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Mate recognition in túngara frogs: a review of some studies of brain, behavior, and evolution*

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Abstract Animal communication systems have been a focus of studies in species recognition, sexual selection, and neuroethology. We and our colleagues have conducted a variety of studies of sexual communication in the túngara frog (*Physalaemus pustulosus*), and we review some of these studies here. Our general approach has been integrative. We investigate the functional significance of the communication system, the neural mechanisms underlying the system, and major patterns of its evolution. Specifically, we have shown how the males' simple (whine-only) and complex (whine plus chucks) calls are used by females to recognize the correct species for mating, and how they lead females to choose among conspecific males. We have identified a major counter-selection force on complex calls, predation by the frog-eating bat (*Trachops cirrhosus*), as well as energetic costs of calling. We have shown the specific features of the call that are critical to elicit female responses, and have made some progress in identifying neural mechanisms underlying call perception. Our comparative studies have shown that preferences for complex calls existed prior to the evolution of complex calls. This supports the hypothesis of sensory exploitation, which states that males evolve traits to exploit preexisting female preferences. Combining comparative studies with artificial neural network simulations, we also argue that the decoding strategies that females use to recognize whines are influenced by the decoding strategies used by their ancestors. Besides reviewing the details of this communication system, we also emphasize the necessity of incorporating different levels of analysis to obtain a deeper understanding of the mechanisms, function, and evolution of complex phenotypes [*Acta Zoologica Sinica* **49** (6): 713－726, 2003].

Key words Animal communication, Túngara frog (*Physalaemus pustulosus*), Sexual selection

一种泡蟾的配偶识别：脑、行为和进化研究*

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摘　要　综述了对泡蟾（*Physalaemus pustulosus*）的性通讯所进行的各种研究。本文作者研究了通讯系统的功能重要性、通讯的神经机制以及通讯进化的格局，特别研究了雌蛙如何使用简单和复杂的叫声来识别可以交配的物种，以及如何识别同种的不同雄性个体。本文确定了复杂叫声的反选择力、一种食蛙蝠——缨唇蝠（*Trachops cirrhosus*）以及叫声的能量消耗，并阐述了引起雌性反应的重要特征。作者的比较研究表明，对复杂叫声的偏好在复杂叫声进化前就有了，这支持感官开发假说（Hypothesis of sensory exploitation，雄性特征进化的结果是开发雌性预先存在的对雄性的偏好）。结合比较研究和人工神经网络的模拟，作者认为雌蛙用来识别雄性简单叫声的解码策略受其祖先解码策略的影响［动物学报 **49**（6）：713－726，2003］。

关键词　动物通讯　泡蟾　性选择

1 Introduction

Mate recognition is a key behavior in sexual reproduction (Darwin, 1871; Andersson, 1994). One consequence of mate recognition is species recognition. Mate recognition usually facilitates matings between conspecifics rather than heterospecifics. Another consequence is sexual selection. Mate recogni-

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** Corresponding author. E-mail: mryan@mail.utexas.edu tion can result in variation in male mating success if females find some males more attractive than others. Both species recognition and preferences among conspecifics could be targets of selection or be incidental consequences of it.

A full understanding of mate recognition requires analyses at several levels of investigation. Tinbergen suggested four aims in ethology: the acquisition of behavior (e.g. genetics, learning, and development), the mechanisms controlling behavior (e.g. neurobiology and physiology), the current function of behavior (adaptive significance), and the evolution of behavior (phylogenetic patterns) (Tinbergen, 1963). Sometimes disagreements about animal behavior have resulted from contrasting interpretations derived from different levels of analysis (Reeve *et al.*, 1993). Alternatively, it has been argued that it is difficult to understand the function and evolution of behavior without integrating data and interpretations across levels of analysis (Ryan *et al.*, 1998; Autumn *et al.*, 2002). We have taken this more integrative approach in our studies of mate recognition in túngara frogs (*Physalaemus pustulosus*).

Our approach has embraced to varying degrees three of Tinbergen's four questions: function, mechanisms, and evolution. Primarily, we have focused on the behavior aspects of the sexual communication system from aspects of sexual selection and species recognition. In collaborations with Dr. Walt Wilczynski we have tried to understand some of the underlying neural mechanisms that guide females to acoustic stimuli. While in collaborations with Dr. David Cannatella we have analyzed the phylogenetic relationships of the túngara frogs and their close relatives to provide a historical background that allows explicit testing of evolutionary hypotheses. Besides investigating this system at different levels of analysis, we attempt to integrate information across these levels to gain a synergistic understanding of function and evolution that would not be possible with a more restricted analysis.

Our purpose here is to review these studies of mate recognition in túngara frogs, emphasizing how the integration of brain, behavior, and evolution can give us a deep understanding of communication in this model system. We do not attempt to put these studies in the context of the very rich literature of mate recognition. Recent reviews of much of this literature can be found in excellent texts by Greenfield for insects (Greenfield, 2002), and Gerhardt and Huber for insects and frogs (Gerhardt *et al.*, 2002) as well as many informative review papers. We have published a variety of different types of studies on túngara frogs (*Physalaemus pustulosus*) and our goal here is to review many of these studies and in an attempt to integrate them into a coherent whole.

2 Sexual selection

The túngara frog is a small frog, about 30 mm in snout-vent length, that is a member of the large family Leptodactylidae. Its range extends from just north of Veracruz, Mexico, south throughout Middle America, and into South America in Colombia, Venezuela, Guyana and Trinidad (Ryan, 1985; Ryan *et al.*, 1996). The call of the túngara frog, which is described in detail below, is similar to our ear throughout its range. But detailed analysis of calls from 30 populations along a 5 000 km transect from northern Mexico to Venezuela showed statistically significant call differences among populations. Many call variables exhibit clinal variation. Geographic proximity is a better predictor than allozymes of similarity of call differences among populations. Interestingly, calls also differ among the two allozymes groups of túngara frogs. These two groups might have resulted from multiple invasions of Middle America that had isolated the two groups for 4 – 4.5 million years before the final invasion that coincided with the closure of the Panamian land bridge 2.4 million years ago (Ryan *et al.*, 1996).

Throughout its range túngara frogs are commonly found in a variety of habitats in the lowland tropics, and is quite common in disturbed habitats. In Panama, where most of our studies have been conducted, breeding occurs during the rainy season, which typically extends from April to December. Males usually call in choruses whose size can range from just a few males to over 100 (Ryan, 1985). Males also can be found calling in isolation. Females come to the chorus to choose a mate. Males remain relatively stationary while calling. Although they do not defend territories, they do maintain inter-individual distances in the chorus. Females are free to move throughout the chorus with little interference from males. Females appear to sample males in the chorus, sitting in front of one male for up to several minutes before moving on to another new male or returning to a previously sampled one before she initiates mating. She does so by making physical contact with the male. Upon contact, the male clasps the female dorsally, a state known as amplexus. The pairs remains in amplexus for a few hours or so before they begin to construct a foam nest. To construct the nest the male beats the jelly matrix of the eggs with his hind legs, the resulting foam is reminiscent of the meringue that forms when beating the white of a chicken egg. The eggs hatch in a few days into the pool of water and the tadpoles metamorph to froglets in a few weeks (Ryan, 1985).

Male frogs produce a species-specific advertisement call to attract females for mating, and females usually only approach calls (exhibit phonotaxis) when searching for a mate (Ryan, 1991; Gerhardt *et al*., 2002). The túngara frog has a call of varying complexity. The key to understanding the túngara frog system is the fact that male túngara frogs facultatively alter call complexity. The advertisement call of this species has two components. The whine is a frequency sweep with substantial energy in up to five harmonics and is typically from 200 – 300 ms in duration (Fig. 1). The whine can be produced alone (simple call) or can be followed by one-six chucks (complex calls). The chuck is much shorter in duration, typically from 20 – 40 ms, and richer in harmonic structure, with substantial energy in as many as 15 harmonics (Fig. 1). The whine is necessary and sufficient for attracting a female but males add chucks when vocally competing with other males (Rand *et al*., 1981).

Our earliest studies addressed the evolution of complex vocal signals in the túngara frog, and most of our studies have been conducted at the Smithsonian Tropical Research Institute in Panama. We examined the significance of signal variation, mate choice, and sexual selection.

When males are calling alone or in smaller groups they tend to produce simple calls (Rand *et al*., 1981). In a larger chorus, however, males add chucks to their calls. Playbacks to males in nature showed that vocal interactions with other males mediate call complexity (Rand *et al*., 1981). Although a simple call is sufficient to elicit phonotaxis from females, experiments showed that they favor complex calls when given a choice (Rand *et al*., 1981; Ryan, 1985b). This raised the question as to why males do not always produce complex calls, and suggested a cost to call complexity. The cost is not energy. Although we showed that there is a substantial aerobic cost to calling, neither the rate of oxygen consumption nor accumulation of lactic acid (indicative of a cost of anaerobic metabolism) increase with increasing call complexity (Bucher *et al*., 1982; Ryan *et al*., 1983; Ryan, 1985a).

The critical cost of complex calling is predation. The frog-eating bat (*Trachops cirrhosus*) eats frogs and locates them passively, relying on the frog's call for locational cues (Tuttle *et al*., 1981; Ryan *et al*., 1981). These bats have a suite of behavioral and morphological specializations that make them acutely sensitive to the low-frequency sounds that characterize frog calls (Bruns, 1989; Ryan *et al*., 1983). Like female frogs, frog-eating bats approach simple calls, but experiments both in the laboratory and the field show that the bats, again like the female frogs, prefer complex calls (Ryan *et al*., 1982). Thus call complexity in túngara frogs seems to have evolved under the conflicting forces of sexual selection and natural selection.

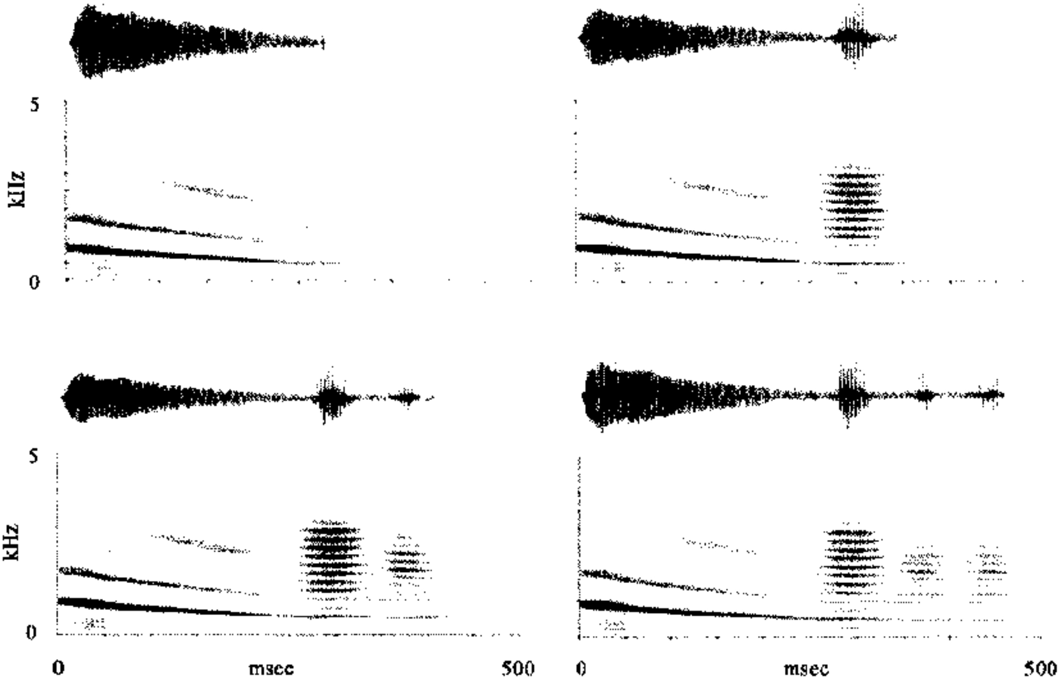

Fig. 1 An example of the call complexity series of the túngara frog (*Physalaemus pustulosus*)

From the left to right, top to bottom, we illustrate a waveform (top) and spectrogram (bottom) of a whine with no chucks, and a whine with one, two and three chucks

It appears that all male túngara frogs are capable of producing calls with chucks, although we know little about variation in the tendency of males to escalate call complexity. Female choice of mates can be more subtle than preference for complex calls, however. An exhaustive study of male mating success in nature showed that females are more likely to choose larger males as mates (Ryan, 1980, 1983, 1985b). Larger males also produce chucks with lower frequencies, probably because the vocal masses are larger and thus vibrate at a lower frequency. When given a choice between chucks of different fundamental frequencies, females preferred the somewhat lower ones over some higher ones within the natural variation in chuck frequency (Ryan, 1980, 1983, 1985b). These early studies were conducted with relatively crude synthetic stimuli that did not precisely mimic the natural energy variation among chuck harmonics. We repeated these experiments with stimuli more faithful to the natural chuck, and achieved a similar result. Females significantly preferred lower to higher frequency chucks, although the strength of preference was weaker than previously thought (Wilczynski *et al.*, 1995; Ryan *et al.*, 2001).

Female túngara frogs gain a reproductive advantage by choosing larger males. In nature, about 10% of a female's several hundred eggs are not fertilized. As in many species of frogs, female túngara frogs are larger than males. When females choose males closer to their own size, which usually means larger males, more eggs are fertilized (Ryan, 1983, 1985b). This probably results from a mechanical advantage, as suggested by Davies and Halliday in their seminal study of toads (Davies *et al.*, 1977). When male and female size differences are large their cloacas are not juxtaposed and fewer eggs are fertilized. A similar effect has been found in other frogs as well (Bourne, 1997).

We can summarize some aspects of sexual selection in túngara frogs up to this point with a chain of correlational and causal statements: larger males have greater mating success because larger males are preferred as mates by females. Larger males produce lower-frequency chucks because their larynx is larger, and females prefer larger males because they prefer lower frequency chucks. Females gain a reproductive advantage in exercising this choice.

3 Sexual selection and neuroethology

As mentioned above, the anuran advertisement call is an important mate recognition character, and as a premating isolating mechanism it figures prominently in the process of speciation. The anuran mate recognition system has also served as an important model for understanding how the brain decodes biologically relevant acoustic stimuli. Capranica and his many students championed this attempt to explain how the female frog's neural mechanisms guide it to the conspecific mating call in preference to other competing sounds (Capranica, 1977). In collaboration with Dr. Walt Wilczynski, we adopted this neuroethological paradigm of species recognition to identify some of the neural mechanisms that result in female frogs exhibiting phonotactic preferences among conspecific calls.

Unlike most other vertebrates, frogs have two inner ear organs that are sensitive to air-born sounds. The amphibian papilla (AP) is most sensitive to sounds below ca 1 500 Hz, while the basilar papilla (BP) is most sensitive to higher frequency sounds. Capranica suggested that the processing of species-specific sounds begins in the auditory periphery. His matched filter hypothesis predicts a correspondence between the tuning of the auditory end organs and the emphasized frequencies in the calls. This hypothesis was supported by his data from bullfrogs, their call has frequencies that stimulate both the AP and BP. Most frogs, however, produce a more narrow range of frequencies that tend to fall within the most sensitive regions of only one inner ear organ. Gerhardt and Schwartz summarized data on auditory end organ sensitivity and call frequency that offered strong support for the matched filter hypothesis (Gerhardt *et al.*, 2001).

A typical túngara frog whine has a dominant frequency of 700 Hz, which is derived mostly from the fundamental frequency of the frequency sweep (Fig. 2). A typical chucks's dominant frequency is 2 500 Hz, which is usually about the 10 – 12th harmonic of the fundamental (Ryan *et al.*, 1990). The tuning of the AP and BP tend to coincide with the emphasized frequencies of the whine and chuck (Fig. 2). The AP is most sensitive to ca. 700 Hz and the BP to ca. 2 200 Hz (Ryan *et al.*, 1990). The upper harmonics of the whine and the lower harmonics of the chuck are not attended to by females during phonotaxis and the effects of the whine and the chuck can be mimicked with use of simple tones (two for the whine and one for the chuck) within the sensitivity region on the AP and BP, respectively (Rand *et al.*, 1992; Wilczynski *et al.*, 1995). Thus processing of the whine seems to be initiated by the AP and processing of the chuck seems to be initiated by the BP.

Although the chuck always follows the whine in nature, this temporal sequence is not critical for the females to perceive the call as complex (Wilczynski *et al.*, 1999). In phonotaxis experiments, the temporal relationship of the whine and chuck were varied. Females preferred most of these calls to a simple whine-only, although there were not necessarily as

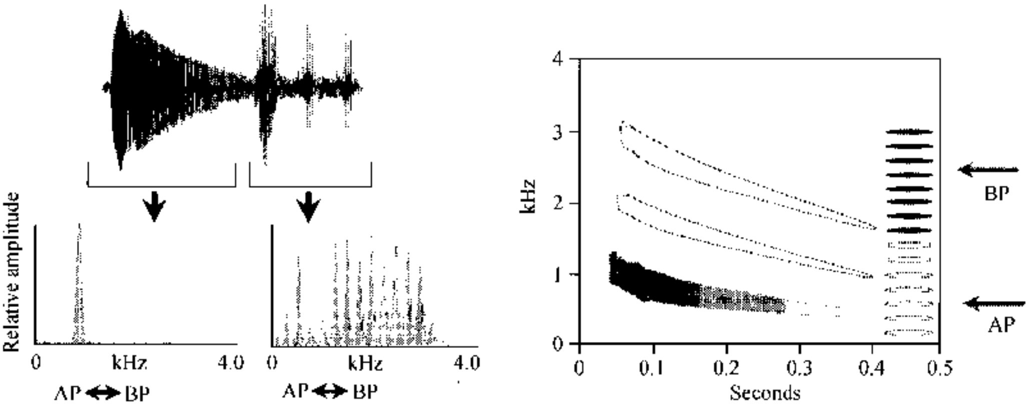

Fig. 2　Call frequencies and auditory tuning

In the top graph we show a waveform of a typical whine with three chucks. In the middle graph we show the power spectrum of the whine and the there chucks. On the X axis of this graph we estimate the frequency ranges to which the amphibian papilla (AP) and the basilar papillae (BP) are most sensitive. For most frogs the AP is most sensitive to frequencies < 1.5 kHz and the BP to frequencies > 1.5 kHz. In the bottom panel we show a sonogram of a typical whine with one chuck. Arrows indicate the frequencies to which the AP and BP are most sensitive for this species. The shading in the sonograms represents the salience of different parts of the call. The darkest shading indicates parts that are necessary or sufficient for females to respond to the whine or the chuck. No shading indicates that these parts of the call have no influence on female phonotaxis. Light gray shading indicates portions that will enhance call attractiveness but by themselves are not able to elicit phonotaxis

attractive as a normal whine-chuck. One position in which the chuck did not enhance the attractiveness of the whine was when is it was placed 50 ms after the whine's onset. In this position females responded to the call as if it were a whine-only. This is a segment of the whine that is critical for whine recognition, thus in this position the chuck might be interfering with whine recognition (Wilczynski *et al*., 1995) (Fig. 2). The fact that the temporal sequence of the whine and chuck is flexible when it comes to eliciting phonotaxis means that there is also flexibility in the temporal order in which a complex call stimulates the AP and BP when eliciting phonotaxis.

The perceptual flexibility for the temporal relationship of the whine and chuck is also paralleled, quite amazingly, by perceptual flexibility for the spatial relationship of the whine and chuck. The ability of an animal to assign different sounds, such as words of a sentence in humans, to the appropriate source is called auditory grouping (Bregman, 1990). If sounds originate from the same location in space they are more likely to be produced from the same source. This will be true when a male túngara frog produces a whine and chuck, since he remains stationary while calling. But the whine and chuck need not originate from the same source to be grouped together by females. Although a female will not approach a chuck-only, she will approach a chuck broadcast with a whine from another location. The chuck can be spatially separated from the whine by as much as 135° and still elicit phonotaxis from the female (Farris *et al*., 2002). At this point, we have no idea why females would group call components from such disparate locations as if they were coming from the same male.

In the previous section we noted that larger male túngara frogs have greater mating success because they are preferred by females, and that females prefer larger males because they prefer lower frequency chucks. There is a slight mismatch between the tuning of the BP (2 130 Hz) and the chuck's dominant frequency (2 550 Hz). We tested the hypothesis that this slight mismatch between tuning and dominant frequency is the neural basis for preference of low-frequency chucks. We constructed a computer model of the BP's tuning and quantified how much energy from a chuck would pass through this filter. We then shifted the frequency spectrum of the chuck and repeated the procedure and again measured the energy that passed through the BP filter. We used chucks from 50 calls randomly chosen from a breeding population. Chucks with lower frequencies than average passed more energy through the BP filter, and we assume would elicit greater neural stimulation from the auditory periphery (Ryan *et al*., 1990). These results suggest a neural mechanism underlying the female's preference for lower-frequency chucks.

These neurophysiological results allow us to expand our interpretation of sexual selection. Larger males have greater mating success because larger males are preferred as mates by females. Larger males produce lower-frequency chucks because their larynx is larger, and females prefer larger males because they prefer lower frequency chucks. Females prefer lower frequency chucks, at least in part, because they better match the tuning of the auditory system. Females gain a reproductive advantage in exercising this choice.

It is not known why there is a mismatch between BP tuning and chuck dominant frequency. It might be an adaptive compromise between signal detection at two distances. Females rely on the male's call not only to choose among males at a close distance but also to locate males and choruses from farther distances (Marsh *et al.*, 2000). The chuck attenuates more quickly with distance than does the whine, and this is due primarily to excess attenuation of the higher frequencies in the chuck compared to the lower frequencies in the whine (Ryan, 1986). Thus the frequency spectrum of a chuck close to the male will have more high-frequency energy than the spectrum of the same chuck farther from the male. This makes it impossible for females to have BP tuning optimal for call detection across all distances. Sun *et al.* (2000) used computer models of BP tuning combined with naturally attenuated calls and showed that the tuning of the BP strikes such a compromise between short and long distance signal detection. Whether the BP evolved such tuning in response to the chuck, or if the chuck evolved this frequency spectrum due to the BP tuning is addressed below.

4 Sexual selection, neuroethology and phylogenetics

The studies we just reviewed tell us something about the current function of the túngara frog's mate recognition system and the neural mechanisms controlling it. Such data can allow one to interpret how adaptive evolution proceeded. The chain of logic about low-frequency chuck preference, for example, could be expanded by one more statement. "... Thus females evolved the tuning of the BP because it guides them to larger males and results in a reproductive advantage". Interpretations about evolution, however, can sometimes be better informed when made with some knowledge of a group's phylogenetic relationships.

Phylogenetics has long played a role in studies of behavior but fell out of favor with the advent of sociobiology. But phylogenetics went through a revolution in North America when Hennig's *Phylogenetic Systematics* became available in English in 1966. Hennig offered a new philosophy, as well as new techniques, to allow a more objective analysis of character evolution. These new approaches to phylogenetics proved critical for testing patterns of behavioral evolution (Ryan, 1996).

An initial phylogeny of the *Physalaemus pustulosus* species group based on examination of some morphological characters was proposed by Cannatella and Duellman (Cannatella *et al.*, 1984). This phylogeny recognized four species in the species group: *P. pustulosus*, *P. petersi*, *P. pustulatus* and *P. coloradorum*. The main conclusions were that the species group is monophyletic and that the phylogenetic relationships within the group are congruent with their biogeography. *P. pustulosus* and *P. petersi*, which like most *Physalaemus* congeners are found east of the Andes (*P. pustulosus* alone is also found in Middle America), are sister taxa, as are *P. pustulatus* and *P. coloradorum*, which are the only taxa found on the western side of the Andes.

We explored the phylogeny in more detail using morphological characters, allozymes, sequences of subunits of two mitochondrial genes, and measures of mating calls (Cannatella *et al.*, 1998). In the interim, our field research identified several new species of *Physalaemus* that have yet to be formally defined but are denoted as species a, and species b (Fig. 3). Furthermore, the taxon *P. petersi* appears to be two species, the former in the northern part of its range in Ecuador, and what we now refer to as *P. freibergi* in Peru. The main conclusion of this phylogenetic analysis supports the previous contention of monophyly of the species group and the two clades within it. One clade contains *P. pustulosus*, *P. petersi* and *P. freibergi*, which are all on the eastern side of the Andes and (*P. pustulosus*) in Middle America. The other clade contains *P. coloradorum*, *P. pustulatus* and one undescribed species and these are all on the western side of the Andes (Fig. 3). Another conclusion of this analysis is that different data sets are not always congruent. When we analyzed the data from DNA sequences, morphology and allozymes there is some variation in the phylogenetic hypotheses that results, but they are all more or less similar to the phylogeny that results from the combined data set. But when the call data are analyzed separately a quite different phylogeny is proposed. This suggests that the mating calls of these species might have evolved more rapidly, and thus generated more homoplasy than the other characters used in the analysis.

We used the best supported hypothesis of phylogenetic relationships to reconstruct the pattern by which the túngara frog's chuck and the female's preference for chuck evolved (Fig. 3). We mapped onto the phylogeny of the species group the presence of complex calls, that is a whine with a facultatively added, acoustically distinct secondary component. All populations of túngara frogs studied to date exhibit complex calls (Ryan *et al.*, 1996). Complex calls, calls with a second component, are variably present in *P. petersi*-*P. freibergi* (Ryan *et al.*, 1993b). Populations we studied in Ecuador lack complex calls, but populations in southern Peru (R. Cocroft unpublished data) and western Brazil (A. Cardoso unpublished data) and components that we refer to as squawks. Recently K. Boul (in press) uncovered populations in

Amazonian Ecuador within 20 km of one another that vary in the presence of complex calls. Complex calls have not been reported in other members of the *Physalaemus pustulosus* species group (Ryan *et al.*, 1993b) or in any of the other ca. 25 species of the genus we have surveyed (A. Cardoso *et al.*, unpublished data). Thus parsimony suggests that the chuck either evolved once in a common ancestor of *P. pustulosus* and *P. petersi-P. freibergi* and then was lost in some populations in the latter clade, or it evolved several times independently. In either scenario the chuck is a derived feature of the *P. pustulosus-P. petersi clade*. In Fig.3 we depict a single derivation of complex calls, which implies that it must have been secondarily lost in some populations of *P. petersi-P. freibergi*. This merely represents our bias at the moment, the multiple evolution of chucks/squawks in the *P. pustulosus-P. petersi* clade is equally parsimonious. Both scenarios are equally supportive of our interpretation of signal-receiver evolution that we now develop.

Many studies of sexual selection, and indeed of animal communication in general, assume that there is very tight coevolution between signal and receiver, trait and preference. How can a signal evolve if the receiver does not respond to it? Various models have suggested how the congruence between signal and receiver is maintained. One suggests pleiotropic effects of a common gene that mediates a central pattern generator (Hoy *et al.*, 1977; Doherty *et al.*, 1985; Boake, 1991). Such a gene could control both signal production and signal recognition if, for example, it generates a temporal pattern that serves as a template by which sound producing organs are pulsed and against which the receiver compares incoming signals. Another possibility is that signals and receivers are under separate genetic control but that there are statistical correlations between genetic variation in signal and receiver; that is, they are in genetic disequilibrium. This genetic mechanism is the basis for Fisher's theory of runaway sexual selection (Fisher, 1930; Kirkpatrick, 1982; Lande, 1980; Kirkpatrick *et al.*, 1991).

An alternative to tight coevolution is sensory exploitation (Ryan, 1990). This hypothesis suggests that there are sensory biases (Endler *et al.*, 1988) that form the basis of preexisting preferences, and males that evolve traits that exploit such preferences are favored by sexual selection. These two scenarios can be distinguished by deducing the phylogenetic pattern by which signals and receivers evolve. The critical predictions of sensory exploitation are that females prefer sexually selected traits present in other species but absent in their own, that this preference is shared through a common ancestor, and the trait was not secondarily lost (Shaw, 1995; Endler *et al.*, 1998; Ryan, 1998).

We tested this hypothesis by asking if females of a species in the western Andes clade, *P. coloradorum*, would more strongly prefer their own male's call if túngara frog chucks were added. When females were given a choice between their own normal, conspecific whine and the same call to which chucks were digitally added, females preferred the latter. The preference for chucks in *P. coloradorum* suggests that the preference for chucks existed prior to the divergence of the *P. pustulosus-P. petersi* clade from the western Andes clade. So if we are correct in interpreting when complex calls evolved and when the preference for complex calls evolved, we conclude that the preferences for this sexually selected trait existed prior to the trait itself (Ryan *et al.*, 1993a, 1993b).

The preexisting preference for chucks could characterize the *Physalaemus pustulosus* species group or the entire genus *Physalaemus*. We tested that hypothesis by determining if female *P. enesefae*, one of the species we used for outgroup analysis, would prefer their male's call with túngara frog chucks added (Tárano *et al.*, 2002). The answer was no. The *P. enesefae* call with chucks was not preferred to the normal chuck-less calls. *P. enesefae* and *P. pustulosus* are sympatric, but it seems that the lack of chuck attractiveness does not result from female *P. enesefae* avoiding a component of the heterospecific call since chucks neither made the calls more or less attractive. Thus the preference for chucks seems to have arisen after the evolution of the genus but prior to the evolution of the species group.

It might seem odd to have a preexisting preference for a chuck. But a number of experiments have shown that the addition of a variety of sounds can increase call attractiveness as effectively as a chuck (Ryan *et al.*, 1990). This is true for males as well as females, as the addition of the same sounds also causes males to escalate call complexity as they do in response to calls with chucks (Ryan *et al.*, 1998). Thus the preexisting preference for chucks is probably part of a preexisting preference for a larger set of sounds, and chucks and squawks are the sounds that male *Physalaemus* just happened to evolve.

We also utilized information on phylogenetic relationships to investigate the evolution of neural correlates of female preference. As mentioned above, the BP is generally tuned to the dominant frequency of the chuck, and behavioral experiments show that it is the harmonics in the sensitivity region of this inner ear organ that are attended to by females rather than the lower-frequency harmonics (which have much less energy) that fall within the most sensitive region of the other inner ear organ, the AP. Furthermore, we had argued that since the tuning of the BP better matched chucks of lower-than-average dominant frequency, BP tuning plays a role in the female's preference for lower frequency chucks. Since females gain a reproductive advantage from mating with larger males, which make lower frequency chucks, we can ask if the tuning of the BP evolved under selection to garner this fitness effect. The answer is no. We compared the tuning of the AP and BP of *P. pustulosus* and its close relatives (Fig. 4) (Wilczynski *et al.*, 2001). We must remember that for the species whose call is only a whine, that is, all species but *P. pustulosus* and some *P. petersi*-*P. freibergi*, the BP is probably not involved in communication. For the most part there is no significant difference in tuning among species. The only exception is that *P. pustulatus* has a BP tuned to higher frequencies than the other species. These data allow us to reject the hypothesis that the tuning of the BP evolved with reference to the chuck, either to better detect it or to prefer lower frequency chucks. Therefore, both BP tuning and the preexisting preference for chucks are sensory biases that males exploited in the evolution of complex calls.

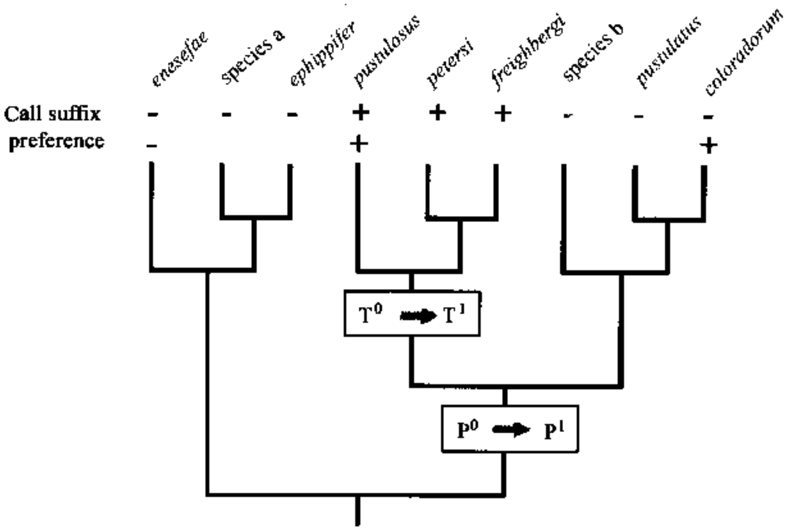

Fig. 3 The phylogenetic relationships of the *Physalaemus pustulosus* species group and three outgroup taxa (Cannatella *et al.*, 1998)

We note species that facultatively add a second syllable to the call (+, chuck in pustulosus, squawk in petersi-freibergi) and species we have studied and that have shown no evidence of adding a second syllable (−). We also indicate those taxa in which females are known to be attracted to a complex versus a simple call (+) and those that are not (−). Most species have not been tested in phonotaxis experiments. Using these data, we plot on the phylogeny our interpretation of when the trait (T), the call suffixes, and preferences for the trait (P) evolved

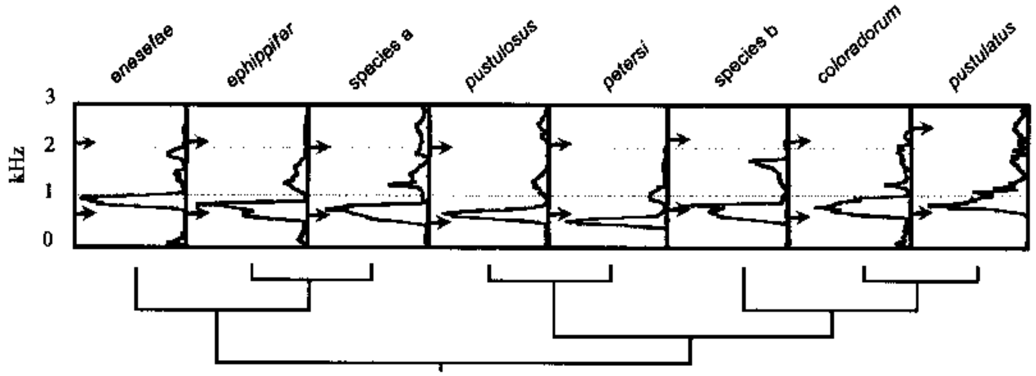

Fig. 4 The phylogenetic relationships of members of the *Physalameus pustulosus* species group

For each species we show a power spectrum of the whine-like advertisement call. For species that add secondary call components, we show only the whine. The power spectra show the relative amount of energy in the call from 0 (bottom) to 3 kHz (top). The bottom arrow in each panel shows the frequency to which the AP of that species is most sensitive and the top arrow shows the frequency to which the species' BP is most sensitive (redrawn after Wilczynski *et al.*, 2001)

5 Estimating ancestral calls and simulating brain evolution

We have been emphasizing the function and evolution of the chuck because it influences the female's choice of mates among conspecifics. Females use the whine for species recognition. All species in the *Physalaemus pustulosus* species group produce whines, and the whines of all the species differ statistically from one another. Túngara frogs are not sympatric with any other species of *Physalaemus* except for a small area of overlap with *P. enesefae* in the llanos of Venezuela. So female túngara frogs do not need to discriminate between the conspecific call and that of other *Physalaemus* throughout most of their range, and certainly in Panama. Nevertheless, most species prefer their own signals over those of other species, regardless of whether they are allopatric or sympatric. Thus we determined the degree to which túngara frogs respond to their own call and those of other close relatives. One main concern was to understand if past history had an influence on species recognition mechanisms.

To explore species recognition in túngara frogs we performed two kinds of phonotaxis experiments. First, we asked if females prefer the conspecific call to other calls, similar to many of the experiments described above. We refer to these as discrimination experiments. Second, we presented females with only the call of another species to determine if she would respond to this call as if it signified an appropriate mate. We refer to these as recognition experiments. In these experiments the null hypothesis of no recognition is that females would encounter a speaker broadcasting a test call with the same probability as if would she randomly wander through the arena and come in contact with a silent speaker. We determined this probability as 0.10; 2 of the 20 females tested encountered a silent speaker.

Initially, we conducted these experiments with heterospecific calls of the species group and the three species we had used for outgroup analysis: *P. enesefae*, *P. ephippifer*, and species a. In the discrimination experiments females always showed a preference for the conspecific over the heterospecific signal. In the recognition experiments, however, there were some heterospecific calls that resulted in significant phonotaxis from females (Fig. 5). We surmised that this false recognition results from two factors. First, there is no selection against responding to these calls since they are not sympatric. Second, female túngara frogs share certain characteristics of the auditory system and brain (some of which we have shown above) through common ancestors (Ryan *et al*., 1999a, 1999b), thus they are susceptible to respond to signals of close relatives.

We further explored this possible influence of history by producing ancestral advertisement calls

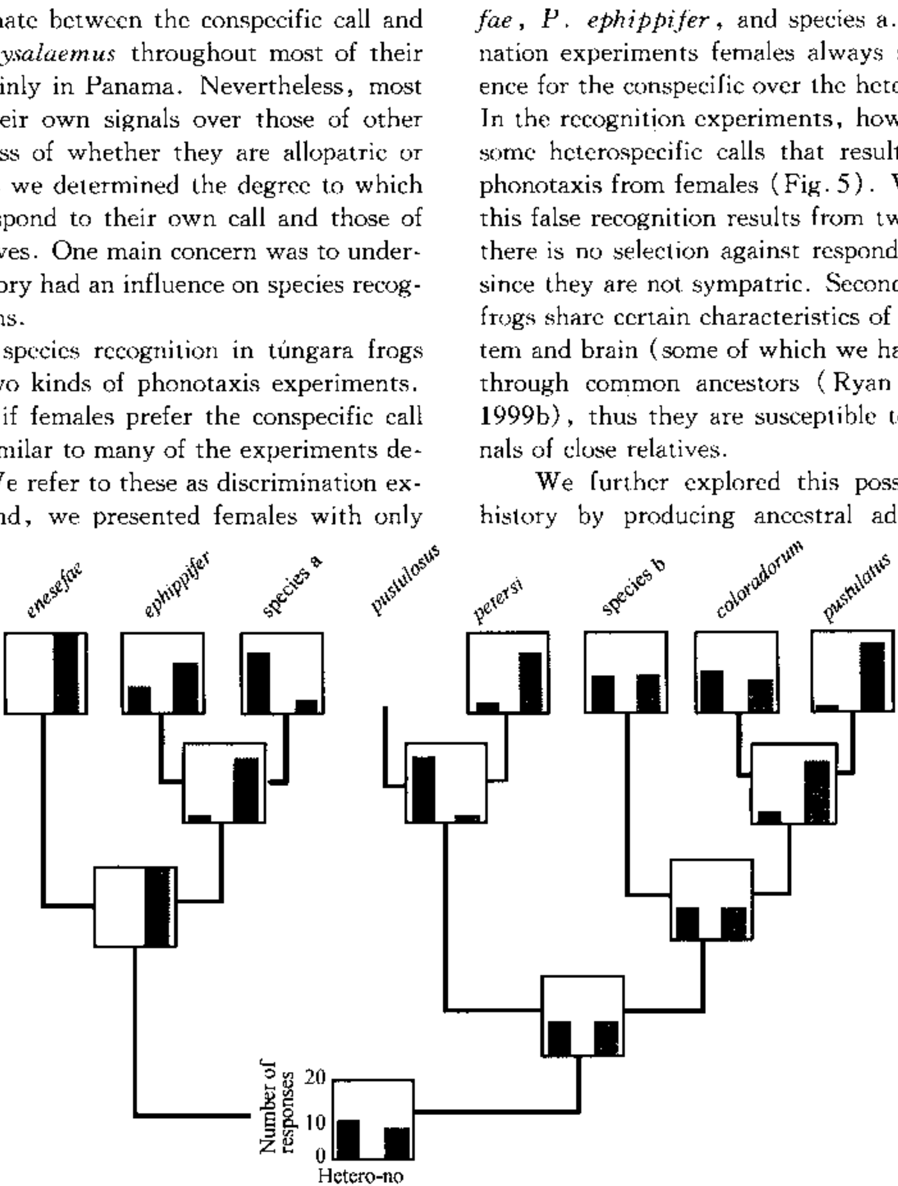

Fig. 5 The number of túngara frog females ($n = 20$) attracted to the heterospecific and ancestral calls (black bars) versus those that showed no response to the same call (gray bars)

The calls illustrated in Fig. 6 correspond to the test results in this figure. If a female did not exhibit phonotaxis a "no response" was only recorded if the female then responded to a conspecific call. Thus "no response" should include only females who found the call not meaningful and should not include females not physiologically motivated for phonotaxis (redrawn after Ryan *et al*., 1995)

(Ryan *et al.*, 1995, 1999a, 1999b). We measured a number of call variables for all of the species' calls tested (Fig. 6). These are the same call variables which in túngara frogs form the parameters of a synthetic call that the females do not discriminate from conspecific calls. For each of these call parameters we estimated their values at all of the ancestral nodes on the phylogenetic tree. We used a variety of models and assumptions to do this, but they were all based on the assumption of parsimony; specifically, all of these estimates were an attempt to minimize the total amount of evolutionary change that took place. When these models yielded somewhat different calls for the same ancestor we repeated the phonotaxis experiments. Once all the call variables were estimated at all the nodes we synthesized the calls (Fig. 6). A caveat is necessary here. We are not suggesting any certainty about what individual ancestors might have sounded like. But we think these estimates are a useful method for estimating the kinds of sounds that relatives of túngara frogs heard.

We repeated the discrimination and recognition experiments described above with the ancestral calls (Fig. 5). The results were similar to what was found with the heterospecific calls. In all but one case, females preferred the conspecific call to the ancestral calls. The exception was the call at the node of *P. pustulosus*-*P. petersi*. Although this call was statistically significantly different from the *P. pustulosus* call, the females did not recognize it as such. Also, despite the prevalence of preference for the conspecific call, we see that the strength of this preference varies among the ancestral calls. In the recognition experiments, there were a variety of calls that elicited recognition (Fig. 5).

Túngara frogs are allopatric with the species (including ancestors) we tested, thus we would not expect processes such as reproductive character displacement to influence how female túngara frogs respond to foreign calls. We thought two variables should be important in predicting the strength of recognition for foreign calls: how similar the foreign call is to the conspecific call (acoustic similarity), and the evolutionary relationship between the foreign taxon and the túngara frog (history). Females should be responsive to foreign calls that are more similar to the túngara frog call. We also predicted that that the strength of response should be stronger when a taxon is phylogenetically closer to the túngara frog. Acoustic similarity and history are not confounding variables since, as noted above, calls are not good predictors of phylogenetic relationships.

We analyzed the degree to which both phylogeny and call similarity predict female response with a stepwise multiple regression analysis in which female response (the numbers of females that responded to the

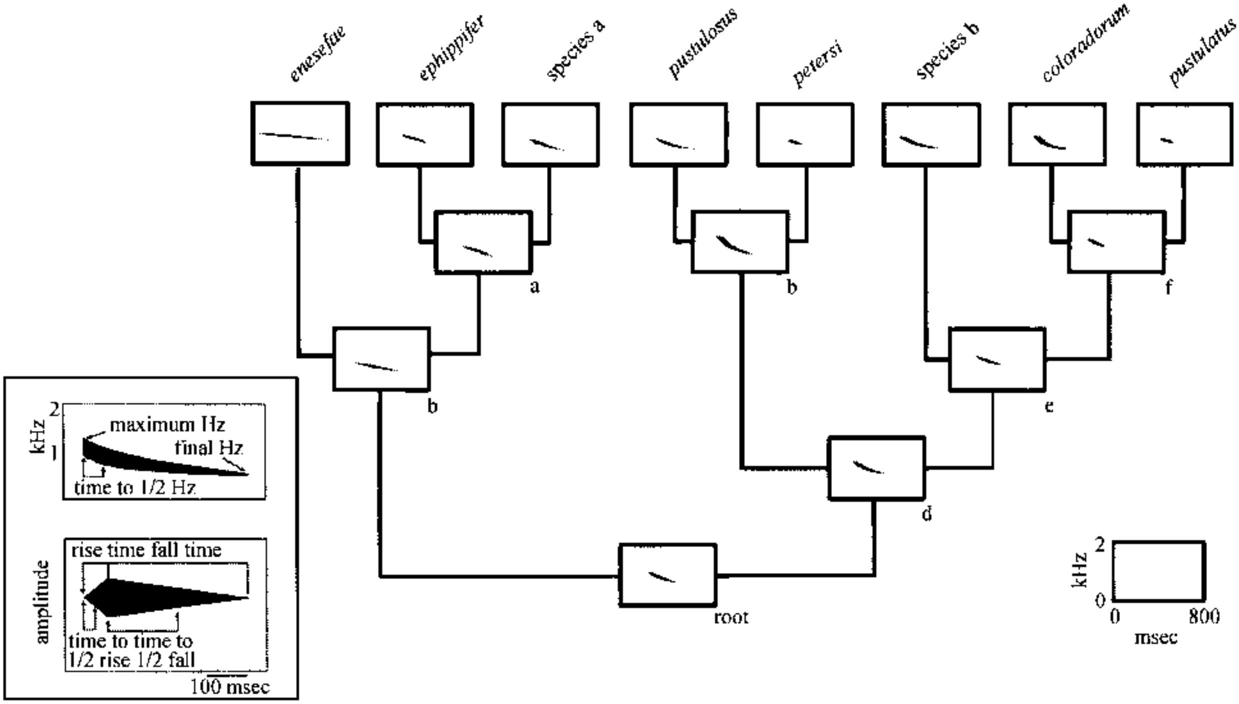

Fig. 6 The phylogeny of the *Physalaemus pustulosus* species group and outgroups

Each box in the tree contains a sonogram of the synthetic call for each species and ancestral node. The insert (bottom, left) shows the spectral (sonogram, top) and temporal (waveform, bottom) call parameters that are measured for all species, and are then estimated for ancestral calls and used in call synthesis (redrawn after Ryan and Rand, 1995) foreign call of a sample size of 20) was the dependent variable, and the phylogenetic distance from the tested taxon to the túngara frogs (estimated by the number of sequence base changes) and the overall similarity (euclidean distances computed from all call variables) of the tested call to the túngara frogs calls were the independent variables. When we conduct these analyses we see that history has an effect independent of call similarity in both discrimination and recognition experiments. In the discrimination experiments, call similarity explained 18% of the variation in female responses to calls, while phylogeny explained 39%. In the recognition experiments each independent variable explained a similar amount of the variation in responses to calls: call similarity 31% and phylogeny 38%.

The above study suggests that the response of females to foreign calls is influenced by past history. This should be expected. There are innumerable ways to recognize a sound, and it is not surprising that the way females recognize their calls is influenced by the way their ancestors recognized calls. The alternative is that with each speciation event females evolve newly derived computational strategies for signal decoding instead of merely adjusting the strategy already in place.

We further tested the role of history by simulating brain evolution through different call histories (Phelps *et al*., 1998, 2000; Phelps *et al*., 2001). To do this, we used artificial neural networks. A neural net is an array of computational units arranged in layers. One layer, the input layer, interacts with the (digitized) stimulus under investigation. Typically, these input units are connected to all units in a middle layer and all the units converge on a single output unit. These connections all have weights, typically varying between -1 and $+1$. The neural nets will give different outputs for different signals if these signals stimulate the units in the input layer differently. In addition, the same net will give different outputs to the same stimuli if the weighting of the connections change. Nets can be trained to recognize signals by varying the weights between the connections until some optimal response is achieved.

Initially, we trained artificial neural networks to recognize túngara frog calls (Phelps *et al*., 1998). We constructed 100 neural networks with weights of all the connections assigned randomly. A net was stimulated with a túngara frog call and noise (a scrambled version of the call). A fitness value was assigned to each net based on how well it discriminated between the call and noise. A high fitness value was assigned if the response to call and noise was very different and a low fitness value was assigned if there was little difference. Nets were then subject to selection. The probability of a net being selected to pass on to the next generation was based on its fitness. One hundred nets were selected with replacement in this way to constitute the next generation. Once selected the nets underwent mutation. Weights were selected randomly with some "mutation" probability. If a weight was selected its value was randomly changed. This process was continued until an arbitrary fitness criterion was achieved; specifically, when the fittest individual in the population had a fitness of at least 0.90 and the average fitness was 0.75.

We used this approach further to study the effect of history on recognition strategies (Phelps *et al*., 2000; Phelps *et al*., 2001). We had three treatments that varied in call history. In the first, the "mimetic" history, nets were selected to recognize the ancestral call at the base of the phylogeny. Once recognition was achieved, the nets were then trained to recognize the call that was the immediate descendent on the evolutionary path to the túngara frog (Fig.7). This was continued until the nets were selected to recognize the túngara frog call. The second treatment is the "random" history treatment. Like the first treatment, the nets are selected to recognize three calls before being selected to recognize the túngara frog call. These three calls, however, are chosen randomly from the calls in the phylogeny (Fig. 7). We had 20 different random histories to which nets were trained. The third treatment is referred to as the "mirror" histories. These calls were constructed by flipping the coordinates of the mimetic history in acoustic space to create something analogous to a multidimensional mirror image of the mimetic history. This controls for the path length of the historical trajectory, unlike the random histories. But like the random histories, nets receive a history that is different than the 'mimetic' history. Once the fitness criteria were reached we asked if the nets in the different treatments differed in their ability to predict the responses of real females. Previously, we had tested females with a variety of stimuli besides the conspecific call. We plotted the strength of response of females to those calls (the proportion of females that exhibited phonotaxis to those stimuli in a phonotaxis test) versus the strength of average response of the neural networks to the same stimuli.

In all treatments the nets were able to evolve to recognize the túngara frog call. Thus the history of the neural networks does not constrain their evolving effective signal recognition. But there were differences in how well the networks in each treatment predicted the response of females to other stimuli. The correlation between the response of the nets and the response of the females varied among treatments (histories): mimetic, $r = 0.56$; random, $r = 0.32$; mirror, $r = 0.20$ (Fig.7). Paired t tests of the residual error in each correlation showed that the mimetic history predicted female response significantly better than did the random ($P < 0.05$) or the mirrored history ($P < 0.01$).

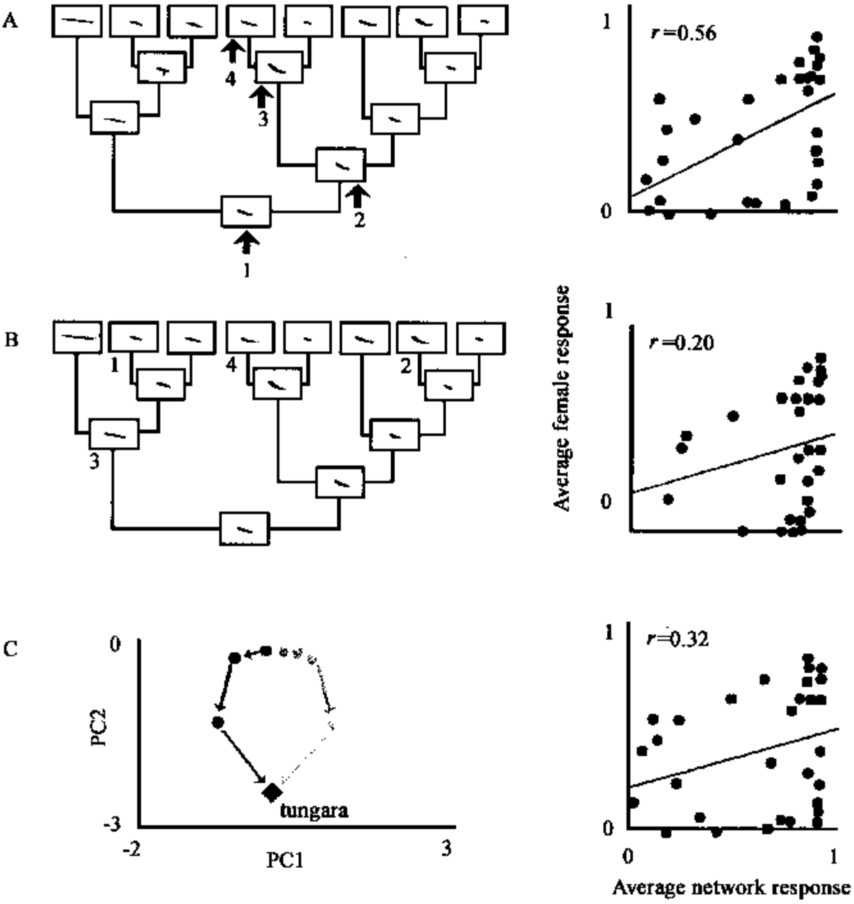

Fig.7 The three treatments to which populations of artificial neural networks were subjected during training and the correlation in the relative strength of response of female túngara frogs and neural networks to the same stimuli (A, mimetic history; B, random history; C, mirror history)

A. The numbers indicate the order of the calls to which networks were trained, note calls 1 – 3 are on the direct pathway of descent from the call at the base of the phylogeny (1, root) to the túngara call (4). B. An example of one of the 20 random histories used. The first three calls to which the nets were trained were chosen at random from the phylogeny. The fourth and final call to which the net were trained was the túngara frog call (4), as in A, C. In the mirrored history the first three calls to which the networks are trained are essentially the mirror images of calls 1 – 3 in A. These were constructed by rotating the calls variable 180 degrees in multivariate space. The black circles are calls 1 – 3 from A (from top to bottom) and the gray circles are their analogous mirror images. As in the mimetic and random histories the final call to which the networks were trained is the túngara frog call

These simulations with artificial neural networks strongly suggest that past evolutionary history leaves a footprint on the brain, and that tasks that needed to be solved by ancestors influence how a species solves similar tasks today. They also emphasize the general point we have made throughout, that current behaviors need to be interpreted in the context of their past evolutionary history.

6 Summary

We have reviewed many of our studies of the sexual communication system of túngara frogs. Our approach has been integrative. We feel that our understanding of this system is richer due to the integration of various levels of analysis, in this case sensory mechanisms, adaptive significance, and patterns of evolution. We also feel that in some cases our interpretations would have been incorrect if we had focused on only one level of analysis. For example, our current view of the role of sensory exploitation in the evolution of complex calls in these frogs is derived from our knowledge of the behavioral significance of complex calls in this species, the importance of various auditory end-organs in processing whines and chucks, and information on the behavioral response and neural tuning of other closely related species. We suggest such integration is valuable to obtain a deeper understanding of the mechanisms, function, and evolution of complex phenotypes.

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