

Effect of anomalous pulse timing on call discrimination by females of the gray treefrog (*Hyla versicolor*): behavioral correlates of neurobiology

Joshua J. Schwartz^{1,*}, Kenneth Huth², Raymond Huncce¹ and Brandon Lentine¹

¹Department of Biology and Health Sciences, Pace University, Pleasantville, NY 10570, USA and ²The Wildlife Conservation Society, Bronx, NY 10460, USA

*Author for correspondence (jschwartz2@pace.edu)

Accepted 9 March 2010

SUMMARY

Research has demonstrated that certain midbrain neurons of anurans ‘count’ interpulse intervals (IPIs). Some neurons fire after exposure to fewer intervals than do others. Counting can be reset to zero if an IPI falls outside the cell’s tolerance range. We tested female gray treefrogs for behavioral correlates of these neural response patterns using phonotaxis tests in order to gain a better understanding of the mechanistic bases of female responses to calls. For example, previous work demonstrated females often prefer longer to shorter pulsed advertisement calls, even when the former occur at lower rates. Call attractiveness can also be reduced when pulse duration and timing have been manipulated experimentally or disrupted by acoustic interference. In this study, female responses were consistent with neural data, emphasizing the importance of IPIs. Females discriminated in favor of calls with normal interpulse timing relative to those in which a single IPI was too long or too short. Our data suggest that neural resetting of interval counting by inappropriate intervals may more strongly influence females than reduced firing in response to such intervals on an individual basis. Data also suggest a transition point between 125 ms and 175 ms at which an interval between pulse strings is treated as an interval between calls.

Key words: anuran, gray treefrog, auditory system, communication, pulse-timing.

INTRODUCTION

A fundamental goal of neuroethology is to integrate findings from both behavior and the neurobiology of the peripheral and central nervous system (CNS) in order to improve understanding within these fields and guide future research [see Konishi (Konishi, 2006) for excellent classic examples]. Over the past 30–40 years, the communication biology of anuran amphibians has proved to be an especially edifying area for such an integrative approach (Capranica and Moffat, 1983; Gerhardt and Huber, 2002; Simmons, 2003). Cells exhibiting a variety of response characteristics exist at higher levels in the anuran auditory system and the structure of calls has been linked to these characteristics (Feng and Schellart, 1999; Gerhardt and Huber, 2002; Rose and Gooler, 2007). Of particular interest has been ongoing research on neurons in the torus semicircularis, a center of the midbrain homologous to the mammalian inferior colliculus. Neurons in this part of the CNS exhibit a variety of filter types for different rates of amplitude modulation as well as sensitivity differences to other signal attributes (Rose et al., 1985; Diekamp and Gerhardt, 1995). Pulsatile structure (a form of amplitude modulation=AM) is an important feature in the calls of many anurans, and the calls of different species as well as intraspecific call types in many cases differ primarily in the presence (or absence) and rate of AM (reviewed in Gerhardt and Huber, 2002) (Wells, 2007).

Rose and his colleagues have found that certain neurons in the torus semicircularis of two anuran species, *Hyla regilla* and *Rana pipiens*, respond only after exposure to a sufficient portion of the AM (i.e. pulsed) call (Alder and Rose, 1998; Edwards et al., 2002). These long-latency (i.e. integrating) neurons exhibit the following intriguing response property. Rather than fire after a certain number of pulses in the call (irrespective of interpulse intervals, IPIs), the

cells appear to count IPIs of appropriate duration. If the gap between pulses is either too short or too long, the integration process is reset (to a count of zero). For example, a neuron that reliably fired following presentation of a 9-pulse call (containing eight normal IPIs with separation between pulses of 10 ms) failed to fire when two normal 8-pulse strings were separated by 20 ms. If the length of the trailing pulse string was lengthened to 9 pulses, the cell again fired after what had become a 17-pulse call [see fig. 3 of Edwards et al. (Edwards et al., 2002)]. Whole-cell patch recordings indicated that inhibition together with a reversal of activity-dependent enhancement of excitation contribute to this resetting for long intervals. In the case of short intervals, excitatory inputs to cells may fail to fire to each call pulse (Edwards et al., 2007) (G. J. Rose, personal communication). In both species, different call types differ in their temporal structure (e.g. AM rate) and thus it is reasonable to suppose that the aforementioned neural responses are at least a partial substrate for recognition and differential behavioral response to call types during natural interactions. Although their contributions to call recognition and discrimination have been inferred (Alder and Rose, 1998; Edwards et al., 2002; Edwards and Rose, 2003), the extent to which ‘interval counting’ by a subset of resettable toral neurons specifically influences natural behavior (such as female call discrimination or preference reflected by phonotaxis) remains to be more directly explored.

In the present study we illuminate how neurobiological data pertaining to the processing of call pulse structure might be relevant to anuran behavior. Specifically, we used a female choice phonotaxis paradigm to evaluate the effect of anomalous IPIs in the male advertisement call on signal discrimination by females of *H. versicolor*. Females of *H. versicolor* are especially well suited for this study for a variety of reasons. Although the resettable interval-

counting neurons found in *H. regilla* and *R. pipiens* have not been reported in the literature for *H. versicolor*, such cells have been identified in work underway (G. J. Rose, unpublished data). In addition, there are a wealth of data obtained from both two-speaker call discrimination and single-speaker call recognition tests of female phonotaxis that illustrate sensitivity to the pulse structure and pulse timing of the call (e.g. Diekamp and Gerhardt, 1995; Gerhardt and Schul, 1999; Gerhardt, 2001; Bush et al., 2002; Schul and Bush, 2002). At equal call rates, females of *H. versicolor* also show a robust preference for calls with more pulses to those with fewer pulses and will approach the former more quickly (Gerhardt, 2001; Bush et al., 2002). This pattern of discrimination helped us assess the effect of interval manipulations on call attractiveness in a subset of our tests in which we offered an anomalous call containing more pulses against a shorter call alternative with normal IPIs. Finally, resettable interval-counting neurons might represent the physiological substrate of the following intriguing finding. Females of *H. versicolor* often discriminate in favor of longer calls over shorter calls even when the 'pulse effort' (mean call rate \times mean pulses per call) of alternative stimuli are equivalent or nearly so (Gerhardt et al., 1996; Klump and Gerhardt, 1987; Schwartz et al., 2001; Schwartz et al., 2008). As discovered by Rose and colleagues, interval-counting neurons differ in the number of intervals required to elicit a response (Edwards et al., 2002) (e.g. see their fig. 1c showing neurons responding after 2–8 intervals). In the case of *H. versicolor*, longer calls could recruit more neurons and elicit more spikes (our Fig. 1A) from these cells than would shorter calls that are presented more often (Fig. 1B).

MATERIALS AND METHODS

General procedures

We captured amplexed, gravid females of *Hyla versicolor* Le Conte 1825 during evenings at a pond within the Blue Mountain Reservation (Peekskill, NY, USA) during May and June of 2004, 2005 and 2009. After separation from their mates, the females were placed in plastic containers, returned to our laboratory (Pace University, Pleasantville, NY, USA) and held until testing (usually the next day) either in an ice-filled cooler or refrigerator in order to postpone oviposition. Following tests of phonotaxis, we returned the treefrogs to their pond (typically within 2–3 days post capture). The handling and use of study animals were approved by the Pace University Animal Care and Use Committee.

We performed our phonotaxis experiments within a temperature-controlled ($\sim 20^\circ\text{C}$) chamber (inner dimensions: 255 cm long \times 225 cm wide \times 195 cm high; Ultimate Walk-Ins, Inc.; Airmont, NY, USA) floored with waterproof low-pile carpet and walled with echo-attenuating acoustic foam (Silent Source HFX-4; Silent Source, Northampton, MA, USA). We observed female movements under infrared illumination (IR Lighting Kit, part # 11665OP; Marlin P. Jones and Assoc. Inc., Lake Park, FL, USA) on a video monitor outside the chamber following their release (via an externally controlled pulley) from an acoustically transparent cage on the floor of the chamber. Choice stimuli offered to the females were synthetic advertisement calls (8-bits per sample; 20 kHz sampling rate) that were modeled after the natural calls of *H. versicolor* calling at our testing temperature (J.J.S., unpublished software). Female gray treefrogs do not discriminate between natural advertisement calls and such synthetic models (Gerhardt, 1978). The test calls (50 ms rise time) were formed from sequences of individual pulses (20 ms linear rise, 5 ms concave down fall; pulse duty cycle=50% unless otherwise noted) with each 25 ms pulse created by first summing and then envelope shaping a 2200 Hz and a 1100 Hz (-6 dB relative

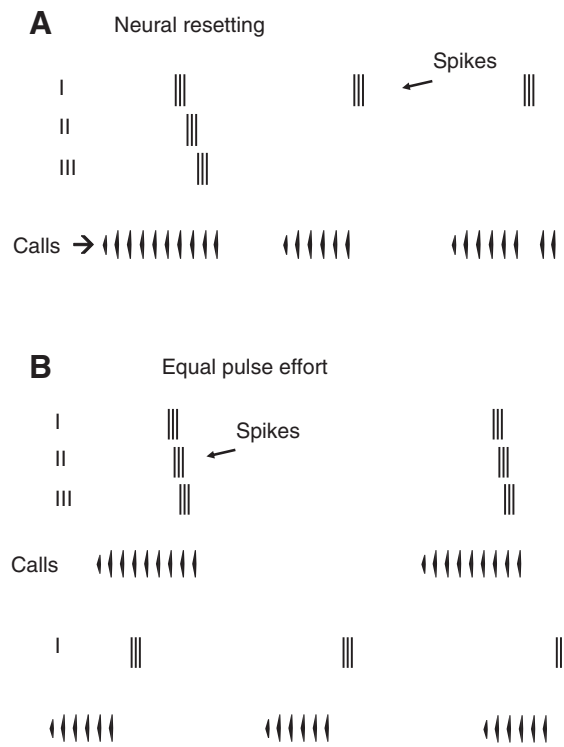


Fig. 1. (A) Illustration of how neurons in the mid-brain of *Hyla versicolor* could respond to presentations of the conspecific advertisement call without (left, middle) or with a larger than normal interpulse interval (IPI). These non-phasic neurons are long-latency (integrating) units that reset following anomalous IPIs (either too long or too short). Neurons I, II and III fire after detection of pulse strings with five, six or seven correct IPIs, respectively. Note that neuron I, with the briefest integration window, is the only one that fires in response to all three calls because there are sufficient numbers of normal intervals presented for only this cell (e.g. the anomalous gap in the call on the right occurs too early for neurons II and III). The strength of the response reflects the total number of responding neurons. (B) Illustration of how neurons of A fire following call sets of equal pulse effort (two 9-pulse calls above and three 6-pulse calls below). Note that a larger population of neurons (I, II and III) fires in response to the longer calls presented at lower rate than in response to the shorter calls presented at higher rate (only neuron I fires).

amplitude) sinusoid (0 deg phase offset). In most tests, one of the alternative stimuli had an interval between a pair of pulses altered (see below). Pulse duration was not altered although changes in interval duration did change the pulse duty cycle (pulse duration divided by pulse period) in a section of the modified calls (see Figs 2–5). Stimulus alternatives were uploaded into FutureSound software (Applied Visions, Inc., Medford, MA, USA) and played, in alternating fashion, at a natural rate (15 stimuli per minute per speaker) from a Commodore Amiga 600 computer (Commodore Business Machines; West Chester, PA, USA) using a Realistic SA-10 amplifier and Realistic Minimus 0.3 speakers (RadioShack; Fort Worth, TX, USA). We adjusted sound amplitudes to 85 dB SPL (flat weighting, fast root-mean-square, dB re. 20 μPa) at the central female release position (1 m from each speaker at opposite sides of the arena) with a calibrated Gen Rad 1982 Precision Sound Level meter (IET Labs; Westbury, NY, USA). Each female was exposed to the call stimuli for 30 s prior to release from the cage and a choice recorded if she came within 10 cm of a speaker within 10 minutes. Fewer than 10% of females were non-responsive. No female was used more than once per test although most females were used in

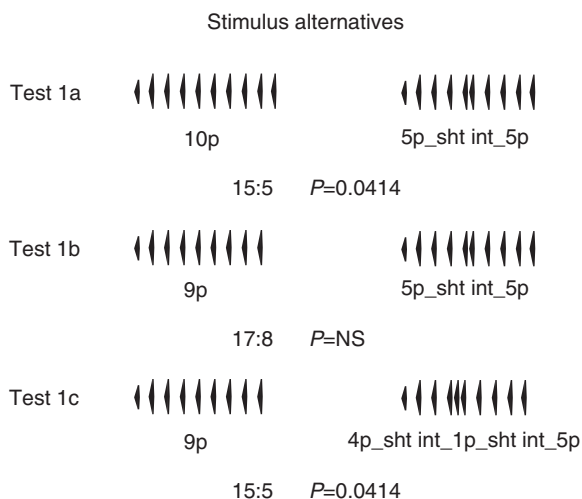


Fig. 2. Representatives of stimulus alternatives (left) of 10, nine and nine pulses with normal interpulse intervals offered against alternatives (right) containing one (tests 1a and 1b) or two short intervals (test 1c). The number of females choosing the call illustrated on the left and right are shown below each pair of calls along with the *P*-level for a two-tailed binomial test.

five to seven tests, including some not reported in this study. To the best of our knowledge, choice behavior of female gray treefrogs in phonotaxis experiments is not influenced by their choice behavior in earlier tests (Gerhardt, 1981; Gerhardt et al., 2000), obviating a need for counterbalancing. The inter-test interval for all females tested more than once was a minimum of 9 min. During this time females were acoustically isolated from the test stimuli and held individually at the testing temperature in the dark within plastic containers. We used two-tailed binomial tests ($\alpha=0.05$) to evaluate the null hypothesis that females were equally likely to approach the speakers broadcasting alternative stimuli.

Because of the large number of tests, in the next section we present additional details about specific choice stimuli along with predicted and actual results. We use the term ‘gap’ to refer to the silent interval between the end of one pulse and the beginning of the next pulse within a call.

‘IPI’ refers to the time interval between the initiation (i.e. onset) of one pulse and the next pulse (Edwards et al., 2008). Because all pulses in our stimuli were 25 ms in duration, IPIs were always 25 ms longer than gaps. The term ‘discrimination’ refers to a behavioral choice during two-stimulus tests of phonotaxis. It is, of course, possible that females detect differences among stimuli to which they do not discriminate in this manner. We make the assumption that, given a choice, females will discriminate during phonotaxis in favor of an advertisement call stimulus that elicits a significantly stronger neural response relative to a stimulus that elicits a weaker response.

RESULTS

Specific tests, predictions and results

We knew from previous work (Schwartz et al., 2010) that increasing the gap between one pair of pulses by 25 ms (to 50 ms, in middle of call) resulted in significant discrimination in favor of a ‘normal’ call (both call alternatives=10 pulses). Here, we first tested (tests 1a–1c) whether abbreviating the gap between pulses (1 ms gap=pulses nearly abutting) could also reduce relative call attractiveness. Recall an abnormal call IPI, within the integrating time-window of an interval-counting neuron, will reset the pulse

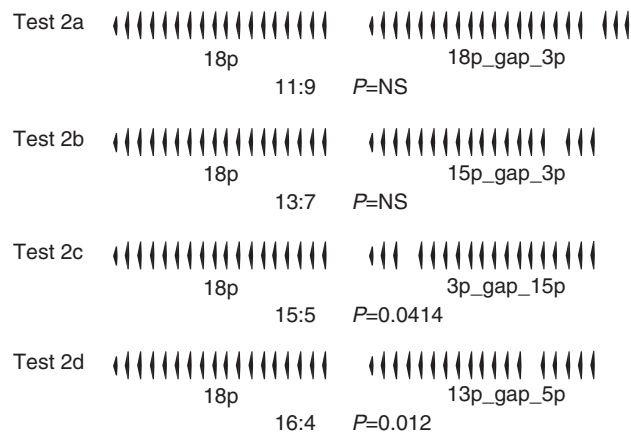


Fig. 3. Representatives of stimulus alternatives offered to females in tests 2a–2d. Anomalous calls all contained a long interval created by removing a single pulse (long gap=75 ms). The number of females choosing the call illustrated on the left and right are shown below each pair of calls along with the *P*-level for a two-tailed binomial test.

interval count to zero and result in no or reduced firing to the call by the neuron. In test 1a, stimulus alternatives (Fig. 2) had an equal number of pulses (10) but an unequal number of normal duration gaps (normal call=9 *versus* abnormal call=8). We predicted that females would discriminate in favor of the normal call because the normal call should not elicit neural resetting. However, the neural response to the abnormal call could be similar to that of two 5-pulse calls, provided the anomalous IPI in the middle of the 10-pulse call resets integrating neurons to a count of zero. Such extremely brief calls are expected to elicit little attractive potential in choice tests (e.g. Schwartz et al., 2001). We observed significant discrimination against the abnormal call, which attracted just 25% of the subjects. In test 1b, stimulus alternatives had an unequal number of pulses (9 *versus* 10) but an equal number of normal duration gaps. Again, more than twice as many females chose the normal calls over the calls with abnormal pulse timing. However, the results were not statistically significant ($P=0.1078$). Finally, in test 1c we also offered females a choice between a normal 9-pulse call (with 8 normal gaps) and a 10-pulse alternative. However, the longer abnormal call had two abbreviated gaps (with 7 normal gaps). Such a structure might increase the likelihood of neural resetting, should it occur. In this situation, females discriminated significantly against the abnormal call.

We next employed stimuli with abnormally long gaps. Again, one alternative was a normal call (here with either 17 or 18 pulses). In the first test of this series (test 2a), we offered females a choice between a normal 18-pulse call and an anomalous 21-pulse call. The 21-pulse call consisted of a normal sequence of 18 pulses followed by a 75 ms gap (created by removing one pulse) followed by a sequence of three pulses (18p_gap_3p, Fig. 3). Based on previous results [see fig. 15 in Gerhardt (Gerhardt, 2001)] using normal calls, either a 21-pulse call or a 20-pulse call should be preferred to an 18-pulse call. We found however, in agreement with a behavioral impact of neural resetting, that females failed to discriminate but rather split in their choices nearly evenly (11:9). The preference for the normal call became stronger or significant when the two alternatives both contained 18 pulses (Fig. 3: tests 2b–2d). Here the long gap in the anomalous call was positioned either after the 15th pulse (15p_gap_3p, test 2b), the 3rd pulse (3p_gap_15p, test 2c) or the 13th pulse (13p_gap_5p,

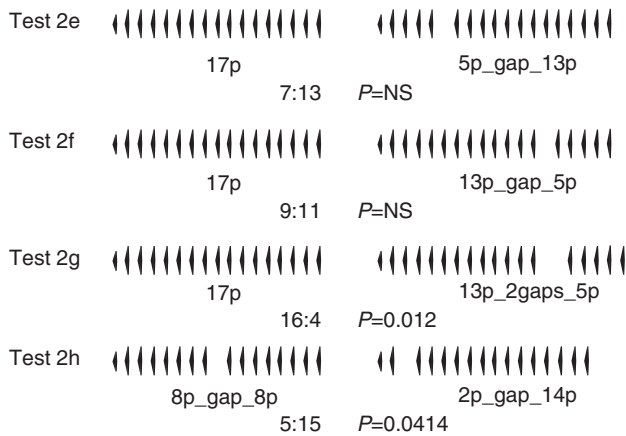


Fig. 4. Representatives of stimulus alternatives offered to females in tests 2e–2h. Anomalous calls all contained a long interval created by removing one (tests 2e, 2f and 2h) or two pulses (test 2g; gap=125 ms). The number of females choosing the call illustrated on the left and right are shown below each pair of calls along with the P -level for a two-tailed binomial test.

test 2d). The number of normal pulse intervals in the long and the short pulse strings on either side of the long gap are identical or very similar in the modified calls and therefore the neural response to them contributed by resettable interval-counting neurons should be similar. Indeed, the results to these three tests do not reveal significant effects of gap location (note that the choice splits are quite similar; d.f.=2, $\chi^2=1.19$, $P=0.551$). In tests 2b–2d, the anomalous calls contained both a long gap and fewer normal IPIs than the normal 18-pulse call. To assess whether discrimination would be maintained when call alternatives had the same number of normal IPIs, we conducted two additional tests with anomalous calls with mirror-image long gap locations. In these tests (tests 2e and 2f; Fig. 4) the normal call contained only 17 pulses. In both tests, females failed to discriminate significantly. Based on neurophysiological results with *R. pipiens* and *H. regilla* we reasoned that if the long gap in the abnormal call was made even longer, it might be more effective in resetting the interval-counting process. Indeed, when we repeated test 2f using an anomalous call in which the gap was lengthened to 125 ms (equivalent to two missing pulses; 13p_2gaps_5p, test 2g, Fig. 4), females showed significant discrimination against the anomalous call even though it contained more pulses (16:4). This result offers strong support for a behavioral impact of neural resetting as does the penultimate test of test series 2. In test 2h (Fig. 4), both alternatives contained 75 ms gaps and 14 good IPIs. However, in one stimulus (8p_gap_8p) the 75 ms gap was positioned in the middle of the call and in the other stimulus (2p_gap_14p) the 75 ms gap occurred following the second pulse. We predicted that, with neural resetting, the neural response of females to the 8p_gap_8p call should be similar to the response to two 8-pulse calls. The neural response to the 2p_gap_14p call should be similar to the response to a 2-pulse call and a 14-pulse call. In test 2h, females showed a significant preference (5:15) for the call (2p_gap_14p) likely to elicit firing by a larger population of integrating (resettable) neurons than the alternative stimulus. Indeed, the response was similar to that obtained when pairs of separate calls (8-pulse and 8-pulse versus 2-pulse and 14-pulse; 2 s interval from call midpoint-to-call midpoint within

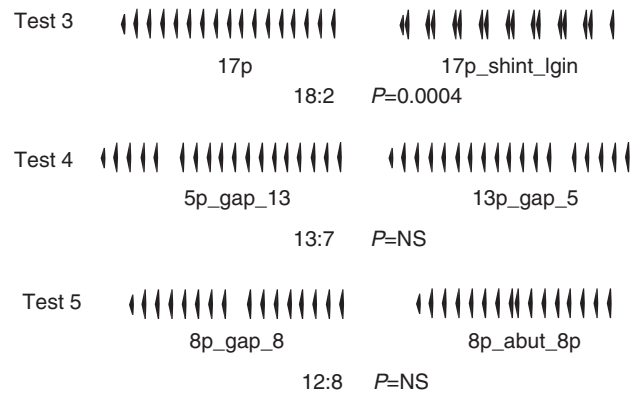


Fig. 5. Representatives of stimulus alternatives offered to females in tests 3, 4 and 5. In test 3, the anomalous call (right) containing both short and long intervals had a mean interpulse interval of a normal call. In test 4, the long interval was created by removing one pulse. In test 5, the long interval in the call on the left was created by removing one pulse. In the call on the right, the central pulses were separated by ~1 ms and were thus nearly abutting. The number of females choosing the call illustrated on the left and right are shown below each pair of calls along with the P -level for a two-tailed binomial test.

each alternative) were offered as stimulus alternatives (3:17 in favor of the 2-pulse and 14-pulse calls; test 2i; figure not shown).

Additional related tests

Edwards et al. tested their interval-counting hypothesis and the central importance of temporal constraints by recording neural responses when subjects were exposed to either normal calls or anomalous calls with the same mean IPI as the normal calls (Edwards et al., 2002). The interval-counting hypothesis predicts that the latter stimulus (with a mix of alternating shorter and longer than normal IPIs) will be ineffective. Their results were consistent with this prediction. We conducted a similar test at the level of behavior by offering females a choice between a normal 17-pulse call and a 17-pulse call in which the position of alternate pulses was shifted so as to nearly abut the previous pulse (gap=1 ms; test 3; Fig. 5). Thus, the mean IPI of the two calls was equal (both calls: 16 IPIs; abnormal call: 8 short + 8 long intervals). Females overwhelmingly chose the normal call; thus, emphasizing the importance of IPIs that fall within specific temporal bounds. However, an unexpected result was that two of the females tested approached the anomalous call (see Discussion).

Does the location of the anomalous gap make a difference when, unlike in test 2h, neural resetting predicts equivalent responses? The choice results from tests 2b and 2c as well as tests 2e and 2f suggest that, in mirror-image anomalous stimuli, the position of the long gap is irrelevant (note the similar response ratios). However, the results of Schwartz and Marshall suggested that disruption (via call overlap) of the temporal structure of a call is more detrimental when it occurs near the beginning rather than near the end of a call (Schwartz and Marshall, 2006). When we paired two anomalous 18-pulse calls against one another (test 4; 5p_gap_13p versus 13p_gap_5p; Fig. 5) that differed only in long gap position, we found no significant difference in female responses.

Given that both reducing and increasing gap duration can reset interval-counting neurons and our findings that they can influence female behavior, in test 5 (Fig. 5), we directly compared the impact of these changes with one another. A 16-pulse call (8p_gap_8p) with a central longer than normal gap (75 ms) was offered as an

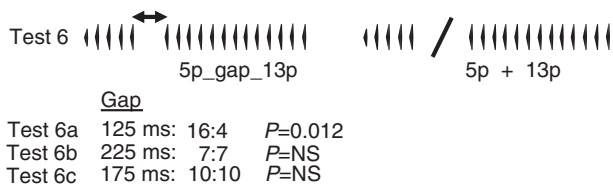


Fig. 6. Representatives of stimulus alternatives offered to females in tests 6a–6c. The choice alternative on the right consisted of two normal calls separated by an interval (call midpoint-to-call midpoint) of 2 s (indicated by solidus). The anomalous alternative was a call with a long interpulse interval of 125 ms (test 6a), 225 ms (test 6b) or 175 ms (test 6c). The double arrow indicates that a range of gap durations were used. The number of females choosing the call illustrated on the left and right are shown below each pair of calls along with the *P*-level for a two-tailed binomial test.

alternative to a 16-pulse call (8p_abut_8p) with a central shorter than normal gap (1 ms ~ abutting pulses). Females failed to discriminate between these two anomalous stimuli (12:8).

Our results support the hypothesis that an anomalously large gap within a call reduces its attractiveness relative to a call lacking such a gap. It is nevertheless unclear, in the case of such an anomalous stimulus, whether the ‘call’ is treated (or perhaps perceived) as a single entity (e.g. one call) or two separate calls. It seems reasonable to hypothesize that there could be a gap interval shorter than a typical inter-call interval that would be sufficient to elicit the latter percept. We tested this idea by offering females a choice between two calls broadcast from one speaker (a 5-pulse call and a 13-pulse call separated by an intercall interval of 2 s (call midpoint-to-call midpoint) and a single 18-pulse call with a gap between the 5th and 6th pulses of 125 ms (equivalent to two missing pulses; test 6a, Fig. 6), 225 ms (equivalent to four missing pulses; test 6b) or 175 ms (equivalent to three missing pulses; test 6c) broadcast from the other speaker. We knew from previous tests that a 125 ms gap was sufficient to elicit discrimination when paired against a normal call without a gap of this duration. We found that although females significantly preferred the 125 ms gap stimulus in our test, they split evenly between the two-call stimulus and the gap stimulus when the gap was 175 ms or 225 ms. This suggests that there is a rather abrupt transition point at which an interval between pulse strings is treated as an intercall interval.

DISCUSSION

We emphasize that our behavioral results reveal intriguing correlates of the response properties of a subset of midbrain neurons rather than an air-tight linking of cause and effect. The ascending auditory pathway in the CNS of anurans contains neurons that exhibit a range of response properties (Rose and Gooler, 2007) and we also stress these are likely to shape the behavioral response of females to sounds to varying degrees. Indeed, much needs to be learned about the relative contribution of such cells to behavior and precisely how they, their associated neural circuits and combined responses influence phonotaxis to natural vocalizations (Gerhardt and Huber, 2002; Endepols et al., 2003). Our data are, however, clearly consistent with predictions based on the response properties of long-latency integrating neurons reported by Rose and colleagues (Edwards et al., 2002) as well as findings of Schul and Bush (Schul and Bush, 2002), in that they indicate the importance of appropriate IPIs in eliciting behavioral responses. Neural resetting of interval counting by inappropriate intervals, rather than cells simply not firing in response to such intervals, also appears to strongly influence

females. Integrating neurons may therefore, under some circumstances, exert a more powerful role in shaping behavior than short-latency units (referred to as ‘long-interval’ neurons) (Edwards et al., 2008) (formerly referred to as short-latency recovery type) (Rose and Gooler, 2007) that fire phasically following individual IPIs of appropriate (i.e. normal or potentially longer than normal) duration. A failure of females to discriminate in favor of the 75 ms gap-containing 21-pulse call over the 18-pulse normal call in test 2b supports this interpretation because the response of such phasic cells should have been, even with the presence of a single longer IPI, stronger to the 21-pulse call. Discrimination in test 2h, in which alternative stimuli differed only in the location of the abnormal gap, reinforces this view. In this test, the response of such phasic units to the stimulus alternatives should have been similar in contrast to the response of many integrating cells. The behavior of females in test 2h was also inconsistent with the hypothesis that the filter-like responses, *per se*, of neurons tuned to species-specific rates of AM (Rose et al., 1985) were most strongly influencing choice behavior. Pulse rates were identical in the stimulus alternatives and thus would not be expected to differentially affect such neural responses.

The results of test 3, in which the normal and anomalous call had the same number of pulses and the same mean IPI also support the hypothesis that what is being counted by at least a subset of neurons in the CNS of *H. versicolor* females is the correct IPIs rather than just the number of pulses within the particular integration time windows of counting neurons. Interestingly, Schul and Bush found in single-speaker tests (pulse duration=25 ms) that calls with a full set of IPIs similar to that of either the long or the short (abutting pulses) interval of our anomalous stimulus elicited phonotaxis (Schul and Bush, 2002). However, our test 3 used a call with a mix of intervals and involved discrimination with respect to a normal call alternative. All other anomalous stimuli with larger than normal gaps in our experiments fell outside the recognition space delimited by the results of Schul and Bush (Schul and Bush, 2002). Our results, including the choice by two females of the anomalous stimulus in test 3, and those of Schul and Bush (Schul and Bush, 2002) suggest that there are subsets of neurons with different resetting requirements. In fact, Edwards et al. report that their interval-counting neurons exhibit a range of resetting intervals. Some neurons were fairly labile with respect to their ‘tolerance’ of IPIs and an abnormally brief interval would not reset the counting process (Edwards et al., 2002). The tolerances of other neurons were much more constrained and would reset if an interval deviated only slightly from an optimal duration (e.g. by just 10 ms). Neural sensitivity to such fine-scale temporal changes could be the substrate for relatively sharp interval discrimination at the level of behavior.

Our data on *H. versicolor* indicate behavioral discrimination in response to IPI changes briefer than that accompanying the removal of 1 pulse (Schwartz et al., 2010) and thus are consistent with neurophysiological findings reported from *H. regilla* and *R. pipiens*. However, a major caveat in applying the results of Rose and colleagues to our findings is that our data and those published by the former group were obtained from different species. It would be naïve to assume that there are not species idiosyncrasies. These could occur at the neuronal level as well as be reflected in how neural responses contribute to behavior. For example, work on *H. regilla* and *R. pipiens* (Alder and Rose, 1998) found that interval duration but not pulse duty cycle were crucial to interval counting. In *H. versicolor* the single-speaker tests of Schul and Bush suggest that choice tests in which both pulse duration and interval duration are manipulated would be desirable (Schul and Bush, 2002). In fact, preliminary data from our laboratory indicate that a single longer

than normal pulse (125 ms) can reduce the relative attractiveness of a call, although the effect may not be as strong as that of an abnormally long interval. Another caveat is that the long-latency neurons of *H. regilla* and *R. pipiens* respond best to high pulse repetition rates (above 60 Hz) but not to those below 50 Hz. The mean AM rate of advertisement calls of *H. versicolor* is about 20 Hz (at 20°C). Clearly, continued neurophysiological studies of *H. versicolor* are warranted to further test for the presence and specific response properties of neurons like those described in the other species of frogs. However, given that *H. regilla* and *H. versicolor* are congeners in which pulsatile calls form an important component of the vocal repertoires, it is not surprising that integrating neurons are present in the midbrain of gray treefrogs (G. J. Rose, unpublished data). It would also not be surprising if the specific quantitative characteristics (e.g. best pulse repetition rate) of such units were shifted in *H. versicolor* relative to other species. In fact, interspecific differences in the sensitivities/selectivities of units to signal temporal features are known in the torus semicircularis and elsewhere in the auditory system (Edwards et al., 2005; Rose and Gooler, 2007).

Edwards et al. point out that the selectivity for correct IPIs in the counting neurons of *H. regilla* and *R. pipiens* could facilitate discrimination of advertisement from aggressive calls (Edwards et al., 2002). Such a contribution is supported by male responses during playbacks to *H. regilla* using synthetic calls incorporating alternating advertisement and encounter call interpulse (onset-to-onset) intervals (Rose and Brenowitz, 2002). What are the implications of our results for communication of gray treefrogs in the wild? Perhaps most significantly, they may provide a proximate-level explanation for the preference of longer calls relative to shorter calls even when the former are presented at a lower call rate (Fig. 1B). Previous research has also demonstrated that acoustic interference can have a negative impact on the relative attractiveness of a male's calls (Wells and Schwartz, 2007). This can come about (a) when the background din produced by the chorus at large masks (e.g. increases detection thresholds of) calls, and (b) when the vocalizations of neighboring individuals overlap and so disrupt the temporal structure of calls. Angular separation of a signaler and the source(s) of interference can assist receivers in detecting important temporal information within signals (e.g. Schwartz and Gerhardt, 1989; Schwartz and Gerhardt, 1995; Bee, 2007). However, when directional cues are inadequate to enable females to resolve overlapped calls as separate signals, the anomalous pulse intervals perceived may reset neurons that count. With the caveats mentioned in the previous paragraph in mind, our results suggest that a single short interval can trigger this resetting (test 1a) and that a single inserted pulse (thus, replacing one normal interval with two short intervals) can significantly reduce call attractiveness (test 1c). In choruses of the gray treefrog, call overlap would typically subject calls to considerably higher levels of temporal disruption (Schwartz et al., 2002) than mimicked in such test stimuli. In such cases, the degree of call overlap and the location of the disrupted section of the call will determine the magnitude of the impact on call relative attractiveness (Schwartz and Marshall, 2006).

Although anomalous intervals created by removing one (tests 2c and 2d) or more pulses [test 2g (Schwartz et al., 2010)] reduced call attractiveness, the location of the abnormal gap can sometimes make a difference. For example, a long gap near the middle of a call may be more detrimental than one near the beginning of a call (test 2h). A long gap near the end of the call was roughly equivalent to one near the beginning of a call [test 4; see Gerhardt et al. (Gerhardt et al., 2007) for examples in which location of a long gap

followed or led by an 'acoustic appendage' does make a difference]. With the reminder that we conducted just one test (5) of this type, we also found that shortening the interval between two pulses was equivalent to lengthening this interval (from 25 ms to 75 ms). Short intervals can occur as a result of call overlap; however, it is unlikely that calls incorporating brief (longer than normal) gaps are given frequently in nature. Although males may terminate a call and rapidly reinitiate it following an interruption, such responses were relatively rare during male–male interactions in choruses monitored in an artificial pond (Schwartz et al., 2002). What is particularly interesting is that an interval of just 175 ms was treated as equivalent to an intercall interval (test 6). However, pulse strings interrupted by 125 ms were treated differently (preferred) from pulse strings timed as two separate calls. 125 ms is the interval that would result from the removal of two pulses. This brief interval, however, was sufficient to reduce call attractiveness (as was a gap of 50 ms) (Schwartz et al., 2010). Within the limits of temporal resolution set by our experimental stimuli and our assessment paradigm, it could be that the perception of females is categorical. Categorical perception is a form of perception that has only recently (Baugh et al., 2008) been reported for anurans (see also Gerhardt, 1978; Ryan et al., 2003). Of course it is also possible that the call alternatives with gaps of 125 ms, 225 ms and 175 ms were all perceived as single calls and that the unattractiveness of the different gaps themselves influenced whether or not there was discrimination with respect to the separate calls offered as the alternative. Our results are, however, consistent with variation in the resetting properties across interval-counting neurons; integration may be reset in some cells after gaps of ~50–75 ms whereas others may require as much as 125–175 ms. However, we emphasize that determining whether this is so and whether perception in this temporal domain is truly categorical will require additional tests of behavior and neurophysiological investigation. Categorizing intervals and thus broken pulse strings as belonging to one longer or two shorter calls could sometimes help females evaluate the vocal performance of potential mates based on call duration. In addition, if males evaluate and categorize intervals (as females might), it could help males alternate calls during pair-wise interactions. It will therefore be important to conduct playback tests with males to determine whether this is the case.

Because the aggregations in which treefrogs often communicate may generate high-levels of ambient noise, it is interesting to note that a negative, albeit attenuated, effect of abnormal intervals on call preferences can arise even if anomalous calls incorporate or are overlapped by filtered noise (Schwartz et al., 2010). Nevertheless, additional work will be required to identify the range of naturalistic acoustic conditions under which neural interval-resetting and associated behavioral consequences occur.

ACKNOWLEDGEMENTS

This material is based upon work supported by the National Science Foundation under Grant No. 0342183. The handling and use of study animals was approved by the Pace University IACUC (Protocol No. 2004-1). We are grateful to Gary Rose, Carl Gerhardt and Martha Tobias for helpful discussions and specific feedback on the manuscript. All experiments complied with the 'Principles of Animal Care', publication No. 86-23, revised 1985 of the National Institute of Health, and also with the current laws of the United States.

REFERENCES

- Alder, T. B. and Rose, G. J. (1998). Long-term temporal integration in the anuran auditory system. *Nat. Neurosci.* **1**, 519–522.
- Baugh, A. T., Akre, K. L. and Ryan, M. J. (2008). Categorical perception of a natural, multivariate signal: Mating call recognition in túngara frogs. *Proc. Natl. Acad. Sci. USA* **105**, 8985–8988.
- Bee, M. A. (2007). Sound source segregation in grey treefrogs: spatial release from masking by the sound of a chorus. *Anim. Behav.* **74**, 549–558.

- Bush, S. L., Gerhardt, H. C. and Schul, J.** (2002). Pattern recognition and call preferences in treefrogs (Anura: Hylidae): a quantitative analysis using a no-choice paradigm. *Anim. Behav.* **63**, 7-14.
- Capranica, R. R. and Moffat, A. J. M.** (1983). Neurobehavioral correlates of sound communication in anurans. In *Advances in Vertebrate Neuroethology* (ed. J. P. Ewert, R. R. Capranica and D. J. Ingle), pp. 701-730. New York: Plenum Press.
- Diekamp, B. and Gerhardt, H. C.** (1995). Selective phonotaxis to advertisement calls in the gray treefrog, *Hyla versicolor*: behavioral experiments and neurophysiological correlates. *J. Comp. Physiol. A* **177**, 173-190.
- Edwards, C. J. and Rose, G. J.** (2003). Interval-integration underlies amplitude modulation band-suppression selectivity in the anuran midbrain. *J. Comp. Physiol. A* **189**, 907-914.
- Edwards, C. J., Alder, T. B. and Rose, G. J.** (2002). Auditory midbrain neurons that count. *Nat. Neurosci.* **5**, 934-936.
- Edwards, C. J., Alder, T. B. and Rose, G. J.** (2005). Pulse rise time but not duty cycle affects the temporal selectivity of neurons in the anuran midbrain that prefer slow AM rates. *J. Neurophysiol.* **93**, 1336-1341.
- Edwards, C. J., Leary, C. L. and Rose, G. J.** (2007). Counting on inhibition and rate-dependent excitation in the auditory system. *J. Neurosci.* **27**, 13384-13392.
- Edwards, C. J., Leary, C. L. and Rose, G. J.** (2008). Mechanisms of long-interval selectivity in midbrain auditory neurons: roles of excitation, inhibition, and plasticity. *J. Neurophysiol.* **100**, 3407-3416.
- Endepols, H., Feng, A. S., Gerhardt, H. C., Schul, J. and Walkowiak, W.** (2003). Roles of the auditory midbrain and thalamus in selective phonotaxis in female gray treefrogs (*Hyla versicolor*). *Behav. Brain Res.* **145**, 63-77.
- Feng, A. S. and Schellart, N. A. M.** (1999). Central auditory processing in fish and amphibians. In *Comparative Hearing: Fish and Amphibians* (ed. R. R. Fay and A. N. Popper), pp. 218-268. New York: Springer-Verlag.
- Gerhardt, H. C.** (1978). Discrimination of intermediate sounds in a synthetic call continuum by female green tree frogs. *Science* **199**, 1089-1091.
- Gerhardt, H. C.** (1981). Mating call recognition in the green treefrog (*Hyla cinerea*): Importance of two frequency bands as a function of sound pressure level. *J. Comp. Physiol.* **144**, 9-16.
- Gerhardt, H. C.** (2001). Acoustic communication in two groups of closely related treefrogs. *Adv. Study Behav.* **30**, 99-167.
- Gerhardt, H. C. and Huber, F.** (2002). *Acoustic Communication in Insects and Frogs: Common Problems and Diverse Solutions*. Chicago: University of Chicago Press.
- Gerhardt, H. C. and Schul, J.** (1999). A quantitative analysis of behavioral selectivity for pulse rise-time in the gray treefrog, *Hyla versicolor*. *J. Comp. Physiol. A* **185**, 33-40.
- Gerhardt, H. C., Dyson, M. L. and Tanner, S. D.** (1996). Dynamic acoustic properties of the advertisement calls of gray tree frogs: patterns of variability and female choice. *Behav. Ecol.* **7**, 7-18.
- Gerhardt, H. C., Tanner, S. D., Corrigan, C. M. and Walton, H. C.** (2000). Female preference functions based on call duration in the gray tree frog (*Hyla versicolor*). *Behav. Ecol.* **11**, 663-669.
- Gerhardt, H. C., Humfeld, S. C. and Marshall, V. T.** (2007). Temporal order and the evolution of complex signals. *Proc. R. Soc. Lond. B. Biol. Sci.* **274**, 1789-1794.
- Klump, G. M. and Gerhardt, H. C.** (1987). Use of non-arbitrary acoustic criteria in mate choice by female gray treefrogs. *Nature* **326**, 286-288.
- Konishi, M.** (2006). Behavioral guides for sensory neurophysiology. *J. Comp. Physiol. A* **192**, 671-676.
- Rose, G. L. and Brenowitz, E. A.** (2002). Pacific treefrogs use temporal integration to differentiate advertisement from encounter calls. *Anim. Behav.* **63**, 1183-1190.
- Rose, G. J. and Gooler, D. M.** (2007). Function of the amphibian auditory system. In *Hearing and Sound Communication in Amphibians* (ed. P. M. Narins, A. S. Feng, A. N. Popper and R. R. Fay), pp. 250-290. New York: Springer-Verlag.
- Rose, G. J., Brenowitz, E. A. and Capranica, R. R.** (1985). Species specificity and temperature and temperature dependency of temporal processing by the auditory midbrain of two species of treefrogs. *J. Comp. Physiol. A* **157**, 763-769.
- Ryan, M. J., Rand, W., Hurd, P. L., Phelps, S. M. and Rand, A. S.** (2003). Generalization in response to mate recognition signals. *Am. Nat.* **161**, 380-394.
- Schul, J. and Bush, S. L.** (2002). Non-parallel coevolution of sender and receiver in the acoustic communication system of treefrogs. *Proc. R. Soc. Lond. B. Biol. Sci.* **269**, 1847-1852.
- Schwartz, J. J. and Gerhardt, H. C.** (1989). Spatially-mediated release from masking in an anuran amphibian. *J. Comp. Physiol. A* **166**, 37-41.
- Schwartz, J. J. and Gerhardt, H. C.** (1995). Directionality of the auditory system and call pattern recognition during acoustic interference in the gray treefrog, *Hyla versicolor*. *Aud. Neurosci.* **1**, 195-206.
- Schwartz, J. J. and Marshall, V. T.** (2006). Forms of call overlap and their impact on advertisement call attractiveness to females of the gray treefrog, *Hyla versicolor*. *Bioacoustics* **16**, 39-56.
- Schwartz, J. J., Buchanan, B. and Gerhardt, H. C.** (2001). Female mate choice in the gray treefrog (*Hyla versicolor*) in three experimental environments. *Behav. Ecol. Sociobiol.* **49**, 443-455.
- Schwartz, J. J., Buchanan, B. and Gerhardt, H. C.** (2002). Acoustic interactions among male gray treefrogs (*Hyla versicolor*) in a chorus setting. *Behav. Ecol. Sociobiol.* **53**, 9-19.
- Schwartz, J. J., Brown, R., Turner, S., Dushaj, K. and Castano, M.** (2008). Interference risk and the function of dynamic shifts in calling in the gray treefrog (*Hyla versicolor*). *J. Comp. Psychol.* **122**, 283-288.
- Schwartz, J. J., Huth, K., Jones, S. H., Brown, R., Marks, J. and Yang, X.** (2010). Tests for call restoration during signal overlap in the Gray Treefrog, *Hyla versicolor*. *Bioacoustics* (in press).
- Simmons, A. M.** (2003). Perspectives and progress in animal acoustic communication. In *Acoustic Communication* (ed. A. M. Simmons, A. N. Popper and R. R. Fay), pp. 1-14. New York: Springer-Verlag.
- Wells, K. D.** (2007). *The Ecology and Behavior of Amphibians*. Chicago: University of Chicago Press.
- Wells, K. D. and Schwartz, J. J.** (2007). The behavioral ecology of anuran communication. In *Hearing and Sound Communication in Amphibians* (ed. P. M. Narins, A. S. Feng, A. N. Popper and R. R. Fay), pp. 44-86. New York: Springer-Verlag.