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Experience-based plasticity of acoustically evoked aggression in a territorial frog

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Abstract Territorial male bullfrogs (*Rana catesbeiana*) display lower levels of aggression toward familiar territorial neighbors compared to unfamiliar individuals based on the perception of individual differences in vocalizations. Stimulus-specific habituation elicited by repeated exposures to a neighbor's vocalizations is believed to play a role in mediating the low levels of aggression between neighbors. The present study describes habituation in multiple components of the bullfrog territorial aggressive response and examines whether these separate response components habituate at similar or different rates. In response to repeated broadcasts of synthetic bullfrog advertisement calls in a field playback experiment, the numbers of aggressive calls and movements, the latency to the first aggressive call, and the distance approached toward the playback speaker exhibited significant response decrements that developed at nearly identical rates. All four responses exhibited recovery upon subsequent broadcasts of a novel stimulus simulating a different individual. In contrast, the number of advertisement calls did not exhibit significant decrements with repeated playbacks. The patterns of stimulus-specific response decrement were inconsistent with explanations such as sensory adaptation, effector fatigue, non-specific changes in motivation, and a simple form of auditory habituation. Possible mechanisms for the observed plasticity in aggression are discussed in the context of anuran communication.

Keywords Acoustic communication · Behavioral plasticity · Habituation · *Rana catesbeiana* · Territorial aggression

Abbreviations *DFDL* different frog/different location · *DFSL* different frog/same location · *HP* habituation phase · *SFDL* same frog/different location · *SFSL* same frog/same location · *TP* test phase

Introduction

The simplest and most taxonomically widespread form of behavioral plasticity contingent upon experience is habituation, in which responses to a salient stimulus exhibit marked decrements to repeated or continuous stimulus presentations (Thorpe 1963; Hinde 1970; Macphail 1993). Response habituation is a centrally mediated process that can be observed in diverse behavioral response systems, from those mediated by simple reflex circuits (e.g., Groves and Thompson 1970; Carew 1984; Wiel et al. 2001), to more complex behaviors such as swimming (Brown 1998), predation (Peeke 1995), and predation-avoidance responses (Hinde 1954; Pedreira et al. 1995; Wyttenbach and Hoy 1997), as well as social behaviors such as courtship displays (Peeke and Figler 1997; Jenkins and Rowland 2000; Rowland 2000) and territorial aggression (Peeke 1984; Petrinovich 1984; Bee and Gerhardt 2001a). Not surprisingly, habituation remains a model phenomenon for studying the physiological bases of learning and memory in a variety of organisms (e.g., Rose and Rankin 2001; Wiel et al. 2001). Unfortunately, however, there is often little overlap between the simple response systems usually chosen for study by neuroscientists interested in elucidating the physiological mechanisms of habituation and the more complex response systems chosen by ethologists and behavioral ecologists interested in understanding habituation as an adaptive behavior (Worden 1973). Hence, important questions remain concerning how the physiological mechanisms elucidated from studies of relatively simple circuits, usually in invertebrates, might function in the habituation of more complex behaviors, such as vertebrate social behaviors (Macphail 1993). Clearly, an important first step toward

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understanding the relationships between learned changes in behaviors and their underlying neural bases is to fully characterize the learned changes in behavior.

One behavior that may offer opportunities to study the mechanisms of habituation within an evolutionary and ecological framework is territorial aggression (Peeke 1984). In territorial species, which occur in all classes of vertebrates, individuals establish and defend areas from which they exclude other conspecifics using stereotyped aggressive displays that often consist of separate visual, acoustic, and tactile elements. In many taxa, territory holders exhibit a form of social recognition in which they display lower levels of aggression toward their nearby territorial neighbors compared to unfamiliar conspecifics (reviewed in Ydenberg et al. 1988; Temeles 1994). This behavior, often referred to as “the dear enemy effect” or “neighbor recognition,” is often mediated by long-distance communication signals (e.g., birdsong, Stoddard 1996). Displays of relatively lower aggression toward established territorial neighbors are believed to function as an adaptation that allows territory holders to avoid the costs of fighting (e.g., increased risk of injury, increased energy expenditure, decreased mate attraction effort, or decreased vigilance against predators) that may be incurred during repeated aggressive interactions with individuals that pose little threat to territory ownership. Stimulus-specific habituation to an established neighbor or its communication signals is hypothesized to result in a decrease in aggressive responsiveness that would allow territory holders to exhibit low levels of aggression toward their nearby neighbors, while retaining the ability to respond aggressively to unfamiliar individuals that encroach upon their territory (Peeke 1984). Previous studies in fish (reviewed in Peeke 1984) and songbirds (reviewed in Petrinovich 1984), and more recent studies in frogs (Owen and Perrill 1998; Bee and Gerhardt 2001a, 2001c, 2002), provide support for this hypothesis.

Here I report results from a behavioral field study that investigated habituation of territorial aggression in the North American bullfrog (*Rana catesbeiana*, Anura, Ranidae). Territorial aggression in bullfrogs has been well characterized in a number of previous field studies (Emlen 1968, 1976; Wiewandt 1969; Howard 1978; Ryan 1980). During their breeding season, male bullfrogs establish territories in permanent bodies of water. From within their territories, males emit “advertisement calls” to attract gravid females and to repel rival males (Fig. 1A). In response to a perceived challenger, territorial males emit advertisement calls and a distinct aggressive vocalization termed an “encounter call” (Fig. 1B), leave their territory to engage the challenger, often by swimming distances of several meters, and display stereotyped aggressive movements, such as hops, jumps and lunges toward and onto an opponent. Importantly, these four types of behavior—advertisement calling, encounter calling, stereotyped movements, and approaches toward an opponent—constitute distinctly different responses to perceived challengers that

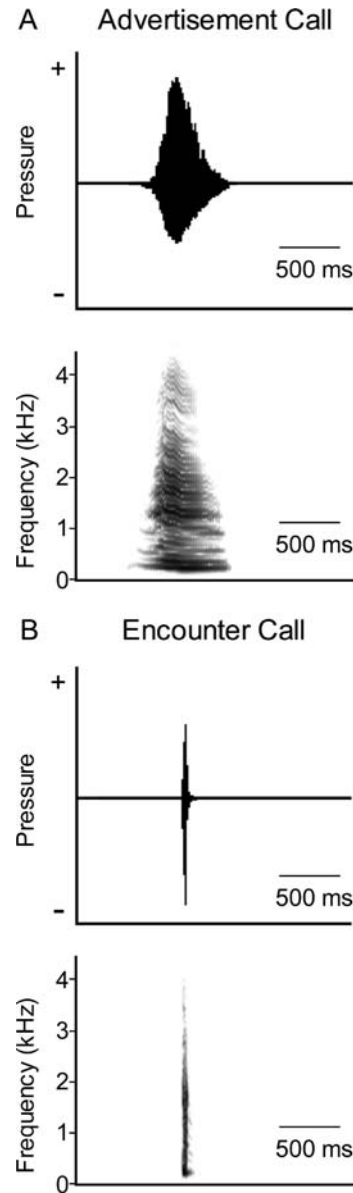


Fig. 1 Oscillograms (*top traces*) and sonograms (*bottom traces*) of **A** a one-note advertisement call and **B** an encounter call. *Horizontal bars* indicate the time scale

can occur independently of one another. That is, individuals are able to produce each of the response components alone without concurrently exhibiting the other behaviors (M.A. Bee, unpublished data). Field playback experiments have also established that territorial males respond aggressively toward a speaker broadcasting either natural, pre-recorded advertisement calls (Emlen 1968; Wiewandt 1969; Davis 1987) or computer-generated models of advertisement calls (Davis 1988; Bee and Gerhardt 2001a, 2001c, 2002).

Previous fieldwork with bullfrogs has also demonstrated that territorial males display lower levels of aggression in response to broadcasts of the vocalizations of familiar territorial neighbors from the direction of the neighbor’s territory compared to broadcasts of the

vocalizations of neighbors from novel locations or the vocalizations of unfamiliar individuals (Davis 1987). The role of habituation in mediating reduced levels of aggression between established neighbors in this species was investigated in a series of recent field studies (Bee 2001, 2002; Bee and Gerhardt 2001a, 2001c, 2002). These studies demonstrated that territorial aggression evoked by acoustic playbacks simulating a new territorial neighbor exhibits many of the characteristics typical of response habituation that were originally enumerated in the seminal review by Thompson and Spencer (1966). The properties of habituation demonstrated thus far for aggression in bullfrogs include exponential response decrements, spontaneous recovery, dishabituation, long-term retention, stimulus-specificity and stimulus-generalization, a direct relationship between stimulus repetition rate and the rate of habituation, and an inverse relationship between stimulus intensity and the rate of habituation (Bee 2001; Bee and Gerhardt 2001a, 2001c, 2002). More important, Bee and Gerhardt (2001a, 2001c) demonstrated that habituation of territorial aggression is specific to fine-temporal or spectral properties of advertisement calls that are individually distinct (Bee and Gerhardt 2001b). One important limitation of these studies, however, has been the use of multivariate statistical methods (e.g., principal components analysis) to measure the level of aggression as a composite measure based on a subset of several different response components (e.g., aggressive vocalizations, movements, tendency to approach the speaker). Such an approach ignores potentially important information about the rates of habituation in separate response components that may shed some light on the underlying mechanisms of changes in aggressive responsiveness.

Because social behaviors, such as territorial aggression, can involve multiple displays mediated by separate motor systems, one important question concerning the physiological basis of habituation in social behaviors is whether response decrements develop at similar or different rates in different response components. The purpose of the present study was to extend previous work with bullfrogs by investigating stimulus-specific habituation in different components of the territorial aggressive response. The primary aims of this study were (1) to describe the changes in separate components of bullfrog territorial behavior that occur with repeated stimulation by the advertisement calls of a simulated unfamiliar frog, (2) to determine whether these separate components exhibit similar patterns of stimulus specificity, and (3) to assess whether habituation of these separate response components develops at similar or different rates. I report results from a field playback experiment in which I repeatedly broadcast synthetic acoustic communication signals to territorial male bullfrogs simulating the arrival of a new neighbor on an adjacent territory. Thus, all of the sensorimotor pathways that mediate the separate components of aggressive responses shared the common sensory input of an acoustic stimulus. I examined response decrements, and the

stimulus-specificity of response decrements, in five different components of the bullfrog aggressive response: advertisement calling, aggressive calling, stereotyped aggressive movements, phonotaxis toward the sound source, and response latency. This study has an advantage over some previous laboratory studies of habituation in simpler response systems in that I repeatedly presented biologically meaningful stimuli at behaviorally relevant stimulus rates and intensities to freely behaving animals in their natural environment and recorded changes in multiple components of the elicited response.

Materials and methods

Playback experiments

Between 10 May and 1 August 2000 I conducted a field playback experiment in a set of experimental ponds at the Little Dixie Lake Conservation Area (Callaway County, Mo., USA) under ambient light conditions (see Bee and Gerhardt 2001a for additional details about the study site). Playback tests were conducted between 9:30 p.m. and 7:00 a.m., Central Daylight Time, and usually commenced between 10:00 p.m. and midnight each night after bullfrogs began actively calling. On a night prior to that of testing, each subject had been captured and individually marked for visual identification from distances of 2–3 m using the protocol described by Bee and Gerhardt (2001a).

Equipment

The digital-to-analogue output of portable notebook computers (Dell Inspiron 3500 or 5000, Dell Computer Corporation, Round Rock, Tex., USA) and battery-powered Rockford Fosgate 2.6× amplifiers (Rockford Corporation, Tempe, Ariz., USA) were used to broadcast acoustic stimuli through 25-cm Optimus speakers (Radio Shack Corporation, Fort Worth, Tex., USA) mounted in wooden boxes and floated on Styrofoam platforms covered in black plastic. The frequency response of each speaker was determined in a semi-anechoic sound chamber at the University of Missouri-Columbia and was flat (± 4 dB) over the range of frequencies in the stimuli. Playback levels were measured and calibrated in the field with a GenRad 1982 sound-level meter (GenRad, Westford, Mass., USA) or a Radio Shack sound level meter (Radio Shack Corporation, Fort Worth, Tex., USA) calibrated against the GenRad sound-level meter.

Stimuli

As a stimulus, I broadcast synthetic advertisement calls at a sound pressure level (SPL) of 87 dB measured at a distance of 1 m (re. 20 μ Pa, fast RMS, C-weighted), which reflects the upper end of the range of variation in the SPL of natural calls (M.A. Bee unpublished data; Megela-Simmons 1984). SPLs typically varied between 86 and 88 dB at 1 m, and between 70 and 72 dB at the frog's location at the start of a test. Stimuli were generated at a sampling rate of 20 kHz with 16-bit resolution using custom-designed software. All values of stimulus properties fell within the range of natural variation for this species (Bee and Gerhardt 2001b). A stimulus consisted of five consecutive advertisement calls separated by 30-s inter-call intervals (call duty cycle = 0.17; Fig. 2A). Each call within a stimulus consisted of five identical notes that were 700 ms in duration, had symmetrical linear rise and fall times of 300 ms duration, and were separated by 700-ms inter-note intervals (note duty cycle = 0.50). Each note within a call consisted of a series of ten harmonics (f_2 – f_4 and f_{10} – f_{16}) that were integer multiples of the fundamental frequency (f_1) and had the same starting phase

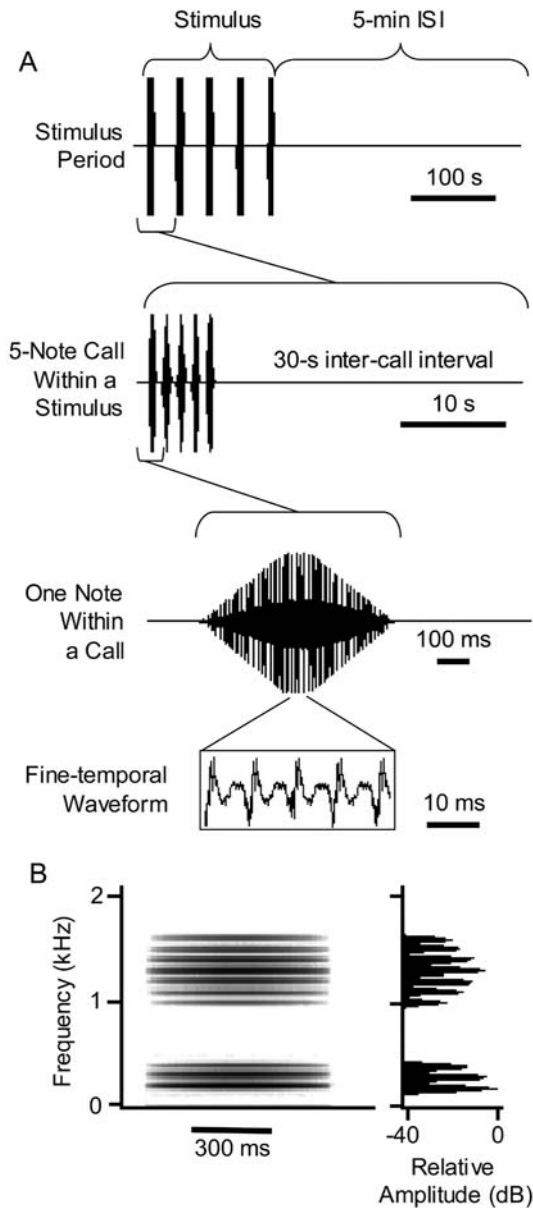


Fig. 2A,B Synthetic bullfrog advertisement calls used as stimuli in this study. **A** One “stimulus period” comprised of one “stimulus” with five consecutive 5-note calls followed by a 5-min “interstimulus interval” (ISI) (top); one 5-note call followed by the 30-s inter-call interval (middle); a single note from the stimulus (bottom) with inset showing the fine temporal waveform. During playback tests, the stimulus period shown in (A, top) was repeated as a continuous loop. **B** Sonogram (left) and power spectrum (right) of a single note from a stimulus used in playback tests ($f_1 = 95$ Hz). Horizontal bars indicate time scales

relationships of 0° (Fig. 2B). The dominant frequency was the second harmonic (f_2). All other harmonics were attenuated by 5–20 dB in relation to the dominant frequency in order to create a frequency spectrum representative of a bullfrog advertisement call. In order to compensate for small differences in the frequency responses of the speakers used in playback tests, the amplitude of each harmonic was appropriately adjusted using the synthesis software so that the resulting frequency spectra of the stimuli differed by 0–2 dB when broadcast through different speakers in a semi-anechoic environment. The fifth and final call of the stimulus

was followed by a silent interstimulus interval (ISI) of 5 min. The combination of the stimulus and the ISI was broadcast repeatedly as a continuous loop using the sound-editing software GoldWave 4.02. Together, a stimulus and the subsequent ISI constitute what is hereafter referred to as a “stimulus period”.

As a habituation stimulus, I broadcast one of two stimuli comprised of synthetic advertisement calls that simulated either a “large” bullfrog with a low fundamental frequency ($f_1 = 95$ Hz) or a “small” bullfrog with a high fundamental frequency ($f_1 = 125$ Hz). The intensity of aggressive responses and the rates of response decrement elicited by playbacks of these two stimuli do not differ (Bee 2002). Therefore, differences in response to the large and small simulated challengers were not examined here. Based on the regression of fundamental frequency on body size (Bee 2002), the large and small stimuli acoustically represented frogs with snout-vent-lengths of 177 mm and 117 mm, respectively. Subjects in this study had snout-vent lengths ranging from 115 mm to 169 mm (mean \pm SD = 141 ± 12.6 mm; population range = 109–181 mm; M.A. Bee, unpublished data).

Experimental protocol

The experiment consisted of a “habituation phase” (HP) followed immediately by a “test phase” (TP). During the HP, I simulated the arrival of a new territorial neighbor by broadcasting one of the two habituation stimuli differing only in fundamental frequency (and correlated spectral differences) from an unoccupied position adjacent to the subject’s territory. An equal number of subjects (total $n = 48$) heard a habituation stimulus that simulated either the large bullfrog ($n = 24$) or the small bullfrog ($n = 24$). These two stimuli were presented as the habituation stimulus to different frogs in random order (see below). I repeatedly broadcast the habituation stimulus for 30 stimulus periods during the HP, which was equivalent to approximately 3.75 h of repeated playbacks. During the TP, which lasted for three consecutive stimulus periods and began immediately after the end of the last stimulus period of the HP, I manipulated the location of the stimulus (same/different) and the “identity” of the stimulus (same/different) in a 2×2 factorial design. I manipulated location by presenting stimuli from a second speaker located in a novel position. Identity was manipulated by broadcasting the acoustic stimulus that was not used as the habituation stimulus during the HP. Previous work has shown that fundamental frequency is the most individually distinctive acoustic property of bullfrog vocalizations (Bee and Gerhardt 2001b). Hence, novel stimuli with different fundamental frequencies had particularly important biological relevance because they effectively simulated vocalizations by a different frog than that simulated by the habituation stimulus (Bee and Gerhardt 2001a, 2001c, 2002). The four treatment groups in the TP are denoted as follows: same frog/same location (SFSL, $n = 12$, control group), same frog/different location (SFDL, $n = 12$), different frog/same location (DFSL, $n = 12$), and different frog/different location (DFDL, $n = 12$). Playbacks occurred in six temporal blocks of eight randomly ordered tests. Both acoustic stimuli were used as the habituation stimulus four times in each block, once for each of the four experimental treatments in the TP (2 HP stimuli \times 4 TP treatments \times 6 temporal blocks = 48 subjects).

Prior to beginning a test, I placed speakers at a distance of 6 m from the subject along the pond bank. Typical distances between adjacent territorial males range between 3 and 16 m (Emlen 1976; M.A. Bee, unpublished data). I preferentially tested males that did not have nearest neighbors within 10 m in any direction to avoid interference from other territorial males. For control tests (SFSL) and tests involving only a change in frog identity (DFSL), a single speaker was placed in the direction of the subject’s most distant neighbor. If there were no nearby neighbors (< 15 –20 m away), speaker position (left or right relative to the frog) was randomly determined. For tests involving changes in location (SFDL and DFDL), two speakers were placed on opposite sides of the frog (12 m and 180° apart), and the speaker used during the HP was placed in the direction of the subject’s closest neighbor. Once

subjects resumed normal calling, I waited 10–20 min before beginning the HP.

Data analysis

During each stimulus period of the HP and TP, one or two observers seated near the pond bank (usually < 5 m from the subject at all times) counted the numbers of (1) advertisement calls, (2) encounter calls, and (3) stereotyped aggressive movements, (4) determined with a stop watch the latency to the first encounter call produced in each stimulus period, measured from the beginning of the stimulus period, and (5) determined the subject's closest approach toward the speaker (to the nearest 0.5 m) from its original position at the start of the HP (0 m = at the original position; 6 m = at the speaker). For the analyses described below, if no encounter call was produced during a stimulus period I assigned a latency value of 452 s, which was equivalent to the duration of the stimulus period.

Four frogs were excluded from statistical analyses because they never responded to the stimulus by approaching the playback speaker (two subjects each in the SFSL and DFDL groups). Separately for each of the 44 remaining subjects, I standardized the values for advertisement calls, encounter calls, response latency, movements, and approach distance as a percentage of the subject's maximum response for each variable in any stimulus period during the HP (maximum value = 100%). Before standardizing response latency, I expressed latency values as $[1 - (\text{latency}/452)]$, so that increases in response latency would be reflected as a decrease in response magnitude. Percentage values were log-transformed [$Y' = \log_{10}(Y+1)$] to improve linearity (Zar 1984). I used linear regression to examine the relationships between the stimulus period number during the HP and the mean magnitude of each standardized and log-transformed response variable averaged over all 44 frogs (Petrinovich and Widaman 1984). To compare the rates of response decrements among the five response variables, I separately regressed the log-transformed percentages from each frog on the repetition number of the stimulus period during the HP (1–30). I compared the slopes of the best-fit regression lines for the response variables within individuals using a large sample approximation of Friedman's repeated measures analysis of variance by ranks. I determined whether response magnitudes at the end of the HP were different from those at the beginning of the HP by comparing initial responses (averaged over stimulus periods 1–3) to the ending responses (averaged over stimulus periods 28–30). To examine the stimulus specificity of habituation in separate response components, and also to verify that changes in response magnitudes during the HP were not the result of sensory adaptation or effector fatigue, I compared the magnitude of responses during the TP (averaged over all three stimulus periods of the TP) to the magnitude of responses at the end of the HP (averaged over stimulus periods 28–30). To determine whether the magnitudes of responses in the TP differed from initial response magnitudes, I compared the average response during the TP to the initial response during the HP (averaged over stimulus periods 1–3). Analyses of initial HP, ending HP, and TP responses were performed separately for the four treatments in the TP using Wilcoxon's matched-pairs tests to compare the standardized raw response magnitudes (i.e., the untransformed percentages based on maximum responses). Because these tests involved two comparisons each for initial HP, ending HP and TP responses, I used a significance criterion of $\alpha = 0.025$ in these tests. For all other tests, I used a significance criterion of $\alpha = 0.05$. All statistical analyses were performed using Statistica 5.5 (Statsoft 2000).

Results

Prior to the beginning of playback tests, the vast majority of males produced exclusively advertisement

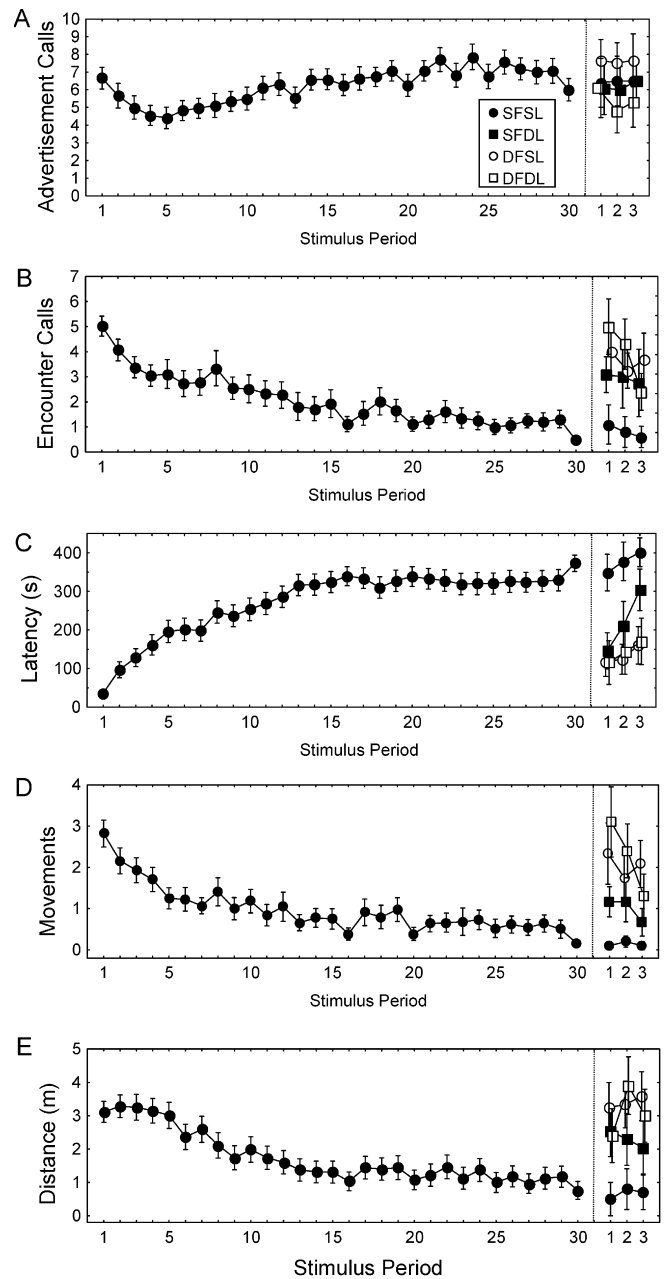


Fig. 3A–E Changes in multiple components of the aggressive response with repeated playbacks during the 30 stimulus periods of the habituation phase (HP) and the three stimulus periods of the test phase (TP). **A** advertisement calls; **B** encounter calls; **C** latency to the first encounter call; **D** stereotyped aggressive movements; and **E** maximum approach toward the playback speaker. During the 30 stimulus periods of the HP, points depict mean (\pm SE) responses during each stimulus period averaged over 44 subjects. See legend in **A** for a description of symbols for the 3 stimulus periods of the test phase (TP); points for the TP depict mean (\pm SE) responses for subjects in the same frog/same location (SFSL), same frog/different location (SFDL), different frog/same location (DFSL), and different frog/different location (DFDL) groups (see text for sample sizes)

calls and remained within a small area (e.g., < 0.5 m²) at their originally identified calling site. Only rarely were encounter calls and movements observed prior to the

Fig. 4 A Response magnitudes for the numbers of advertisement calls and encounter calls, the latency to the first encounter call, the number of aggressive movements, and the maximum approach distance. Each variable was standardized by the maximum responses during the HP. See legend for a description of symbols. Points depict the means (\pm SE) averaged over 44 subjects. **B, C** Regression slopes calculated over all 30 stimulus periods (**B**), and over the first 15 stimulus periods (**C**), of the HP. The graphs depict the medians (line), interquartile range (box), and the 10th and 90th percentiles (bars). *AC* advertisement calls, *EC* encounter calls, *L* response latency, *M* aggressive movements, *D* approach distance

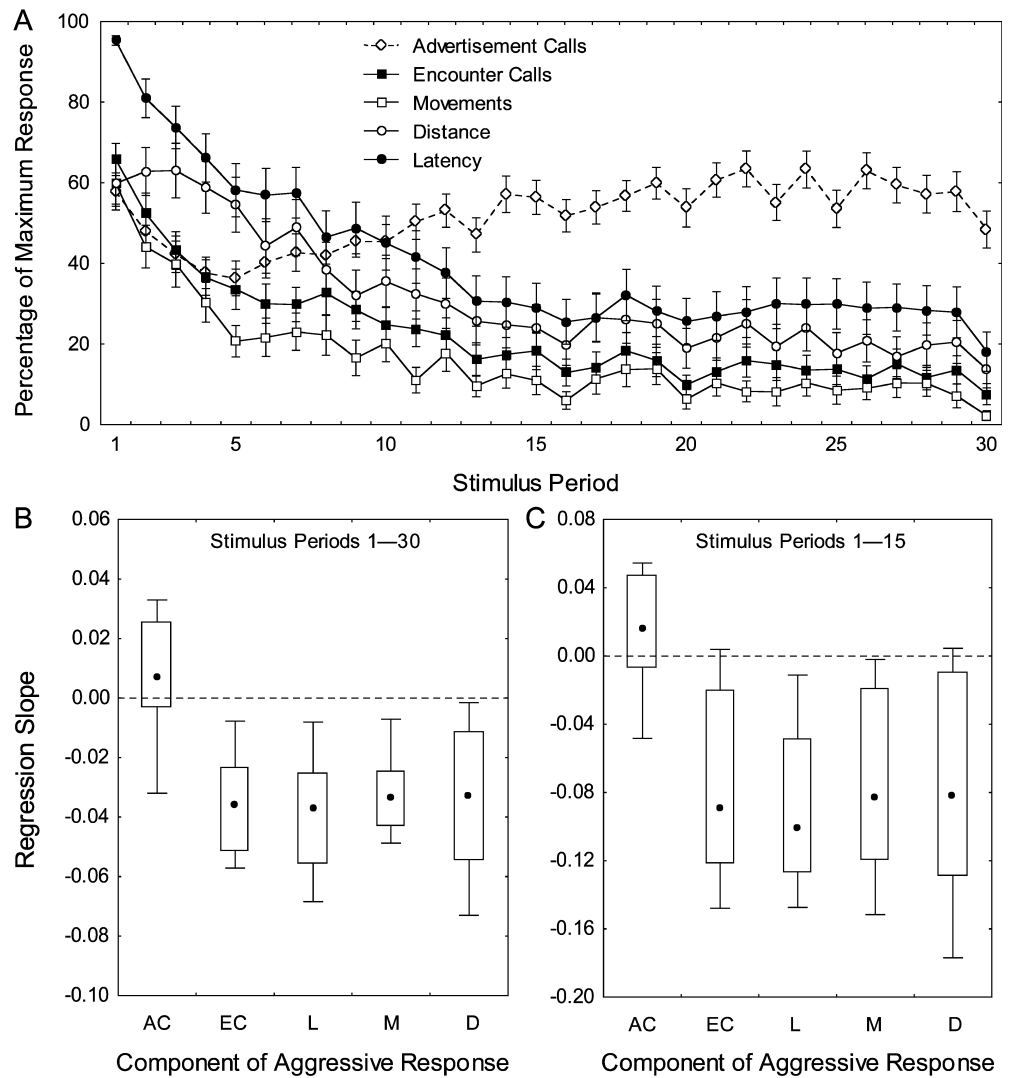


Table 1 Results from regression analyses of the standardized and log-transformed response variables averaged over 44 subjects. Results are shown separately for regressions on all 30 stimulus periods and the first 15 stimulus periods of the habituation phase (HP)

Response variable	Stimulus periods 1–30				Stimulus periods 1–15			
	$F_{1,28}$	P	β^a	R^2	$F_{1,13}$	P	β^a	R^2
Advertisement calls	10.3	0.0033	0.52	0.27	2.5	0.1359	-0.40	0.16
Encounter calls	90.4	<0.0001	-0.87	0.76	151.2	<0.0001	-0.96	0.92
Response latency	87.2	<0.0001	-0.87	0.76	220.6	<0.0001	-0.97	0.94
Aggressive movements	60.6	<0.0001	-0.83	0.68	66.5	<0.0001	-0.91	0.84
Approach distance	112.0	<0.0001	-0.89	0.80	242.0	<0.0001	-0.97	0.95

^aStandardized regression coefficient

onset of the acoustic stimulus, and these were never directed toward the silent playback speaker. In response to broadcasts of the stimulus, frogs oriented toward the speaker, produced advertisement calls, encounter calls, approached the speaker, and exhibited stereotyped aggressive movements. Frogs often repeatedly charged toward the speaker during the broadcast of the stimulus and returned to their original calling site during a subsequent ISI. Similar aggressive behavior was described

by Emlen (1968), Wiewandt (1969), and Ryan (1980). With repeated broadcasts of the habituation stimulus, aggressive responsiveness declined and the majority of frogs eventually returned to within 0.5 m of their original calling position at the start of the HP and resumed producing exclusively advertisement calls.

During the HP, the number of advertisement calls initially decreased and subsequently increased with repeated stimulation, while the numbers of encounter calls

Table 2 Mean Pearson product-moment correlations (r) between aggressive response variables during the 30 stimulus periods of the HP averaged over the correlations generated separately for each of the 44 subjects

Response variable	Response variable		
	Response latency	Aggressive movements	Approach distance
Encounter calls	-0.77*	0.69*	0.68*
Response latency		-0.58*	-0.60*
Aggressive movements			0.63*

* $P < 0.05$

and aggressive movements, and the maximum distance approached toward the playback speaker exhibited marked decrements, and the latency to the first encounter call increased (Figs. 3 and 4A). The regression analyses of the standardized and log-transformed response variables averaged over all 44 frogs are summarized in Table 1. There was an overall significant positive relationship between stimulus period number (1–30) and the number of advertisement calls. There were significant negative relationships between stimulus period number (1–30) and the magnitude of response strength for encounter calls, response latency, movements, and approach distance. The magnitude of the four response variables that exhibited marked declines during the HP reached an asymptote after about 15 stimulus periods (Figs. 3 and 4A). Regression analyses indicated even stronger negative relationships between response magnitude and stimulus period number over the first 15 stimulus periods for encounter calls, response latency, movements, and approach distance. Linear regression did not reveal any significant effect of stimulus period number on the number of advertisement calls during the first 15 stimulus periods.

Table 3 Results of statistical tests (P values shown) comparing response magnitudes at the beginning of the HP (Initial HP), the end of the HP (Ending HP), and in the test phase (TP)

TP stimulus	Response variable	Wilcoxon's matched pairs tests		
		Initial HP versus Ending HP	Initial HP versus TP	Ending HP versus TP
SFSL	Advertisement calls	0.9594	0.8785	0.3743
	Encounter calls	0.0051*	0.0069*	0.7532
	Response latency	0.0051*	0.0069*	0.7532
	Aggressive movements	0.0117*	0.0244*	0.2851
	Approach distance	0.0117*	0.0173*	— ^a
SFDL	Advertisement calls	0.7537	0.7989	0.8589
	Encounter calls	0.0022*	0.6836	0.0367**
	Response latency	0.0022*	0.0037*	0.0619
	Aggressive movements	0.0033*	0.0077*	0.1282
	Approach distance	0.0022*	0.1095	0.1235
DFSL	Advertisement calls	0.1095	0.1823	0.8589
	Encounter calls	0.0022*	0.8445	0.0022*
	Response latency	0.0022*	0.2132	0.0022*
	Aggressive movements	0.0022*	0.7897	0.0059*
	Approach distance	0.0254**	0.9292	0.0663
DFDL	Advertisement calls	0.8590	0.2845	0.4446
	Encounter calls	0.0051*	0.9594	0.0284**
	Response latency	0.0051*	0.9594	0.0469**
	Aggressive movements	0.0069*	0.9188	0.0173*
	Approach distance	0.0069*	0.9188	0.0251**

* $P < 0.025$; ** $P < 0.05$)

^a AP value could not be calculated due to ties for 9 of the 10 subjects in the SFSL treatment. Eight of these 9 subjects were calling from their original location at the Ending HP and did not approach the speaker during the TP
DFDL different frog/different location, DFSL different frog/same location, SFDL same frog/different location, SFSL same frog/same location

The decreases that occurred in the numbers of encounter calls and movements, response latency, and approach distance developed at similar rates, and the rates of change in all four response variables differed from those of advertisement calls (Fig. 4). A comparison of regression slopes from individual frogs across all five response variables revealed a significant difference when slopes were calculated across stimulus periods 1–30 ($\chi^2 = 62.75$, $P < 0.01$, $df = 4$, $n = 44$, Fig. 4B) and stimulus periods 1–15 ($\chi^2 = 48.34$, $P < 0.01$, $df = 4$, $n = 43$, Fig. 4C). These differences can be attributed to the fact that slopes for advertisement calls were positive, while those for the other response variables were negative. A comparison of slopes for encounter calls, response latency, movements, and approach distance indicated that all four of these response variables habituated at similar rates across periods 1–30 ($\chi^2 = 4.42$, $P = 0.22$, $df = 3$, $n = 44$, Fig. 4B), and across stimulus periods 1–15 ($\chi^2 = 6.63$, $P = 0.10$, $df = 3$, $n = 43$, Fig. 4C). (One additional frog was excluded from the analyses of stimulus periods 1–15 because it approached all the way to speaker during the first stimulus period and did not return to its calling site during the first 15 stimulus periods.) Not surprisingly, there were strong correlations between the raw magnitudes of encounter calls, response latency, movements, and approach distance across the 30 stimulus periods of the HP (Table 2). The numbers of encounter calls and stereotyped movements and approach distance were positively intercorrelated; all three of these responses were negatively correlated with response latency.

The decrements that occurred during the HP were significant, or nearly significant ($P = 0.0254$), for encounter calls, response latency, movements, and approach distance in all four groups that received different treatments during the TP (Table 3, Initial HP versus

Ending HP). However, in no case was there a significant difference in the number of advertisement calls between the initial and ending responses during the HP. In the group of subjects that experienced the same stimulus during the HP and the TP (the SFSL control group), there was no significant recovery during the TP in any of the response variables measured, and the response magnitudes for encounter calls, response latency, movements, and approach distance during the TP were significantly lower than the initial responses during the HP (Fig. 3 and Table 3). Hence, no recovery was observed in the TP when the same stimulus was broadcast in the HP and the TP. In contrast, for subjects in the SFDL, DFSL, and DF DL groups, in which subjects were presented with a novel stimulus during the TP, responses exhibited recovery (Fig. 3, Table 3). For subjects in the SFDL group, which heard the habituation stimulus broadcast from a novel location, response recovery was intermediate between the control group (SFSL) and the groups that heard a different acoustic stimulus during the TP (DFSL and DF DL). The intermediate degree of recovery is indicated by the marginally significant results for encounter calls ($P=0.0367$) and response latency ($P=0.0619$) in comparisons of responses in the TP to the end of the HP. In addition, the magnitudes of responses for encounter calls and approach distance during the TP were not significantly different from initial response magnitudes in the HP, even though both of these responses exhibited significant response decrements during the HP, while response latency and the number of movements were significantly lower in the TP compared to the beginning of the HP. In the two groups that heard a novel acoustic stimulus in either the original location (DFSL) or a novel location (DF DL), most response variables exhibited significant ($P<0.025$) or marginally significant ($P<0.05$) increases during the TP compared to the end of the HP (Fig. 3, Table 3). The single exception was the tendency of frogs to approach the speaker in response to the DFSL treatment. While on average the frogs in this group approached more closely to the speaker during the TP than at the end of the HP (Fig. 3E), this trend was not significant ($P=0.0663$). In both the DFSL and DF DL groups, the response magnitudes for encounter calls, response latency, movements, and approach distance in the TP were not significantly different from those at the beginning of the HP.

Discussion

The number of advertisement calls evoked during the initial periods of the HP started high and decreased during the first five stimulus periods (= 37.5 min), but subsequently increased over the remainder of the HP. These data reflect the fact that steady calling activity began around 9:00–10:00 p.m., increased in intensity over the next several hours, reaching a peak between

1:00 a.m. and 3:00 a.m., and then steadily declined until dawn (M.A. Bee, unpublished data). Hence, the playbacks initially evoked advertisement calling from subjects when the overall level of advertisement calling in the chorus was low, and this evoked vocal response showed initial signs of response decrement, but advertisement calling later increased as the overall calling activity in the chorus increased. These results reflect the presumed dual function of advertisement calls, which are likely used to attract females and repel rival males. These results are also in agreement with findings from a previous study of habituation in bullfrogs, in which Megela and Capranica (1983) demonstrated habituation, dishabituation, and spontaneous recovery of evoked advertisement calling when a naturally recorded advertisement call was repeatedly presented to two males housed in a laboratory colony. In that study, evoked advertisement calling exhibited marked decrements within 10 min of repeated stimulation. In a more natural setting, such as the chorus environment in which the present study was conducted, and over a much longer period of stimulation (nearly 4 h), I was unable to demonstrate that advertisement calling exhibits exponential response decay and response recovery elicited by novel stimuli.

In contrast to advertisement calling, the number of encounter calls, the latency to the first encounter call, the number of stereotyped aggressive movements, and the tendency to approach the playback speaker, exhibited significant response decrements with repeated stimulation during the HP. Moreover, these response measures exhibited recovery during the TP. Responses to the novel stimulus simulating the same frog in a novel location (SF DL) exhibited moderate levels of response recovery in the TP. In response to the novel acoustic stimuli simulating a different frog (DFSL and DF DL), the magnitudes of responses during the TP were similar to those elicited during the initial periods of the HP, suggesting that responses had fully recovered. These results clearly rule out effector fatigue as an explanation for the observed response decrements.

The present study extends previous work with bullfrogs (Megela and Capranica 1983; Bee 2001, 2002; Bee and Gerhardt 2001a, 2001c, 2002) by examining in detail how separate components of an aggressive territorial response change with repeated stimulation. My results indicate that encounter calling (both the number of calls and call latency) and stereotyped aggressive movements in response to synthetic advertisement calls, as well as the tendency to approach the sound source, exhibit characteristics of stimulus-specific habituation. All of these response measures reached asymptotic levels in about 15 stimulus periods (Figs. 3 and 4A), which was equivalent to nearly 2 h of repeated stimulation, and the magnitudes of these responses exhibited highly significant correlations across the 30 stimulus periods of the HP (Table 2). More important, my results indicate that response decrements develop at the same rate in these separate response measures (Fig. 4). An important

question, then, is what sorts of physiological mechanisms might account for the similarity in the rates of habituation of different response components?

Behavioral data and neurophysiological recordings from the bullfrog auditory nerve can rule out the hypothesis that the changes that underlie the response habituation reported here occur in the auditory periphery, for example in the form of sensory adaptation or selective “gating” by afferent inhibition (Worden 1973). In bullfrogs, spectrally complex communication signals are reliably encoded in the auditory nerve by a temporal code of action potentials that phase-lock to peaks in the repeated fine-temporal waveform of the advertisement call (Schwartz and Simmons 1990; Simmons et al. 1992, 1993; Simmons and Ferragamo 1993). Phase-locking is usually strongest to the fundamental frequency, even in the presence of background noise or when the fundamental frequency is absent from the frequency spectrum. Importantly, the harmonic fine-structure of the call is not reflected in the profile of spike discharge rates, and units arising in both the amphibian papilla and the basilar papilla continue to phase-lock to the fundamental or other low-frequency peaks when higher harmonic components of the stimulus are near the fiber’s best excitatory frequency. These data suggest that the bullfrog auditory periphery encodes spectral information using a temporal code and not a rate-place code. In addition, Megela and Capranica (1983) reported that single units in the auditory nerve showed no evidence of response decrements when repeated series of 750-ms tone bursts at the unit’s best frequency and 10 dB above threshold were presented at a rate of 1 tones per second. It seems unlikely that response depression in 8th nerve fibers might be observed at much slower stimulus repetition rates similar to those used in the present study. In fact, we can be sure that information about the fundamental frequency of a stimulus continues to be reliably encoded in the auditory periphery following repeated stimulus exposures because large differences between the fundamental frequencies of the habituation and novel stimuli elicited significant response recovery during the TP in the present study (Fig. 3, Table 3). Previously, Bee and Gerhardt (2001a, 2001c) reported that changes in fundamental frequency as small as 5–10% were sufficient to elicit response recovery.

A second hypothesis for why separate components of the aggressive response might habituate at the same rate is that response decrements resulted from non-specific changes in an animal’s motivational state. This hypothesis predicts that following repeated stimulus exposures, subject’s should be less responsive to calling by any conspecific individual and not just toward that individual simulated by playbacks during the HP. Two of the treatments in the TP were explicitly designed to test this prediction. In the DFSL and DFDL conditions of the TP, I presented a novel stimulus that acoustically simulated a different individual (Bee and Gerhardt 2001b). The habituated responses of males in these groups were stimulus-specific and recovered during the

TP to levels that were not significantly different from those elicited at the beginning of the HP (Fig. 3, Table 3). These data exclude the hypothesis that similarities in the rates of habituation of different response components resulted from non-specific or stimulus-general changes in overall arousal or aggressive motivation during the HP. The fact that advertisement calling did not habituate during the HP is also consistent with the conclusion that habituation in other response variables was not the result of decreases in overall arousal.

The finding that non-specific changes in motivation cannot explain aggressive response decrements in bullfrogs is consistent with data from recent field playback studies of plasticity of aggression in two other frog species, the Pacific treefrog (*Hyla regilla*, reviewed in Brenowitz et al. 2001) and the spring peeper (*Pseudacris crucifer*, Marshall et al. 2003). In both species, a male’s motivation to respond aggressively to a stimulus was assessed by measuring the stimulus amplitude required to elicit aggressive calling. Repeated broadcasts of the advertisement calls of a new neighbor above a subject’s “aggressive threshold” led to significant increases in these thresholds, which subsequently returned to baseline levels after short retention intervals without stimulation (i.e., exhibited spontaneous recovery). With repeated stimulus presentations, aggressive responses exhibited decrements characteristic of response habituation. For both species, the decrease in the magnitude of aggressive responses is specific to the advertisement call; presentations of an aggressive signal are able to elicit renewed aggressive signaling (Brenowitz et al. 2001; M.A. Bee, S.C. Humfeld and V.T. Marshall, unpublished data). In addition, for Pacific treefrogs the elevated aggressive thresholds themselves exhibit stimulus-specificity to the type of call that is broadcast (Brenowitz et al. 2001). Aggressive thresholds were increased for the specific type of call used as a repeated stimulus (either advertisement calls or aggressive calls) and aggressive thresholds for responding to the other type of call remained largely unaffected. Thus, changes in the aggressive thresholds of Pacific treefrogs do not reflect non-specific changes in motivation, but rather are specific to the signaling context (i.e., either advertisement calling or aggressive calling by a neighbor). What is not yet known for these species is how males respond to a second calling individual in the vicinity. It will also be important to investigate the stimulus specificity of shifts in aggressive thresholds in bullfrogs.

A third hypothesis for why four of the response measures habituated at the same rate is that decreases in acoustically-mediated responses occur as a result of auditory habituation, or decreases in acoustically-evoked neural responses in the auditory system (reviewed in Worden 1973). In addition to investigating auditory habituation in bullfrog eighth nerve fibers, Megela and Capranica (1983) examined neural habituation to acoustic stimuli in two additional loci in the bullfrog’s ascending auditory pathway, the principal nucleus of the torus semicircularis and the dorsal

thalamus. Using two-tone combination stimuli, they found that evoked potentials in the torus semicircularis exhibited significant habituation at a stimulus repetition rate of 1/s, but not at slower rates of 1 stimulus presented every 2, 3, or 5 s. Evoked potentials recorded in the central nucleus of the dorsal thalamus habituated at rates of 1 stimulus every 3, 5, and 10 s, but not at rates as slow as 1 stimulus every 20 or 30s. On the one hand, these data clearly indicate that decreases in neural responsiveness in the bullfrog's ascending auditory pathway can result from repeated exposures to acoustic stimuli. On the other hand, however, these data suggest that auditory responses to two-tone combination stimuli in the torus semicircularis and the dorsal thalamus do not exhibit habituation at slower stimulus repetition rates similar to the stimulus rates used in the present study. While the consecutive notes in a bullfrog advertisement call, which consist of 1–22 notes (typically 4–6), can occur at rates as fast as one note per second (Bee and Gerhardt 2001b), consecutive calls are separated by much longer intervals that can range from 20–30 s during periods of very active calling (Emlen 1976), to intervals of 5–15 min or longer during periods of less active calling (M.A. Bee, unpublished data). Indeed, Megela and Capranica (1983) have argued that male bullfrogs produce advertisement calls at rates that allow them to avoid auditory habituation. Hence, it is unclear to what extent neural habituation in the ascending auditory system, which appears to occur only at relatively fast stimulus rates compared to normal rates of acoustic signaling in bullfrogs, might be able to account for habituation of acoustically-evoked aggressive responses. Furthermore, the finding reported here and elsewhere (Bee and Gerhardt 2001c, 2002), that presentation of the same habituation stimulus from a novel location in a test phase results in recovery of aggressive responsiveness, argues against the hypothesis that response decrements result from a simple form of neural habituation in the ascending auditory system.

The hypothesis that aggressive responses evoked by the vocalizations of a neighbor habituate as a result of decrements in neural processing by the auditory system also appears inconsistent with results from a number of studies on selective attention in frogs. Studies of signal timing interactions among anurans demonstrate that male frogs usually pay attention to the signaling behavior of a small number of their closest or loudest neighbors and attempt to time their own signals in relation to those of their neighbors (Brush and Narins 1989; Schwartz 1993; Greenfield and Rand 2000). In a recent study of bullfrog chorusing behavior, Boatright-Horowitz et al. (2000) demonstrated that male bullfrogs attend to the calls of both nearby and more distant neighbors, and suggested that males might adjust the timing of their own calls in order to avoid producing calls that follow those of a nearby neighbor too closely in time. In addition, Boatright-Horowitz et al. (2000) showed that males produced calls following those of a nearby neighbor significantly less often than expected by

chance. This result is consistent with a mechanism of selective inhibition by a neighbor's calls, and appears inconsistent with the hypothesis of simple auditory habituation, which would predict that males should produce calls following those of a nearby neighbor at chance levels. Together, the data on signal timing interactions in anurans indicate that, in fact, vocalizing males both hear and attend to the calls of their nearby neighbors. These findings appear inconsistent with the hypothesis that the auditory system simply habituates to the calls of a neighbor as a result of repeated exposures.

Based on presently available data, what differentiates bullfrogs from other frogs is that changes in aggressive responsiveness to repeated stimulation have been demonstrated to be long-term (lasting between nights of chorusing) and specific to both the stimulus fundamental frequency, which is an individually distinctive acoustic property of advertisement calls, and to the location of the sound source (Bee and Gerhardt 2001a, 2001b, 2001c, 2002). The present study extends these findings by demonstrating that separate components of the territorial aggressive response habituate at the same rate. These patterns of response decrement and stimulus-specific recovery are inconsistent with explanations such as sensory adaptation, effector fatigue, non-specific changes in motivation, and auditory habituation. However, these data are consistent with the hypothesis that bullfrogs are able to form an enduring representation of a neighbor's calls that is associated with information about the location of the neighbor's territory. This hypothesis is also consistent with an influential comparator theory of habituation (Wagner 1976, 1979), which postulates that long-term habituation is mediated by the formation of associations between a representation of the stimulus (e.g., the neighbor's vocalizations) and salient contextual stimuli (e.g., the location of the neighbor's territory). Of course, in real territorial encounters, we might expect that male bullfrogs learn considerably more about their neighbors than just the sound of the neighbor's voice and the location of its territory; for example, males likely learn about the size and fighting ability of a new neighbor and retain information about previous encounters with the neighbor.

Three important questions that should be addressed in future behavioral and neurophysiological studies of bullfrogs are: (1) how does the nervous system encode information about the acoustic properties of the advertisement calls of other individuals that are heard repeatedly over behaviorally relevant intervals?; (2) how is information about sound source location encoded in the nervous system?; and (3) how do these sources of encoded information interact with response systems that mediate aggressive interactions? Recent work is beginning to elucidate the neural pathways and mechanisms in the anuran midbrain that integrate auditory information with acoustically guided behaviors, such as evoked vocalizations and phonotaxis (Walkowiak and Luksch 1994; Luksch and Walkowiak 1998; Endepols

and Walkowiak 1999; Walkowiak et al. 1999). Future studies that use acoustic communication signals presented at species-typical calling rates to examine neural plasticity in the ascending auditory system and centers of audio-motor integration that control acoustically evoked motor responses may provide for a better understanding of the physiological basis of experience-based plasticity in the acoustically evoked behavior of anurans. Indeed, a better understanding of both the evolution of behavioral plasticity and its physiological bases will be achieved by examining plasticity in a number of different taxonomic groups and behavioral response systems, including complex social behaviors. The analysis of changes in territorial aggression that occur as a result of repeated exposures to conspecific communication signals represents one such potentially informative response system.

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