect perceptions of size and dominance in synthetic human vocalizations [18,32], we tested whether these perceptual effects generalize to other mammal vocalizations. Indeed, dominance-like behaviours have been described as a trait of individual temperament in dogs [36,37], and can occur during the early development of pre-weaned canids, including puppies (e.g. during play with littermates) [36,38]. We thus predicted that NLP would increase perceived size and dominance. Finally, under the assumption that the interspecific function of infant distress vocal signals may be particularly important in caregiving contexts (e.g. for breeders and veterinarians), we replicated our experiment on a distinct sample of professionals with extensive experience in puppy caregiving to test whether such experience modulates the perceived effects of NLP.

## 2. Material and methods

Our experimental protocol included (1) audio recording natural distressed puppy whines, (2) creating synthetic whine stimuli based on these recordings of natural vocalizations, and (3)

**Table 1.** Overview of the experimental design used for playback experiments.

		playback experiment 1 ( $n = 206$ ) and experiment 3 ( $n = 55$ ) <sup>a</sup>	playback experiment 2 ( $n = 221$ )
total number of original synthetic prototypes		20	40
manipulations	types of NLP <sup>b</sup>	none, frequency jumps, subharmonics, sidebands, chaos	none, subharmonics, sidebands, chaos
	duration of NLP (% of whine duration)	20–40% <sup>c</sup>	50% or 80%
	levels of $f_0$	low, medium, high	low, medium, high
total number of synthetic stimuli		300	840

<sup>a</sup>Experiments 1 and 3 shared an identical design and identical whine stimuli.

<sup>b</sup>Level of manipulation 'none' indicates vocal stimuli without NLP.

<sup>c</sup>Values drawn at random within this range.

online playback experiments in which we respectively tested 206 and 221 human listeners from the general population (experiments 1 and 2), and 55 listeners selected for their extensive experience in puppy caregiving (experiment 3). All participants judged the perceived level of distress, body size, and dominance conveyed by each synthesized puppy whine. While the first and third playback experiments tested the perceptual effects of the presence or absence of NLP, the second experiment also tested the perceptual effects of their duration (table 1).

### (a) Acoustic recordings

We recorded whines of 32 beagle puppies from 6 different litters at roughly 1, 3, 6 and 9 weeks of age (electronic supplementary material, table S1) at two different breeding facilities (France). We used a Sennheiser MKH70 directional microphone connected to an audio recorder Zoom H4n (44.1 kHz, 24 bit) and positioned at approximately 30 cm from the animal's mouth.

Mothers and their litter stayed in the same enclosure during the nine first weeks of the puppies' lives. To record natural whines, each puppy was placed alone in a pen in a separate room (roughly 100 × 50 cm). Each puppy was thus recorded in a separation behavioural context, without visual and vocal contact with its mother and littermates. This method of recording distress vocalizations has been widely applied in vertebrates [22]. Our recordings started once the puppy had produced its first whine and did not last for more than 5 min to limit stress potentially induced by our experimental conditions. At the breeding facilities, bitches are habituated to being temporarily separated from their litter (e.g. during daily walks) and accustomed to breeders manipulating puppies during daily care (e.g. weight measurements evaluating physical condition of puppies). It was thus unlikely that we induced stressful behaviours in mothers while recording their puppies. In addition, when bringing the puppy back to its mother, the observation of normal maternal behaviours such as grooming or breastfeeding [34] indicated low stress levels.

### (b) Stimulus creation

From this pool of acoustic recordings of natural puppy vocalizations, we used 10 whine sequences at each age category (i.e. 1, 3, 6 and 9 weeks old, 40 sequences in total) from 26 different individuals as templates, to create their associated fully synthetic copy (hereafter synthetic prototype). We choose whine sequences composed of three repeated whines without NLP to facilitate their synthesis with the R package *soundgen* [31].

For each synthetic prototype, we prepared sets of vocalizations with systematic manipulations of NLP and  $f_0$  (table 1) for playback experiments. Specifically, we created synthetic whine stimuli at low, medium, and high levels of  $f_0$ , with or without different types of NLP added at each  $f_0$  level, based on those that naturally occur in puppy whines, including frequency jumps or varying proportions of subharmonics, sidebands, and chaos (figure 1). Manipulations of NLP and  $f_0$  were applied to the three repeated whines composing the entire synthetic prototype.

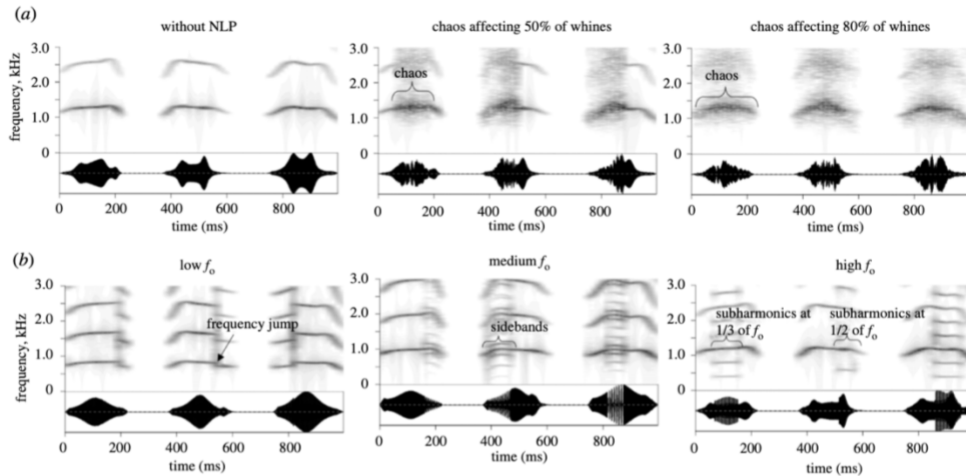
To inform these manipulations, we first performed detailed acoustic analyses on our recorded whines to characterize the natural vocal variation in puppy whines (see electronic supplementary material). We manually measured onset time and proportion of NLP as well as the  $f_0$  of 4841 natural whines containing NLP. This allowed us to create highly realistic variants that remained within the range of natural production, thus increasing the ecological validity of our experimental method and reliability of our results.

In total, there were 300 stimuli in experiments 1 and 3: 20 prototypes × 5 NLP conditions (none, frequency jump, subharmonics, chaos, sidebands) × 3  $f_0$  levels (low, medium, high). Experiment 2 included 840 stimuli: 40 prototypes × 7 conditions of NLP duration (none + 50% or 80% of the whine duration affected by subharmonics, sidebands, chaos = 1 + 2 × 3 = 7) × 3  $f_0$  levels (table 1). All audio stimuli and R code for their synthesis are available for download at <https://osf.io/tp7m8/>.

#### (i) Manipulations of nonlinear phenomena

Experiments 1 and 3 tested perceptual effects of the presence or absence of NLP in puppy whines. We manually coded frequency jumps (figure 1b) as instantaneous changes in  $f_0$  occurring at the maximum  $f_0$  slope. Indeed, as the production of NLP is typically associated with increases in subglottal pressure, leading to a relatively high  $f_0$  and amplitude, and ultimately, to an unstable vocal system [3,39], NLP are expected to occur in sections of calls where  $f_0$  is relatively high [40]. We also added sidebands, subharmonics, and chaos to whines by randomly selecting the onset time from the observed natural distribution. Durations of these types of NLP were selected at random within an interval corresponding to the most commonly produced NLP durations in natural whines, i.e. 20% to 40% of the whine's duration (electronic supplementary material, figure S1).

Experiment 2 tested perceptual effects of NLP duration. Here, the duration of NLP was fixed at either 50% (hereafter medium proportion) or 80% (hereafter high proportion) of the variant's duration (figure 1a), both of which remained within the natural



**Figure 1.** Spectrograms of variants of the same whine prototype synthesized with or without NLP (frequency jumps, subharmonics, sidebands, chaos) at different levels of (a) NLP duration (NLP affecting none, 50% or 80% of the whine's duration, as illustrated with the addition of chaos) and (b)  $f_0$  (low, medium, high). While every NLP manipulation was tested at every  $f_0$  level, here we show a range of NLP manipulations at each  $f_0$  level as examples.

range (electronic supplementary material, figure S1). The onset time of NLP was also randomly selected from the observed natural distribution, but under the constraint that the onset time should be within a specific interval (medium proportion: [0; 50] % of the whine duration, high proportion: [0; 20] % of the duration).

We synthesized subharmonics, sidebands, and chaos using a method allowing for natural variability. Specifically, to synthesize subharmonics, the integer ratio of subharmonic frequencies to  $f_0$  was drawn from a truncated Poisson observed distribution (mean = 1 and range = [2, 5]), typically resulting in additional spectral components (subharmonics) at  $f_0/2$  or  $f_0/3$ . We created sidebands by adding amplitude modulation, the frequency of which was selected from a Gaussian distribution (mean  $\pm$  s.d. =  $170 \pm 20$  Hz) characterizing sidebands in natural puppy whines. The amplitude of subharmonics and sidebands, expressed as the proportion of the amplitude of  $f_0$ , was randomly selected from a Gaussian distribution with a mean  $\pm$  s.d. of  $50\% \pm 10\%$  [3] and  $80\% \pm 10\%$ , respectively. Finally, we created chaos by adding jitter (i.e. changes in  $f_0$  periodicity) with values of  $3 \pm 0.6$  semitones in experiments 1 and 3, and  $1.2 \pm 0.3$  semitones in experiment 2, such that chaotic episodes sounded natural.

### (ii) Manipulations of $f_0$

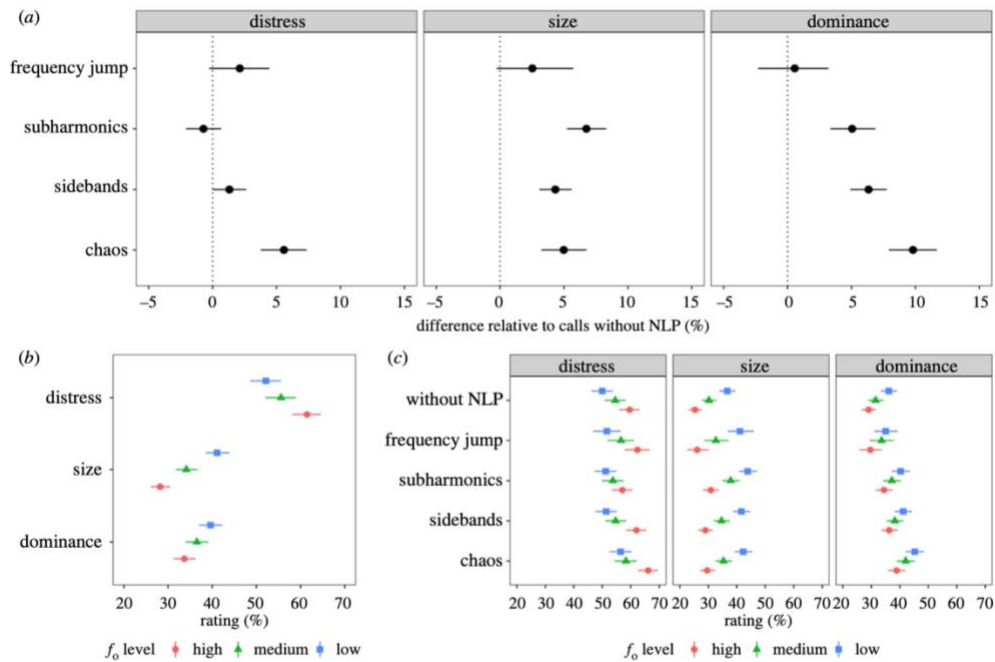
Because variations in  $f_0$  can affect perceptions of distress [41–44], size [45,46] and dominance [47], we also tested whether perceptual effects of NLP vary across  $f_0$  levels. From our  $f_0$  measurements of natural whines containing NLP, we identified three main  $f_0$  categories that roughly correspond to quartiles of the natural distribution with a mean  $\pm$  s.d. of  $790 \pm 27$  Hz (low  $f_0$ ),  $972 \pm 27$  Hz (medium  $f_0$ ), and  $1170 \pm 27$  Hz (high  $f_0$ ), hereafter termed target region. We thus synthesized stimuli at each  $f_0$  level by first randomly selecting a target  $f_0$  value from each target region. This allowed for natural variability in  $f_0$  across variants within each target region. Second, we calculated a coefficient of correction as the ratio between the target  $f_0$  value and average  $f_0$  across whines in the original synthetic prototype. Third, the  $f_0$  of the prototype was corrected by this coefficient, such that the mean  $f_0$  of the new variant was equal to the target  $f_0$  value.

### (c) Playback experiments with human listeners

We performed online playback experiments on 482 human adult listeners ( $n = 206$  in experiment 1;  $n = 221$  in experiment 2;  $n = 55$  in experiment 3). Before each experiment, participants completed a short questionnaire indicating their sex, age, profession, level of comfort with puppies and experience with puppy-ownership. They were also informed that they would hear distressed puppy whines recorded during separation from mothers and littermates. Because the term 'puppy' can refer to both pre- and post-weaned dogs, we defined a puppy as a dog younger than 3 months of age in our online questionnaire. Participants listened to three natural whine sequences containing NLP to familiarize themselves with nonlinear puppy whines and to adjust their sound level to a comfortable volume, which was then kept constant for the experiment. We asked participants to use headphones when completing the experiment in a quiet environment.

Playback experiments comprised three blocks testing perceived distress, size or dominance. The order of blocks and stimuli within blocks were randomized. Each block contained 20 (experiments 1 and 3) or 40 (experiment 2) whine stimuli, such that each prototype was tested once per block with a randomly chosen level of  $f_0$  and NLP condition for a given participant who listened to a total of 60 stimuli (experiments 1 and 3) or 120 stimuli (experiment 2) (table 1). Following each whine exposure, participants rated the whine by indicating how distressed/dominant/large the puppy sounds, using a continuous sliding scale ranging from 0 (not at all distressed/dominant or extremely small) to 100 (extremely distressed/dominant/large). Participants could listen to stimuli several times and could adjust their rating if needed.

In experiments 1 and 2, we respectively excluded 6 and 4 participants who completed less than 80% of the experiment or otherwise failed to engage with the study. No participant from experiment 3 was excluded. Statistical analyses were thus performed on 472 participants ( $n = 200$  in experiment 1;  $n = 217$  in experiment 2;  $n = 55$  in experiment 3), aged  $28 \pm 11$  years old (mean  $\pm$  s.d.) (range [18–74]), of whom 40% self-reported as female, 60% as male, and less than 1% as 'other'. Nearly all participants in experiments 1 and 2 (409/417) did not professionally work with puppies but felt comfortable in their presence (381/417), and 65% had owned a puppy within the past 5 years. In



**Figure 2.** Main effects of (a) NLP, (b)  $f_0$  and (c) interactions between NLP and  $f_0$  on perceived distress, size, and dominance ( $n = 417$ , experiments 1 and 2). Solid markers correspond to medians of posterior distribution with their 95% credible intervals. (Online version in colour.)

experiment 3, we specifically selected participants for their extensive experience in puppy caregiving, and thus their regular exposure to whines. Most of these participants professionally worked with puppies, as out of 55, 40 were dog breeders, 12 were veterinarians and 2 were dog trainers.

#### (d) Data analysis

Data from playback experiments were analysed using Bayesian multilevel models fitted with the *brms* R package [48] using default priors. The response variable of models corresponded to ratings re-coded from 0 to 1 and modelled with zero-one-inflated beta distribution [49]. To investigate whether the presence of NLP affected human perceptions of puppy whines and whether these effects of NLP varied with whine  $f_0$ , we first fitted a model including data from experiments 1 and 2, testing the general population ( $n = 417$  participants). This model included a three-way interaction between the tested perceptual scales (3 levels), NLP condition (5 levels) and  $f_0$  (3 levels). We allowed effects of NLP on each scale to vary across participants and prototypes by fitting the interaction between NLP and scale as random slopes for all participants and prototypes. We also assumed that each stimulus would have its unique rating on each scale. This model resulted in the following *brms* syntax:

$$\text{Rating response} \sim \text{scale} \times \text{NLP} \times f_0 + (\text{NLP} \times \text{scale} | \text{participant}) + (\text{NLP} \times \text{scale} | \text{prototype}) + (\text{scale} | \text{stimulus}).$$

To examine whether experience in puppy caregiving influences the effects of NLP, we fit an analogous model to the data collected from experienced caregivers ( $n = 55$  participants in experiment 3).

Finally, we tested whether the duration of NLP reinforced their perceptual effects by fitting a second model with data

from experiment 2 ( $n = 217$  participants). This model included a two-way interaction between rating scales and duration of NLP (7 levels). The model followed this *brms* syntax:

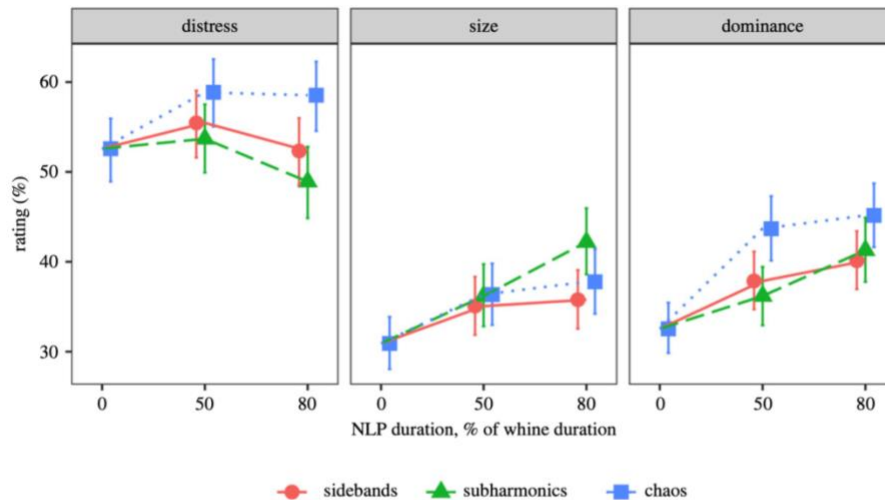
$$\text{Rating response} \sim \text{scale} \times \text{duration} + (\text{scale} \times \text{duration} | \text{participant}) + (\text{scale} \times \text{duration} | \text{prototype}) + (\text{scale} | \text{stimulus}).$$

In this model, we captured an assumed variability of predictors *scale* and *duration* across participants and prototypes, and we allowed for ratings of each scale to vary across each unique stimulus. Posterior distributions of model parameters were summarized by their median and 95% credible interval.

### 3. Results

We predicted that the presence and duration of NLP in puppy whines would increase perceptions of distress, size, and dominance by human listeners. With the exception of frequency jumps, all NLP affected listeners' ratings on one or more of these perceptual scales (figures 2a and 3; electronic supplementary material, figure S2a).

Specifically, in the general population (experiments 1 and 2), we found that perceptions of distress were primarily affected by chaos, which increased distress ratings by 5.6% [3.8, 7.4] compared to identical whines without NLP. By contrast, distress ratings were not noticeably affected by the addition of subharmonics ( $-0.7\%$  [ $-2.1, 0.7$ ]) nor sidebands (1.3% [0, 2.6]). Experienced puppy caregivers (experiment 3) also rated whines with chaos as more distressed-sounding, but unlike the general population, they furthermore associated sidebands and subharmonics with higher distress (see



**Figure 3.** Effects of NLP duration on perceived distress, size and dominance in experiment 2 ( $n = 217$ ). A duration of zero corresponds to whines without NLP. Solid markers correspond to medians of posterior distribution with their 95% credible intervals. (Online version in colour.)

electronic supplementary material for details, including figure S2a).

Moreover, we predicted that longer episodes of subharmonics, sidebands and chaos in puppy whines would increase their perceptual salience and thus, enhance their effects on perceived distress. Surprisingly, we found that increasing the duration of chaos from 50% to 80% of the whine's duration did not further increase perceived distress ( $-0.4\%$  [ $-3.0, 2.3$ ]), and that increasing the duration of subharmonics and sidebands reduced perceived distress by 4.8% [2.2, 7.5] and 3.1% [0.8, 5.4], respectively (figure 3).

While effects of NLP were relatively stable across each  $f_o$  level, our results confirm an independent effect of  $f_o$  on distress ratings in participants for all three experiments (figure 2b,c; electronic supplementary material, figure S2b,c). Indeed, puppies producing whines with higher levels of  $f_o$  were rated as more distressed (9.3% [8.1, 10.6] in participants of experiments 1 and 2; 8.3% [5, 11.6] in participants of experiment 3), compared to the same whines synthesized at lower  $f_o$  levels (figure 2b; electronic supplementary material, figure S2b).

In addition to effects on perceived distress, our results show that the general population (experiments 1 and 2) rated size and dominance about 4% to 6% higher in whines containing subharmonics, sidebands, or chaos, compared to whines without NLP (figure 2a). However, these effects were absent or limited in experienced puppy caregivers (electronic supplementary material, figure S2a).

We also predicted that increasing the duration of subharmonics, sidebands, or chaos from 50% to 80% of the whines' durations would reinforce their effects on perceived size and dominance. Our results indeed show that whines containing 80% of subharmonics (but not chaos and sidebands) increased perceived size by 6% [3.0, 8.9] and perceived dominance by 5.1% [2.3, 7.7] compared to the same whines with only 50% of these NLP (figure 3).

Finally, we found no effect of the interaction between NLP and  $f_o$  levels in the general population (figure 2c), instead

confirming an independent effect of  $f_o$  on perceived size and dominance (figure 2b): whines with a lower  $f_o$  were systematically associated with larger (12.9% [14.1, 11.7]) and more dominant (5.9% [4.8, 7]) sounding puppies, relative to the same whines synthesized at higher levels of  $f_o$ . Similar results were found for experienced puppy caregivers (electronic supplementary material, figure S2b,c).

## 4. Discussion

We investigated the independent perceptual effects of non-linear phenomena (NLP) that occur naturally in a non-human animal vocalization, namely in distressed puppy whines. By applying an innovative method of parametric sound synthesis in the context of interspecific vocal communication, we show that the presence and duration of different NLP types in puppy whines generally increase their perceived distress, size or dominance, as judged by human listeners.

### (a) Deterministic chaos increases perceptions of distress

We found that the presence of chaos in puppy whines, but not the addition of frequency jumps, sidebands, or subharmonics, increased perceptions of distress in human listeners representing the general population. From a biomechanical perspective, the production of NLP, particularly chaos, is typically associated with high subglottal pressure and strong muscle tension on vocal folds [39]—configurations of the vocal apparatus that are likely to arise in distressed animals with highly aroused physiological states [1]. Chaos is indeed a characteristic of calls associated with distress in a wide range of mammals [10,15,16,21,23] (this study). Additionally, at the perceptual level, Blumstein & Récapet [24] have shown that white noise (which shares some perceptual elements with deterministic chaos [50]) added to natural alarm calls of marmots induces potentially costly responses in these animals (reduced foraging activity). An earlier study also showed that chaos added to

human nonverbal vocalizations increases the intensity of perceived negative, rather than positive, affective states [51]. Together, our findings thus support that chaos is perceived as a vocal indicator of higher levels of distress in mammals.

As increases in subglottal pressure, characterised by high amplitude and  $f_{ov}$ , often lead to vocal instability [3,39], NLP are expected to occur when  $f_o$  is relatively high. For instance, the onset of NLP corresponds with the maximum  $f_o$  in chimpanzees' calls [40]. As such, it is difficult to disentangle the effect of  $f_o$  from the effect of NLP when performing playback experiments using natural calls [9,24,25], as these acoustic parameters may covary. To overcome this limitation, we used synthetic stimuli varying in the presence and duration of NLP at different  $f_o$  levels. While we confirmed that perceived distress increases independently with  $f_{ov}$  as reported in numerous previous studies [41–44], the effect of NLP on the perception of distress was relatively stable across all  $f_o$  levels. This suggests that perceptual effects of  $f_o$  and NLP are independent and cumulative, and that both are salient vocal indicators of distress.

Contrary to our prediction, whines containing relatively longer episodes of chaos did not increase perceived distress compared to whines with less chaos, suggesting that a ceiling effect may have been attained with chaos affecting only half of the call duration. Indeed, listeners may have applied a categorical decision (no chaos = relatively low distress, chaos = relatively high distress) when exposed to whines with or without chaos. Moreover, for sidebands and subharmonics (whose presence did not affect perceived distress in the general population), we found that perceived distress was lower in variants containing longer episodes of these NLP. We suggest that bifurcations between the tonal and non-tonal sections of the calls may be less salient in calls with a largely dominant nonlinear regime. In a recent study, we showed that subharmonics and sidebands added to human nonverbal vocalizations lowered their perceived pitch [18]. As such, when exposed to subharmonics or sidebands affecting 80% of the whine duration, listeners may have perceived these calls with a relatively high tonality at a lower  $f_o$  and may thus have associated these lower-pitched sounds with behavioural contexts of aggression, rather than distress. This would follow Morton's motivation-structural rules stating that aggressive animals typically produce sounds with a low  $f_{ov}$ , whereas distressed, fearful animals produce sounds with a high  $f_o$  [28].

### (b) Nonlinearities increase perceptions of size and dominance

Experimentally adding subharmonics, sidebands and chaos—but not frequency jumps—to whines increased the puppy's perceived body size and dominance. Increasing the duration of subharmonics further increased perceived size and dominance. These results are consistent with recent work on human nonverbal vocalizations [18,32]. Our results also confirm the well-established perceptual effect of  $f_o$  on size and dominance [45–47]—whines with a relatively low  $f_o$  were systematically perceived as produced by larger and more dominant-sounding puppies, independent of the effects of NLP on perceived size and dominance. Although we show that  $f_o$  directly affects perceptions of size and dominance, we cannot completely rule out the possibility that NLP independently lower the perceived pitch of whines (as they do in

human vocalizations) [18]. This pitch-lowering effect may thus be one of the perceptual mechanisms whereby NLP cause puppies to sound larger and more dominant. This prediction could be directly tested in future work by simultaneously assessing the effect of NLP manipulations on auditory (pitch, timbre) and ecological (distress, dominance, etc.) dimensions.

The pitch-lowering effect of NLP has been hypothesized as a possible strategy for animals to exaggerate perceived size and dominance [4,5]. Indeed, frequency components such as formant frequencies and/or  $f_o$  mediate the vocal communication of size and dominance, which are crucial factors affecting the outcome of social interactions, such as mating, sexual competition, or territorial defence [2]. Many species have developed strategies to exaggerate their apparent size and, potentially, also their dominance (reviewed in [27] and [52]), with specific adaptations to their vocal apparatuses such as a descended and mobile larynx [53]. Because there is no clear biological function for a puppy to sound larger and more dominant to humans while producing a distress vocalization, we suggest here that the perceptual effects of NLP on size and dominance are likely to be the consequence of a general perceptual bias linking 'harsh' with 'large and dominant' [18].

### (c) Effect of experience on listener perceptions of puppy whines

While infant distress calls conveying need to parents are ubiquitous in vertebrates [22], in the context of domestication, these distress calls may also function to communicate need to heterospecific caregivers. Indeed, human baby pain cries are often characterised by very high levels of NLP [16] and human listeners pay attention to these acoustic features to assess a baby's level of distress and need [16]. As such, the production of whines, and within them the production of NLP may have been encouraged by dog domestication, with puppies exploiting human sensitivity to chaos in offspring calls. Breeders indeed do intervene and provide care to puppies [35] (e.g. in the case of mother neglect, aggression or rejection [34]). Consistent with this, when asked to characterize how they react when exposed to puppy whines in their normal professional activities, more than 50% of our experienced puppy caregivers reported being negatively affected by whines, paying attention to their harshness and roughness, and using these acoustic features as cues to distress in order to adjust the level of care they provide to the puppies. Interestingly, while experienced caregivers were as sensitive to chaos as the general population when rating perceived distress, they also associated subharmonics and sidebands with higher levels of distress (unlike the general population), as also observed in other species (e.g. subharmonics in meerkats [9]). Together these results suggest that human sensitivity to chaos, the most severe form of NLP, has a universal base when assessing distress in puppy whines, but that sensitivity to other NLP can also arise with extensive experience. To further investigate the possibility that domestication may have emphasized whining and NLP within puppy whines as a consequence of sensory exploitation of caregivers (or as a consequence of relaxed predation pressures [54]), future studies should contrast the production of NLP within the whines of domestic dog puppies and wolf pups (*Canis lupus*).

Finally, NLP had little effect on perceptions of size and dominance in experienced caregivers, suggesting that their extensive experience allows them to correct for the perceptual bias identified in our general population. Yet, experienced caregivers remained sensitive to whine  $f_0$  when assessing distress, size and dominance. This suggests that  $f_0$  may function as a reliable index of these traits in puppy vocalizations (as identified for distress and size in adult dog whines [55,56] and in the calls of other mammals [1,2]), a hypothesis that we are currently investigating.

#### (d) Perspectives

It remains to be established whether NLP signal distress information in puppy-mother vocal interactions, as they do to human listeners. Indeed, a recent study reports a positive relationship between the level of jitter (noisiness) in natural distress puppy whines and the strength of adult dogs' behavioural responses [57]. Furthermore, although whines are typically associated with distress contexts, the domestic dog's vocal repertoire also includes vocalization types that are produced in both positive (e.g. playful) and negative (e.g. agonistic) contexts, such as growls or barks [58,59]. In addition to signalling relatively stable physical attributes (e.g. size) [60], these vocalizations can vary acoustically between positive and negative behavioural contexts [56,61]. For example, high-pitched and tonal barks are produced in playful contexts, whereas low-pitched and harsh, chaotic barks are more commonly produced during territorial defence [56]. Nonlinear phenomena in barks or growls may thus function to communicate aggressive intent (rather than play) and, in such an agonistic context, may also function to exaggerate size and dominance. This prediction may be tested in the contexts of both inter- and intra-specific vocal

communication. We thus suggest that using methods of parametric sound synthesis, as reported here, could be generalized to a wider range of species and contexts to directly test the communicative function of NLP in animal vocal communication.

**Ethics.** Acoustic recordings of puppies were performed under the ethical approval no. E-42-218-0901 (ENES lab agreement, Direction Départementale de la Protection des Populations, Préfecture du Rhône). All human participants provided informed consent, and ethical approval for performing perceptual experiments with human subjects was provided by the Comité d'Éthique du CHU de Saint-Etienne (IRBN692019/CHUSTE).

**Data accessibility.** All audio stimuli, R codes for synthesizing stimuli, html code for running psychoacoustic experiments, datasets as well as R codes for analysing data can be downloaded from <https://osf.io/rp7m8/>.

The data are provided in electronic supplementary material [62].

**Authors' contributions.** M.M.: conceptualization, data curation, formal analysis, methodology, writing—original draft; A.A.: conceptualization, formal analysis, methodology, writing—review and editing; K.P.: conceptualization, methodology, writing—review and editing; K.R.: conceptualization, methodology, writing—review and editing; N.M.: conceptualization, methodology, writing—review and editing; D.R.: conceptualization, methodology, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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## Electronic Supplementary Materials

# Nonlinear vocal phenomena affect human perceptions of distress, size and dominance in puppy whines

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### Acoustic analysis of nonlinear puppy whines

We performed a detailed acoustic analysis in order to then synthesize and manipulate nonlinear phenomena (NLP) and fundamental frequency ( $f_0$ ) based on their natural production in puppies. This allowed us to create a large dataset, from which we selected values to create highly realistic synthetic whines at three  $f_0$  levels (i.e., low, medium, high levels within the natural puppy range), with or without NLP that typically occur in natural whines (i.e., frequency jumps, subharmonics, sidebands and chaos).

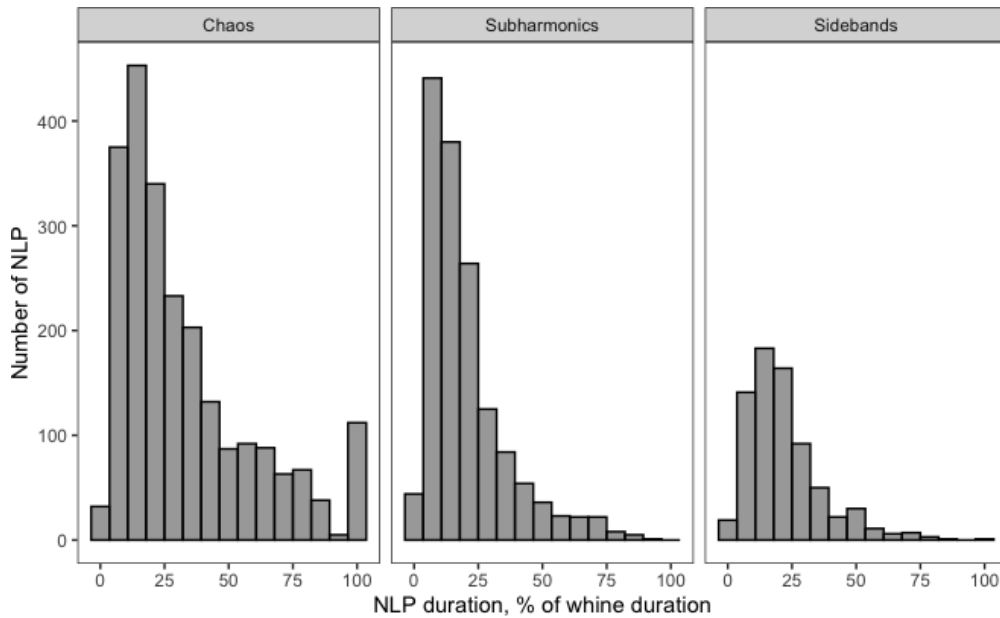
We analysed recordings of 17 different puppies that were randomly selected for each age class (i.e., 1-, 3-, 6-, 9-week-old) from our total pool of whine recordings (table S1), allowing us to create a balanced dataset across ages. In total, 68 recordings were thus acoustically analysed.

Litter	Age of puppies			
	1 week	3 weeks	6 weeks	9 weeks
#1			6	6
#2		5	4	4
#3	8	8	8	8
#4	4	4	4	4
#5		4	4	4
#6	5	5	5	
<b>Total number of individuals</b>	<b>17</b>	<b>26</b>	<b>31</b>	<b>26</b>

**Table S1:** Overview of acoustic recordings from puppies. Numbers indicate the litter size, and thus the total number of recorded puppies in each respective age class.

We visually inspected spectrograms of 13344 natural puppy whines in Praat [1] (Gaussian window, window length = 0.01 sec, dynamic range = 100 dB). Of these, 4841 whines contained one or several types of NLP. We manually annotated the onset time and duration of each episode of NLP within these calls. Chaos was the most common NLP type produced by puppies, as it occurred in 45% of whines that contained any NLP. Additionally, frequency jumps, subharmonics, and sidebands appeared in 37%, 29% and 14% of calls containing NLP, respectively. Because the duration of whines with NLP varied widely

(mean  $\pm$  SD = 315  $\pm$  165 ms, range [125, 1674]), we converted the absolute duration (ms) of chaos, subharmonics, and sidebands into a proportion of call duration (figure S1). The typical  $f_0$  contour of nonlinear whines was also measured by automatically extracting and averaging  $f_0$  contours using the `analyze()` function in the *soundgen* R package [2]. Finally, we performed additional acoustic measurements on 30 natural puppy whines containing subharmonics or sidebands (15 calls with subharmonics and 15 calls with sidebands). Specifically, we extracted values corresponding to the ratio of subharmonics to  $f_0$  (e.g.,  $f_0/2$ ,  $f_0/3$ ) and to the lower frequency component in sidebands.



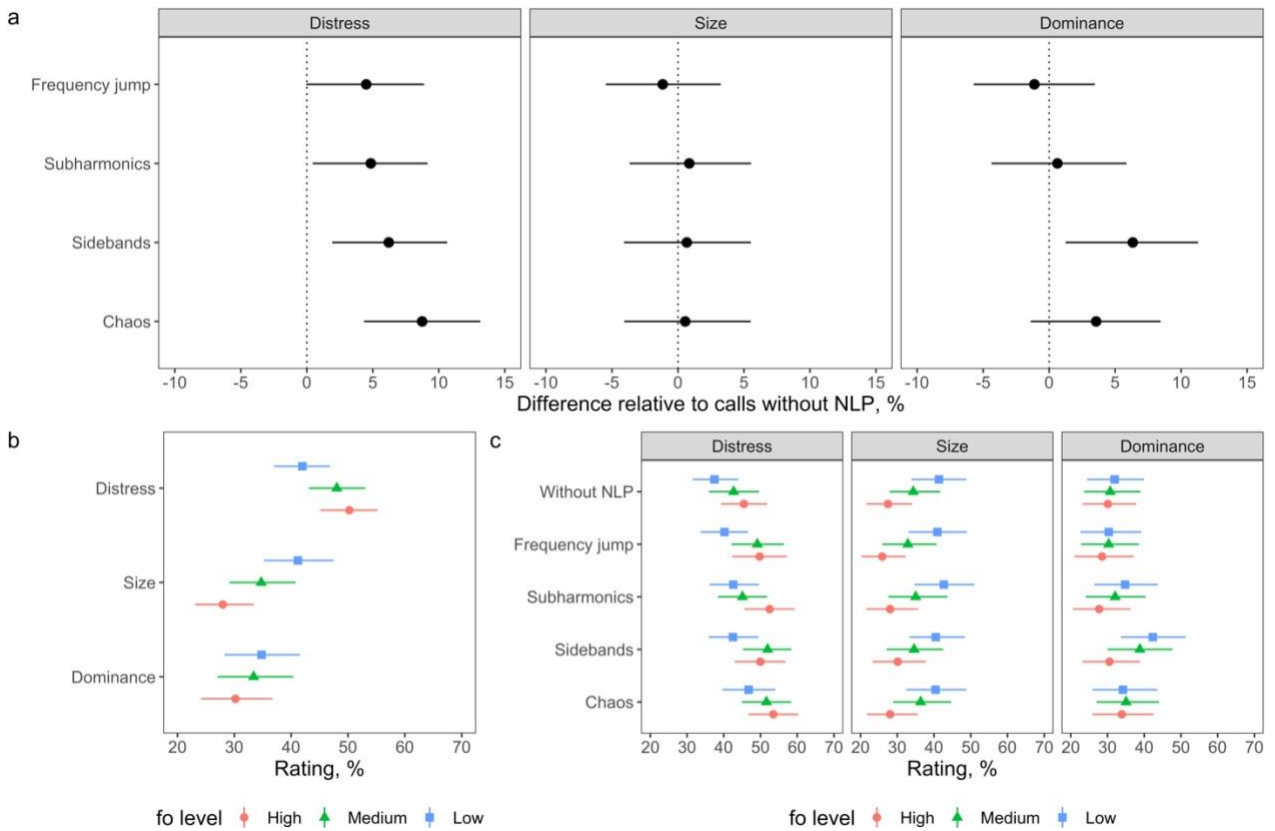
**Figure S1:** Natural distribution of the duration of chaos, subharmonics, and sidebands, expressed as the proportion of NLP affecting natural puppy whines.

### **Effects of NLP and $f_0$ on perceptions of distress, size, and dominance by experienced puppy caregivers**

In listeners with extensive experience in puppy caregiving (hereafter, experienced caregivers, such as dog breeders and veterinarians), we found that the presence of chaos in puppy whines increased perceptions of distress by 8.7% [4.3, 13.1] (median [95% CI]) compared to the same whines without NLP (figure S2a). Frequency jumps (4.5% [0, 8.9]) had no noticeable effects, and sidebands (6.2% [1.9, 10.6]) and subharmonics (4.8% [0.4, 9.1]) had mild effects on perceived distress. We also found no effect or mild effects of NLP

on perceived size and perceived distress (figure S2a).

Finally, our results confirm the absence of an interaction between NLP and  $f_0$  on perceived distress, size, or dominance in experienced caregivers (figure S2c), and confirm a main, independent effect of whine  $f_0$  on perceptions of all three dimensions (figure S2b). Whines with a lower  $f_0$  were systematically rated as produced by less distressed (8.3% [5, 11.6]), larger (13.2% [10, 16.4]), and more dominant (4.5% [1.4, 7.7]) puppies, compared to whines with a relatively lower  $f_0$ .



**Figure S2:** Main effects of (a) NLP, (b)  $f_0$ , and (c), and the interaction between NLP and  $f_0$  on perceived distress, size, and dominance in human listeners with extensive experience in puppy caregiving, i.e., experienced caregivers such as dog breeders or veterinarians (N=55 participants from Experiments 3). Solid markers correspond to medians of posterior distribution with their 95% credible intervals.

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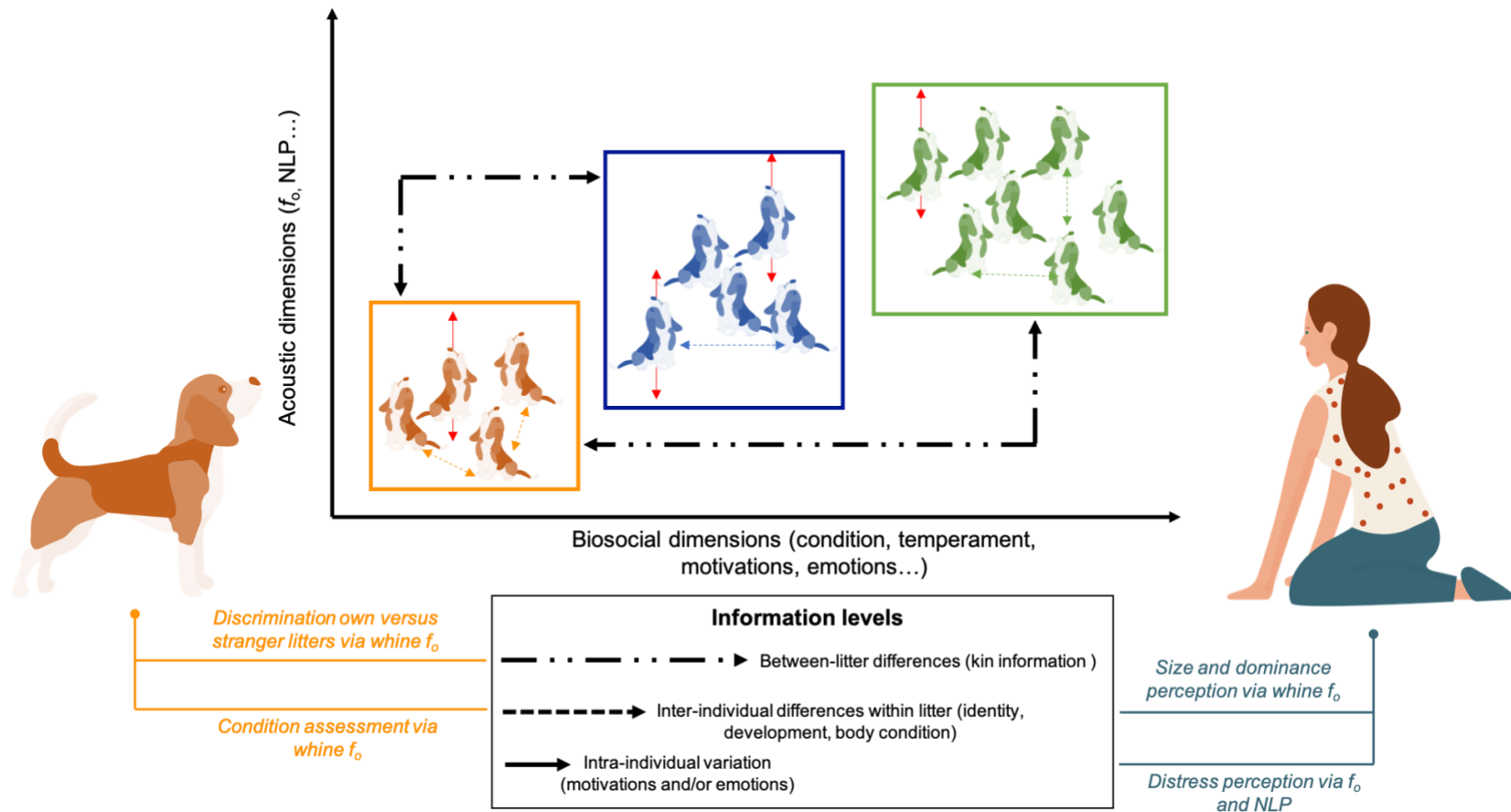
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# General discussion

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## Key findings



In this thesis, I show that fundamental frequency ( $f_0$ ) and nonlinear phenomena (NLP) are key features of puppy whines that simultaneously encode static and dynamic information at different levels (individual, litter, kin). I suggest that the communication of this multilevel information supports the provision of optimal care by mothers and human caregivers.



In vertebrates, rearing several offspring exposes parents to a problem for simultaneously assessing kin, individual identity, condition and short-term need. As such, litters constitute an excellent model for studying the, as of yet largely under-investigated, communication of multilevel information within offspring vocalisations. The aim of this thesis was to fill this gap, by investigating the production and perception of multilevel information in the offspring calls of the domestic dog, a large litter-rearing mammal. More specifically, I examined what vocal cues encode for static and dynamic information in puppy calls and tested their perception by dog mothers, and also human caregivers. I found that a unique call type, the puppy whine, can convey rich information to mothers and human caregivers, including litter, individual and/or emotional information. I suggest that this multilevel communication supports provision of optimal care to puppies and ultimately their survival. Below I discuss and connect the key findings of my thesis, while considering their wider implications and suggesting future research directions.

## **Communication of litter information**

In Chapter 1, I first demonstrated that puppy whines produced during separation from their mother were different between litters, and that  $f_0$  (affecting the perceived pitch) was a key contributor to these differences. I thus confirmed that  $f_0$  can be an important vocal cue for signalling litter (or brood) identity in the calls of young vertebrates (Monk, Koenig and Koenig, 1997; Illmann *et al.*, 2002; Briefer and McElligott, 2012). At 3-week-old, between-litter differences in whine  $f_0$  were mediated by puppy body weight variation (a proxy for body size and condition in vertebrates, Ronget *et al.*, 2018, and specifically domestic dog puppies, Dikmen, Elmaz and Askoy, 2008). Indeed, I found a positive relationship between average litter weight and whine  $f_0$ , where puppies from relatively heavier litters produced whines with a significantly higher  $f_0$  than puppies from relatively lighter litters (Chapter 1). Because theories of acoustic allometry predict that relatively larger animals should have bigger lung capacities (Bartlett and Areson, 1977), allowing them to increase subglottal pressure to levels needed for the production of relatively higher  $f_0$  calls (Titze, 1994), my results suggest that the relationship between litter body weight and litter  $f_0$  may be mediated by variation in lung capacity.

Due to genetic relatedness between puppies and their parents or littermates, this positive correlation may be genetically determined. Thus, I also tested whether parents' identity explained

variation in whine acoustics and in weight between litters sharing one or two parents. I found that fathers' identity predicted differences in litter weight and in whine  $f_0$  (as well as in duration and frequency modulations). The statistical power of my analyses was however too low to provide clear conclusions regarding the contribution of mother's identity on weight and whine acoustic variation between litters, as only small sets of two or three litters shared a same mother. Although these preliminary results suggest that "litter signatures" have a genetic component (as previously suggested in goat kids (*Capra hircus*): Briefer and McElligott, 2012), further analyses are need to confirm these observations, while also investigating whether the body weight of parents predicts litter whine  $f_0$ .

Because my findings show that each litter is characterised by a specific frequency range, I then investigated whether mothers use  $f_0$  to discriminate their litter by the means of playback experiments of resynthesized whines. I showed that mothers provided more care in response to their puppies' whines when these were resynthesized to fall within their litter-specific  $f_0$ , thus indicating that they depend on whine  $f_0$  to discriminate puppies as theirs at a litter level (Chapter 1). As such, these results provide evidence for reliable communication of litter identity (and overall body condition) via whine  $f_0$  in mother-puppies vocal interactions.

## **Communication of individual information**

### *Whine $f_0$ as a vocal cue to body condition in 3-week-old puppies*

To optimise the provision of parental care to multiple young, offspring recognition is expected to not only push for acoustic differentiation of offspring calls different between litters as shown above, but also, within groups, between individuals (within their litter). Beyond contributing to the acoustic distinctiveness of litters, I found that whine  $f_0$  also contributed to vocal differences between puppies within the same litter. Indeed, at 3 weeks, I found that differences in whines between littermates were also driven by weight variation, but that in contrast to the positive weight-effect across litters, body weight was negatively correlated to  $f_0$  within litters (Chapter 1). This suggests that acoustic allometry, whereby relatively larger animals are expected to have longer vocal folds and produce lower-pitched vocalisations (as reviewed in Taylor and Reby, 2010; Charlton *et al.*, 2020), can also explain inter-individual differences in call  $f_0$  – as was previously

determined in other young vertebrates including bird and mammal species producing single (Gladbach *et al.*, 2009; Wermke and Robb, 2010; Shinya *et al.*, 2014) or several offspring (Weary, Lawson and Thompson, 1996).

Together, my results thus show that, at 3 weeks old, differences in whine  $f_0$  between litters and littermates are driven by two opposing mechanisms, that may reflect variation in body condition (Fischer *et al.*, 2004; Taylor and Reby, 2010; Charlton *et al.*, 2020). I propose that effects of body weight between-litter and within-litter are respectively reflecting variation in the lung capacities and in the vocal fold size of puppies. As such, these mechanisms (lung capacities *versus* vocal fold size of individuals) using slightly different magnitudes of  $f_0$  variation, appear to support the simultaneous acoustic encoding of litter and individual information within a single call type. I suggest that this multilevel encoding functions to enable caregivers to assess kin and condition at both litter and individual levels in a species that rears multiple offspring.

I then moved on to testing the communicative function of these individual weight-related acoustic cues contained in whines. To do this, I conducted playback experiments exposing caregivers, i.e., dog mothers and humans to puppy whines and found that they use whine  $f_0$  to assess puppies' body condition (Chapters 1 and 3). Consistent with observations in birds (Caro *et al.*, 2016) or piglets (*Sus domesticus*) (Weary, Lawson and Thompson, 1996), I demonstrate that mothers provide significantly more maternal care in response to relatively high  $f_0$  whines indicating smaller/weaker puppies (Chapter 1). In addition, human listeners, including professional caregivers (e.g., dog breeders or veterinarians), also rated whines synthesized at higher  $f_0$  levels as smaller, needier sounding puppies compared to lower-pitched whines (Chapter 3). Together, my findings suggest that the communication of kin and/or body condition via whine  $f_0$  supports the provision of optimal care to puppies in contexts of inter and intra specific interactions. Future studies could aim at disentangling the mechanisms underlying vocal kin discrimination in a wider range of avian and mammal species that rear multiple offspring, in order to better understand the evolutive origins of such complex multilevel communication systems.

Finally, kin discrimination can also be mediated via olfaction in dogs (Hepper, 1994). As such, multimodal approaches could also be used to unravel the respective contributions of vocal and olfactory channels in mother-puppy interactions, as has been conclusively conducted in pinnipeds (Charrier, Pitcher and Harcourt, 2022).

*Whine  $f_0$  as a vocal cue to individual temperament but not testosterone in 9-week-old puppies*

Because the outcome of sibling competition can be predicted by offspring temperament (e.g., dominance) and/or testosterone levels (Roulin and Dreiss, 2002; Benhaiem *et al.*, 2012; Hofer *et al.*, 2016; López-Jiménez *et al.*, 2016; Bebbington *et al.*, 2017), parents may be able to perceive interindividual differences in vocal cues to temperament/testosterone to reduce costs associated to sibling rivalry and adjust provided care (as suggested in spotted hyenas (*Crocuta crocuta*): White, 2008).

I thus investigated in older puppies, whether individual temperament and circulating testosterone levels explain variation in whines produced during a separation from their mother and littermates. I found that interindividual differences in puppy whines appear to reflect variation in temperament of dominance and sociability independent of circulating testosterone levels (Chapter 1). Specifically, at 9 weeks, puppies characterized by a relatively high dominance and low sociability (with humans) tend to produce lower  $f_0$  and more tonal whines, compared to subordinate and sociable littermates respectively. In terrestrial mammals, low harmonicity and high  $f_0$  are considered as possible indicators of heightened arousal of vocalisers (Briefer, 2012, 2020). As such, I suggest that low tonality in whines given by relatively sociable puppies and high  $f_0$  in whines of relatively subordinate may reflect higher level of arousal experienced by individual during a separation from their mother and littermates.

In Chapter 3, I also tested communicative function of whine  $f_0$  in signalling dominance to humans (both professional puppy caregivers and non-caregivers) and found that listeners rated whines synthesized with low  $f_0$  as produced by more dominant-sounding puppies, compared to higher  $f_0$  whines (Chapter 3). Together, my findings confirm dominance encoding and decoding via  $f_0$  in terrestrial mammal calls (as extensively demonstrated in humans (*Homo sapiens*): Puts *et al.*, 2007; Pisanski and Bryant, 2016; Puts *et al.*, 2016; Stern *et al.*, 2021). While communication of dominance should be important when siblings strongly compete for parental resources, sibling rivalry is however relatively low in dogs (Arteaga *et al.*, 2013). Indeed, there is no evidence for individual puppies' nipple preference and aggressive-related behaviours (e.g., puppies dislodging a suckling littermate) when suckling. As such, in a context of mother-puppy interactions, vocal signalling of dominance via whine  $f_0$  is expected to play a rudimentary role in supporting mothers' care adjustment. On the other hand, communication of dominance cues could be involved in vocal

interactions between littermates during play-fighting – a common interaction in 9-week-old puppies. In addition, during these play-fighting interactions, puppies typically emit growls or barks. Therefore, vocal information related to dominance, but also to testosterone level, could be encoded in other call types than whines. Finally, I suggest that the absence of relationship between circulating testosterone and whine acoustics in 9-week-old puppies may reflect too low levels of androgens to significantly predict variation in dominance and vocal fold length of puppies (Evans *et al.*, 2008). Future experiments in adult, sexually mature dogs should verify the implication of androgens in mediating individual differences in dominance and call  $f_0$ .

### *NLP as possible vocal cues to puppies' size and dominance*

Nonlinear phenomena (NLP, such as chaos, subharmonics and amplitude modulation) can lower perceived pitch (Anikin *et al.*, 2021), and ultimately emphasize perception of formant frequencies by increasing spectral density (as suggested by Fitch, Neubauer and Herzel, 2002). Because fundamental and formant frequencies represent key and well-described vocal indicators of size and dominance in terrestrial mammals (Charlton *et al.*, 2020), it has been suggested that NLP may signal (and/or exaggerate) size and dominance of emitters (Fitch, Neubauer and Herzel, 2002).

In Chapter 3, I found that presence of chaos, subharmonics and amplitude modulation in synthetic whines increased perceived size and dominance by human listeners characterising the general population. Nonlinear phenomena had however weak effects on size and dominance perceptions in professional puppy caregivers. As there is no clear biological function of size and dominance exaggeration in distress vocalisations, I interpreted the effects of NLP identified in the general population as consequences of a perceptual bias associating “low” with “large and dominant” that extensive experience of puppy caregiving allows to correct for. As such, my findings suggest that NLP in puppy whines are probably not reliable vocal cues to size/condition and dominance in the context of human-dog vocal interactions. However, in the context of mother-puppy vocal interactions, I cannot exclude the hypothesis that NLP may play a key role. Indeed, owing that increases in subglottal pressure usually leads to the production of high  $f_0$  calls and to vocal instabilities (Berry *et al.*, 1994; Titze, 1994), NLP are expected to occur in relatively high  $f_0$  vocalisations. According to this mechanistic theory, it is thus reasonable to predict that, at 3 weeks, puppies from relatively heavy litters should produce whines affected by higher extent of NLP

compared to puppies from relatively lighter litters. In addition, mothers could use NLP to recognise their litter, as they similarly do with whine  $f_0$ . These hypotheses could be tested in future studies to investigate whether NLP encode for dual-information related to litter's identity, but also to puppies' emotional state (as I discuss below).

## **Communication of varying emotional state in puppy whines**

While parents must assess kin and condition among their offspring to avoid provision of misdirected, poor or excessive care, it is also crucial for them to detect intra-individual variation in arousal to adjust care accordingly to motivational or emotional state level of their young (e.g., hunger-related need, distress). In terrestrial mammals, increases in the vocaliser's arousal is typically associated with increases the subglottal pressure and muscle tensions that can ultimately affect the regular pattern of vocal fold vibration (Berry *et al.*, 1994). As reviewed in a wide range of infant mammal species (Lingle *et al.*, 2012; Briefer, 2020), highly aroused animals produce vocalisations that are usually characterized by a high  $f_0$ , amplitude, duration and/or affected by NLP (i.e., frequency jumps, subharmonics, biphonation, chaos). Therefore, it has been suggested that NLP (especially chaos) can signal heightened level of offspring need, while also reducing habituation and facilitating attention grabbing in caregivers by making vocal signals unpredictable (Fitch, Neubauer and Herzel, 2002).

Extensive descriptions of NLP and acoustic correlates in natural vocal communication systems are very scant. As such, mechanistic and functional theories that suggest NLP as vocal cues to arousal lack strong foundations and ecological validity. In Chapter 2, I filled this gap by investigating temporal dynamics of NLP occurrence (and their acoustic correlates) during a short separation period from their mothers and littermates. Specifically, alongside an increase in whining, I showed that the proportion of whines containing NLP, especially chaos, increased with time since separation. However, there was no clear increases of whine  $f_0$  and duration as separation became longer, suggesting that NLP may take over  $f_0$  and duration for the communication of puppies' emotional state. To confirm the communicative function of NLP as possible cues to arousal, I am now investigating relationships between the production of whines containing NLP and behavioural indicators of heightened level of arousal, such as locomotor activity of puppies (reviewed in Briefer, 2012). With an increasing period of separation, I predict that locomotor

activity would also increase along with the number of whines affected by NLP. Moreover, I am also currently examining the acoustic characteristics of whines produced in negatively valenced contexts that elicit arousal varying from mild to more extreme levels. Indeed, during my thesis, I also collected whines given by 3-week-old puppies (N=183 individuals from 34 litters) exposed to two different conditions of arousal in a negatively valenced context of separation from their mother and littermates. Specifically, puppies were either recorded without manipulation from the experimenter (same method as presented in Chapter 2) (i.e., relatively low arousal) or with manipulation from the experimenter who gently maintained the individual on its back for one minute (i.e., relatively high arousal). I expect puppies to produce higher-pitched and longer whines, while also emitting more calls affected by NLP when experiencing high arousal compared to low arousal.

While my findings suggest that NLP function as possible vocal indicators heightened levels of distress, I also tested their effects on human perception of distress (Chapter 3). Nonlinear phenomena, especially chaos, increased perceived distress in both puppy caregivers and non-caregivers. Interestingly, we found an effect of caregiving experience, where professional breeders or veterinarians were even more affected by NLP than the general population. Therefore, this result indicates that caregivers have probably learned to assess distress in puppy whine, as do humans with baby cries when assessing level of pain (Corvin *et al.*, 2022).

Among vertebrates, it is now well established that offspring calls can encode information related to their emotions and motivations via  $f_0$ , duration and or NLP (Briefer, 2012, 2020; Lingle *et al.*, 2012; Klenova, 2015). Yet, most investigations of parents' perception of need/distress levels in their offspring calls reported field observations or tested this hypothesis using natural sound playbacks (black caimans (*Melanosuchus niger*): Vergne *et al.*, 2011; reed warblers (*Acrocephalus australis*): Kilner, Noble and Davies, 1999; tree swallows (*Tachycineta bicolor*): Leonard and Horn, 2001; Wilson's storm petrels (*Oceanites oceanicus*): Quillfeldt, 2002; Quillfeldt, Masello and Hamer, 2004; zebra finches (*Taeniopygia guttata castanotis*): Perez *et al.*, 2016; grey seals (*Halichoerus grypus*): Smiseth and Lorentsen, 2001; meerkats (*Suricata suricatta*): Manser *et al.*, 2008; humans: Corvin *et al.*, 2022). As such, these studies could not control for potentially covarying acoustic parameters (e.g.,  $f_0$ , amplitude, NLP) and thus determine what vocal cues parents used to assess intra-individual variation of need/distress in their offspring calls. Yet, until recently, a major limitation to these experimentations has been the absence of tools for the

synthesise of naturalistic sounds, allowing for controlled manipulations of key vocal features such as NLP (Anikin, 2019). As successfully used in Chapter 3 and in earlier studies (Anikin, 2020; Anikin, Pisanski and Reby, 2020; Anikin *et al.*, 2021), I thus applied these methods of parametric sound synthesis to test whether NLP in puppy whines signal heightened levels of emotions or motivations to mothers. I am indeed currently performing playback experiments exposing mothers to their puppy whines synthesized with or without NLP and quantifying their behavioural (N=26) and physiological (N=7) responses. I predict that females would provide more maternal care and exhibit stronger stress-related behavioural and physiological responses (increased heart beating rate and respiration rate, Briefer 2012) to whines with NLP, compared to whines without NLP.

While vocal communication of need or distress has been described as honest signalling (Smiseth and Lorentsen, 2001; Quillfeldt, 2002; Sacchi, Saino and Galeotti, 2002; Klenova, 2015; Davis *et al.*, 2019), several studies have also provided evidence for vocal exaggeration of such information (as reviewed in birds by Kilner and Hinde, 2008). Indeed, parent-offspring conflict predicts that arm race dynamics should lead to a system where offspring exaggerate their vocal communication of need to grab their parent's attention and receive care, but also where parents develop a fine-tuned detection of such deceptive signals (Trivers, 1974; Dawkins and Krebs, 1978; Searcy and Nowicki, 2010). Specifically, in litter or brood rearing species, theory states that offspring enter in conflicts brood or littermates for the division of parental resources (Macnair and Parker, 1979; Mock and Parker, 1997). As such, vocal exaggeration of need/distress should arise when sibling rivalry is relatively high, as demonstrated in birds commonly parasitized by cuckoos (Kilner and Hinde, 2008). As mentioned earlier, there is no clear evidence that puppies compete with their littermates for suckling (Arteaga *et al.*, 2013). Therefore, I suggest that vocal communication of need/distress (via  $f_0$  or NLP in whines) should reflect an honest, rather than dishonest, signalling in domestic dog puppies, although this remains to be experimentally verified.

### **Future research directions: comparative studies among canids**

Because selective breeding has favoured behavioural differences between dog breeds, artificial selection could have influenced mother-puppies vocal communication systems. However, inter-breed differences in maternal behaviours are marginal (Santos, Beck and Fontbonne, 2020), thus suggesting that effects of selective breeding on vocal interactions between mother and their



puppies are unlikely. Future studies could yet replicate my thesis work in a different breed than Beagles to test this hypothesis.

Furthermore, I cannot exclude the hypothesis that dog domestication by humans may have influenced mother-puppy vocal interactions, compared to the wolf (*Canis lupus lupus*), their wild ancestor. Indeed, compared to dogs, wolves should be under stronger natural selection pressures, as pups are exposed to higher risks of aggressions from predators or pack members. As such wolves provide an excellent contrast to investigate the effects of domestication on vocal interactions between caregivers and offspring. Specifically, while wolves present a presumably similar context for parent-pup interactions (where only one pair of individuals usually reproduces within the pack), provision of care is however supported by both parents (as opposed to dogs where only the mother provides care) (Lord *et al.*, 2013). Indeed, wolf mothers usually stay to nurse newborns in a den, while males protect and provide food to females and/or pups via regurgitation (Lord *et al.*, 2013). Crucially, wolf pups also produce whines shortly after birth (Cohen and Fox, 1976) that may attract parent's attention as they do in dog mothers.

Therefore, similar to dogs, I would expect in wolves multilevel encoding of information related to kin, individuality, condition and motivations/emotions in pup whines; but I would also predict stronger behavioural responses to whines in wolf males than in dog males, due to the relative absence of care provisioning by dog males. Furthermore, in domestic dogs, asymmetries in parental investment between males and females may have shaped important differences in their vocal interactions with puppies. Indeed, it has been recently demonstrated that non-breeding females display stronger behavioural responses to puppy whines than males (Root-Gutteridge *et al.*, 2021). However, due to biparental care in wolves, I expect that wolf males would respond as strongly as females to their own pup vocalisations. Finally, to further investigate whether domestication have emphasized whining behaviour as a consequence of sensory exploitation of human caregivers and mothers (due to relaxed selective pressure), future studies should also contrast the whine acoustics (including NLP) of domestic dog puppies and wolf pups.

## Conclusion

In this thesis, I report a series of studies investigating vocal communication between multiple young and mother in the domestic dog, human's "best companion". Surprisingly, puppies' vocal behaviour had never been investigated, despite the fact that this behaviour is expressed in our own homes, and possibly partially directed at us. Like the offspring of other domesticated animals (e.g., pigs, cats, rabbits), puppies are born and raised in litters, and thus occupy a specific niche in the animal kingdom. I investigated the production and perception of their distress call, the whine, and demonstrated that several vocal features, including  $f_0$  and NLP, conveyed multilevel information about puppies' litter and individual identity, condition and emotional states. I also showed that this information, encoded within a single call type was perceived by the puppies' mothers as well as by human caregivers. Infant mammals typically have a poorly developed vocal repertoire and often produce stereotypical, simple calls (Lingle *et al.*, 2012). Here, I show that complex information can be encoded within a single call type, in a way that resembles the multilevel encoding of information in human babies' cries (Gustafsson *et al.*, 2013; Koutseff *et al.*, 2018; Bouchet *et al.*, 2020; Corvin *et al.*, 2022). My thesis work thus fills gap in our understanding of the function of parent-offspring communication in vertebrates by revealing a multilevel-encoding mechanism supporting optimal interactions with caregivers. More systematic and comparative studies should investigate multilevel encoding in offspring calls of a wider range of domestic and wild vertebrate species in order to uncover under which conditions such complex encoding evolves.

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