

RESEARCH ARTICLE

Differential encoding of signals and preferences by noradrenaline in the anuran brain

Sabrina S. Burmeister[‡], Verónica G. Rodríguez Moncalvo^{*} and Karin S. Pfennig

ABSTRACT

Social preferences enable animals to selectively interact with some individuals over others. One influential idea for the evolution of social preferences is that preferred signals evolve because they elicit greater neural responses from sensory systems. However, in juvenile plains spadefoot toad (*Spea bombifrons*), a species with condition-dependent mating preferences, responses of the preoptic area, but not of the auditory midbrain, mirror adult social preferences. To examine whether this separation of signal representation from signal valuation generalizes to other anurans, we compared the relative contributions of noradrenergic signalling in the preoptic area and auditory midbrain of *S. bombifrons* and its close relative *Spea multiplicata*. We manipulated body condition in juvenile toads by controlling diet and used high pressure liquid chromatography to compare call-induced levels of noradrenaline and its metabolite MHPG in the auditory midbrain and preoptic area of the two species. We found that calls from the two species induced different levels of noradrenaline and MHPG in the auditory system, with higher levels measured in both species for the more energetic *S. bombifrons* call. In contrast, noradrenaline levels in the preoptic area mirrored patterns of social preferences in both *S. bombifrons* and *S. multiplicata*. That is, noradrenaline levels were higher in response to the preferred calls within each species and were modified by diet in *S. bombifrons* (with condition-dependent preferences) but not *S. multiplicata* (with condition-independent preferences). Our results are consistent with a potentially important role for preoptic noradrenaline in the development of social preferences and indicate that it could be a target of selection in the evolution of condition-dependent social preferences.

KEY WORDS: *Torus semicircularis*, Mating preferences, Preoptic area, Norepinephrine, Species recognition

INTRODUCTION

Social preferences enable animals to selectively interact with some individuals over others and such preferences underpin a range of social interactions including parental care and mating. There are two divergent perspectives for how social preferences become manifest. An emphasis of sensory ecologists is on bias in sensory systems driving the expression of preferences (Fuller et al., 2005). For example, Rodd et al. (2002) proposed that selection for a preference for orange-coloured prey led to the evolution of a preference for orange-coloured conspecifics in guppies. Indeed, there is some

support for preferred signals evoking greater responses by sensory systems (Monbureau et al., 2015; Sandkam et al., 2015; Woolley and Doupe, 2008). Another perspective is that social preferences emerge from valuation-sensitive brain circuits after social signals are represented by sensory systems (Chakraborty and Burmeister, 2015; Goodson and Wang, 2006; Bloch et al., 2018). While these need not be competing hypotheses, and the processes of representation and valuation can interact (Maney, 2013), they make distinct predictions about the neural mechanisms of social decision making. If social preferences are mediated primarily by sensory processes, then one would predict preferred signals to evoke greater responses in sensory systems, perhaps because the sensory systems are tuned to preferred signals. In contrast, if social preferences are mediated by downstream processes that assign value to signals, then one would predict social preferences to become manifest in integrative brain regions such as the basal forebrain.

In anurans, there has been a historical focus on sensory mechanisms for mediating social preferences (Ryan and Rand, 1990; Ryan et al., 1992) with a more recent emphasis on downstream processes (Burmeister et al., 2017; Burmeister, 2017). Selective phonotaxis – a measure of social preferences in anurans – emerges in the juvenile stage in both males and females and typically reflects the same preferences as adults (Baugh and Ryan, 2010; Pfennig et al., 2013). That is, juveniles will associate preferentially with the call type that is preferred by adults, indicating that the neural substrates for phonotaxis are functional early in life (Horowitz et al., 2007). One challenge in studying neural mechanisms of social preferences in anurans is the difficulty in disentangling stimulus from preference, as they tend to be confounded, i.e. while the strength of female preferences vary, they do not generally switch preferences (e.g. Lynch et al., 2006). As a result, one cannot independently evaluate signal processing from signal valuation. The condition-dependent mating preferences of plains spadefoot toads [*Spea bombifrons* (Cope 1863)], in contrast, provide an opportunity to dissociate preference from stimulus: when in good body condition, *S. bombifrons* females prefer *S. bombifrons* males in fast-drying ponds but when in poor body condition, they prefer males of their close relative the Mexican spadefoot toad [*Spea multiplicata* (Cope 1863)] because of the advantages of heterospecific matings (Pfennig, 2007; Chen and Pfennig, 2020). In this species, adult preferences likely stem, in part, from effects of diet in juveniles (Pfennig et al., 2013), which can easily be manipulated in the laboratory. Furthermore, we previously found that in *S. bombifrons*, conspecific calls evoked a greater response from the auditory midbrain of juveniles while response patterns in the preoptic area mirrored social preferences of adults (Burmeister et al., 2017). Our previous results support distinct roles for the auditory system in encoding call type, but not preference, and the preoptic area in encoding preference, but not call type (Burmeister et al., 2017). However, whether this separation of signal processing from signal valuation generalizes to other anurans is unknown. If it is, this would have implications for how social preferences evolve.

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To address this gap, we contrasted the relative contributions of noradrenergic signalling in the preoptic area and auditory midbrain of *S. bombifrons* with those of its congener *S. multiplicata*. We focused on the preoptic area and auditory midbrain because they are central to the expression of social preferences in anurans (Burmeister, 2017). The auditory midbrain (i.e. torus semicircularis; homologue of the inferior colliculus) receives ascending auditory projections from the superior olive and projects to the basal forebrain (e.g. preoptic area, hypothalamus, septum) and telencephalon (e.g. medial pallium) via the thalamus (reviewed in Burmeister, 2017). Because the auditory midbrain receives substantial descending projections, including from the preoptic area, and, in turn, has descending projections onto motor control regions of the hindbrain, many consider the function of the anuran auditory midbrain as an audio-motor integrator underlying selective phonotaxis (Walkowiak and Luksch, 1994). Like other vertebrates, the preoptic area of anurans collects information about socially relevant stimuli (i.e. mating calls; Allison, 1992; Allison and Wilczynski, 1991), is reciprocally modulated by physiological state (Urano, 1988; Andersen et al., 1988) and has descending projections that could regulate behavioural circuits (Sánchez-Camacho et al., 2001). In male anurans, the preoptic area regulates acoustically evoked calling (Schmidt, 1984; Zornik and Kelley, 2011). In female anurans, the preoptic area is required for phonotaxis (Walkowiak et al., 1999) and its activity correlates with mating preferences (Chakraborty and Burmeister, 2015).

Highly conserved among vertebrates, the primary source of central noradrenaline (NA) is the locus coeruleus in the brainstem, which has far-reaching ascending and descending projections. Broadly considered a neuromodulator, NA can modify sensory responses according to the animal's arousal state or context (Hurley et al., 2004). In mammals, NA is released in response to social signals from males (Fabre-Nys et al., 1997) and stimulates the luteinizing hormone surge underlying ovulation (Szawka et al., 2013). In the auditory forebrain of songbirds, NA is critical for discriminating between conspecific and heterospecific song (Appeltants et al., 2002; Vyas et al., 2008). We previously found that NA release in the auditory midbrain of *S. bombifrons* differed in response to conspecific (*S. bombifrons*) and heterospecific (*S. multiplicata*) mating calls (Burmeister et al., 2017), suggesting that a role for NA in auditory processing of social signals may be conserved. Interestingly, levels of NA in the preoptic area of *S. bombifrons*, in contrast, were modified by diet in a manner that mirrored adult social preferences (Burmeister et al., 2017), an effect that is broadly consistent with a role for NA in behavioural flexibility and motivation (Jahn et al., 2018; Lapid and Morilak, 2006; Cieślak et al., 2017).

To further our understanding of the role of NA in the preoptic area and auditory midbrain in mediating social preferences in anurans, we compared NA response patterns in *S. bombifrons* with those in *S. multiplicata*. We chose *S. multiplicata* because of its close relationship to *S. bombifrons*, their partially overlapping distributions (Stebbins, 2003), and the fact that we can use the same calls to represent conspecific and heterospecific signals that were used in our original study (Burmeister et al., 2017). Importantly, unlike those in *S. bombifrons*, preferences in *S. multiplicata* are condition independent: females prefer *S. multiplicata* males regardless of body condition and pond depth (Pfennig, 2007).

Our hypothesis was that patterns of NA in the preoptic area reflect social preferences while NA in the auditory midbrain reflects sensory representation of calls independent of whether they are preferred. To test our hypothesis, we manipulated body condition through diet

measured NA responses in the brains of *S. bombifrons* and *S. multiplicata* (Fig. 1). We predicted that, for the preoptic area, diet would reverse the NA response to calls in *S. bombifrons* (species with condition-dependent mating preferences) but not *S. multiplicata* (species with condition-independent mating preferences; Fig. 1). Alternatively, if the preoptic area is sensitive to diet cues for reasons unrelated to social preferences, then we would expect diet to influence responses to calls in both species. For the auditory midbrain, we predicted that patterns of NA would reflect the call received, independent of diet treatment. If NA levels in the auditory midbrain are sensitive to call characteristics that distinguish the species' calls (e.g. call rate), we predict similar responses in *S. bombifrons* and *S. multiplicata* (Fig. 1). However, if NA in the auditory midbrain is tuned to species-specific calls, then we predict *S. bombifrons* and *S. multiplicata* will demonstrate opposite patterns of call-evoked NA levels.

MATERIALS AND METHODS

Subjects

We focused on juveniles in this study as it allowed us to experimentally control for body condition experienced in early life, a factor that affects the developmental emergence of social preferences in *S. bombifrons* (Pfennig et al., 2013). To generate our subjects, we bred three pairs of *S. multiplicata* to compare with the subjects generated from four pairs of *S. bombifrons* that were previously reported (Burmeister et al., 2017). While data are reported separately, subjects from the two species were processed simultaneously. All adults were wild-caught from sympatric populations near Portal, AZ,

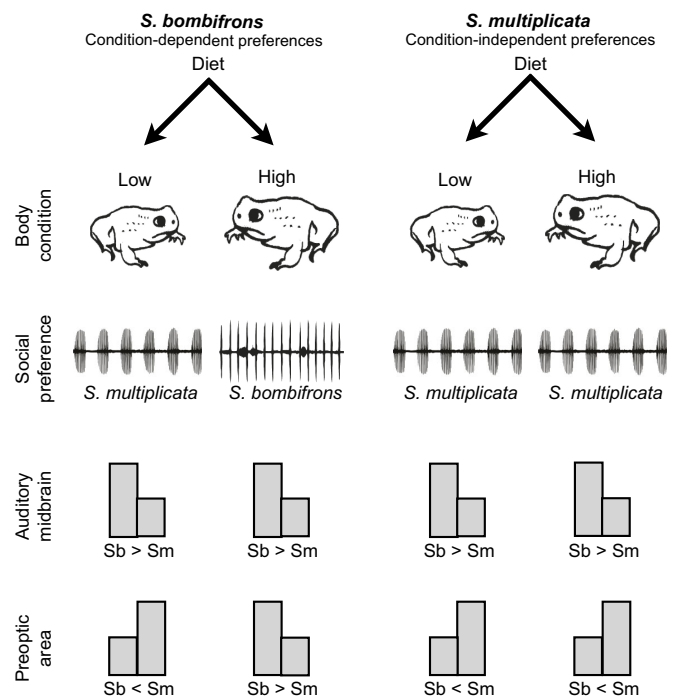


Fig. 1. Predicted noradrenergic responses in the auditory midbrain and the preoptic area for two species of spadefoot toads that vary in condition-dependent social preferences. If noradrenaline (NA) in the auditory midbrain reflects acoustic features of calls, we predict similar responses in the two diet treatments across species. In contrast, if NA responses in the preoptic area reflect the salience of social signals, we predict diet treatment to modify acoustically evoked NA responses in *Spea bombifrons* but not *Spea multiplicata*. Social preferences for natural calls (Sb, *S. bombifrons*; Sm, *S. multiplicata*) are based on previous work (Pfennig, 2007; Chen and Pfennig 2020).

USA. We reared the resulting tadpoles in the laboratory, as described previously (Burmeister et al., 2017; Rodriguez Moncalvo et al., 2013). Briefly, tadpoles were fed tadpole chow *ad libitum*. At metamorphosis, juvenile *S. multiplicata* ($n=31$) and *S. bombifrons* ($n=30$) were randomly assigned to either a high-food or low-food diet. Because live juveniles cannot be sexed, both males and females were randomly included in our sample (sex ratios of sibships are generally 50:50 in both species; K.S.P., unpublished data). Sibships were evenly divided between diet treatments. Individuals were size matched so no initial mass differences existed between diet treatments. From metamorphosis until 6 weeks of age, juveniles on the high-food diet were fed five ~3 mm crickets daily while those on the low-food diet were fed five ~3 mm crickets 3 times per week. At 6 weeks of age, we measured body mass and snout–vent length (SVL). The University of North Carolina Institutional Animal Care and Use Committee approved all animal procedures.

At 6 weeks post-metamorphosis, we presented individual toads from both species with either 40 min of *S. bombifrons* mating calls or 40 min of *S. multiplicata* mating calls in a dark acoustic chamber as previously described (Rodriguez Moncalvo et al., 2013). We tested animals in a dry environment (on moist towel, but no standing water) which, in *S. bombifrons* (but not *S. multiplicata*), would tend to elicit heterospecific preferences in poor-condition females (Pfennig, 2007). The call stimuli were identical to those used previously (Burmeister et al., 2017; Pfennig, 2007), and consisted of calls that were average for the call characters of each species (Fig. 2; Pfennig, 2000, 2007). The calls of each species are readily distinguished, especially by call rate: *S. bombifrons* males produce many more calls per minute (and therefore invest more energy in calling) than *S. multiplicata* (Fig. 2; Pfennig, 2000).

Quantification of NA

Immediately following the 40 min sound exposure, we decapitated each animal, quickly dissected its cranium, embedded it in OCT embedding medium (Sakura, Finetek, Torrance, CA, USA), and froze it in liquid nitrogen (see Rodriguez Moncalvo et al., 2013, for details). We stored craniums at -80°C until sectioning. We sectioned craniums in a caudal-to-rostral direction at -15°C , obtaining two 300 μm brain sections for microdissection of the auditory midbrain and preoptic area.

We collected 350 μm diameter punches from the auditory midbrain and preoptic area following Rodriguez Moncalvo et al. (2013). For the auditory midbrain, we combined punches from the two hemispheres. During punching, we maintained the section at -20°C using a cold stage (Thermoelectric Cold Plate, TCP-2; Thermoelectrics Unlimited, Inc.). In all cases, we expelled the tissue punches into microcentrifuge tubes containing 100 μl of mobile phase and 0.1 $\text{pg } \mu\text{l}^{-1}$ of an internal standard (see Rodriguez Moncalvo et al., 2013, for details) and rapidly froze them on dry ice. We stored all samples at -80°C until analysis.

As in Rodriguez Moncalvo et al. (2013), we used high pressure liquid chromatography (HPLC) with electrochemical detection to determine tissue levels of NA and its metabolite MHPG (3-methoxy-4-hydroxyphenylglycol). Tissue levels of NA reflect amounts that are both stored (in cells and fibres) and released, whereas levels of MHPG are believed to reflect NA release (Heal et al., 1989). Thus, concomitant changes in NA and MHPG levels can be interpreted as changes in NA release/signalling. We were not able to normalize NA levels per microgram of protein because the protein content of each sample was too low to be detected using standard protein assays. However, treatment identity was unknown at the time of punching, and samples of the treatment were interspersed during processing so that all samples were processed blind and randomly in time with respect to treatment group. Thus, any variation in our measures due to variation in punching would be distributed randomly among groups. If anything, such error would decrease our ability to detect differences among treatments. Thus, our results are likely conservative estimates of differences among groups.

Statistical analysis

We calculated body condition for individuals relative to that of others by regressing mass on SVL and using the resulting residuals as our measure of body condition (Pfennig, 2007). We used ANOVA to examine the effects of species, diet and their interaction on change in final length (SVL) and body condition. For NA and MHPG, we analysed the preoptic area and auditory midbrain separately as follows. For the auditory midbrain, we used a general linear model (GLM) to test the hypothesis that call type and species affected NA levels (and separately MHPG levels) while accounting for variation due to the combination of other factors (diet alone and all interactions). For the preoptic area, we constructed an interaction term to test the specific hypothesis that diet reversed the NA response to calls in *S. bombifrons* but not *S. multiplicata* while taking the overall species differences in NA levels into account and while accounting for variation due to other factors (call type alone, diet alone and all two-way interactions). We did not statistically examine MHPG in the preoptic area because levels for *S. bombifrons* were near or below the detectable limit.

RESULTS

Six-week old toads on the high-food diet were longer (diet: $F_{1,57}=69.5$, $P<0.001$) and had better body condition (diet: $F_{1,57}=16.1$, $P<0.001$) than toads on the low-food diet (Fig. 3). As expected, the two species differed in final length (Pfennig and Pfennig, 2005). Nevertheless, they responded similarly to the diet manipulation (diet \times species: SVL, $F_{1,57}=1.9$, $P=0.18$; condition, $F_{1,57}=2.5$, $P=0.12$; Fig. 3).

In the auditory midbrain, we found a strong relationship between the call presented and levels of both NA and MHPG (Fig. 4), suggesting an increase in both production and release. In particular,

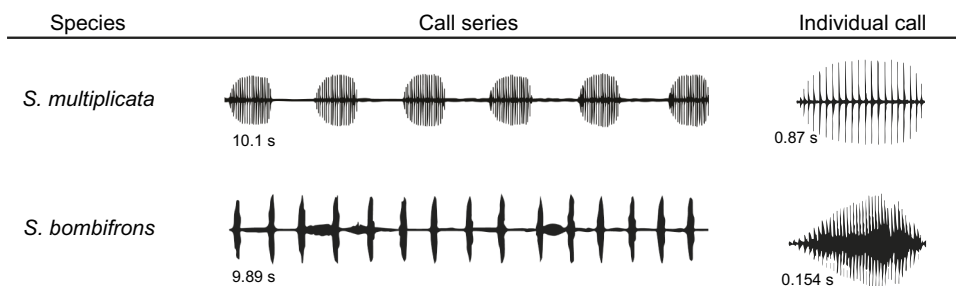


Fig. 2. Mating calls of the two species are temporally distinctive in sympatric populations. While the species' calls overlap in dominant frequency, the calls of *S. bombifrons* have higher call rate and pulse rate compared with the calls of *S. multiplicata*.

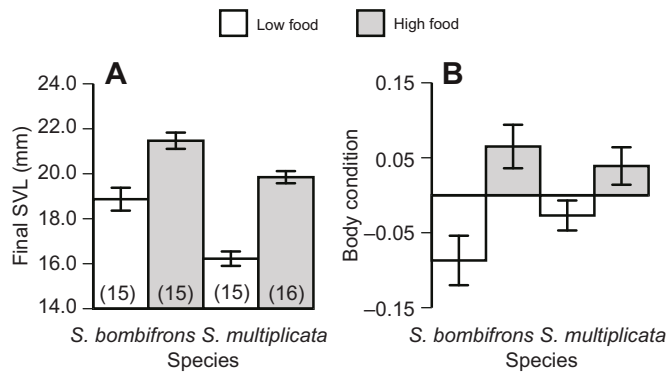


Fig. 3. Diet manipulation affects growth and body condition similarly in the two species. Compared with those on the low-food diet, juvenile spadefoot toads on the high-food diet reached a longer final length (final snout-vent length, SVL: $F_{1,57}=69.5$, $P<0.001$; A) and had better body condition (residual from regression of mass on SVL: $F_{1,57}=16.1$, $P<0.001$; B). Sb, *S. bombifrons*; Sm, *S. multiplicata*. Data are means \pm s.e.m. Sample sizes are shown in parentheses.

our GLMs supported the hypothesis that call and species affected NA and MHPG levels (Table 1).

Diet reversed the relationship between call and NA levels in the preoptic area of *S. bombifrons* but not *S. multiplicata* (Fig. 5, Table 2). Specifically, call-evoked NA levels in *S. bombifrons*, the species with condition-dependent mate choice, were higher in response to the *S. bombifrons* call in the high-food diet treatment but, in the low-food diet treatment, NA levels were higher in response to the *S. multiplicata* call (Fig. 5). In contrast, in *S. multiplicata* the species with condition-independent mate choice, preoptic NA and MHPG levels were higher in response to the *S. multiplicata* calls in both diet treatments (Fig. 5).

DISCUSSION

Sensory ecologists studying social preferences in anurans have emphasized the role that sensory systems play in evoking social preferences (e.g. Ryan and Rand, 1990). In *S. bombifrons*, however, we found that neural encoding of signals and valuation of signals could be separated (Burmeister et al., 2017). Here, we extend that study to *S. multiplicata*, a close relative with condition-independent mating preferences. We found that, in both species, NA signalling in the auditory midbrain was higher in response to the *S. bombifrons* call, a finding that is inconsistent with the interpretation that the preferred call is simply more effective at eliciting a response in the auditory system. Furthermore, our results suggest that NA signalling in the auditory midbrain may underlie initial processing and differentiation of species' call in both species. In the preoptic area, we found that call-evoked NA levels were sensitive to diet in *S. bombifrons*, the species with condition-dependent mating preferences, but not in *S. multiplicata*. That is, NA responses of the preoptic area more closely reflected social preferences, rather than call type, in both species. Thus, we conclude that, for the

Table 1. Results of the general linear model testing the effect of species and call on noradrenaline (NA) and MHPG levels in the auditory midbrain

| | d.f. | NA | | MHPG | |
|------------------|------|------|--------|------|--------|
| | | F | P | F | P |
| Overall | 7 | 2.51 | 0.0260 | 2.17 | 0.0519 |
| Species and call | 2 | 6.62 | 0.0027 | 5.05 | 0.0098 |
| Other | 5 | 0.93 | 0.4713 | 10.4 | 0.4062 |

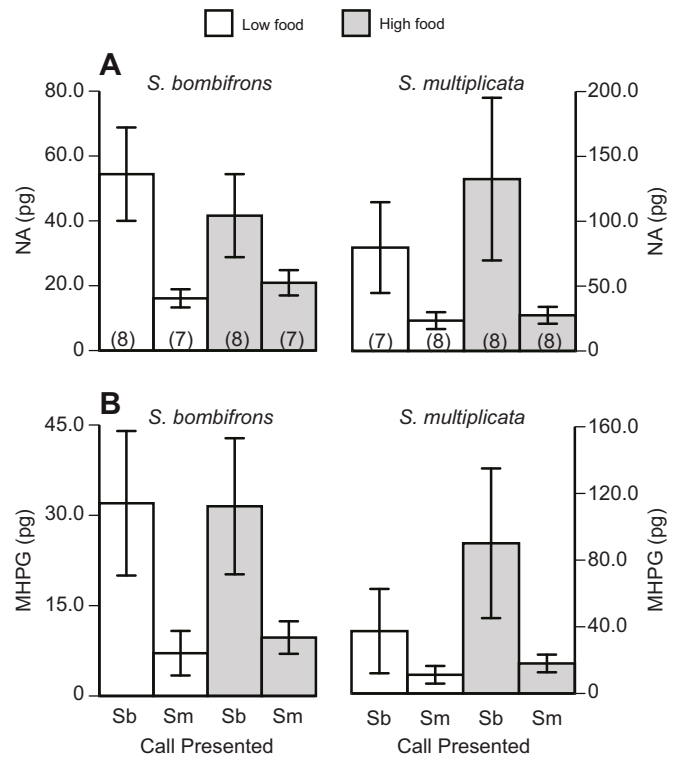


Fig. 4. Effects of mating calls on NA and MHPG in the auditory midbrain of two species of spadefoot toad. Independent of diet treatment, the more highly energetic *S. bombifrons* call (Sb) elicited more NA ($F_{2,54}=6.6$, $P=0.003$; A) and MHPG ($F_{2,54}=5.1$, $P=0.01$; B) than the *S. multiplicata* call (Sm) in both species. Data are means \pm s.e.m. Sample sizes are shown in parentheses.

auditory midbrain, NA responses are not modulated by valuation circuits, but reflect sensory processing. For the preoptic area, NA responses appear to reflect valuation. Thus, while these two brain regions are part of a broader neural circuit mediating social behaviour in anurans, they appear to make distinct contributions. Taken together, our results indicate that a separation of signal processing from signal valuation may be more broadly true in anurans than previously appreciated, and that the neural substrates are already in place and functional at early developmental stages.

Using HPLC, we measured tissue levels of NA and its primary metabolite MHPG integrated over 40 min of exposure to social signals. Synaptic signalling of catecholamine neurotransmitters, such as NA, is terminated primarily by metabolism (rather than reuptake). Thus, high levels of MHPG reflect active synaptic release of NA (Heal et al., 1989). Interpreting changes in NA levels over this time course is more complicated. Increasing the release of NA, without concomitant increases in production, would be associated with lower levels of tissue NA due to the conversion of synaptic NA to MHPG. In contrast, higher levels of NA could reflect increased synthesis but less release, as NA is stored and builds up, or more NA production and release. Given our time scale of 40 min, which is long enough for neurons to alter neurotransmitter production to meet demands, and the coordinated changes in NA and MHPG that we found, the increases in tissue levels of NA in our study likely reflect increased NA signalling.

We chose to investigate the neural mechanisms of social preferences by examining response properties of the NA system because of its well established role in regulating arousal, behavioural flexibility and processing of salient sensory events (Hurley et al., 2004). In songbirds, NA has been implicated in the auditory discrimination of potential

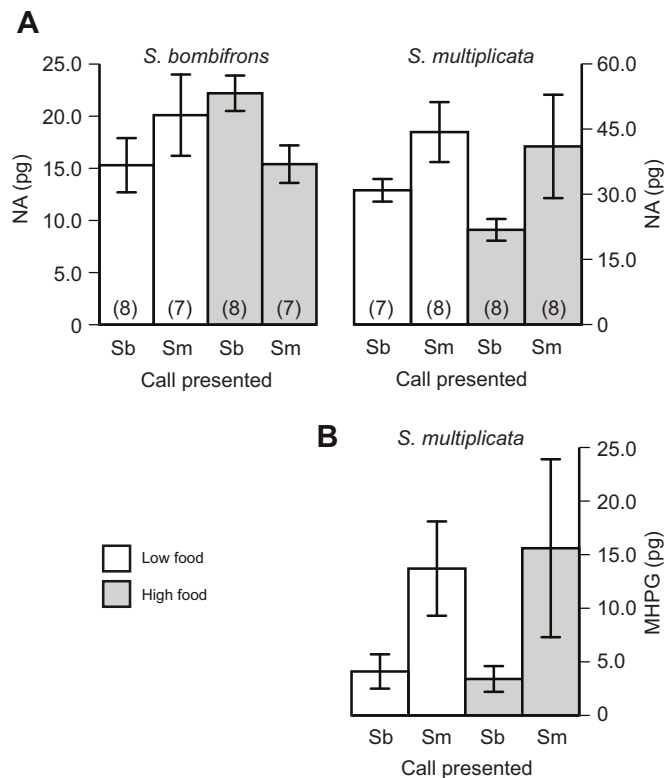


Fig. 5. Effects of diet and mating call on NA and MHPG in the preoptic area of two species of spadefoot toad. (A) Diet affected call-evoked NA levels in the preoptic area differently in *S. bombifrons* and *S. multiplicata* (GLM: $F_{1,53}=7.8$, $P=0.007$). In *S. bombifrons*, with condition-dependent preferences, diet reversed the relationship between call presented and NA levels in a manner that reflects preferences of adults. In *S. multiplicata*, with condition-independent mating preferences, call-evoked NA levels were not modified by diet treatment. (B) In *S. multiplicata*, MHPG levels were also not modified by diet treatment. Note, data are not shown for *S. bombifrons* as MHPG levels in the preoptic area were near or below the detectable limit. Sample sizes are shown in parentheses. Sb, *S. bombifrons*; Sm, *S. multiplicata*.

mates. Indeed, lesions of NA systems impair the ability of females to discriminate between the songs of conspecifics and heterospecifics (Appeltants et al., 2002), likely through effects on the auditory forebrain (Lynch and Ball, 2008; Cardin and Schmidt, 2004). Less is known about effects of NA on auditory processing in mammals. However, in rats, the inferior colliculus has reciprocal connections with the locus coeruleus (Hormigo et al., 2012). To our knowledge, our studies in *Spea* are the first to examine a functional relationship between NA and auditory processing in anurans, although an anatomical basis for locus coeruleus modulation of the auditory midbrain has long been established (González and Smeets, 1993). However, unlike NA in the auditory forebrain of birds, NA levels in the auditory midbrain of *Spea* do not reflect social preferences or call

Table 2. Results of the general linear model testing the prediction that diet reverses the relationship between call and NA levels in the preoptic area in *S. bombifrons* but not *S. multiplicata* (represented by the interaction term)

| | d.f. | F | P |
|-------------|------|-------|--------|
| Overall | 7 | 4.11 | 0.0011 |
| Species | 1 | 16.87 | 0.0001 |
| Interaction | 1 | 7.83 | 0.0072 |
| Other | 5 | 0.68 | 0.6386 |

category (conspecific versus heterospecific) but, rather, appear to encode, or respond to, particular aspects of the call. The calls of *S. multiplicata* and *S. bombifrons* differ in a number of features (Pfennig, 2000; Fig. 2). Most notably, while the dominant frequencies overlap, the calls of *S. multiplicata* are longer and are produced with slower call rates with audible gaps between pulses compared with those of *S. bombifrons* (Stirman and Pfennig, 2019; Fig. 2). Possibly, the greater NA signalling in the auditory midbrain in response to *S. bombifrons* is a result of greater overall call energy.

The vertebrate preoptic area receives socially relevant sensory information, responds to changes in physiological state, and simultaneously regulates behavioural circuits and gonadal activity. In addition, it is one of the many ascending targets of the locus coeruleus (Berk and Finkelstein, 1981). In birds, NA in the basal forebrain, which includes the preoptic area, contributes to sexual motivation (Riters and Pawlisch, 2007). In rodents, NA appears to have dose-dependent effects on sexual performance of males (Hull and Dominguez, 2007). In females, NA stimulates gonadotropin release (Szawka et al., 2013), likely facilitating ovulation. The anuran preoptic area is conserved in a number of respects, including receiving social stimuli (Allison, 1992; Allison and Wilczynski, 1991), responding to the gonadal hormones (Chakraborty and Burmeister, 2010; Chakraborty and Burmeister, 2015) and modulating behavioural circuits (Sánchez-Camacho et al., 2001). While the anuran preoptic area almost certainly contributes to the expression of sexual behaviour, including social preferences (Walkowiak et al., 1999), we know much less about the details of this contribution compared with that in other vertebrates. Our data from *Spea* provide further evidence for a possible role of the preoptic area in social preferences of anurans.

How and why social preferences evolve remain open areas of inquiry, and contrasts such as those used in our study can provide important insights into the neural basis of social preferences that could be the targets of selection. Social preferences can depend on an individual's internal state, along with a complex set of cues from both the individuals with which interactions take place and the external environment. Future work is needed to better understand what neural or molecular characteristics determine how such cues impact signalling in key brain areas and modify neural activity. Critically, developing this understanding in a comparative context will provide key insights into how social preferences evolve.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.S.B., K.S.P.; Methodology: V.G.R.M., K.S.P.; Formal analysis: S.S.B.; Investigation: V.G.R.M.; Writing - original draft: S.S.B., V.G.R.M.; Writing - review & editing: S.S.B., K.S.P.; Visualization: S.S.B.; Supervision: K.S.P.; Project administration: K.S.P.; Funding acquisition: K.S.P.

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