EVOLUTION OF SIGNAL DIVERGENCE AND BEHAVIOR IN

CYPRINELLA GALACTURA, THE WHITETAIL SHINER

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EVOLUTION OF SIGNAL DIVERGENCE AND BEHAVIOR IN *CYPRINELLA GALACTURA*, THE WHITETAIL SHINER

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EVOLUTION OF SIGNAL DIVERGENCE AND BEHAVIOR IN *CYPRINELLA GALACTURA*, THE WHITETAIL SHINER

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DISSERTATION ABSTRACT

EVOLUTION OF SIGNAL DIVERGENCE AND BEHAVIOR IN *CYPRINELLA GALACTURA*, THE WHITETAIL SHINER

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Acoustic signaling in anurans, insects, birds, and mammals has played an important role as an interspecific isolating mechanism and is also believed to be a mechanism of sexual selection in intraspecific mate choice. A similar mechanism in North American freshwater fishes has been virtually unexplored. Before the evolutionary significance of signal divergence in fishes can be determined, a detailed description of variation within and between species must be made. *Cyprinella galactura*, the whitetail shiner, is known to produce sounds during the breeding season, and due to its disjunct distribution and complex acoustic repertoire, was the perfect model for a study of this type. Acoustic signals were thoroughly described detailing a complexity in signal structure never before documented in freshwater fishes. In addition, the role of acoustic signaling in agonistic and courtship behavior was examined. Sound was found to be frequent during low and moderate level male displays and decreased during the highest levels of male motivation for both contexts. Geographic signal variation was examined in detail among four populations of *C. galactura* separated by the Mississippi embayment. Significant population-level differences in both temporal and spectral parameters were found, however, pulse parameters in every method of analysis contributed to the most divergence. Adjacent populations are more similar for courtship signal parameters, but not for agonistic signal parameters. A combination of geographic isolation and genetic drift may contribute to these differences. Furthermore, acoustic variation was examined at five different levels: within a signal, within individuals, within a population, within a species, and among species. Agonistic signals were more divergent and more variable than courtship signals both within a population and within a species. In addition, static and dynamic signal properties were defined. Burst duration and burst interval were more variable at the within-a-signal level and are considered dynamic properties. Pulse duration, pulse interval, and pulse rate are more variable at the within a species level and are considered static properties. The role these variations may play in the evolution of acoustic signaling is discussed. This study paves the way for further studies of signal variation within the genus *Cyprinella*.

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INTRODUCTION

The study of acoustic communication is an excellent model for examining the evolution of behavior. Acoustic signals are usually species-specific (Ballantyne and Colgan, 1978; Deily and Schul, 2004) and may play a crucial role in mate recognition (Ryan and Rand, 1993; Shaw, 2000) and mate choice (Searcy and Anderson, 1986). If species are “groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups (Mayr, 1942)”, then for speciation to occur, there has to be an initial decrease in reproductive interaction, which may result in decreased genetic exchange. Small reproductively isolated groups may evolve at a quicker rate than large groups with gene exchange. In the case of acoustic communication, changes in courtship signals could act as a pre-mating isolating mechanism. By examining intraspecific variation in acoustic signaling, and especially, in courtship signals, we can gain insight into the processes that may lead to changes in these signals, and possibly, speciation.

Several processes may act to drive the evolution of acoustic signals, including natural selection (Podos, 2001), sexual selection (Ryan, 1990), accumulation of mutations (Coyne, 1992), migration (Tregenza et al., 2000), and genetic drift (Carson and Templeton, 1984; Tregenza et al., 2000). For natural selection to occur, heritable genetic changes must exist that result in increased fitness for the individual. Sexual selection is a special kind of natural selection in which physical or behavioral changes enhance reproductive success of the individual (Darwin, 1871). Sexual selection may lead to divergence of courtship signals in isolated populations. If the signal properties affected by sexual selection are involved in mate recognition, then reproductive isolation and subsequent speciation is possible (West-Eberhard, 1983; 1984). Sexual selection can also act on those signal properties not used in species identification. This may cause signal divergence, but not result in speciation. This was found to be the case in populations of the planthopper, *Nilaparvata bakeri* (Claridge and Morgan, 1993).

Genetic drift can also play a major role in speciation. One way random genetic drift can occur is as the result of a vicariant event. Small isolated populations may have a different genetic makeup, which may not be representative of the larger population. A combination of genetic drift and repeated founder events has resulted in acoustic divergence in populations of the meadow grasshopper, *Chorthippus parallelus* (Tregenza et al., 2000).

The study of the evolution of acoustic behavior can be difficult since the actual process can involve changes that are made over significantly long periods of time. There are a couple of comparative approaches researchers employ to aid in the further understanding of this process. The first involves examining and comparing the variation among similar species. This approach is especially informative when a widely accepted morphological or genetic phylogeny is available for comparison. This has been useful in examining the evolution of acoustic communication in anurans (Cocroft and Ryan, 1995), insects (Gogala and Trilar, 2004), birds (Winkler and Short, 1978; Thomassen and Povel, 2006), and mammals (Peters and Tonkin-Leyhausen, 2004; Shelley and Blumstein, 2005). Patterns of acoustic signals in the genus *Bufo* and *Pseudacris* were coded and mapped onto phylogenetic trees based on morphological and biochemical data. In doing so, Crocroft and Ryan (1995) discovered that call characters could evolve independently of one another and at different rates. One downfall with this comparative among-species approach is that it may be difficult to know for certain if the divergence being observed arose before or after the speciation event (Tregenza et al., 2000).

A second approach is to examine variation in behavior among populations within a species. In the past, researchers have treated behavior as if it is static and purposely minimized the effect of variation in the experimental setup because it complicated the outcome (such as only collecting data from a single site). However, behavior is not a static trait (Foster and Endler, 1999; Bell, 2005) and, in eliminating data from more than one location, the entire picture is not being told. By studying intraspecific differences in behavior, we may gain insight into the processes that cause changes in acoustic signaling and may eventually lead to speciation.

The study of geographically isolated populations with known communication systems may provide insight into these types of speciation events. Although well studied in birds (Marler and Tamura, 1962; Kroodsma et al., 1999; MacDougall-Shackleton and MacDougall-Shackleton, 2002; Slabbekoorn and Smith, 2001; Leader et al., 2002), anurans (Snyder and Jameson, 1965; Wilczynski et al., 1992; Littlejohn et al., 1993; Gergus et al., 2004), insects (Clardridge and Morgan, 1993; Wells and Henry, 1992; Pinto-Juma et al., 2005), and mammals (Esser and Shubert, 1998; Cerchio et al., 2001), geographic variation in acoustic communication has not been widely investigated using freshwater fishes as a model. With low dispersal abilities, freshwater fishes are ideal models for investigations of this type, because without gene flow, high levels of divergence are possible.

Acoustic communication in fishes is used during territory defense (Lugli, 1997) and accompanying elaborate visual displays during courtship and reproduction (Delco, 1960; Winn and Stout, 1960; Stout, 1975). With over 2000 species, cyprinids comprise the largest family of freshwater fishes in North America and, arguably, the second largest family in the world (Helfman et. al., 1997). The widely distributed and large genus *Cyprinella*, with 27 (Mayden, 1989) to 30 (Broughton and Gold, 2000) species may provide insight into the evolution of acoustic signaling in freshwater fishes. Sound production has been described for three species of *Cyprinella* (Delco, 1960; Winn and Stout, 1960; Stout, 1975), but all are believed to produce sounds (Johnston, unpubl.; Phillips and Johnston, unpubl.).

Cyprinella are known crevice spawners (Johnston and Page, 1992) in which males use sound to vigorously defend their established territories (Stout 1975, Johnston, unpubl.). *Cyprinella* signals are typically low frequency, non-harmonic, and vary by behavioral context (Stout, 1975; Johnston, unpubl.). These signals contain many temporal and spectral parameters and these components may, as in other taxa, respond separately to selective pressures. For example, Ryan (1991) found great variation within signal components of anuran calls that suggest different functions for these components. Furthermore, components can be associated with morphology (frequency) and conserved across species, or controlled by physiology (temporal patterning) and diverge across species (Crocroft and Ryan, 1995). In these ways, components of acoustic signals may serve as pre-mating isolation mechanisms and aid in sexual selection (Bradbury and

Vehrencamp, 1998), as call components that reveal male condition (temporal patterning) are chosen.

Cyprinella galactura, the whitetail shiner, was chosen as a model for the study of intraspecific acoustic communication, as it has a disjunct distribution with populations occurring on both sides of the Mississippi embayment. These populations have been separated since the Pleistocene, at least 15,000 years ago (Thornbury, 1965), and it is possible that divergence in acoustic signal structure has occurred. Furthermore, populations on both sides of the embayment are known to be morphologically similar (Gibbs, 1961). *Cyprinella galactura* are known to produce sounds during both courtship and agonistic contexts which allows predictions to be made as to the rate of evolution of the different contexts.

The purpose of this study is to 1) describe the signals and contexts of sound production in *C. galactura*, 2) assess geographic variation among populations in agonistic and courtship signals, and 3) investigate levels of variability in signal structure in *C. galactura* and relate this to signal properties that may be used for different roles (such as reflect male quality and may be used for assessment).

First, a description of acoustic signals in *C. galactura* is needed, including a method of dissecting and measuring the fine-scale as well as gross-scale acoustic properties. Although, gross-scale temporal structure has been described in *C. venusta, C. lutrensis* (Delco, 1960), and *C. analostana* (Winn and Stout, 1960; Stout, 1975), fine-scale temporal structure has never been fully described in this genus. These fine-scale pulse properties are often the parameters that convey important species-specific information in anurans (Ryan, 1990; Bush et al., 2002) and insects (Higgins and

Waugaman, 2004; Pinto-Juma et al., 2005). Furthermore, a detailed description of the signals of this species, with a methodology for data extraction that can be replicated in the signal analysis of other *Cyprinella,* is required before future comparative studies can occur within the genus. The specific role acoustic signaling plays during behavioral interactions in the species is also examined. For example, when do *C. galactura* produce acoustic signals? Do differences exist in the signals between aggressive and courtship contexts?

Geographic signal divergence in courtship and agonistic acoustic signals among four populations of *Cyprinella galactura* is examined. Populations were selected due to location and the proposed drainage history, with two populations from the Ozarks and two populations from the Appalachians. Within-species acoustic differentiation in *C. galactura* populations is analyzed and proposed methods of evolution are discussed. Adjacent drainages are predicted to be more similar to one another than to a non-adjacent drainage. Furthermore, courtship signals are predicted to be more conserved among populations due to their presumed importance in mate recognition.

The next chapter takes the question of signal divergence one step further, examining variation at different levels. Acoustic properties are categorized and variation is examined at five levels of comparison: within a signal, within individuals, within a population (among individuals), within a species (among populations) and among several species. Three additional acoustically described species of *Cyprinella* are used for species comparisons: *C. gibbsi*, *C. trichroistia,* and *C. callisema*. A description of the comparison of the signals in these species is included in chapter 3.

The typical acoustic signal is composed of different components, both temporal and spectral, that may evolve at different rates. A method was developed in anurans to observe how signals may evolve by examining signal temporal and spectral property variation at different levels of analysis (Gerhardt, 1991). Gerhardt (1991) described two patterns of variation present in acoustic signals in several species of treefrogs (Hylidae) and he called these static and dynamic properties. In order to determine if signal properties are static or dynamic, he analyzed signals at three levels within treefrogs, among-species, among-male (in one population) and within-male, and then he compared coefficients of variation among those levels. Static properties are defined as those signal parameters which change little during a breeding season, while dynamic signal properties can change markedly during a single bout of calling (Gerhardt, 1991). Static properties are signal components that are associated with morphology and are more conserved. While static properties are generally less variable overall, there is a gradient of variability that exists when comparing different levels of analysis. On this gradient, static properties typically are most variable at the within-species level, and least variable at the within-individual level. These properties may relay information such as species identification. Dynamic properties are those signal components that are associated with physiology or behavior and are less conserved. While these properties are generally more variable overall, a gradient of variability exists when comparing different levels of analysis, and dynamic properties typically are more variable at the within-individual level. These signal properties may relay information about the quality or condition of the sender.

This study design allowed me to assess both fine and coarse scale geographic variation in acoustic signaling and take a detailed look at signal variation in *Cyprinella* *galactura*. This is only the second study to examine geographic signal divergence in freshwater fishes (Johnson, 2001), and the first to examine acoustic signal divergence in the large family Cyprinidae. In addition, I examined differences in signal structure among species of *Cyprinella*. It may clarify the role of geographic isolation in producing behavioral diversity, and give insight into the process of speciation as it pertains to reproductive isolation and acoustic signaling. The results of this investigation will pave the way for further work on the dynamics of female choice, signal evolution, and speciation in this species and in the genus *Cyprinella*.

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CHAPTER 1

SOUND PRODUCTION AND ASSOCIATED BEHAVIORS IN *CYPRINELLA GALACTURA*

Although described for other species in the genus, sound production has not been investigated in *Cyprinella galactura,* the whitetail shiner. Furthermore, no thorough description of signal structure exists for any species of *Cyprinella*. In this study, *Cyprinella galactura* produced sounds during agonistic and courtship interactions associated with the breeding season. Females did not produce sounds. Signals were complex and could be monophasic (one call type/call), diphasic (two call types/call), or triphasic (three call types/call). Three call types (knocks, short knocks, and pulses) were identified and characterized by differences in duration and dominant frequency. All call types were non-harmonic, low frequency, and could occur in trains. Signals contained both gross-scale (require no magnification to measure), and fine-scale (require magnification to measure) temporal signal parameters. The exact mechanism of sound production in this species is unknown. Sounds are most frequent during low and moderate level displays, and decrease during the highest level of motivation under both contexts. Agonistic signals are significantly more variable than those produced during courtship, and the two contexts can be distinguished on the basis of temporal and spectral parameters. Larger males and territory owners win the majority of contests for spawning sites.

INTRODUCTION

Sound production in many fishes is a mechanism of communication used during territory defense (Lugli, 1997) and accompanying elaborate visual displays during courtship and reproduction (Delco, 1960; Winn and Stout, 1960; Stout, 1975; Lugli et al., 1996). In addition, sound production may play a role in species recognition (Myrberg et al., 1978) and mate choice (Myrberg et al., 1986). Sound production is only beginning to be described in North American freshwater fishes. Examples of sound documentation in this group include some percids in the subgenus *Catonotus* (Johnston and Johnson, 2000), centrarchids (Gerald, 1971; Ballantyne and Colgan, 1978), fundulids (Drewry, 1962), ictalurids (Rigley and Muir, 1979; Fine et al., 1997) and cyprinids in the genus *Pimephales* (Johnston and Johnson, 2000), *Cyprinella* (Delco, 1960; Winn and Stout, 1960; Stout, 1975), and *Codoma* (Johnston and Vives, 2003).

With over 2000 species, cyprinids comprise the largest family of freshwater fishes in North America and arguably the largest family in the world (Helfman et. al., 1997). Little is known about the significance or distribution of sound production within this group. The widely distributed and large genus *Cyprinella*, with 27 (Mayden, 1989) to 30 (Broughton and Gold, 2000) species may provide insight into the evolution of acoustic signaling in freshwater fishes. Sound production has been described for three species of *Cyprinella* (Delco, 1960; Winn and Stout, 1960; Stout, 1975), but all are believed to produce sounds (Johnston, unpubl.; Phillips and Johnston, unpubl.).

Sound production has not been documented in *Cyprinella galactura*, the whitetail shiner. Classification of signals can be difficult as signals in some *Cyprinella* can be extremely variable, often containing multiple call types, which vary by context as well as motivation of the male (personal observation). It is often difficult to determine where the signal starts and ends (unless behavior is closely associated). Although gross temporal structure has been described in *C. venusta*, *C. lutrensis* (Delco, 1960), and *C. analostana* (Winn and Stout, 1960; Stout, 1975), fine scale temporal structure has not been fully described in this genus. This study intends to provide a method of dissecting complex *Cyprinella* signals that can be used in a thorough acoustic description of the species, and as a type of roadmap for describing other complicated species in the genus.

Cyprinella galactura was also chosen as a model for an investigation of the role of sound production and behavioral context within a species. Unlike some other species of *Cyprinella*, *C. galactura* produce sounds during both agonistic and courtship displays (Phillips and Johnston, submitted). *Cyprinella galactura*, like other species of *Cyprinella*, lay eggs in crevices found in submerged logs and bedrock (Outten, 1961; Pflieger, 1975). Male *Cyprinella* often establish dominance hierarchies around these territories where the dominant male will defend the crevice from intruders (personal observation) and also recruit and spawn with females. The agonistic interactions between males involve a series of graduated displays ranging from chases to lateral displays and circle swims. Courtship behaviors are also associated with visual displays such as male-female orbiting, where the male will rapidly circle the female. Both agonistic and courtship displays are coupled with acoustics in many of the contexts (personal observation). Differences in signal structure and variability among the contexts will be examined.

This study describes acoustic signal structure in *C. galactura*. A method was established for data extraction of the complex, variable signals found in *C. galactura* that is applicable to other species of cyprinids. Correlations between temporal and spectral signal parameters are also discussed. Agonistic and reproductive behavior in *Cyprinella galactura* is described, including one behavior undescribed in minnows. In addition, the role of male body size and territory ownership on eliciting agonistic behaviors and on conflict outcome is examined. The role of sound production during behavioral context is examined.

METHODS

During the spawning season 2002 (May – August) and 2003, *Cyprinella galactura* were collected with seines. Collections were made from: Nottely River (Tennessee River drainage), Hwy 180, just E of Hwy 129/19, Union County, Georgia, 30 May 2002; Lick Creek (Duck River drainage), 6 mi. SW of Greenbrier, Maury County, Tennessee, 12 June 2002; Crooked Creek (White River drainage), 5 mi. E of Harrison, Blount County, Arkansas, 9 July 2002; Piney Fork (Black River drainage), 4 mi. NW of Evening Shade, Sharp Co., Arkansas, 14 June 2003. (State permits were issued to CEJ and CTP; AUM animal protocol number 2004-0663).

Fish were transferred to the laboratory and placed in 84 l aquaria. Fish were fed a diet of bloodworms and commercial fish flakes daily. Their photoperiod was regulated at 10-12 hours of light. All behavioral trials were performed between 30 May - 2 August 2002 and 17 June - 30 July 2003. The trial aquaria were kept in a separate insulated room and isolated from the lab table by a layer of foam. Each aquaria contained a sand substrate and an artificial crevice nesting cavity. Both male-male and male-female trials were set up to gather agonistic and courtship data. For male-male trials, one male, usually fairly large (> 80.0mm), was placed in an aquarium with 2-4 females. This male was given time to acclimate, achieve dominance and establish a territory (minimum of 3 hours). A second male, either smaller in size, of similar size, or of larger size was added to stimulate agonistic behavior. Interactions between males were recorded and the winner of any dominance dispute was noted. The winner was identified as the male that successfully established and defended the crevice territory after a period of male-male dispute. The loser was identified as the individual that no longer engaged in displaying and remained motionless on the bottom of the tank. In the case of intense fighting where no winner was established after approximately 1-3 trials, the introduced male was removed to avoid unnecessary stress, and no winner was declared. Courtship behavior was usually observed following removal of the secondary male. The number of times each behavior occurred was counted and sound production, if any, was noted. Trials were also simultaneously videotaped. All males used in trials were kept separated in labeled aquaria. Following examination of the population, individual standard lengths (SL, mm) of males were measured with a dial caliper.

Acoustic methods

Sounds were recorded using a Bruel and Kjaer 8103 hydrophone, Bruel and Kjaer 2635 charge amplifier and Sony model TC-D5 Pro II stereo cassette recorder. Sounds were digitized and analyzed using Raven ver. 1.1 (Cornell University). Temporal parameters were measured from the waveform and spectral parameters were measured from the power spectrum. Spectrograms were generated using the following settings: Hanning window, clipping level, filter bandwidth 124 Hz, frame length 512 pts, and 50% overlap. Power spectra were generated using a Hanning window of 1024. Signals were not filtered prior to data extraction as acoustic signal information overlapped in the low frequency range. Signals were sorted by population, male number, and associated behavior.

Acoustic signals that occurred during a particular behavior were considered associated. Observation periods were 30 minutes in length. Due to the reverberant effect of using small tanks (Parvalescu, 1967), the hydrophone was placed within the range of attenuation length from the fish to ensure signal accuracy (Akamatsu et al., 2002; Okumura et al., 2002). Signals contaminated with excess noise or interference were excluded from the analysis. Contamination was assessed through observed interference (fish hitting the hydrophone or crevice) or by examination of the waveform and spectrogram (low frequency electrical interference).

Cyprinella galactura signals were extremely complex and highly variable, unlike previously analyzed species, *C. gibbsi* and *C. trichroistia* (Phillips and Johnston, submitted), but similar to the ornate minnow, *Codoma ornata* (Johnston and Vives, 2003). A uniform signal structure was not immediately obvious in most calls. Some signals were lengthy and visual comparisons between populations were not an option. Signals were first magnified until individual component structure could be recognized. Separate components were identified visually and audibly, by examining the structure in the spectrogram, along with listening to the individual components simultaneously. For example, all components that did not contain pulses and were non-harmonic were identified through structure and sound, and then grouped. Once groups were established, temporal and spectral measurements (Fig. 1-1) were extracted manually using Raven 1.1

(Cornell University). Additional descriptive quantitative parameters can be found in Appendix A.

Data analysis of duration and dominant frequency for the grouped components was used to identify three distinct groups which will be referred to as call types: knocks, short knocks and pulses (Fig. 1-2). These call types could be arranged in different combinations to form a typical *C. galactura* call. Calls usually varied in the number and type of call types present as well as the combinations of call types per signal grouped by behavioral context. Two categories of temporal parameters were defined in these call types: gross-scale temporal structure and fine-scale temporal structure. Gross-scale temporal structure included what was visible to the human eye and could easily be measured without magnification. The three call types were considered to be gross-scale temporal structure (knocks, short knocks, and pulses). Fine-scale temporal structure included the pulses typically found in an organized burst (train of pulses), and although individual pulses could be visible in the typical signal, they were not well defined. Measurements on fine-scale temporal structure required magnification to reveal the pulse mode structure.

Signal call types were not periodic (spaced evenly) within each signal, so intervals directly following each call type were measured to obtain intercomponent durations. Only pulses and pulse intervals within bursts were found to exhibit periodic structure. Behavior at the time of sound production further complicated signal structure dissection as the type and number of components as well as the total call duration would vary based on male escalation (degree of aggressive or courtship pursuit). Call types were categorized by specific behavioral contexts.

A number of temporal and spectral parameters were measured from the signals (Table 1-1, Fig. 1-1). Temporal parameters are measured from the waveform. Total call duration was defined as the time from the beginning to the end of the call as measured from the first mode to the last mode. Knock duration was the time from the beginning to the end of a knock. Knock interval was the time from the end of the knock to the next signal component. Short knock duration was the time from the beginning to the end of a short knock. Short knock interval was the time from the end of the short knock to the beginning of the next signal component. Burst duration was the time from the beginning to the end of one pulse burst (measured from first mode of first pulse to last mode of last pulse). Burst interval was the time from the end of the burst (last mode of last pulse) to the beginning of the next signal component. Burst rate was calculated as the number of bursts divided by the total call duration. Pulse duration refers to fine-temporal structure within a burst. It was measured as the time difference from one mode to the next mode. Pulse interval was measured as the time difference from one peak to the next. Pulse rate was measured as the number of pulses divided by the total burst duration.

Spectral parameters were measured from the power spectrum. Knock dominant frequency was the knock mode with the highest energy. Knock secondary frequency was the knock mode with the second highest energy. Short knock dominant frequency was the short knock mode with the highest energy. Short knock secondary frequency was the short knock mode with the second highest energy. Burst dominant frequency was the burst mode with the highest energy. Burst secondary frequency was the burst mode with the second highest energy. Additional qualitative information was extracted such as the mean number of knocks per call, the mean number of short knocks per call, the number of pulse bursts per call, mean number of pulses per burst, and the mean number of single pulses per call.

Signals within species were analyzed separately according to context (agonistic vs. courtship). Furthermore, agonistic signals were separated by specific context into 4 groups: sedentary aggression, male-male chase, male-female chase, and lateral display. Courtship signals were relatively stereotyped and could be lumped into one comparative category.

Statistical analysis

All statistical analyses were conducted using the SPSS software package (SPSS ver. 13.0, SPSS Inc, Chicago, IL). The use of the behavioral/acoustic term "parameter" is intended to be equivalent to the term "variable" when describing analyzed data. Descriptive statistics, paired-sample t-tests, and analysis of variance (ANOVA) with Tukey’s multiple comparison tests were used to evaluate differences. All components, including fine-scale structure, were measured from all signals to capture true variation within signals and not lose important signal information. Signal parameters for call types within each call were averaged prior to analysis.

Principal components analysis (PCA) was used to reduce the number of parameters. Discriminant function analysis (DFA) on acoustic parameters was used to investigate classification of signals by context. Coefficients of variation (CV=mean/standard deviation x 100) were calculated for each acoustic property to compare parameter variability by behavioral context.

A percent occurrence of sound production was calculated for behaviors from all of the trials. Each obvious behavior was noted, and a percentage of the time a sound was produced during each behavior was calculated (# times sound produced during the behavior/# times behavior observed * 100). These percent occurrences were not calculated for non-obvious behaviors, such as sedentary aggression, as, in this case, one could only designate the behavior after sound was produced.

Mean occurrence was used in lieu of percent occurrence for measuring behaviors during male-male contests. Male-male contests were divided into 5 size classes by difference in standard length between the males (class 1: <5mm difference, class 2: 5-10mm difference, class 3: 10-15mm difference, class 4: >20mm difference). Six behaviors were recorded during each trial (sedentary aggression, male-male chase, lateral display, parallel swim, and lip lock). These behaviors were summed per trial and averaged among the other trials for each size class. This mean occurrence was recorded for each context by size class.

RESULTS

Behavior in *Cyprinella galactura*

Behaviors were grouped into 2 categories, agonistic and courtship. Agonistic or aggressive behaviors occur during dominance establishment, territory defense, and male-male assessment. Courtship behaviors occur during recruitment of females to the nesting site, mate attraction displays, and prior to and during the act of spawning.

These categories can be further subdivided into individual behaviors that vary in level of escalation (Table 1-2). Agonistic behaviors (in order of increasing escalation) include: sedentary aggression, nudge, male-male and male-female chase, parallel swim, lateral display, circle swim, lip lock, and biting. Courtship behaviors (in order of increasing escalation) included: male inspection of crevice, crevice pass, male-female pursuit, male-female approach, male orbits female, male sits on female and spawning.

Agonistic behaviors

Sedentary aggression occurred when the male was not in motion, was located near an established territory and may be threatened by an approaching intruder. This behavior is not especially obvious, but occurred frequently and is recognizable as the agitated male will generally produce sounds in response to the intruder. The "nudge" was a low level aggressive behavior in which the male slowly moves toward another male, but did not complete the chase. The "chase" involved a male chasing a male or a male chasing a female and occurred when the male advanced towards another fish and chased it some distance away from the territory. The "parallel swim" was when two males swam together in alignment with fins erect. This display was used to assess size and subsequently settle the territory dispute. The "lateral display" occurred when two males lined up in opposite directions (head to tail, and tail to head) and swam slowly with fins erect. This display was also used to assess size and settle a dispute. The "circle swim" was a more elevated level of aggression than the "parallel swim" or the "lateral display" and usually occurred after the other displays had been performed with no dispute settlement. The "circle swim" involved two males, usually of similar size, that swam rapidly in a circle (head to tail, head to tail) chasing one another. The "circle swim" could be performed multiple times until one male became tired and the dispute was settled.

One aggressive behavior previously undescribed in cyprinids was observed in *Cyprinella galactura* males. The "lip lock" typically occurred during territory disputes between males of equal or nearly equal body size (<10mm size difference) and degree of coloration. The lip lock display was an elevated level of aggression that occurred usually only with males that were unable to settle the dispute with lateral displays and circle swims. This behavior involved a large degree of bodily contact and began with a head bashing behavior in which two males rapidly spun around, faced one another and charged toward one another bashing heads and then rapidly followed by a circle swim. As the aggression escalated further, the head bashing increased and males would actually clasp lips together and undulate their body from side to side for approximately 1-5 seconds. Lip locks were interspersed with other forms of intense aggression such as biting the opposing male's fins or torso. This stage was decidedly the most elevated form of aggression and could be labeled as a type of fight. Fighting in fishes involves bodily contact and can result in the injury and even the death of the loser.

A total of 2102 obvious agonistic behaviors were observed in 123 *C. galactura* trials. Chases were the most common behavior and occurred 46.5% (n=2102, total agonistic behaviors) of the time. Lateral displays were the second most common and occurred 36.1% (n=2102) of he time. Lip locks were observed 10.8% (n=2102) of the time. Circle swims were observed only 5.2% (n=2102) of the time, and parallel swims were observed only 1.4% (n=2102) of the time.

Courtship behaviors

In courtship there was a behavior that was not easily apparent to the observer, but mimicked sedentary aggression in that the male was not in motion but was located near, or often, in front of, an established territory or nest site. This “behavior” has been labeled “male at crevice” and it was sometimes coupled with acoustic signaling (see next section), and may serve as a form of female recruitment. During early courtship, the male established a nesting territory and proceeded to inspect the crevice for nest suitability. The male would then place his nose in and along the length of the crevice several times while defending the territory from intruders. After the territory was established, the male then proceeded to a "crevice pass" behavior along the crevice. The "crevice pass" behavior was first described in *Cyprinella* by Stout (1975) and occurred when the male would swim along the crevice undulating his body rapidly along the length of the crevice. The "male-female pursuit" was a low-level courtship behavior and mimicked the male-female aggressive chase with a few small distinctions. Like the chase, it occurred when the male chased a female for some distance; however, the chase usually proceeded in any direction in the tank (both away from the territory and toward the territory), with the intention of coaxing the female toward the nesting site. The "male-female pursuit" generally proceeded toward the nesting site. The "male-female approach" occurred when the male positioned himself laterally along side the female. This behavior occurred in close vicinity to the nesting site usually right in front of the crevice. The female had already shown some interest in the crevice before the occurrence of the "male-female approach". In addition, the male performed two behaviors that signaled an escalated courtship response. The first is where the male will orbit the female and the male will swim rapidly around the female usually in an elliptical pattern (versus circular). A similar behavior has been observed in sunfish (*Lepomis*) (Ballantyne and Colgan, 1978). The second behavior occurred when the male hovered on top of the female and pushed down on her back (possibly pushing her towards the nesting site). Spawning followed with the male and female vibrating along the length of the crevice and releasing gametes.

A total of 398 obvious courtship behaviors were observed in 17 *C. galactura* trials. Male-female approaches were the most common behavior and occurred 62.8% (n=398, total courtship behaviors) of the time. Crevice passes were the second most common and occurred 26.6% (n=398) of the time. Spawning was observed only 7.3% (n=398) of the time, and male-female orbiting was observed only 3.3% (n=398) of the time.

Male Contests - Body size and aggressive behavior

Fifty-four trials involving established and challenger male *C. galactura* of varying body size revealed that size can play an important role in the triggering of a particular behavior or level of aggressive escalation (Fig. 1-3). Males were grouped into 5 size classes based on difference in standard length: 1-5mm difference (17 trials), 5-10mm difference (12 trials), 10-15mm difference (6 trials), 15-20mm difference (7 trials) and greater than 20mm difference (12 trials). In general, males that were similar in body size (<10mm difference) were more likely to engage in highly aggressive behaviors such as lateral displays, circle swims, and lip locks. Lateral displays were observed a mean of 25.8 ± 21.6 times (387 displays total, 15 trials) in the 1-5mm group, a mean of 20.0 ± 20.6 times (200 displays, 10 trials) in the 5-10mm group, a mean of 17.2 ± 10.0 times (86 displays, 5 trials) in the 10-15mm group, and only a mean 4.0 ± 0.0 times (8 displays, 2 trials) in the greater than 20mm group. Circle swims were observed a mean of 42.5 ± 10.6 times (85 displays, 2 trials) in the 1-5mm group and a mean of 12.0 ± 14.1 times (24 displays, 2 trials) in the 5-10mm group, while not at all in the other size classes. Lip locks were observed a mean of 72.0 ± 33.9 times (144 displays, 2 trials) in the 1-5mm group and a mean of 41.5 ± 2.1 times (83 displays, 2 trials) in the 5-10mm group, while not at all in the remaining size classes.

Low-level aggressive behaviors (e.g., sedentary aggression and male-male chases) were observed relatively equally in all size classes. Sedentary aggression was observed a mean of 9.2 times (384 displays, 42 trials) in all size classes. Male-male chases were also observed relatively equally, a mean of 14.8 ± 10.7 times (518 displays, 35 trials) in the top 4 size classes (1-5mm: 194 displays, 14 trials; 5-10mm: 173 displays, 6 trials; 10-15mm: 77 displays, 6 trials; 15-20mm: 100 displays, 12 trials) and only a mean 8.3 ± 7.3 times (100 displays, 12 trials) in the greater than 20mm size class. Parallel swims were rare overall and were observed more often in the 1-5mm size class at a mean of 3.3 ± 2.9 times (23 displays, 7 trials).

Male Contests - Established vs. intruder males

Thirty-four agonistic trials between established and challenger males of varying size revealed the value of an established resource such as the crevice nesting territory as well as body size in determining the outcome of a challenge. In 91.2% (number of trials=34) of the trials the established male won. Although the established male was larger in body size in 74.2% (trials = 31) of the trials, for 25.8% (trials=31) of the time where the established male was the winner, the established male was smaller in body size than the intruder. The intruding male only won 8.8% (number of trials=34) of all challenges. In all of these cases, the intruding male was larger in body size than the established male.

Acoustic repertoire

One hundred sixteen, 30 min trials were performed for *Cyprinella galactura* (44-Nottely Rv., 31-Lick Cr., 19 – Crooked Cr., 22- Piney Fk). A total of 42 males were observed (8 - Nottely Rv., 17 - Lick Cr., 8 - Crooked Cr., 9 - Piney Fk.). Fewer males actually produced sounds (33 total: 8 - Nottely Rv., 11 - Lick Cr., 5 - Crooked Cr., 9 - Piney Fk.). A total of 4182 sounds were observed and recorded from those males (1564 – Nottely Rv., 1046 – Lick Cr., 627 – Crooked Cr., 945- Piney Fork). Males that included fewer than 5 signals for the particular context examined were excluded from analysis.

Male *C. galactura* produced complex acoustic signals consisting of 1-3 call types arranged in any combination in a given signal. These call types are called: knocks, short knocks, and pulses. Pulses were usually arranged in organized trains called bursts. The *C. galactura* call was also found to be monophasic, diphasic, or triphasic (Figs. 1-4,1-5). Monophasic signals only contained one call type and could be anything from a single knock to a lengthy train of knocks. Diphasic calls typically contained two call types, and triphasic calls contained all three call types. This depicts a complexity not previously detailed in other studies of freshwater fishes.

All call types were relatively low frequency and non-harmonic. Differences among call types were tested with single-factor ANOVA (Table 1-3). All courtship call types were significantly different from one another in both duration (F=24.218, $p<0.001$) and dominant frequency (F=18.338, $p<0.001$) and all agonistic call types were significantly different in both duration (F=229.430, $p<0.001$) and dominant frequency (F=142.269, $p<0.001$).

Knocks had a mean dominant frequency of 91.8 ± 35.5Hz (n=318; 33 males) and a mean duration of 166.0 ± 62.0ms (n=487; 33 males) for agonistic signals and a mean dominant frequency of 103.9 ± 43.9Hz (n=43; 15 males) and mean duration of 155.0 ± 52.0ms (n=46; 15 males) for courtship signals. Short knocks had a mean dominant frequency of 79.0 ± 28.7Hz (n=142; 28 males) and a mean duration of 69.0 ± 14ms (n=327; 28 males) for agonistic signals and a mean dominant frequency of 73.8 ± 26.6Hz (n=15; 13 males) and 72.0 ± 12.0ms (n=20; 13 males) for courtship signals. Pulses had a mean dominant frequency of 53.8 ± 16.2Hz (n=294; 33 males) and a mean duration of 27.0 ± 14.0ms (n=207; 33 males) for agonistic signals and a mean dominant frequency of 75.4 ± 22.9Hz (n=113; 15 males) and a mean duration of 35.0 ± 14.0ms (n=133; 15 males) for courtship signals. However, pulses were usually grouped into a burst which is an organized train of pulses. Bursts had a mean duration of 321.0 ± 270.0ms (n=489; 33 males) for agonistic signals and a mean duration of 508.0 ± 443.0ms (n=133; 15 males) for courtship signals.

Sound production according to context

Male *C. galactura* produced sounds during a variety of behavioral contexts including both aggression and courtship. All call types were produced in all contexts (Table 1-4), although some call types were more prevalent than others by context. A trend was apparent between the types of sounds produced and levels of increasing aggression or courtship escalation (Table 1-5). During early stages of aggression, such as sedentary aggression, knocks were produced in 50.6% (n=176, number of calls produced during sedentary aggression) of all calls. Short knocks were produced in 40.9% (n=176) of sedentary aggression calls, and bursts were produced in only 35.2% (n=176) of sedentary aggression calls.

Male *C. galactura* used acoustics during male-male and male-female chases. Overall, sounds were produced 89.5% (n=1088, observed number of male-female chases) of the time during male-female chases and 93.5% (n=977, observed number of male-male chases) of the time during male-male chases. All call types were present in both types of chases. Knocks were typically produced in 64.6% (n=289, number of analyzed male-male chase signals) of all male-male chases and in 57.7% (n=220, number of analyzed male-female chase signals) of all male-female chases. Short knocks were produced in 30.8% (n=289) of male-male chases and 37.3% (n=220) of male-female chases and bursts were produced in 43.3% (n=289) of male-male chases and 35.5% (n=220) of male-female chases.

Males produced sounds 60.0% of the time during parallel swims, although this behavior was rarely observed in the laboratory (probably due to tank size). Sounds were most prevalent during lateral displays, being produced 96.7% (n=758, observed number of lateral displays) of the time. Although lateral displays included all call types, knocks and short knocks were only present 31.4% (n=258, number of analyzed lateral display signals) and 32.2% (n=258) of the time, respectively. Bursts were the dominant call type, produced during lateral displays 84.5% of the time (n=258). As aggression escalated to circle swims (0%, n=109, number of observed circle swims) and lip locks (0%, n=227, number of observed lip locks), sound production ceased to exist.

A similar trend is observed during courtship. Sounds were produced only 13.2% (n=106, number of observed crevice passes) during crevice passes. Crevice pass acoustic signals were composed of knocks 50.0% of the time (n=6, number of analyzed crevice pass signals), short knocks only 16.7% (n=6) of the time, and bursts 100.0% (n=6) of the time. During male-female orbiting, sounds were produced 100.0% (n=13, number of observed male-female orbits) of the time and during male-female approaches sounds were produced 98.5% (n=250, number of observed male-female approaches) of the time. The majority of these sounds were bursts produced in 90.3% (n=113, number of analyzed male-female approach signals) of all male-female approach signals. Finally, during spawning, sound was only produced 24.1% (n=29, number of observed spawns) of the time mimicking the trend seen in higher levels of aggression.

Courtship vs. Agonistic Signals

Agonistic signals included those sounds produced during sedentary aggression, male-male and male-female chases, and lateral displays. Agonistic signals could include anything from a simple call of a single knock or train of knocks to a complex call containing knocks, short knocks and bursts of pulses interspersed in one signal. Lateral display agonistic signals (Fig. 1-6) were compared with courtship signals (Fig. 1-6) due to similarity in signal structure (both contain bursts with pulses). Parameters compared include total call duration, average knock duration, burst duration, pulse duration, pulse interval, pulse rate, burst rate, and burst dominant frequency (Table 1-6). Paired t-tests between the contexts revealed significant differences in both temporal and spectral parameters.

All pulse parameters between courtship and agonistic signals were significantly different ($\alpha = 0.006$ based on a Bonferroni correction). Parameters that were not significant include total call duration, knock duration, and burst rate. Mean total call duration was 6.5 ± 5.4sec (n=150, courtship signals) for courtship calls and 7.0 ± 6.6sec (n=257, lateral display signals) for agonistic calls (t= -0.668, p=0.505). Mean knock duration was 155.0 ± 54.0msec (n=45, courtship signals with knocks) for courtship calls and 154.0 ± 56.0msec (n=81, lateral display signals with knocks) for agonistic calls (t=1.267, p=0.226). Mean burst rate was 1.4 ± 0.9 bursts/second (n=133, signals with multiple bursts) for courtship calls and 1.6 ± 1.0 bursts/second for agonistic calls (n=207) (t= -1.925, p=0.057). The other parameters examined displayed significant differences between the contexts. Mean burst dominant frequency was 71.4 ± 54.1 Hz (n=104) for courtship signals versus 54.1 ± 16.6 Hz (n=157) for lateral display agonistic signals (t=6.224, p=0.000). Mean pulse duration was 35 ± 14msec (n=133, number of courtship signals with pulses) for courtship signals versus 27 ± 10msec (n=207, number of agonistic signals with pulses) for agonistic signals (t=5.013, t=0.000). Mean pulse interval was 34 ± 14msec (n=133) for courtship signals versus 28 ± 15msec (n=207) for agonistic signals (t=3.181, p=0.000). Mean pulse rate was 17.6 ± 4.7 pulses/sec (n=132) for courtship calls versus 24.0 ± 15.6 pulses/sec (n=207) for agonistic signals (t=-4.234, p=0.000). Mean burst duration was 508.0 ± 443.0msec (n=133, number of courtship signals with bursts) for courtship signals versus 336.0 ± 270.0msec (n=207, number of agonistic signals with bursts) for agonistic signals (t=2.803, p=0.006).

Factor loadings from principal components analysis (PCA) of courtship parameters (Table 1-7) depict which signal parameters contribute the most to signal variation and resulted in 2 factors accounting for 79.7% of the variation, with 56.4% of the variation represented by the first factor, while 23.1% of the variation represented by the second factor. For courtship characters, factor 1 had the highest loadings for pulse duration (0.975), pulse rate (-0.941), and pulse interval (0.977). Factor 2 had high loadings for burst duration (0.746) and burst dominant frequency (0.756). These parameters were also the ones found to be significantly different between the contexts in previous analysis through paired T-tests.

Factor loadings from principal components analysis (PCA) of agonistic parameters (Table 1-7) depict which signal parameters contribute the most to signal variation and resulted in 2 factors accounting for 73.7% of the variation with 49.2% of the variation is represented by the first factor, while 19.0% of the variation is represented by the second factor. For agonistic characters, factor 1 had the highest loadings for pulse duration (0.951) and pulse interval (0.960). Factor 2 had the highest loading for burst dominant frequency (0.960), also found to be of significance in the paired T-tests between contexts.

There was no complete separation between the two contexts (courtship and agonistic) and a large area of overlap can be seen on the plot of the scores for the first two factors grouped by context (Fig. 1-7). However, factor 1 (t=-36.169, df=234, p<0.001) and factor 2 (t=-17.166, df=234, p<0.001) were significantly different from one another in a paired T-test among contexts.

A discriminant function analysis (DFA) was used to test for context specificity in signals. The same parameters determined to be useful in factor analysis were used for DFA. These included: burst dominant frequency, pulse duration, pulse interval, pulse rate, and burst duration. In a DFA on agonistic and courtship acoustic parameters, 73.7% of the signals were classified into the correct context, with 72.9% of the courtship signals classified correctly (n=98, number of courtship signals) and 74.3% (n=138, number of agonistic signals) of the agonistic signals classified correctly.

Variation between agonistic and courtship signals

Coefficients of variation (CV) were more variable in agonistic parameters than in courtship parameters (Table 1-8). Coefficients of variation for courtship calls ranged from 55.9 for mean single pulse duration to 176.4 for mean knock interval. Coefficients of variation for agonistic calls ranged from 71.4 for mean knock dominant frequency to 152.0 for mean burst interval. The agonistic CVs for total call duration, average knock duration, burst duration, burst interval, pulse duration, pulse interval, single pulse duration, pulse rate, and burst rate were all larger than courtship CVs. The CVs for short knock duration, short knock interval, single pulse interval, short knock dominant frequency, and burst dominant frequency were similar in agonistic versus courtship signals. Only the CVs for mean knock interval and knock dominant frequency were more variable in courtship signals versus agonistic signals.

DISCUSSION

This study described behavior and acoustic signal structure in *Cyprinella galactura*, the whitetail shiner. Agonistic behaviors in order from least escalated to most escalated included: sedentary aggression, male nudge, male-female chase, male-male chase, parallel swim, lateral display, circle swim, and lip lock. Courtship behaviors in order from least escalated to most escalated included: male at crevice, crevice pass, male-female pursuit, male-female approach, male sits on female, male orbits female, and spawning. One newly described highly aggressive behavior, the lip lock, was observed in *C. galactura*. Although never before described in cyprinids, this behavior is well known in male cichlids (Neat et al., 1998), and has been observed in percids in the genus *Etheostoma* (Johnston, personal communication). This behavior, the highest level of aggression and a type of fight, only occurs between males of similar size (<10mm difference) and degree of coloration.

Trials involving established and challenger male *C. galactura* of varying body size revealed that size could play an important role in the triggering of a particular behavior or level of aggressive motivation. Typically, males that were very similar in body size were the ones that exhibited highly elevated levels of aggression such as circle swims and lip locks. This likely occurred as males of similar size were unable to resolve territory disputes under lower levels of aggression such as during male-male chases and lateral displays. Johnston et al. (1996) also found that males of similar size were more likely to parallel swim than males of different size for three species of cyprinids (*Semotilus atromaculatus, Erimyzon oblongus, Luxilus chrysocephalus*).

Factors such as strength and body size are typical measurements males use to settle contests over territories (Krebs and Davies, 1981). Male *C. galactura* engaged in elaborate male contests to win territories (nesting crevices). In early stages of aggression, the defending or established male *C. galactura* knocked or growled at the intruder. Sound production in *C. galactura* may be an indicator of fighting ability. In order to call for a long time, a male would presumably be in good condition. If the challenger is a good match to the defender, then aggression would elevate and they may engage in parallel swims and/or lateral displays. If the males were still equally matched (usually <10mm size difference), aggression then escalated. Males butted heads vigorously, circle swam, and bit one another. Head butts gradually became lip locks where males locked lips and undulated vigorously. These male contests which are similar to those seen in red deer (head butting and then antler locking), *Cervus elaphus* (Clutton-Brock et al., 1979), may be direct or indirect trials of male strength (Krebs and Davies, 1981). Neat et al. (1998) found that mouth locking in a cichlid, *Tilapia zillii*, was either a trial of stamina or a means to assess the relative costs an opponent is prepared to incur.

Although strength or size is one measurement used to settle male contests, resource ownership can also play an important role (Hyman and Hughes, 2006). Turner (1994) found that territory owning mouthbrooding cichlid fish, *Oreochromis mossambicus*, were more likely to win contests with intruders, especially when the resident was larger in size. In *C. galactura*, the larger males usually won fights (76.5%, n=34); however, in 25.8% (n=31) the territory owner won even if he was smaller. Hyman and Hughes (2006) found that territory-owning male song sparrows, *Melospiza melodia*, respond differently to aggressive and nonaggressive neighbors, responding adaptively toward the individual that poses the greatest threat. *Cyprinella galactura* may use both strength/size and territory ownership to determine the outcome of contests between males.

Many of the above mentioned behaviors in *C. galactura* were coupled with acoustic signals. Classification of these acoustic signals is difficult in some *Cyprinella*, including *C. galactura* due to signal variability and complexity. Acoustic signals in *Cyprinella* are not as stereotyped as some terrestrial organisms (cicada – Pinto-Juma et al., 2005; treefrogs – Gerhardt and Doherty, 1988) and even some marine fishes (Myrberg et al., 1978). Myrberg et al. (1978) examined sound production in several species of damselfish (Pomacentridae), which produce sounds by muscular activity associated with the gas bladder, and found highly stereotyped pulsed signals. While gobies can exhibit pulsed signals similar in structure to *Cyprinella*, pulses are often periodic and occur in trains (Lugli et al., 1995). *Cyprinella* signal call types can occur outside of a periodic train. Furthermore, call types such as the complex call type in the common goby (*Padogobius martensii*) always occur in the same pattern (pulsed component and then tonal component) (Lugli et al., 1995). Other sound producing mechanisms, such as the stridulatory calls in the brown bullhead, *Ictulurus nebulosus*, are pulsed and periodic with a fixed pulse and interpulse duration (Rigley and Muir, 1979).

Cyprinella galactura signals consisted of three call types (knocks, short knocks and pulse bursts), which were produced in any combination, for any given context. Each signal contained gross-scale temporal structure and fine-scale temporal structure. Although gross-scale temporal structure has been examined in other species of *Cyprinella* (Delco, 1960; Winn and Stout, 1960; Stout, 1975), fine-scale structure has virtually been ignored in this group. With advances in technology such as acoustic software like Raven (Cornell University) that can aid in magnification and filtering of signals, analyses of sounds can be conducted with significantly greater precision. It is often the fine-scale pulse parameters that prove to be of interest in studying the evolution of acoustic communication in anurans (Ryan, 1990; Bush et al., 2002) and insects (Pinto-Juma et al., 2005; Higgins and Waugaman, 2004).

The signals produced by *Cyprinella galactura* resemble those described in other species such as *C. analostana* (Stout, 1975) and *C. callisema* (Phillips and Johnston, submitted) with signals consisting of knocks and pulse bursts. Calls could be monophasic consisting of one call type, and each call type could be present singly, or in a train (organized series). Furthermore, call types could be present in any combination for any context. *Cyprinella galactura* signals had a relatively low burst dominant frequency of 54.1 Hz during aggression and 71.4 Hz during courtship. Although this is extremely low frequency, some *Cyprinella* (*C. callisema* – 65.3 Hz), and other fishes (Mozambique tilapia, *Oreochromis mossambicus* – 40 Hz; Amorim et al., 2003) exhibit low dominant frequencies in pulsed call types. Although the mechanism of sound production is unknown in *Cyprinella*, low frequency pulsed signals (bursts) are often produced by contraction of muscles associated with the gas bladder (Demski et al., 1973; Fine et al., 1977).

The complex nature of signal structure in *C. galactura* may actually be due to the hypothesized ancestral nature of the signal. *Cyprinella galactura* seem to be less stereotyped and loose in composition and structure. The more derived species of *Cyprinella* actually seem to have more structured signals with fewer or extremely different call types (personal observation). For example, the tricolor shiner, *C. trichroistia*, and the Tallapoosa shiner, *C. gibbsi*, do not produce knocks or short knocks, and produce the fine-scale temporal pulse bursts for all contexts, as well as two unique harmonic call types during limited contexts (Phillips and Johnston, submitted). These fine-scale temporal pulse bursts are extremely structured and stereotyped.

The role of sound production during agonistic and courtship behavior in *C. galactura* was examined. Not all of the described behaviors were associated with sound production. Highly aggressive behaviors such as lip locking and circle swims were not associated with sound. This may be due to an energy tradeoff. Even though one study found that metabolic costs of sound production in the oyster toadfish, *Opsanus tau*, as measured by oxygen consumption, were not extremely energetically demanding (Amorim et al., 2002), sound production, for many taxa, is known to be energetically demanding. Wells and Taigen (1986) found that acoustic signaling in gray tree frogs, *Hyla versicolor*, can be energetically demanding. Taigen and Wells (1985) also found that rates of oxygen consumption are higher during episodes of calling than during episodes of maximum locomotion. Once aggression elevates to a level that may lead to fighting and injury, it may be too risky to spend any extra energy on producing sounds. If the dispute has not been solved though lateral display (which happens to be the most popular use of sound compared to all aggressive behaviors), then why waste more energy on calling during the more escalated aggressive behaviors (like circle swims) when one could conserve that energy for the fight ahead.

Cyprinella galactura were found to produce sounds frequently during courtship behaviors such as male-female orbiting (100%, n=13) and male female approaches (98%, n=250), but sound production was relatively infrequent during crevice passes (13%, n=106) and spawning (24%, n=29). Although other species of *Cyprinella* commonly produce sounds during the actual crevice pass behavior (Stout, 1975; Phillips and Johnston, submitted), sound production in *C. galactura*, and, interestingly, sister species *C. venusta* were absent (personal observation). Other species of *Cyprinella* also produce sounds commonly during spawning; however, spawning in *C. galactura* was seen relatively infrequently and this percentage of sound production during spawning may actually be larger.

Sound production during courtship, as opposed to aggression, may be universal in *Cyprinella* and closely related species, such as *Codoma ornata* (Johnston and Vives, 2003), which produce sounds frequently during courtship even at the most energetically expensive levels, such as immediately prior to and during spawning. Ryan (1988) reviewed calling energetics in several species of frogs and insects and found that though calling can be energetically demanding, females actually prefer components of signals which represent an expenditure of energy as a measure of male quality. This may explain why males produce sounds in all levels of courtship. The tight coupling of sound production to visual behavior cues may demonstrate the importance of courtship signals in species identification or even sexual selection within species.

Although courtship and agonistic signals contained all elements of the *C. galactura* signal repertoire (knocks, short knocks, and pulses), certain contexts were more likely to contain particular call types. Typically, aggressive signals contained more knocks and short knocks. Knocks were present in 51.6% of all aggressive calls versus 31.3% of all courtship calls. Short knocks were present in 34.6% of all agonistic calls versus 13.4% of all courtship calls. Pulse bursts were more common during higher levels of aggression and courtship and were present in 84.5% of all lateral display calls, and 90.3% of all male-female approach calls. Aggressive signals contained more knocks interspersed with bursts, whereas courtship signals contained many long bursts with few knocks. Due to these differences, agonistic and courtship signals often sounded different to the trained human ear.

Significant differences existed between the contexts for one spectral parameter, burst dominant frequency, and several temporal parameters: burst duration, pulse duration, pulse interval and pulse rate. Courtship calls had higher dominant frequencies (71.4 Hz) than agonistic calls (54.1 Hz). Lower frequency signals are known to travel further in water than high frequency signals (Bradbury and Vehrencamp, 1998). This may reflect that courtship calls are typically only used for short-range communication of males toward potentially receptive females. Agonistic calls may be intended for longer range communication with neighbors as well as intruders. Courtship signals also tended to contain larger bursts (508ms) than agonistic signals (336ms), and longer pulses (35ms) than agonistic signals (28ms). The courtship pulse rate is slower at 17.6 pulses/sec versus 24.0 pulses/second for agonistic signals. These signal differences may be important in mate choice, and may be crucial in the design of a playback model for future studies.

Principal components analysis found that the same signal parameters that were significant in ANOVA contributed to the most variation. Although there was no complete separation between the two contexts, factors from PCA were significantly different from one another in paired t-tests (Factor 1, p=0.000; Factor 2, p=0.000).

Discriminant function analysis did group contexts successfully 71.6% of the time using the same significant parameters.

When comparing variability among the contexts, agonistic signal parameters were found to be much more variable than courtship signal parameters. Johnson (2001) also saw greater variability in agonistic pursuit calls versus courtship calls in longear sunfish, *Lepomis megalotis*. *Cyprinella galactura* is sympatric with other sound producing species of *Cyprinella*: *C. spiloptera, C. whipplei,* and *C. vensuta* (Gibbs, 1961), and parameters may be more general as they could be used among heterospecifics as well as conspecifics in territory establishment and defense. Courtship signals are less variable and may encode important species identification information.

This study was the first to examine gross as well as fine-scale signal structure in *C. galactura*. This study revealed a complexity previously unexamined in the genus. An equally detailed and consistent examination will be necessary in the other described and undescribed species of *Cyprinella* before further comparisons within the genus can be made.

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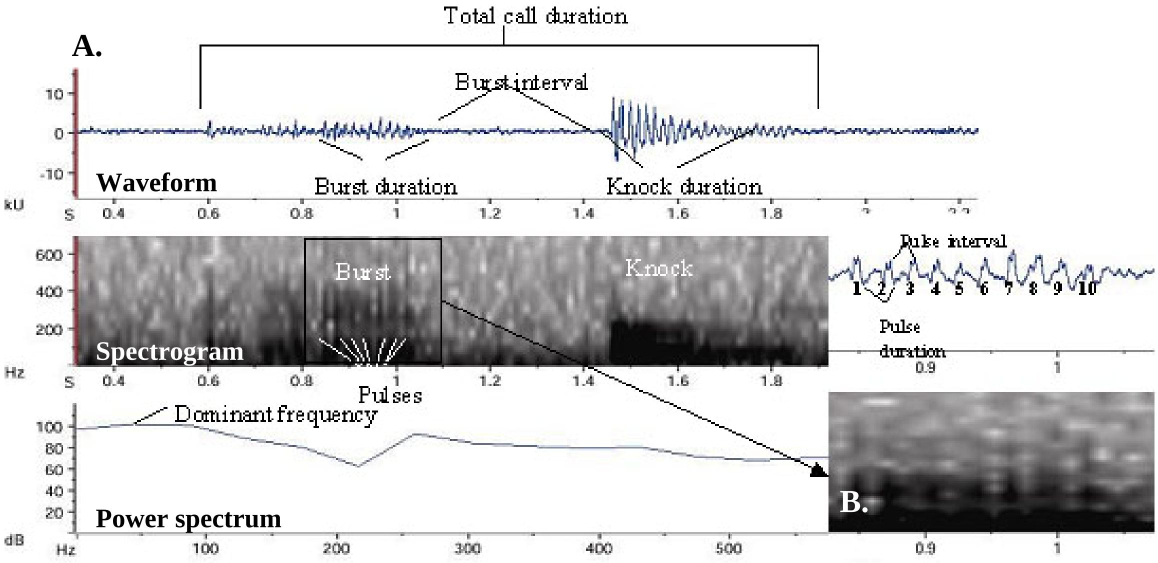

Fig. 1-1. Diagrammed *Cyprinella galactura* signal with measurements labeled. A) Gross-scale temporal structure. B) Fine-scale temporal structure. The waveform depicts amplitude (kU) versus time (sec). The spectrogram depicts frequency (Hz) versus time (sec). The power spectrum depicts amplitude (dB) versus frequency (Hz).

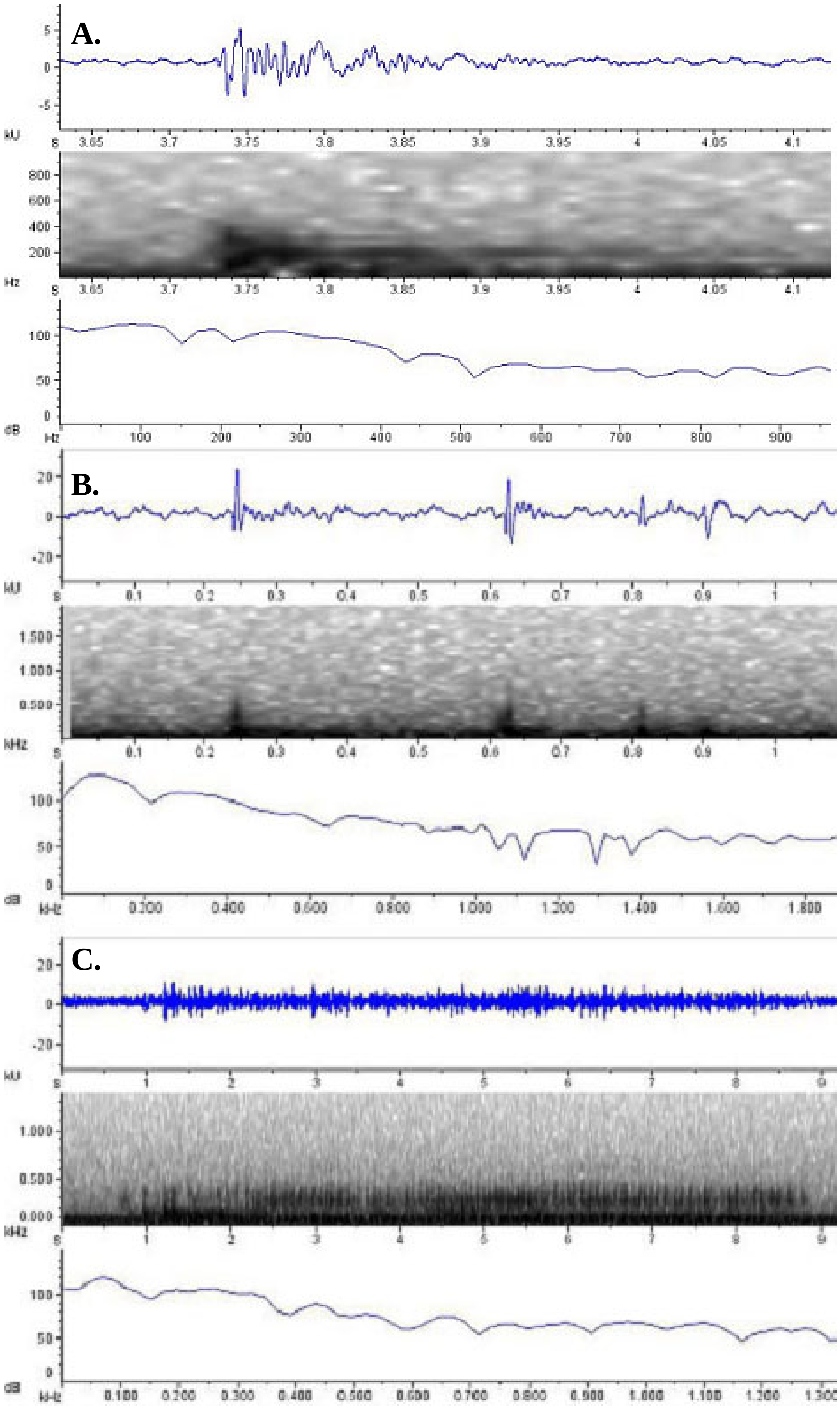

Fig. 1-2. *Cyprinella galactura* call types. A) Knock. B) Short knock. C) Pulse burst.

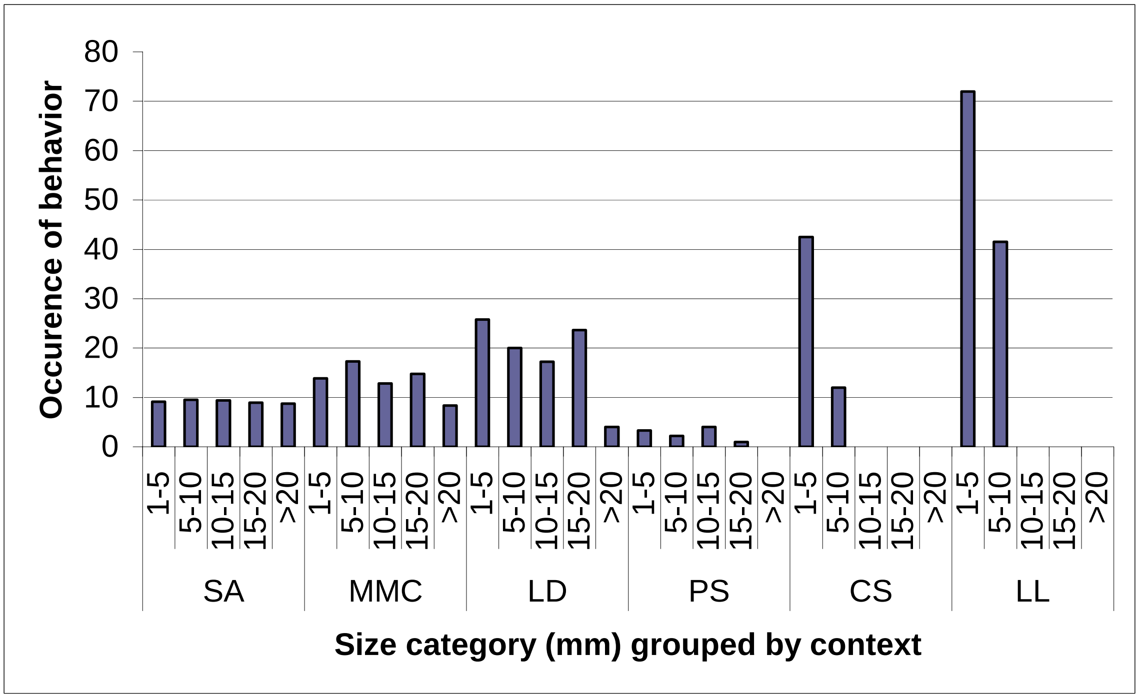

Fig. 1-3. Mean occurrence of behavior during male-male challenges categorized by difference in male size. Size classes include: 1-5mm difference, 5-10mm difference, 10-15mm difference, 15-20mm difference, and >20mm difference. Behaviors include: SA=sedentary aggression, MMC=male-male chase, LD=lateral display, PS=parallel swim, CS=circle swim, LL=lip lock.

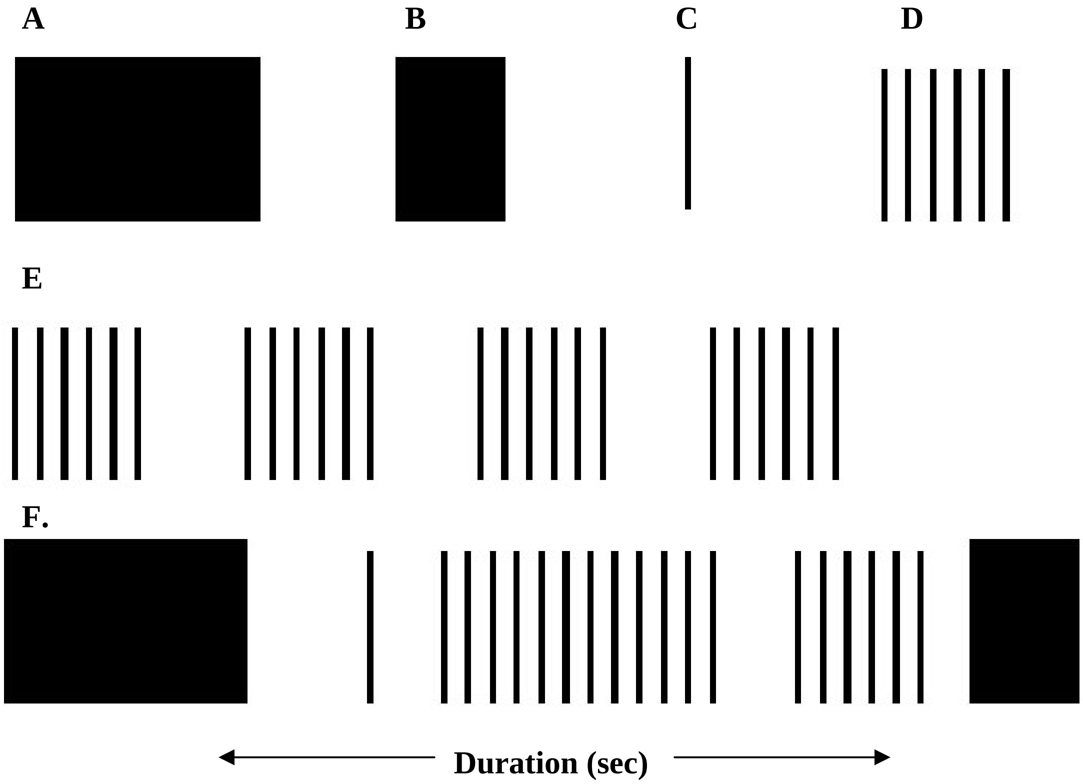

Fig. 1-4. Various types of signals found in *Cyprinella galactura* based on temporal patterns. A) Knock. B) Short knock. C) Pulse. D) Pulse burst. E) Monophasic signal consisting of one call type, in this example, a train of pulse bursts. E) Triphasic signal consisting of three call types, knocks, short knocks, and pulses occurring singly or in bursts.

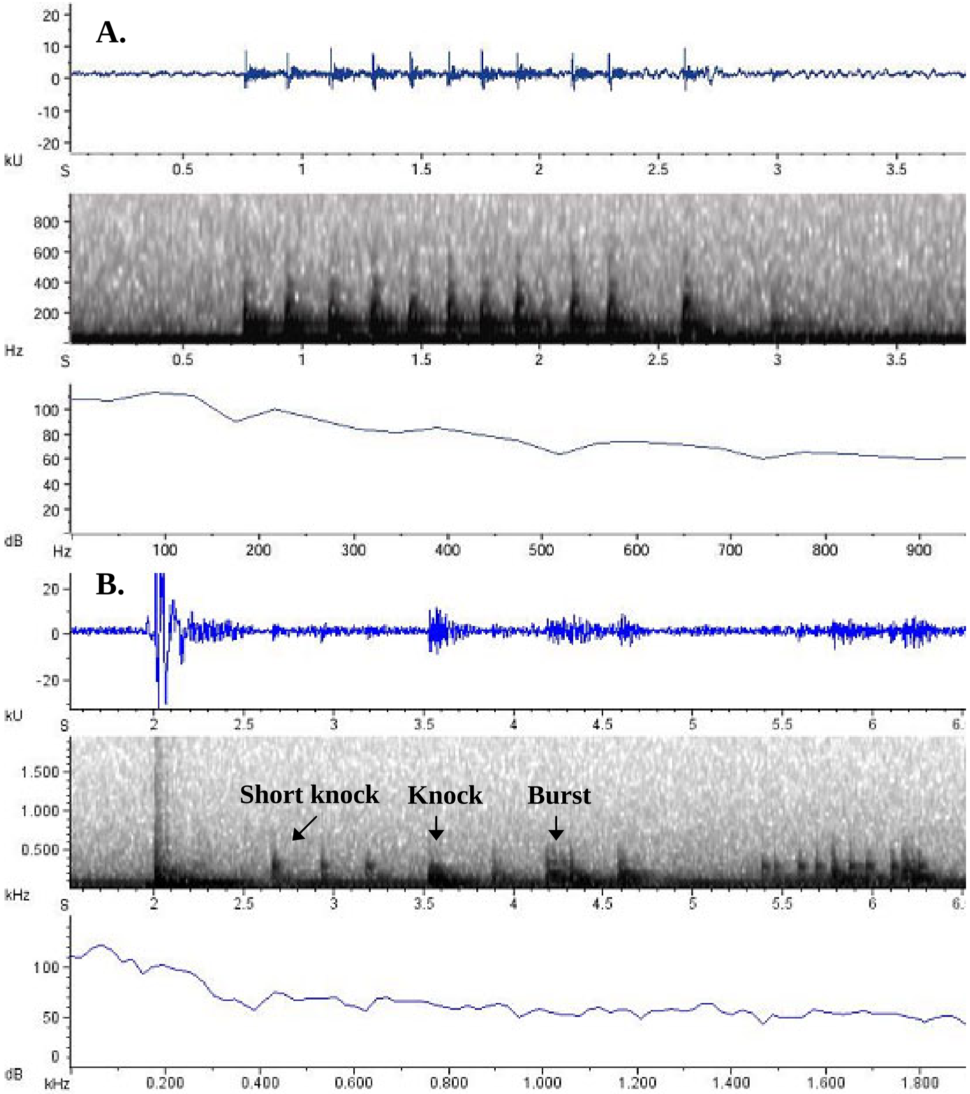

Fig. 1-5. *Cyprinella galactura* A) monophasic, and B) triphasic acoustic signals. The monophasic signal consists of a train of short knocks. The triphasic signal contains knocks, short knocks, and pulse bursts.

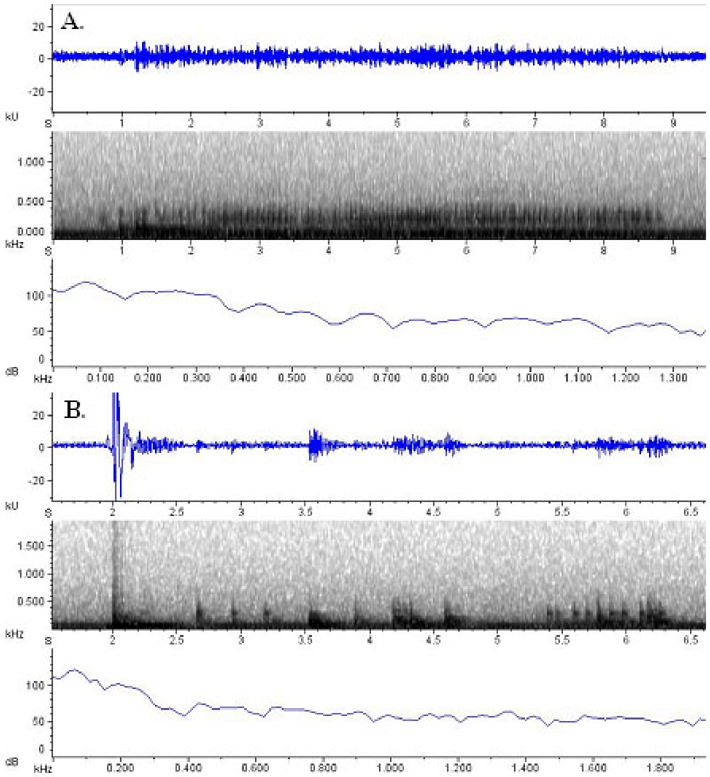

Fig. 1-6. Agonistic and courtship signals produced by male *Cyprinella galactura*. Waveform (top), spectrogram (middle) and power spectrum (bottom). Courtship signal (A) consists of one long burst or train of pulses. Agonistic signal (B) consists of knocks, short knocks and pulses in a burst.

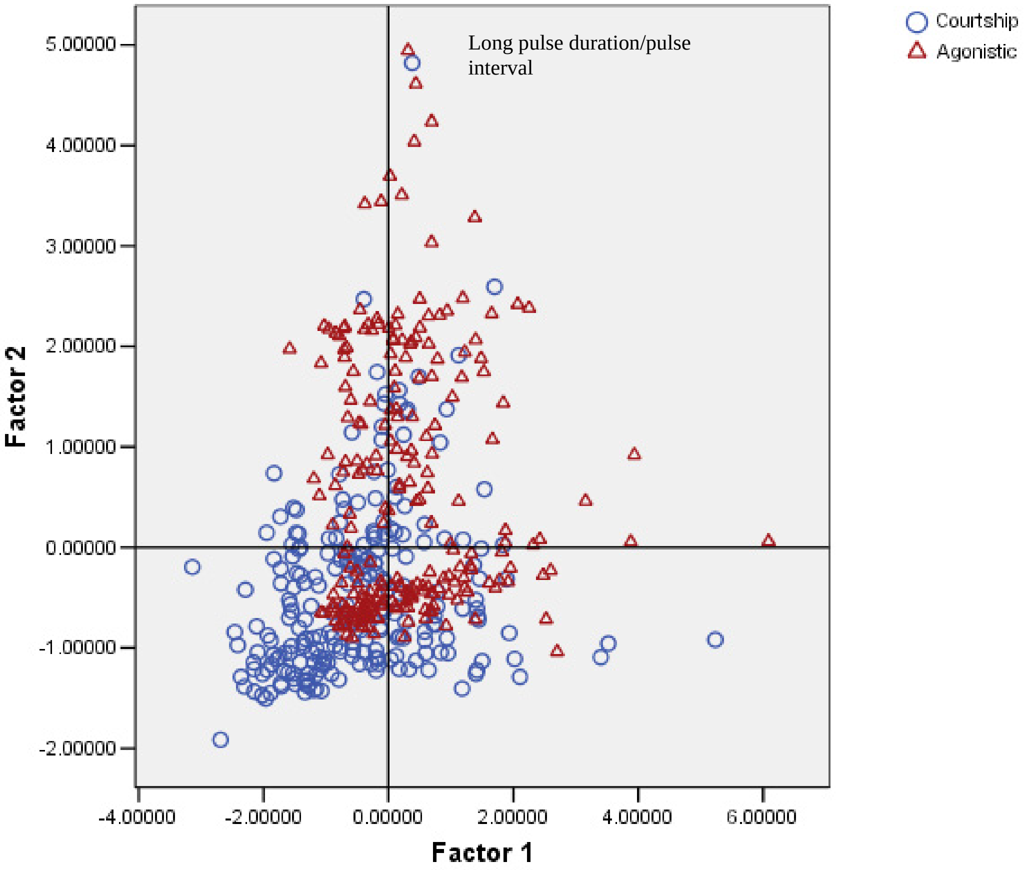

Fig. 1-7. Plot of factor loadings for the first two factors from principal components analysis using means of signal parameters and grouped by context.

TABLE 1-1. SIGNAL PARAMETER TERMINOLOGY AND DEFINITIONS. All temporal components are measured from the waveform. All spectral components are measured from the power spectrum.

Parameters	
Temporal:	
Total call duration	Time from the beginning to end of one call.
Knock duration	Total duration of a knock.
Knock interval	The interval directly following a knock, measured from the end of the knock to the beginning of the next component.
Short knock duration	Total duration of a short knock.
Short knock interval	The interval directly following a short knock, measured from the end of the short knock to the beginning of the next component.
Burst duration	Time from beginning to end of one burst.
Burst interval	The interval directly following a burst, measured from the end of one burst to the beginning of the next component.
Burst rate	The number of bursts/total call duration.
Pulse duration	Time difference from one mode to the next.
Pulse interval	Time difference from one peak to the next.
Pulse rate	The number of pulses/burst duration
Spectral:	
Knock dominant frequency	The mode measured from a knock containing the greatest energy.
Knock secondary frequency	The mode measured from a knock containing the second greatest energy.
Short knock dominant frequency	The mode measured from a short knock containing the greatest energy.
Short knock secondary frequency	The mode measured from a short knock containing the second greatest energy.
Burst dominant frequency	The mode measured from a burst containing the greatest energy.
Burst secondary frequency	The mode measured from a burst containing the second greatest energy.

TABLE 1-2. DESCRIPTIONS OF OBSERVED *Cyprinella galactura* BEHAVIORS.

Behavior	**Description**
Agonistic:	
Sedentary aggression	Male is stationary near an established territory
Nudge	Male slowly advances towards invader
Male-female chase	Male chases female some distance from territory
Male-male chase	Male chases male some distance from territory
Parallel swim	Males swim quickly in parallel alignment with fins erect
Lateral display	Males swim slowly antiparallel with fins erect
Circle swim	Males swim rapidly in a circle in a chasing motion
Lip lock	Males butt heads and briefly clasp lips while undulating the body
Bite	Males bite one another on the torso or fins
Courtship:	
Male at crevice	Male remains stationary in front of the territory in a guarding/recruiting fashion
Crevice pass	Male will swim along crevice undulating his body rapidly (Stout, 1975)
Male-female pursuit	Male slowly chases female with sexual intention
Male-female approach	Male positions himself laterally along side the female
Male sits on female	Male hovers over and subsequently pushes female down towards the substrate
Male circles female	Male swims rapidly around the female in an elliptical pattern
Spawning	Male and female release gametes along the crevice

TABLE 1-3. MEAN DOMINANT FREQUENCY, STANDARD DEVIATION AND DURATION OF SOUND TYPES (REPERTOIRE) PRODUCED BY MALE *Cyprinella galactura*. F-tests from one-way ANOVA between components.

	Knock	**Short knock**	**Burst**	**Pulse**	
AGONISTIC					
Duration	0.166 ± 0.062 (n=487)	0.069 ± 0.014 (n=327)	0.321 ± 0.270 (n=489)	0.027 ± 0.014 (n=133)	**F=229.430, p=0.000**
Dom Freq	91.811 ± 35.510 (n=318)	79.041 ± 28.685 (n=142)	53.764 ± 16.226 (n=294)	---	**F=142.269, p=0.000**
COURTSHIP					
Duration	0.155 ± 0.052 (n=46)	0.072 ± 0.012 (n=20)	0.508 ± 0.443 (n=133)	0.035 ± 0.014 (n=207)	**F=24.218, p=0.000**
Dom Freq	103.923 ± 43.901 (n=43)	73.827 ± 26.590 (n=15)	75.374 ± 22.887 (n=113)	---	**F=18.338, p=0.000**

TABLE 1-4. PERCENT OF CALL TYPE PRODUCED FROM ALL ANALYZED CALLS VS. CONTEXT. More than one call type can be produced per call.

	Knocks	Short knocks	Bursts
SA	50.6 (n=176)	40.9 (n=176)	35.2 (n=176)
MMC	64.6 (n=289)	30.8 (n=289)	43.3 (n=289)
MFC	57.7 (n=220)	37.3 (n=220)	35.5 (n=220)
LD	31.4 (n=258)	32.2 (n=258)	84.5 (n=258)
Bites	57.1 (n=7)	14.3 (n=7)	0 (n=7)
All agonistic	51.6 (n=946)	34.6 (n=946)	51.4 (n=946)
MAC	60.0 (n-5)	20.0 (n=5)	80.0 (n=5)
CP	50.0 (n=6)	16.7 (n=6)	100.0 (n=6)
MFP	52.6 (n=19)	26.3 (n=19)	73.7 (n=19)
MFA	30.1 (n=113)	10.6 (n=113)	90.3 (n=113)
MSOF	14.3 (n=7)	14.3 (n=7)	100.0 (n=7)
All courtship	31.3 (n=134)	13.4 (n=134)	87.3 (n=134)

TABLE 1-5. PERCENT OCCURRENCE OF SOUNDS PRODUCED BY MALE *Cyprinella galactura* GROUPED BY BEHAVIORAL CONTEXT.

Agonistic	Sound	n	Courtship	Sound	n
sedentary aggression	some	---	male at crevice	some	---
nudge	some	---	inspects crevice	some	---
chase	94%	977	crevice pass	13%	106
lateral display	97%	758	male-female pursuit	some	---
parallel swim	60%	30	male orbits female	100%	13
circle swim	0%	109	male-female approach	98%	250
lip lock	0%	227	spawning	24%	29

TABLE 1-6. RESULTS OF PAIRED T-TESTS BETWEEN COURTSHIP AND AGONISTIC ACOUSTIC PARAMETERS IN *Cyprinella galactura*. Significant p-values (α =0.006 based on a Bonferroni correction) in bold.

	Courtship		**Agonistic**		
Parameters	**mean**	**n**	**mean**	**n**	**p-value**
Total call duration	6.515 ± 5.435	150	6.997 ± 6.635	257	0.505
Average knock duration	0.155 ± 0.054	45	0.154 ± 0.056	81	0.226
Burst duration	0.508 ± 0.443	133	0.336 ± 0.270	207	**0.006**
Pulse duration	0.035 ± 0.014	133	0.027 ± 0.010	207	**0.000**
Pulse interval	0.034 ± 0.014	133	0.028 ± 0.015	207	**0.000**
Pulse rate	17.605 ± 4.700	132	24.037 ± 15.553	207	**0.000**
Burst rate	1.357 ± 0.864	133	1.643 ± 1.022	207	0.057
Burst dominant frequency	71.423 ± 21.933	104	54.070 ± 16.547	157	**0.000**

TABLE 1-7. FACTOR LOADINGS OF THE PRINCIPAL COMPONENTS ANALYSIS BASED ON 5 COURTSHIP AND AGONISTIC ACOUSTIC VARIABLES FOR *Cyprinella galactura*.

Variable	**Component 1**	**Component 2**
Courtship		
Burst dominant frequency	-0.120	0.756
Pulse rate	-0.941	-0.199
Pulse duration	0.975	-0.096
Pulse interval	0.977	-0.069
Burst duration	0.186	0.746
Agonistic		
Burst dominant frequency	-0.194	0.945
Pulse rate	-0.573	-0.315
Pulse duration	0.951	0.040
Pulse interval	0.960	0.019
Burst duration	0.498	-0.106

TABLE 1-8. COEFFICIENTS OF VARIATION OF *Cyprinella galactura* ACOUSTIC PARAMETERS BY CONTEXT.

	Coefficients of Variation (CV)			
Parameters	Courtship (n)		Agonistic (n)	
Total call duration	120.6	(150)	191.2	(258)
Average knock duration	73.0	(45)	80.9	(81)
Average knock interval	176.4	(37)	103.1	(64)
Short knock duration	73.1	(20)	74.6	(83)
Short knock interval	106.2	(15)	105.6	(76)
Burst duration	91.0	(133)	127.8	(208)
Burst interval	104.4	(129)	152.0	(195)
Pulse duration	62.7	(133)	104.5	(208)
Pulse interval	62.4	(133)	104.3	(208)
Single pulse duration	55.9	(95)	112.6	(172)
Single pulse interval	147.1	(91)	146.8	(171)
Pulse rate	69.3	(132)	81.3	(208)
Burst rate	101.3	(133)	113.9	(208)
Knock dominant frequency	101.8	(34)	71.4	(52)
Short knock dominant frequency	77.7	(12)	86.7	(52)
Burst dominant frequency	77.1	(104)	73.2	(157)

CHAPTER 2

GEOGRAPHIC SIGNAL DIVERGENCE IN *CYPRINELLA GALACTURA*

Significant amounts of geographic signal divergence were found between four populations of *Cyprinella galactura*. Pulse signal parameters (pulse rate, pulse duration, pulse interval and single pulse duration) were the most important parameters in separating the populations. Courtship signals were found to be less variable and more stereotyped than agonistic signals. Adjacent populations from the Ozark Mountains were found to be more similar to one another than nonadjacent populations. Signal divergence in agonistic signals seems to be more random with adjacent populations clustering with nonadjacent populations. Genetic drift may be responsible for divergence in agonistic signals, while geographic isolation may be responsible for divergence in courtship signals.

INTRODUCTION

Acoustic communication is an excellent model for studying the evolution of behavior. Acoustic signals contain both temporal and spectral signal parameters which may respond separately to selective pressures and evolve at different rates (Gerhardt, 1991). Signal components may be associated with morphology and conserved across species, or controlled by physiology and diverge across species (Crocroft and Ryan, 1995). Acoustic signals are known to play an important role as interspecific isolating mechanisms (Wells and Henry, 1992) or may be used in intraspecific mate choice and sexual selection (Crawford et al., 1997; Ryan and Wilczynski, 1991). Although well studied in birds (Kroodsma et al., 1999; Marler and Tamura, 1962; MacDougall-Shackleton and MacDougall-Shackleton, 2001; Leader et al., 2002; Slabbekoorn and Smith, 2002), anurans (Gergus et al., 2004; Littlejohn et al., 1993; Snyder and Jameson, 1965; Wilczynski et al., 1992), insects (Claridge and Morgan, 1993; Pinto-Juma et al., 2005; Wells and Henry, 1992), and mammals (Esser and Shubert, 1998; Cerchio et al., 2001), the role geographic isolation plays in the evolution of acoustic signaling in fishes has been virtually unexplored.

Variation in an acoustic signal is known as a dialect, and these dialects may maintain cohesion and identity within a group, as well as, aid in mate choice. Many birds, such as the Rufous-sided Towhee (Ewert, 1980) and the White-crowned sparrow (Fugle et al., 1984), have been found to exhibit dialects associated with geographic regions. Song dialects in birds can be important in maintaining species isolation (Marler, 1957, 1960) and in some cases prevent hybridization (Baker and Boylan, 1999). Female white-crowned sparrows, *Zonotrichia leucophrys*, have been shown to exhibit preferences for local dialects (Lampe and Baker, 1994). However, when females are from populations that exhibit multiple dialects, only a preference for the familiar is exposed, not a preference of one dialect over the other (Chilten and Lein, 1996). Territorial male song sparrows have also been found to respond most strongly to playbacks of dialects of conspecifics over that of heterospecifics (Searcy et al., 1997). Similar results were found in white-crowned sparrows (Baker, 1983; Lampe and Baker, 1994). This may show how important local dialects are in the role of establishing territories, thus limiting gene flow between populations with different dialects (Lampe and Baker, 1994). Although the role of geographic variation in acoustic signaling in birds has been well studied, birds as models have two major faults that can greatly complicate studies. Many components of bird song are learned at an early age (although not true for all species, or all signal components), and birds can easily disperse.

Geographic variation has also been examined in many groups of mammals including cetaceans (Cerchio et al., 2001; Weilgart and Whitehead, 1997), seals (Van Parijs et al., 2000), bats (Boughman, 1997; Esser and Schubert, 1998), prairie dogs (Perla and Slobedehileoff, 2002), primates (Boughman, 1997) and in red deer (Reby et al., 2001). These instances of signal divergence may serve a variety of purposes such as the maintenance and interaction of social groups in whales (Cerchio et al., 2001), the result of different environmental challenges, such as acoustic transmission properties and female distribution, in harbour seals (Van Parijs et al., 2000), or a special adaptation to local habitat vegetative cover associated with seasonal changes as in the Gunnison's prairie dog (Perla and Slobodchikoff, 2002). In red deer (Reby et al., 2001) divergence in signals may be important in female choice, as females tend to prefer calls of conspecific stags over those of a neighboring stag. However, like birds, many mammals have learned components to their acoustic repertoires (Esser and Schmidt, 1989) that may complicate the understanding of such divergences in acoustic signals.

Anurans have also shown acoustic intraspecific variation. Giacoma and Castellano (2001) found significant amounts of variation in the acoustic signals of the widely distributed *Bufo viridis* complex. This acoustic variation may also differ between signal parameters. Castellano et al. (2002) found significant variation between populations of *Hyla arborea*, European tree frogs, in two spectral, but not temporal, parameters. Wilczynski et al. (1992) found similar differences in spectral, and not temporal, parameters among populations of cricket frogs, *Acris crepitans*. Gergus et al. (2004) found significant differences in SVL-adjusted dominant frequency and temperature-adjusted call duration in Arizona tree frogs, *Hyla wrightorum*. However, no differences were found in temperature-adjusted pulse rate. Littlejohn et al. (1993) found significant differences in pulse rate and the mean number of pulses per note in the advertisement calls among populations of *Litoria ewingi* (Hylidae) introduced into New Zealand from Tasmania. This study was of particular importance as the introduction dates from Tasmania to each side of the island were known (111 years ago and 38 years ago), and some divergence had already occurred. Additional geographic differences have been found in Pacific tree frogs, *Hyla regilla* (Snyder and Jameson, 1965), and spring peepers, *Pseudacris crucifer* (Sullivan and Hinshaw, 1990).

Insects have also been shown to exhibit geographic divergence in acoustic signaling but the role of these signals can be complicated and has not been established. For example, Simmones et al. (2001) found significant levels of geographic variation in the field cricket, *Teleogryllus oceanicus*. However, female preference was not found to match geographic variation in call structure. This may be due to a tradeoff between female sensory bias and the preference of parasites on vulnerable populations. Wells and Henry (1992) found that song barriers in green lacewings, *Chrysoperia plorabunda*, could be effective reproductive barriers between sympatric morphs and that females tend to duet with songs of males of their own type rather than with songs of other types. Pinto-Juma et al. (2005) found some differences in inter-echeme interval (similar to interburst interval in *Cyprinella*) and other temporal and spectral properties that separated populations of a Mediterranean cicada, *Cicada orni*, in Greece from Eastern Europe. Claridge and Morgan (1993) examined acoustic signals of populations of the planthopper, *Nilaparvata bakeri*, from the Philippines, Indonesia and India and found significant geographic variation in these signals, but also found that mating occurred at random between populations and that the call differences do not play a role in reproductive isolation.

Intraspecific variation of acoustic signaling in fishes has not been widely investigated even though they are good models because signals are not learned (Johnston and Buchanan, 2006) and dispersal is often limited. Two marine studies examined dialects in the damselfish, *Dascyllus albisella* (Mann and Lobel, 1998), and the toadfish, *Opsanus tau* (Fine, 1978). Fine (1978) found small geographic differences, which may not be significant, in the call structure of the oyster toadfish (*Opsanus tau*). Mann and Lobel (1998) found a small degree of geographic variation in pulse duration of signal jump sounds but this was most likely due to varying depths of the recording environment. One recent study by Parmentier et al. (2005) found significant levels of geographic variation in both temporal and spectral call structure among populations in a clownfish, *Amphiprion akallopisos*. While Fine (1978) and Mann and Lobel (1998) attribute lack of geographic divergence to possible larval drift and free dispersal, Parmentier et al. (2005) examined populations from Madagascar and Indonesia that were widely separated by a disjunct distribution in the Indian Ocean which makes dispersal unlikely. Geographic divergence of sound production in freshwater fishes has been virtually uninvestigated. However, freshwater fishes are better models than marine fishes because the impacts of dispersal can be essentially eliminated as many sound-producing freshwater fishes are separated by both drainage and physiographic province.

In one such study, Johnson (2001) discovered geographic variation in signaling of the longear sunfish, *Lepomis megalotis*. Johnson (2001) revealed significant differences in signal structure within individuals, among individuals in the same population, among populations within the same drainage, and between subspecies. These findings would suggest that in other fishes, especially those with disjunct distributions (which may include separation by drainage) the development of call variation and dialects may be more likely than earlier thought.

***Cyprinella galactura* as a model**

Unlike birds, marine mammals, terrestrial organisms, and many marine fishes, freshwater fishes are ideal models for investigating the evolution of acoustic signals, as they are separated by both drainage and sometimes physiographic province which can essentially exclude most forms of dispersal. Also, like anurans and insects, signals are innate (Johnston and Buchanan, 2006, *Codoma ornata*) and not learned as in many birds and mammals. *Cyprinella galactura*, the whitetail shiner, is an ideal model to study the evolution of acoustic signaling. It has a widespread and disjunct distribution in both the Tennessee River drainage (Appalachians) and the White River drainage (Ozarks). This separation dates back to geological glaciation events that took place in the Pleistocene, no less than 15,000 years ago. Also, studies involving populations of *C. galactura* are not complicated by differences in habitat structure or temperature. All populations of *Cyprinella galactura* used in this study were found in cold, fast flowing, deep and rocky mountain streams of similar size, structure and flow. Finally, issues related with temperature and seasonal variation can be eliminated in this study as all males were collected during the limited *C. galactura* breeding season (May – August). *Cyprinella galactura* are already known to produce sounds, although a detailed description of call repertoire, complexity, and behavioral association has not been established (Phillips and Johnston, personal observation). *Cyprinella galactura* also produce sounds during both agonistic and courtship displays, unlike some other species of *Cyprinella* (Phillips and Johnston, submitted). Analysis of dialects in all contexts can be examined and questions regarding the evolution of signaling during courtship vs. agonistic encounters may also be investigated. Furthermore, *C. galactura* interact and spawn readily in aquaria with little to no manipulation, making them the perfect laboratory model for a study of this type.

This study examines acoustic divergence in four populations of *C. galactura*, two from the Appalachian Mountains and two from the Ozark Mountains (Fig 2-1). These populations were chosen due to location and drainage history. One population (Lick Creek) was selected from the Duck River drainage and another (Nottely River) from the main Tennessee River drainage. The Duck River is believed to have been captured by the Tennessee River during the latter half of the Tertiary (Etnier and Starnes, 1993). Arkansas populations were chosen from the Black and White River drainages. The Black River is a major tributary system to the White River. Populations of *C. galactura* were chosen from the Strawberry River drainage (Piney Fork) inside the Black River drainage and from Crooked Creek a White River tributary. This study will test predictions that behavior may evolve before morphology in the evolution of acoustic communication systems in *C. galactura*. It is predicted that behavior is changing even though *C. galactura* on both sides of the embayment are known to be morphologically similar (Gibbs, 1961), and that adjacent populations will be acoustically more similar to one another than that of a non-adjacent population.

METHODS

During the spawning period 2002 (May – August) and 2003 populations of *Cyprinella galactura* were collected from all prospective drainages. Collections were made from: Nottely River (Tennessee River drainage), Hwy 180, just E of Hwy 129/19, Union County, Georgia, 30 May 2002; Lick Creek (Duck River drainage), 6 mi. SW of Greenbrier, Maury County, Tennessee, 12 June 2002; Crooked Creek (White River drainage), 5 mi. E of Harrison, Blount County, Arkansas, 9 July 2002; Piney Fork (Black River drainage), 4 mi. NW of Evening Shade, Sharp Co., Arkansas, 14 June 2003. (State permits were issued to CEJ and CTP; AUM animal protocol number 2004-0663).

Fish were transferred to the laboratory and placed in 84 l aquaria. Fish were fed a diet of bloodworms and commercial fish flakes daily. Their photoperiod was regulated at 10-12 hours of light. The trial aquaria were kept in a separate insulated room and isolated from the lab table by a layer of foam. Each aquarium contained a sand substrate and an artificial crevice nesting cavity. Both male-male and male-female trials were set up to gather agonistic and courtship data. All observed behaviors were noted as well as simultaneously videotaped. Acoustic signals that occurred during a particular behavior were considered associated. Observation periods were 30 minutes. Due to the reverberant effect of using small tanks (Parvalescu, 1967), the hydrophone was placed within the range of attenuation length from the fish to ensure signal accuracy (Akamatsu et al., 2002; Okumura et al., 2002). In addition, although all sounds were noted, signals contaminated with excess noise or interference were excluded from the analysis.

Sounds were recorded using a Bruel and Kjaer 8103 hydrophone, Bruel and Kjaer 2635 charge amplifier and Sony model TC-D5 Pro II stereo cassette recorder. Sounds were digitized and analyzed using Raven ver. 1.1 (Cornell University). Temporal parameters were measured from the waveform and spectral parameters were measured from the power spectrum. Spectrograms were generated using the following settings: Hanning window, clipping level, filter bandwidth 124 Hz, frame length 512 pts, and 50% overlap. Power spectra were generated using a Hanning window of 1024. Signals were not filtered prior to data extraction as acoustic signal information overlapped in the low frequency range.

The following temporal and spectral acoustic parameters were examined: knock dominant frequency (frequency component with the most energy), knock second frequency (frequency component with the second most energy), short knock dominant frequency, short knock second frequency, burst dominant frequency, burst second frequency, total call duration, knock duration, knock interval, short knock duration, short knock interval, burst duration, burst interval, burst rate (# bursts/total call duration, only in calls with >1 burst), the number of bursts per call, pulse duration, pulse interval, pulse rate (#pulses/burst duration, only in bursts with >1 pulse), the number of pulses per burst, and single pulse duration (Fig 2-2). Agonistic and courtship signals within species were analyzed separately. Furthermore, agonistic signals were separated by specific context (ex: male-male chase vs. lateral display vs. sedentary aggression). Signal parameters from separate population were not examined or compared in any way prior to completion of all the populations, so as not to add bias.

Statistical analysis

All statistical analyses were conducted using the SPSS software package (SPSS ver. 13.0, SPSS Inc, Chicago, IL). The use of the behavioral/acoustic term "parameter" is intended to be equivalent to the term "variable" when describing analyzed data. Descriptive statistics, paired-sample t-tests, analysis of variance with Tukey’s and Bonferroni multiple comparison tests, and MANOVA with Tukey’s and Bonferroni multiple comparison tests were performed.

Due to the nature of behavioral experiments, traditional methods of analysis on signal parameters could be problematic. In this case, the experimental setup involves situations in which the same male is used in multiple trials (to record a range of behaviors and signals), or multiple signals must be used from multiple males, instead of simply taking a mean from each male, in order to evaluate systems of variation. Randomization methods were applied to signal parameter variables prior to standard analysis to determine if traditional methods of analysis are valid despite modest violations of parametric assumptions, such as unequal variances or sample sizes. Randomization tests require no assumptions regarding the distribution of data, and can tell us if the pattern we see in our data is or is not likely to have arisen by chance (Manly, 1991). In other words, do the differences between the means in the groups, in this case four populations, reflect true differences among the populations? Single-factor analysis of variance randomization tests with 9999 iterations were computed using the PopTools add-in ver. 2.7.1 for

Microsoft Excel (Hood, 2006). This involved calculating a test statistic, in this case an F-value, and seeing what percentage of times this is exceeded by values from randomized data (Manly, 1991). The Monte-Carlo procedure was used to compare the original data which may exhibit modest parametric violations, to a completely randomized set of data by iteratively recalculating the worksheet many times and comparing the test values of the two ranges.

Pearson r correlation coefficients were used to evaluate the relationship between body size (standard length) and signal parameters. Significant signal correlates were regressed against body size and adjusted values were used. Parameters used in the statistical comparisons between populations include: total call duration, knock duration, short knock duration, burst duration, burst rate, pulse duration, pulse interval, pulse rate, and single pulse duration. Principal components analysis (PCA) was used to reduce the number of parameters into uncorrelated multivariate components. Components were selected with eigenvalues greater than one. Analysis of variance (ANOVA) with multiple comparisons tested whether populations differed from one another in these components. In addition, discriminant function analysis (DFA) on acoustic parameters was used to investigate classification of species-specific signals. Hierarchical cluster analysis (using UPGMA and Euclidean distances) was used to evaluate similarity between species.

Randomization F-tests were compared with parametric F-tests (ANOVA) to determine if the standard method of analysis is sufficient despite differences in variance and sample size (Table 2-1). The results from the randomization test were similar to the parametric F-test indicating that the parametric tests used are robust even when assumptions such as equal variance and sample size are violated. Only one parameter, courtship burst dominant frequency, changed from significant to non-significant following randomization analysis. Therefore, parametric tests were considered to be adequate for analysis and therefore used throughout.

RESULTS

Shared acoustic repertoire

One hundred sixteen, 30 min behavioral trials were performed for *Cyprinella galactura* (44-Nottely Rv., 31-Lick Cr., 19 – Crooked Cr., 22- Piney Fk.). All trials were performed between 30 May 2002 and 2 August 2002 and 17 June 3003 and 30 July 2003. A total of 4182 sounds were observed and recorded (1564 – Nottely Rv., 1046 – Lick Cr., 627 – Crooked Cr., 945- Piney Fork). Signals from thirty-two males from the four populations were analyzed (7-Nottely Rv., 11-Lick Cr., 5-Crooked Cr., 9-Piney Fork). Males that included fewer than 5 signals for the particular context examined were excluded from analysis.

Three distinct call types were established and shared among the four populations: knocks, short knocks, and pulses organized into bursts. All call types were relatively low frequency and non-harmonic. All call types could occur singly or in trains and could occur in any combination together. Knocks had a mean dominant frequency of 91.8 ± 35.5Hz (n=318; 33 males) and a mean duration of 166.0 ± 62.0ms (n=487; 33 males) for agonistic signals and a mean dominant frequency of 103.9 ± 43.9Hz (n=43; 15 males) and mean duration of 155.0 ± 52.0ms (n=46; 15 males) for courtship signals. Short knocks had a mean dominant frequency of 79.0 ± 28.7Hz (n=142; 28 males) and a mean duration of 69.0 ± 14ms (n=327; 28 males) for agonistic signals and a mean dominant frequency of 73.8 ± 26.6Hz (n=15; 13 males) and 72.0 ± 12.0ms (n=20; 13 males) for courtship signals. Pulses had a mean dominant frequency of 53.8 ± 16.2Hz (n=294; 33 males) and a mean duration of 27.0 ± 14.0ms (n=207; 33 males) for agonistic signals and a mean dominant frequency of 75.4 ± 22.9Hz (n=113; 15 males) and a mean duration of 35.0 ± 14.0ms (n=133; 15 males) for courtship signals. However, pulses were usually grouped into a burst which is an organized train of pulses. Bursts had a mean duration of 321.0 ± 270.0ms (n=489; 33 males) for agonistic signals and a mean duration of 508.0 ± 443.0ms (n=133; 15 males) for courtship signals. Bursts could be divided into two types: aggressive growls and courtship purrs. These vary by associated context and were characterized by unique pulse parameters.

Differences among call types were tested with single-factor ANOVA. All courtship call types were significantly different from one another in both duration (F=24.218, $p<0.001$) and dominant frequency (F=18.338, $p<0.001$) and all agonistic call types were significantly different in both duration (F=229.430, $p<0.001$) and dominant frequency (F=142.269, $p<0.001$). All call types were used for all contexts however, some call types were more prevalent in aggressive contexts and some call types were more prevalent in courtship contexts.

Body size adjustment

At the within-species level, standard length was significantly correlated (Pearson’s correlation coefficients) with several temporal and spectral parameters. There was no inverse relationship between standard length and frequency. Standard length was significantly positively correlated with agonistic burst dominant frequency (R=0.269, p=0.001, n=157) and courtship knock secondary frequency (R=0.406, p=0.017, n=34). Standard length was also significantly positively correlated with agonistic burst duration (R=0.159, p=0.022, n=207), and courtship knock duration (R=0.625, p=0.000, n=45) and negatively correlated with agonistic knock duration (R=-0.369, p=0.001, n=81) and short knock duration (R=-0.228, p=0.038, n=83). Larger males produced longer pulse bursts and shorter knocks and short knocks. Total call duration was significantly positively correlated with agonistic burst duration (R=0.209, p=0.003, n=207), pulse duration (R=0.273, p<0.001, n=207), pulse interval (R=0.167, p<0.001, n=207), and single pulse duration (R=0.252, p<0.001, n=171).

To evaluate the effect of body size on signal variation, significant correlates were regressed against body size and adjusted parameters were used in analysis. Although body size did account for a small amount of variation in some signal variables, there was very little effect on the significant differences between populations before and after size adjustment (Table 2-2). Burst dominant frequency changed from significantly different (F=12.081, p=0.001), to not significantly different (F=1.129, p=0.339) between the populations after adjustment. Burst duration changed from not significantly different (F=5.330, p=0.022) to significantly different (F=15.815, p=0.000) between the populations after adjustment.

Acoustic geographic divergence

The four populations examined shared the same acoustic repertoires and produced knocks, short knocks and bursts. Agonistic (Table 2-3) and courtship (Table 2-4) signal descriptives were analyzed by population (comprehensive list of descriptives-appendix B). Even though all populations shared these components, they were significantly different from one another in a number of acoustic variables (Table 2-5).

One-way ANOVAs were performed on signal variables separated into five contexts (courtship, lateral display, male-male chase, male-female chase, and sedentary aggression). Results revealed significant differences among populations in mean knock duration, mean pulse duration, mean pulse interval, and mean single pulse duration for all five contexts. Agonistic ($F=22.035$, $p<0.001$) and courtship ($F=12.012$, $p<0.001$) mean knock duration were significantly different among the four populations. Agonistic ($F=76.816$, $p<0.001$) and courtship ($F=21.472$, $p<0.001$) mean pulse duration were also found to be significantly different among the four populations. Agonistic ($F=17.002$, $p<0.001$) and courtship ($F=22.156$, $p<0.001$) mean pulse interval were significantly different among the four populations. Agonistic ($F=89.708$, $p<0.001$ and courtship ($F=22.976$, $p<0.001$) mean single pulse duration were also significantly different among the populations.

Multiple comparison tests on agonistic parameters found all of the above parameters to be significantly different for all populations except for Nottely River (Appalachians) and Piney Fork (Ozarks). Lick Creek (Appalachians) and Crooked Creek (Ozarks) pulse duration and pulse interval were also not significantly different from one another. Multiple comparison tests on courtship parameters reveal fewer significant differences among the populations. However, populations from the Ozarks (Crooked Creek and Piney Fork) and populations from the Appalachians (Nottely River and Lick Creek) were not significantly different from one another for burst dominant frequency, burst secondary frequency, and knock duration. Appalachian populations were not significantly different from one another for pulse duration, pulse interval, burst duration, and single pulse duration.

Multivariate analysis of variance (MANOVA) was used to determine if population differences existed given multiple signal parameters considered together (Table 2-5). Only the parameters deemed significant from ANOVA were used in MANOVA. Parameters included: burst dominant frequency, knock duration, pulse rate, pulse duration, pulse interval, burst duration, and single pulse duration. For analysis of the contexts sedentary aggression and male-female chase, burst dominant frequency was excluded from analysis due to low number of bursts and burst frequencies for these contexts. Populations were found to be significantly different for all five contexts examined.

Multiple comparison tests from MANOVA (Fig. 2-3) give similar results to those from ANOVA. For agonistic parameters, Nottely River and Piney Fork were not significantly different for every variable except for knock duration. Adjacent populations, Piney Fork and Crooked Creek, and Nottely River and Lick Creek, were not significantly different for knock duration. For courtship parameters, Nottely River and Piney Fork were not significantly different for every parameter measured. Furthermore, adjacent populations Nottely River and Lick Creek were not significantly different for knock duration, pulse duration, pulse interval, and single pulse duration.

Principal components analysis (PCA) was used to reduce the number of parameters and determine which ones account for the most variation by population. Factor loadings from PCA of courtship parameters by population (Table 2-6) depict which signal parameters contribute the most to signal variation and resulted in 2 factors accounting for 72.6% of the variation, with 43.2% of the variation represented by the first factor, while 29.4% of the variation represented by the second factor. Factor loadings were high for some of the parameters. Factor 1 had the highest loadings for pulse rate (-0.983), pulse duration (0.971), and pulse interval (0.958). Factor 2 had high loadings for burst rate (0.879) and burst duration (-0.870).

Factor loadings from principal components analysis of agonistic variables (Table 2-6) depict which signal parameters contribute the most to signal variation. The first 2 factors accounted for 88.4% of the total variation, with 55.7% of the variation represented by the first factor, while 32.7% of the variation was represented by the second factor. Factor 1 had the highest loadings for total call duration (0.970), pulse duration (0.926) and pulse interval (0.973). Factor 2 had the highest loadings for knock duration (0.913), burst duration (0.851) and burst dominant frequency (0.758).

For the PCA of courtship parameters, there was no complete separation between the four populations and a large area of overlap can be seen on the plot of the scores for the first two factors grouped by context (Fig. 2-4). However, in an ANOVA for each factor among the populations, factor 1 (F=14.139, p=0.000, n=944) and factor 2 (F=35.401, p=0.000, n=944), respectively, were found to be significantly different. Multiple comparison tests revealed that Nottely River (Appalachians) and Crooked Creek (Ozarks) were not significantly different from one another for factor 1 and 2. Lick Creek (Appalachians) and Piney Fork (Ozarks) were not significantly different from one another for factor 1.

For agonistic PCA there was no complete separation between the four populations on the plot of the scores for the first two factors grouped by context (Fig. 2-5). However, the area of overlap between the populations was smaller and populations do appear to be more divergent. Factor 1 (F=40.631, p<0.001, n=251) and factor 2 (F=35.007, p<0.001, n=251) were significantly different from one another in an ANOVA between the four populations. Multiple comparison tests revealed that Nottely River (Appalachians) and Lick Creek (Appalachians) were not significantly different from one another for factor 1. Lick Creek (Appalachians), Crooked Creek (Ozarks) and Piney Fork (Ozarks) were not significantly different from one another for factor 2.

A discriminant function analysis (DFA) was used to test for population specificity in signals. The courtship signal parameters that resulted in the best classification included: pulse duration, pulse interval, pulse rate, knock duration and burst duration. The agonistic signal parameters that resulted in the best classification included: pulse duration, pulse interval, pulse rate, burst duration, knock duration and burst dominant frequency.

In a DFA on courtship acoustic signals, 77.8% of the individual signals were classified in the correct population. For Nottely River, 50.0% were correctly classified, 25.0% were classified as Lick Creek and 25.0% were classified as Crooked Creek. For Lick Creek, 75.0% were classified correctly, the remaining 25% were classified as Nottely River. For Crooked Creek, 100% were classified correctly. For Piney Fork, 72.7% were classified correctly, 18.2% were classified into Crooked Creek, and the remaining 9.1% were classified into Lick Creek. In a DFA on agonistic acoustic signals, 84.2% of the individual signals were classified in the correct population. For Nottely

River, 50.0% were classified correctly, 25.0% were classified as Crooked Creek and 25.0% were classified as Piney Fork. For Lick Creek, 50.0% were classified correctly, 25.0% were classified as Crooked Creek, and 12.5% were classified as Nottely River and 12.5% as Piney Fork. For Crooked Creek and Piney Fork, 100.0% of the signals were classified into the correct population.

Two separate hierarchical cluster analyses (using UPGMA and Euclidian distance) from agonistic and courtship acoustic parameters resulted in two dendograms (Fig. 2-6). The courtship dendogram resulted in a cluster between the two Ozark populations, Crooked Creek and Piney Fork. The agonistic dendogram resulted in a cluster between the Appalachian population, Lick Creek, and the Ozark population, Crooked Creek, and other cluster between the Appalachian population, Nottely River, and the Ozark population, Piney Fork.

DISCUSSION

This study was the first to examine acoustic signal divergence in the family Cyprinidae. Populations of *Cyprinella galactura*, even those separated by the Mississippi embayment, are not significantly morphologically distinct (Gibbs, 1961). However, acoustic behavior was divergent in some parameters. Although populations shared the same acoustic repertoire producing knocks, short knocks, and pulse bursts, significant amounts of geographic variation were found among populations of *C. galactura*.

Although body size may have played a role in explaining some signal variation, the effect was negligible in this study. There were no consistent relationships between any one signal parameter and body size. Dominant frequency was negatively correlated with body size in frogs (Castellano et al., 1999), birds (Tubaro and Mahler, 1998) and fish (Myrberg et al., 1993; Johnston and Buchannan, 2006). However, body size in *C. galactura* was positively correlated with agonistic burst dominant frequency (R=0.269, p=0.001, n=157) and courtship knock secondary frequency (R=0.406, p=0.017, n=34). Furthermore, although these correlations were significant, they only account for 16.5% and 7.2% of the variation, respectively. Body size certainly did not account for the majority of the variation found in *C. galactura*. Most acoustic signal parameters of *C. galactura*, even after body size adjustment, still exhibited significant differences among the populations.

Results from ANOVA and MANOVA showed that many acoustic parameters were significantly different among the populations for all five contexts examined. These parameters included: mean knock duration, mean pulse duration, mean pulse interval, and mean single pulse duration. Multiple comparisons from ANOVA and MANOVA on agonistic lateral display parameters found that nonadjacent populations Nottely River (Appalachians) and Piney Fork (Ozarks) were not significantly different for any of the above listed parameters. Furthermore, nonadjacent populations Lick Creek (Appalachians) and Crooked Creek (Ozarks) were also not significantly different from one another in the pulse parameters, mean pulse duration and mean pulse interval. Therefore, adjacent populations were not found to be more similar to one another than non-adjacent populations for the agonistic context.

Multiple comparisons from ANOVA and MANOVA on courtship parameters found that populations from the Ozarks and populations from the Appalachians were not significantly different from one another for burst dominant frequency, burst secondary frequency, and knock duration. In addition, Appalachian populations were not significantly different from one another for burst duration and all of the pulse parameters (pulse duration, pulse interval, single pulse duration). Therefore, adjacent populations were found to be more similar to one another than non-adjacent populations for the courtship context, while non-adjacent populations were found to be significantly different.

Principal components analysis for agonistic and courtship signals revealed that pulse parameters, pulse rate, pulse duration and pulse interval, accounted for the most variation in courtship signals. Signal pulse parameters, pulse duration and pulse interval, as well as total call duration, accounted for the most variation in agonistic signals. There was no complete separation of the four populations for either courtship or agonistic principle components, but populations were significantly different from one another for both contexts in ANOVA of the principle components.

Courtship signal parameters had more overlap among the populations and were less divergent. While non-adjacent populations were significantly different, adjacent populations, Crooked Creek and Piney Fork, were not significantly different for courtship parameters. Agonistic signal parameters had less overlap among the populations and were more divergent. The opposite trend was found in agonistic signals with adjacent populations being significantly different, and the nonadjacent populations, Nottely River and Piney Fork, not significantly different. This seems to indicate that agonistic signals may be evolving faster than courtship signals.

Furthermore, adjacent populations had similar knock durations, and dominant and secondary frequencies for both agonistic and courtship signals. However, the pulse parameters that accounted for the most variation were shared between non-adjacent populations, and seemed to be more divergent among populations than other temporal properties. In fact, in all methods of statistical analysis, the pulse parameters, pulse duration, pulse interval, and pulse rate were found to be very important in contributing to significant differences among populations. Pulse parameters are known to be important in anurans. Pulse rate was the most divergent parameter in the ground-dwelling treefrog, *Litoria verreauxii* (Smith et al., 2003). Parmentier et al. (2005) in his study of geographic variation between populations of the anemonefish, *Amphiprion akallopisos*, also found pulse duration to be most divergent between the populations. Johnson (2001) found pulse duration to be the most important parameter for distinguishing courtship calls among sites of the longear sunfish, *Lepomis megalotis*.

Long periods of isolation can drive divergence. In the tungara frog, *Physalaemus pustulosus*, genetic distance and assortative mating are related to geographic distance, suggesting that divergence may be caused by gradual genetic differences in allopatric populations (Ryan et al., 1996). If signal divergence is a product of geographic isolation, we would predict the nonadjacent populations from the Ozarks and the Appalachians to be the most divergent. This is true for courtship signals. Nonadjacent populations were the most divergent, while adjacent populations were less divergent. Courtship signals were also more stereotyped, less variable, and had a greater degree of overlap among the populations than agonistic signals. Courtship signals may be more important than agonistic signals as they are probably used in mate recognition, therefore selection may be keeping them from drifting (unlike agonistic signals).

Environmental differences can also drive divergence. The greatest differences would be between populations with different ecological selection pressures. Natural selection may cause reproductive isolation between populations exploiting different resource environments (Schluter, 1996). This could reflect anything from structural differences (depth, substrate), competition between males for a limited resource (territories), or the presence of a sympatric species. Differences in ecological selection pressures such as competition for food and various feeding strategies have been known to contribute to divergence in postglacial lake fishes (Schluter, 1996). It is conceivable that differences in breeding habitats could also have a similar effect. A pattern that could support ecological selection was seen in *C. galactura* agonistic signals. Agonistic signal divergence seemed to be more random, as non-adjacent populations were in some cases more similar to one another than adjacent populations. Changes do not reflect evolution by geographic isolation. Agonistic signals were also less stereotyped, more variable, and had a smaller degree of overlap among the populations than courtship signals. This could be due to random genetic drift, but it could also reflect differences between habitat quality. If a limited amount of suitable breeding territories were available at any given time, this could have potentially changed the level of aggression between males. Aggressive individuals that could secure territories in times of resource scarcity would be favored. If territories were abundant, selection on aggressive males may have relaxed. Habitat changes may be a critical factor driving the evolution of agonistic signals.

Likewise, agonistic signal changes between populations may not be due to ecological selection pressures. Random genetic drift may cause enough differences in populations isolated for a long time that eventually populations might be unable to mate if reintroduced. Agonistic signals may not have been as important as courtship signals as they are not likely used in mate recognition, so selection on these signals may have been relaxed and genetic drift could occur.

Although the exact mechanism driving the evolution of acoustic signaling in *C. galactura* cannot be exclusively identified, the differences in divergence for agonistic and courtship signals provide clues towards understanding the process. The unique acoustic signal repertoire specific to *C. galactura*, and present in other *Cyprinella* (Johnston and Phillips, unpubl.), and the fine-scale pulse parameters, may act as species identifiers. Future studies might include playbacks to determine if females can discriminate between males on either side of the embayment, or even males from their own population, or playbacks to determine if males react more aggressively to males from nonadjacent populations, than their own or an adjacent population.

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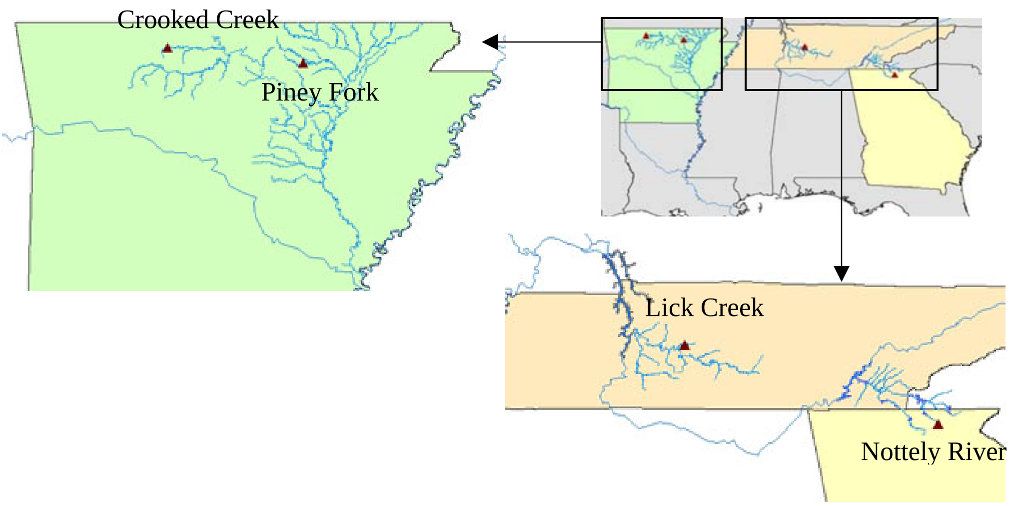

Fig. 2-1. The study populations of *Cyprinella galactura* from the Ozark mountains (Crooked Creek - White River drainage, Piney Fork - Black River drainage) and the Appalachian mountains (Lick Creek - Duck River drainage, Nottely River -Tennessee river drainage).

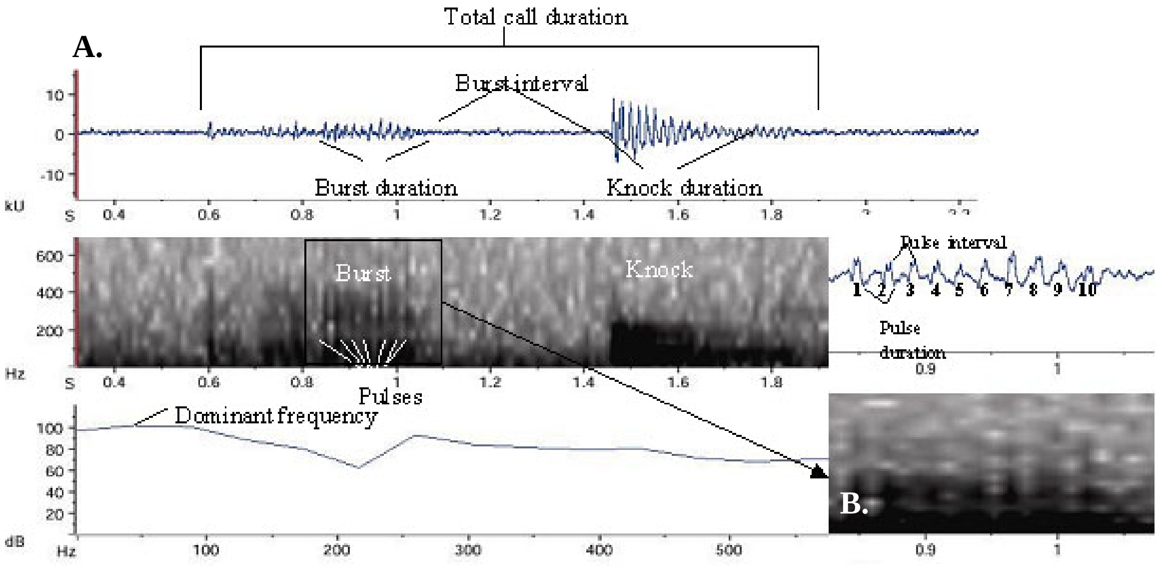

Fig. 2-2. Diagrammed *Cyprinella galactura* signal with measurements labeled. A) Gross-scale temporal structure. B) Fine-scale temporal structure.

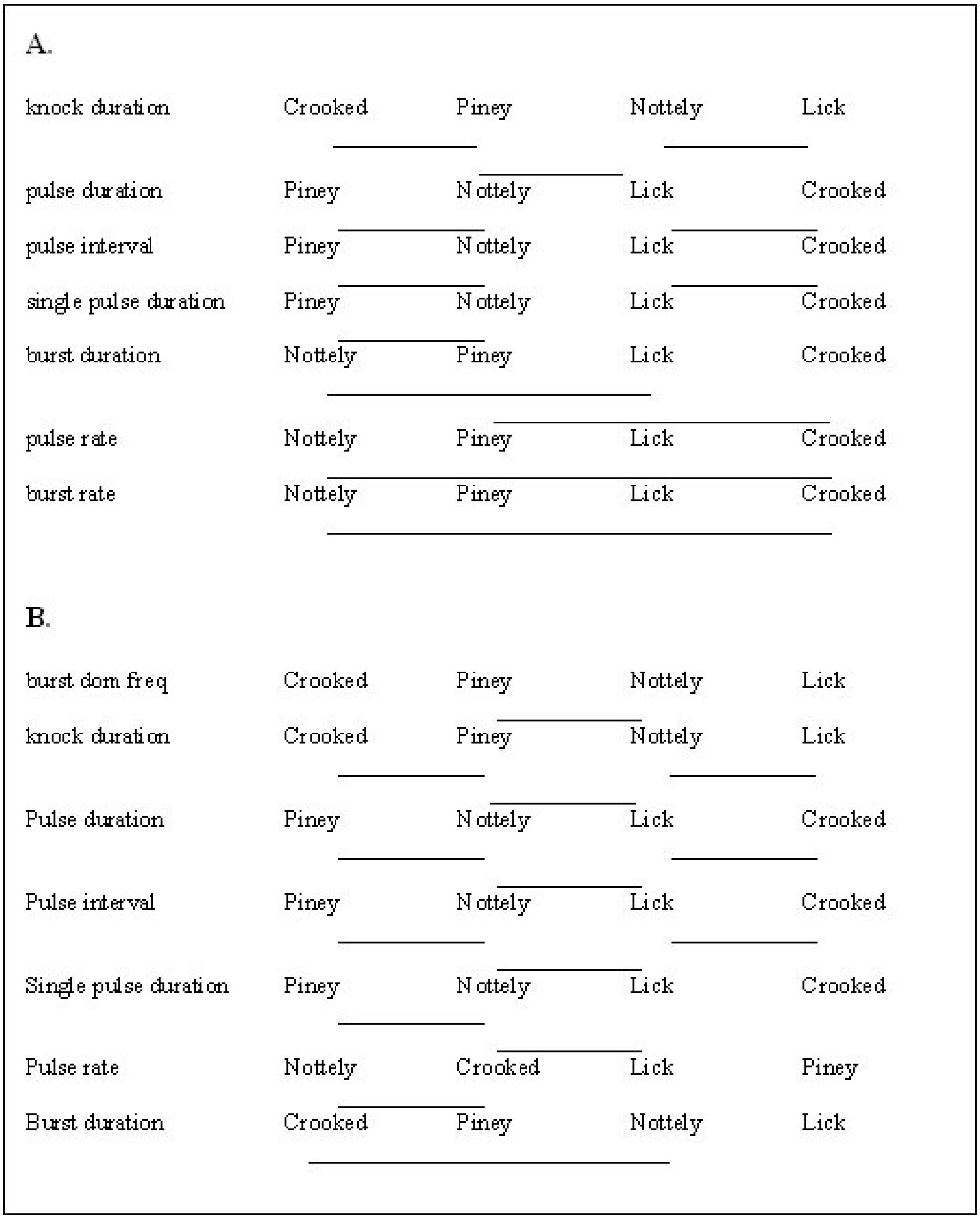

Fig. 2-3. Multiple comparisons tests for MANOVA on agonistic lateral display (A) and courtship (B) signal parameters. Nottely = Nottely River (Appalachians), Lick = Lick Creek (Appalachians), Crooked = Crooked Creek (Ozarks), Piney = Piney Fork (Ozarks).

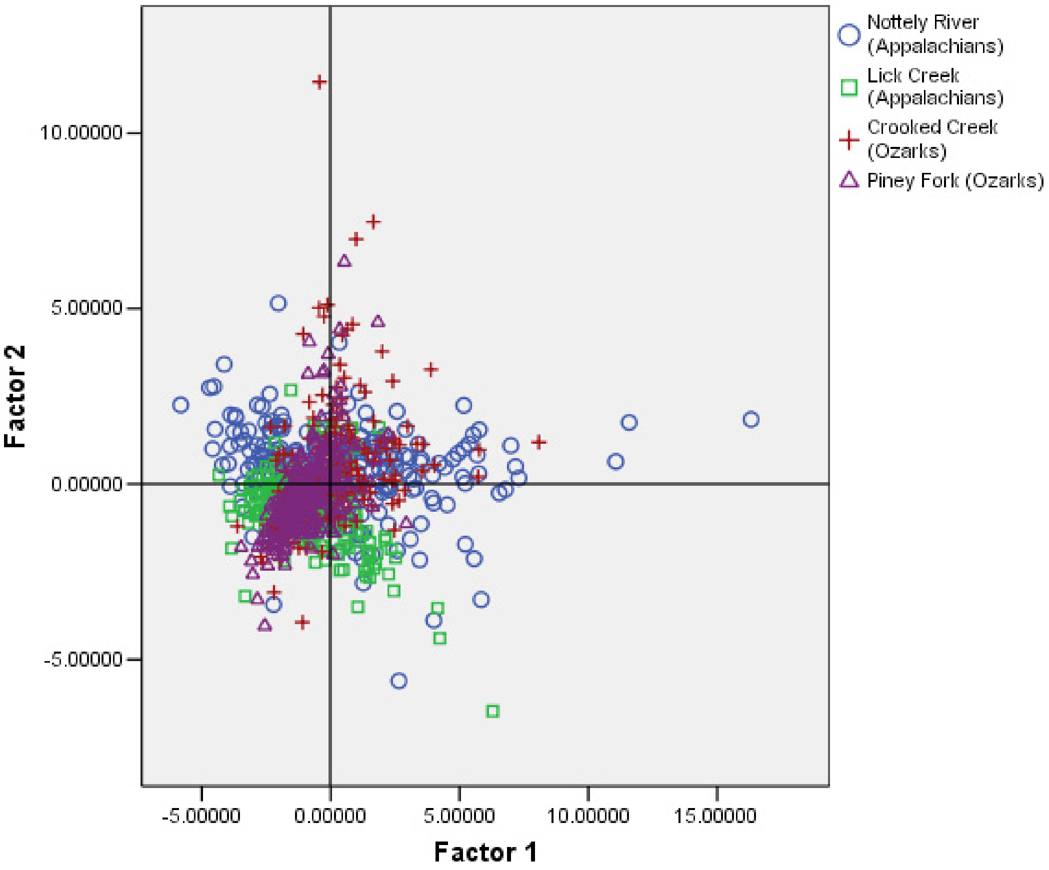

Fig. 2-4. Courtship plot of factor loadings for the first two factors from principle components analysis using means of signal parameters and grouped by population.

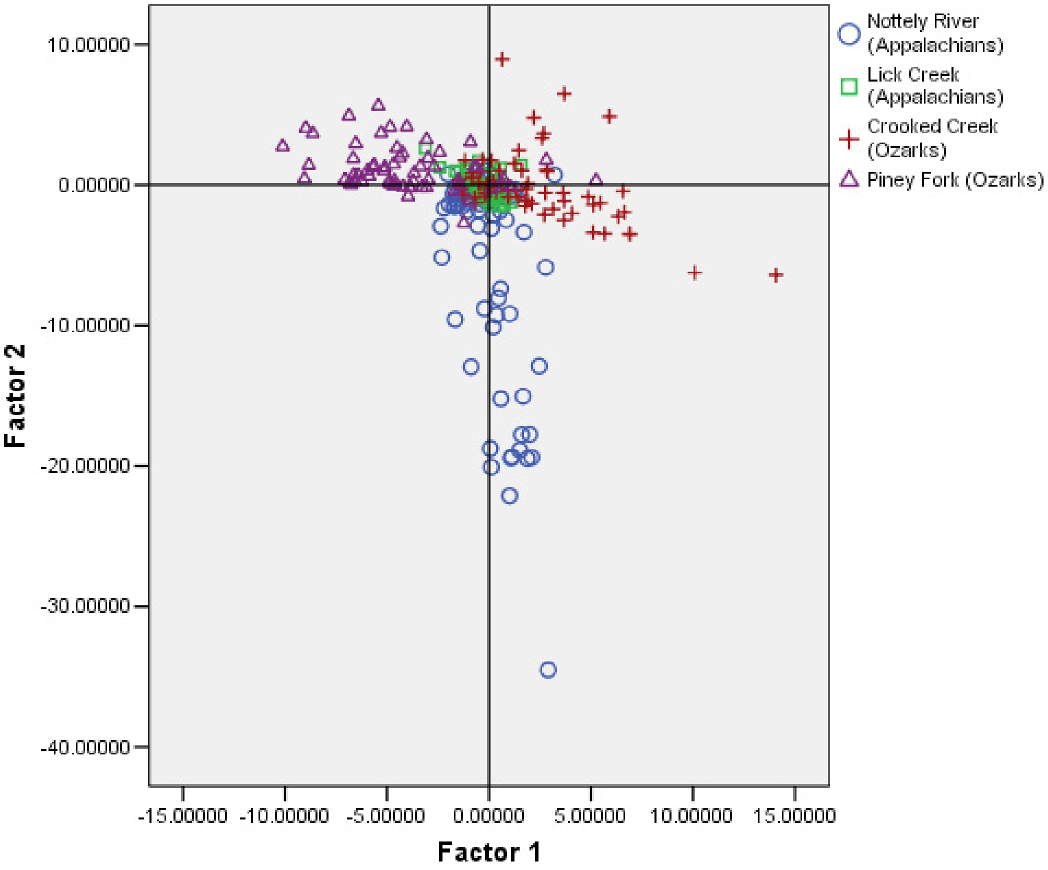

Fig. 2-5. Agonistic plot of factor loadings for the first two factors from principle components analysis using means of signal parameters and grouped by population.

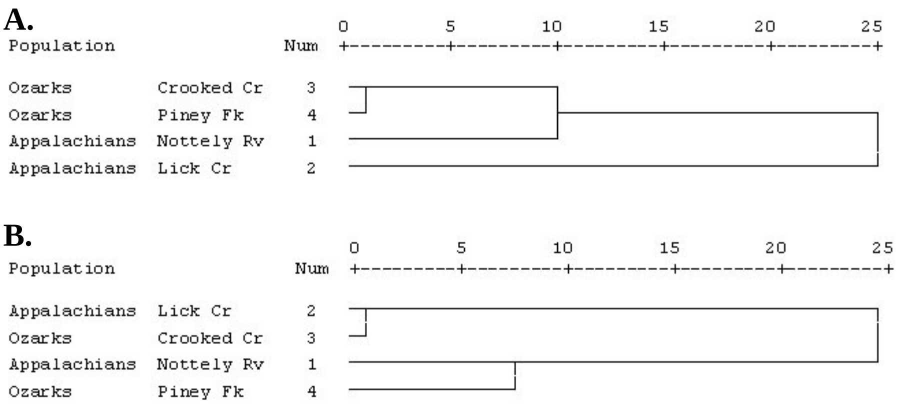

Fig. 2-6. Courtship (A) and agonistic (B) dendogram from cluster analysis.

TABLE 2-1. RESULTS AND COMPARISON OF A STANDARD F-TEST (ANOVA) WITH A RANDOMIZATION F-TEST. Significant p-values for ANOVA (α = 0.004 based on a Bonferroni correction). Asterisks designate changes in significance with the randomization test. Courtship short knock dominant frequency and knock dominant frequency were excluded from analysis.

	Courtship		**Agonistic (LD)**	
Parameters	**F-test**	**R**	**F-test**	**R**
Total call duration	0.363	0.366	0.019	0.019
Average knock duration	0.000	0.000	0.000	0.000
Burst duration	0.000	0.000	0.000	0.000
Pulse duration	0.000	0.000	0.000	0.000
Pulse interval	0.000	0.000	0.000	0.000
Single pulse duration	0.000	0.000	0.000	0.000
Pulse rate	0.000	0.000	0.000	0.000
Burst rate	0.055	0.049	0.000	0.000
Knock dominant frequency	0.015	---	0.302	0.270
Short knock dominant frequency	0.185	---	0.389	0.355
Burst dominant frequency	0.000	0.044*	0.326	0.315

TABLE 2-2. RESULTS FROM ANOVA BETWEEN POPULATIONS BEFORE AND AFTER BODY SIZE ADJUSTMENT. Only parameters in which standard length had a significant correlation are included. Asterisks designate changes in significance after adjustment. Significant p-values α = 0.004 based on a Bonferroni correction. Abbreviations for populations include: LC = Lick Creek, CC = Crooked Creek, PF = Piney Fork.

	Before size adjustment		After size adjustment	
Parameter	**F**	**p**	**F**	**p**
Agonistic				
Burst dominant frequency	12.081	0.001	1.129	0.339*
Knock duration	12.484	0.001	20.444	0.000
Short knock duration	4.446	0.038	8.918	0.000*
Burst duration	5.330	0.022	15.815	0.000*
Courtship				
Knock duration	27.529	0.000	10.645	0.000
Burst interval	6.935	0.010	1.959	0.124
Pulse duration	15.351	0.000	19.385	0.000
Pulse interval	15.336	0.000	20.389	0.000
Pulse rate	11.782	0.001	20.168	0.000

TABLE 2-3. MEAN AGONISTIC SIGNAL PARAMETERS FOR *Cyprinella galactura* BY POPULATION.

	Nottely River	Lick Creek	Crooked Creek	Piney Fork
Agonistic parameters:				
Knock dom. freq.	90.325 ± 42.563 (n=6)	100.077 ± 31.958 (n=11)	83.041 ± 40.665 (n=27)	108.675 ± 21.030 (n=8)
Short knock dom. freq.	69.600 ± 11.728 (n=6)	72.782 ± 26.243 (n=11)	74.000 ± 33.382 (n=12)	85.924 ± 27.532 (n=23)
Burst dom. freq.	49.619 ± 13.956 (n=20)	50.917 ± 15.834 (n=18)	54.595 ± 15.560 (n=92)	57.680 ± 21.273 (n=27)
Total call duration	7.758 ± 7.044 (n=40)	8.055 ± 7.796 (n=52)	5.142 ± 3.794 (n=110)	7.753 ± 9.708 (n=56)
Knock duration	0.168 ± 0.0420 (n=17)	0.207 ± 0.062 (n=20)	0.111 ± 0.020 (n=31)	0.158 ± 0.042 (n=13)
Short knock duration	0.072 ± 0.006 (n=6)	0.077 ± 0.019 (n=17)	0.057 ± 0.014 (n=27)	0.071 ± 0.009 (n=33)
Burst duration	0.565 ± 0.438 (n=34)	0.376 ± 0.244 (n=43)	0.229 ± 0.139 (n=100)	0.373 ± 0.201 (n=31)
Burst rate	1.058 ± 0.444 (n=34)	1.593 ± 1.109 (n=43)	2.006 ± 1.017 (n=100)	1.186 ± 0.878 (n=31)
Pulse duration	0.037 ± 0.010 (n=34)	0.025 ± 0.008 (n=43)	0.022 ± 0.004 (n=100)	0.040 ± 0.010 (n=31)
Pulse interval	0.037 ± 0.009 (n=34)	0.029 ± 0.031 (n=43)	0.023 ± 0.017 (n=100)	0.039 ± 0.009 (n=31)
Pulse rate	16.015 ± 3.291 (n=34)	28.236 ± 30.438 (n=43)	27.461 ± 6.209 (n=100)	15.682 ± 4.226 (n=31)
Single pulse duration	0.040 ± 0.009 (n=17)	0.028 ± 0.010 (n=27)	0.022 ± 0.004 (n=94)	0.042 ± 0.009 (n=34)

Table 2-4. Mean courtship signal parameters for *Cyprinella galactura* by population.

	Nottely River	Lick Creek	Crooked Creek	Piney Fork
Courtship parameters:				
knock dom freq	102.686 ± 46.533 (n=7)	108.275 ± 24.296 (n=4)	113.689 ± 58.055 (n=13)	53.320 ± 11.175 (n=10)
short knock dom freq	57.850 ± 23.264 (n=2)	42.300 ± 0.000 (n=2)	63.167 ± 21.415 (n=3)	88.660 ± 28.956 (n=5)
burst dom freq	55.529 ± 15.986 (n=17)	88.611 ± 20.332 (n=18)	67.452 ± 19.808 (n=40)	74.568 ± 22.031 (n=26)
total call duration	5.636 ± 4.610 (n=35)	8.028 ± 6.487 (n=25)	6.668 ± 5.585 (n=50)	5.999 ± 4.930 (n=37)
knock duration	0.203 ± 0.063 (n=11)	0.198 ± 0.047 (n=5)	0.115 ± 0.018 (n=15)	0.146 ± 0.034 (n=14)
short knock duration	0.072 ± 0.015 (n=2)	0.061 ± 0.004 (n=3)	0.058 ± 0.007 (n=3)	0.078 ± 0.006 (n=12)
burst duration	0.498 ± 0.241 (n=31)	1.036 ± 0.722 (n=24)	0.291 ± 0.133 (n=47)	0.419 ± 0.280 (n=28)
burst rate	1.432 ± 0.565 (n=31)	1.019 ± 0.684 (n=24)	1.561 ± 1.039 (n=47)	1.204 ± 0.919 (n=28)
pulse duration	0.028 ± 0.005 (n=31)	0.033 ± 0.008 (n=24)	0.032 ± 0.005 (n=47)	0.050 ± 0.023 (n=28)
pulse interval	0.028 ± 0.005 (n=31)	0.033 ± 0.008 (n=24)	0.031 ± 0.005 (n=47)	0.049 ± 0.021 (n=28)
pulse rate	20.105 ± 4.580 (n=31)	16.610 ± 3.365 (n=24)	18.607 ± 3.343 (n=47)	13.089 ± 3.915 (n=28)
single pulse duration	0.029 ± 0.005 (n=20)	0.036 ± 0.008 (n=5)	0.033 ± 0.005 (n=46)	0.048 ± 0.015 (n=24)

Table 2-5. Results of one-way ANOVA of *Cyprinella galactura* acoustic parameters. Significant p-values for ANOVA ($\alpha = 0.004$ based on a Bonferroni correction) and MANOVA ($\alpha = 0.05$) in bold. MANOVA analysis only includes the following variables: burst dominant frequency, knock duration, pulse rate, pulse duration, pulse interval, burst duration, and single pulse duration. Burst dominant frequency was excluded from MFC and SA MANOVA (very few bursts in this call type).

Parameters	**C**	**LD**	**MMC**	**MFC**	**SA**
Total call duration	0.363	0.019	0.762	0.422	**0.000**
Average knock duration	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Short knock duration	**0.001**	**0.000**	0.035	**0.000**	0.775
Burst duration	**0.000**	**0.000**	0.221	0.012	0.176
Pulse duration	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Pulse interval	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Single pulse duration	**0.000**	**0.000**	**0.001**	**0.000**	**0.000**
Pulse rate	**0.000**	**0.000**	**0.000**	0.839	0.002
Burst rate	0.055	**0.000**	0.045	0.177	0.208
Knock dominant frequency	0.015	0.302	0.998	0.919	0.012
Short knock dominant frequency	0.185	0.389	0.785	0.713	0.150
Burst dominant frequency	**0.000**	0.326	0.389	0.838	0.844
MANOVA	**0.003**	**0.000**	**0.002**	**0.001**	**0.004**

Table 2-6. Factor loadings of the population principle components analysis for courtship and agonistic acoustic variables for *C. galactura*.

Variable	Component 1	Component 2
Courtship		
Burst dominant frequency	0.313	0.470
Total call duration	0.101	-0.509
Burst rate	-0.250	0.879
Pulse rate	-0.983	0.030
Pulse duration	0.971	0.108
Pulse interval	0.958	0.192
Burst duration	0.156	-0.870
Agonistic		
Burst dominant frequency	-0.355	0.758
Total call duration	0.970	0.240
Burst rate	-0.859	-0.061
Pulse rate	-0.786	0.534
Pulse duration	0.926	0.312
Pulse interval	0.973	0.189
Burst duration	-0.313	-0.851
Knock duration	-0.367	0.913

CHAPTER 3

ACOUSTIC SIGNAL VARIATION IN *CYPRINELLA GALACTURA* – A MULTILEVEL STUDY

Acoustic signal parameters may not evolve as one unit, as parameters may be under different types of selection pressures, and evolve at different rates. Trends were examined in signal parameter variability in *Cyprinella galactura*, the whitetail shiner, at five different levels: among species, within a species, within a population, within individuals, and within a signal. Stereotyped, fine-scale acoustic pulse parameters were found to decrease in variability from the species level to the individual and within-a-signal level. These properties are static, and may be used in species recognition. Variable, gross-scale acoustic parameters, burst duration and burst interval, were found to increase in variability at the individual and within-a-signal level. These properties are dynamic, and may be used in mate choice and may possibly indicate male quality.

INTRODUCTION

Acoustic signals in birds (Marler, 1956), anurans (Bosch et al., 2000; Kime et al., 2004), insects (West-Eberhard, 1984; Rivero et al., 2000), and mammals (Miller and Bain, 2000; Arraut and Vielliard, 2004) are known to contain information that conveys species identification, individual identification, sex, motivation, and possibly mate quality. For some or all of these types of information to be contained in one signal, some signal components must relay different information than others. The typical acoustic signal is composed of different components, both temporal and spectral, and they may evolve at different rates. For example, signal components used in species identification should be more stereotyped than components used in individual identification which need to differentiate them from the general species signal in some way to be meaningful.

A method was developed in anurans to observe how signals may evolve by examining signal temporal and spectral property variation at different levels of analysis (Gerhardt, 1991). Gerhardt (1991) described two patterns of variation present in acoustic signals in several species of treefrogs (Hylidae), which he called static and dynamic properties. In order to determine if signal properties are static or dynamic, he analyzed signals at three levels within treefrogs, among-species, among-male (in one population) and within-male, and then he compared coefficients of variation among those levels. Static properties are defined as those signal parameters which change little during a breeding season, while dynamic signal properties can change markedly during a single bout of calling (Gerhardt, 1991). Static properties are signal components that are associated with morphology and the mechanism of sound production, and are more conserved. While these properties are generally less variable overall, there is a gradient of variability that exists when comparing different levels of analysis. On this gradient, static properties typically are most variable at the within-species level. These properties may relay information such as species identification. Dynamic properties are those signal components that are associated with physiology or behavior and are less conserved. While these properties are generally more variable overall, a gradient of variability exists when comparing different levels of analysis, and dynamic properties typically are more variable at the within-individual level. These signal properties may relay information about the quality or condition of the sender. Gerhardt (1991) categorized these properties by examining coefficients of variation in treefrogs and determining that those signal properties that had a less than 5% coefficient of variation are static, while those properties that had a greater than 15% coefficient are dynamic.

Gerhardt's methods were used to categorize anuran signals in additional studies (Castellano et al., 2002; Giacoma and Castellano, 2001). Castellano et al. (2002) examined four levels of variation in *Hyla arborea*, the European treefrog: within individuals, within populations, among populations of the same species, and among species of the same clade. Signal parameters such as pulse rate, call duration and frequency that are associated with the mechanism of sound production were determined to be static properties (Castellano et al., 2002). Parameters such as bout duration, intercall duration and call rate, which are often produced in response to behavior of other conspecifics, were found to be dynamic properties (Castellano et al., 2002).

Giacoma and Castellano (2001) examined signal variation in the *Bufo viridis* complex at four different levels of analysis: among populations, within populations, within individuals, and within a bout of calling. They identified hierarchical patterns of variation among dynamic and static properties. Static properties were usually the most variable at the among-population level and the least variable at the individual or within a signal level. Dynamic properties exhibit the opposite trend, being more variable at the individual or within a signal level, and less variable at the species level. In the *Bufo viridis* complex, energy dependent traits such as call and intercall duration were found to be dynamic properties, while morpho-physiological traits such as fundamental frequency and pulse rate were found to be static properties (Giacoma and Castellano, 2001).

Examination of acoustic signal variation is not exclusive to anurans and has been described in insects. Pinto-Juma et al. (2005) also looked at acoustic signal variation within a cicada, *Cicada orni*. Temporal parameters (echeme duration/burst duration and interval) were found to be much more variable than spectral parameters (peak frequency). However, unlike anurans, *Cicada orni* signals seem to be much more variable overall with coefficients of variation ranging from 7-75%. Also, unlike anurans, no static or dynamic properties could be identified as all signal properties were less variable at the within-individual level than at the within-population level (Pinto-Juma et al., 2005) and seem to be static. Rivero et al. (2000) examined signal variation in the Wolf Spider, *Hygrolycosa rubrofasciata*. He determined that temporal properties such as pulse rate and pulse rate change were static and spectral properties like peak frequency and peak frequency change were dynamic. Also, the temporal properties were highly stereotyped in terms of repeatability for individual males indicating a possible use in species recognition.

Acoustic signal variation has never been examined in fishes in detail and only a few studies have examined variation within a species (Fine, 1978; Mann and Lobel, 1998; Johnson, 2001; Parmentier et al., 2005). Johnson (2001) was the only one to examine signal variation at any level other than between populations. Her study examined signal variation in longear sunfish, *Lepomis megalotis,* at 4 different levels: within individuals, among individuals from the same population, among populations of the same subspecies, and between subspecies. Johnson (2001) documented significant amounts of signal variation at every level of analysis.

Species in the large genus *Cyprinella* (Cyprinidae) are all believed to produce sounds (Johnston, unpubl.), and are a good system to examine acoustic variation in fishes. This study examines whether static and dynamic properties exist in the acoustic signals of *Cyprinella galactura*, the whitetail shiner. Acoustic properties are categorized and variation is examined at an unprecedented five levels of comparison: within a signal, within individuals, within a population (among individuals), within a species (among populations) and among several species (Fig. 3-1). Three additional acoustically described species of *Cyprinella* are used for species comparisons: *C. gibbsi, C. trichroistia*, and *C. callisema* (Phillips and Johnston, submitted).

Acoustic signals in *C. galactura* are complex and consist of both gross-scale (does not require magnification to measure) and fine-scale (requires magnification to measure) acoustic parameters (Phillips, unpubl.). I predict that fine-scale acoustic parameters which may include pulse rate, pulse duration and pulse interval, will exhibit characteristics of static properties, being more variable at the species level and highly constrained by morphology. I predict that gross-scale acoustic parameters, which may include burst duration and burst interval, will exhibit characteristics of dynamic properties, being less variable at the species level and controlled by male motivation. Possible evolutionary implications for any differential signal variation are discussed.

Cyprinella galactura **as a model**

Cyprinella galactura has a widespread and extremely disjunct distribution in the Tennessee River drainage (Appalachians) and the White River drainage (Ozarks) (Fig 3-2). This disjunction dates back to geological glaciation events that took place in the Pleistocene, no less than 15,000 years ago. Populations for this study were chosen from both sides of the Mississippi embayment (the structural trough that separates the two mountain ranges).

Studies involving populations of *C. galactura* are not complicated with major differences in habitat structure or temperature. All populations of *C. galactura* used in this study were all found in cold, fast flowing, deep and rocky mountain streams of similar size, structure and flow. Finally, issues related with temperature and seasonal variation can be eliminated in this study as all males were collected during the limited *C. galactura* breeding season (May – August).

Cyprinella galactura are already known to produce sounds (Johnston, unpubl. data) although a detailed description of call repertoire, complexity, and behavior association has not been established (Phillips and Johnston, in prep). *Cyprinella galactura* also produce sounds during both agonistic and courtship displays, unlike some other species of *Cyprinella* (Phillips and Johnston, submitted). Analysis of variation in courtship and agonistic contexts can be examined. Furthermore, *C. galactura* interact and spawn readily in aquaria with little to no manipulation, making them the perfect laboratory model for a study of this type.

METHODS

During the breeding season 2002 (May – August) and 2003 populations of *Cyprinella galactura* were collected from all prospective drainages. Collections were made from: Nottely River (Tennessee River drainage), Hwy 180, just E of Hwy 129/19, Union County, Georgia, 30 May 2002; Lick Creek (Duck River drainage), 6 mi. SW of Greenbrier, Maury County, Tennessee, 12 June 2002; Crooked Creek (White River drainage), 5 mi. E of Harrison, Blount County, Arkansas, 9 July 2002; Piney Fork (Black River drainage), 4 mi. NW of Evening Shade, Sharp Co., Arkansas, 14 June 2003. (State permits were issued to CEJ and CTP; AUM animal protocol number 2004-0663).

Fish were transferred to the laboratory and placed in 84 l aquaria. Fish were fed a diet of bloodworms and commercial fish flakes daily. Their photoperiod was regulated at 10-12 hours of light. The trial aquaria were kept in a separate insulated room and isolated from the lab table by a layer of foam. Each aquarium contained a sand substrate and an artificial crevice nesting cavity. Both male-male and male-female trials were set up to gather agonistic and courtship data. All observed behaviors were noted as well as simultaneously videotaped. Acoustic signals that occurred during a particular behavior were considered associated. Observation periods were 30 minutes. Due to the reverberant effect of using small tanks (Parvalescu, 1967), the hydrophone was placed within the range of attenuation length from the fish to ensure signal accuracy (Akamatsu et al., 2002; Okumura et al., 2002). In addition, although all sounds were noted, signals contaminated with excess noise or interference were excluded from the analysis.

Sounds were recorded using a Bruel and Kjaer 8103 hydrophone, Bruel and Kjaer 2635 charge amplifier and Sony model TC-D5 Pro II stereo cassette recorder. Sounds were digitized and analyzed using Raven ver. 1.1 (Cornell University). Temporal parameters were measured from the waveform and spectral parameters were measured from the power spectrum. Spectrograms were generated using the following settings: Hanning window, clipping level, filter bandwidth 124 Hz, frame length 512 pts, and 50% overlap. Power spectra were generated using a Hanning window of 1024. Signals were not filtered prior to data extraction as acoustic signal information overlapped in the low frequency range.

Signals were grouped by context prior to analysis. Both courtship and agonistic signals were analyzed. Although there are several aggressive contexts (sedentary aggression, male-male chase, male-female chase, lateral display), the lateral display aggressive context was selected for comparison because it resembles courtship signals in structure with frequent pulse bursts. The following temporal and spectral acoustic parameters were examined: knock dominant frequency (frequency component with the most energy), knock second frequency (frequency component with the second most energy), short knock dominant frequency, short knock second frequency, burst dominant frequency, burst second frequency, total call duration, knock duration, knock interval, short knock duration, short knock interval, burst duration, burst interval, burst rate (# bursts/total call duration, only in calls with >1 burst), the number of bursts per call, pulse duration, pulse interval, pulse rate (# pulses/burst duration, only in bursts with >1 pulse), the number of pulses per burst, the number of single pulses, single pulse duration, and single pulse interval.

All statistical analyses were conducted using the SPSS software package (SPSS ver. 13.0, SPSS Inc, Chicago, IL) using descriptive statistics, paired-sample t-tests, analysis of variance with Tukey’s multiple comparison test, and MANOVA with Tukey’s multiple comparison test. The use of the behavioral/acoustic term "parameter" is intended to be equivalent to the term "variable" when describing analyzed data. Pearson correlations of signal parameters with standard length (SL) were used to evaluate the effect of body size and determine which parameters were significantly correlated. Parameters were regressed against standard length and adjusted parameter values from regression were used in analysis.

Coefficients of variation were calculated (CV=standard deviation/mean x 100) for each level of analysis to quantify acoustic signal variation. Variation at one level is more easily compared to the next level by calculating ratios between the CVs of the two levels. This was used to compare among-species variation to within-species variation, within-species variation to within-population variation, within-population variation to within-individual variation, and within-individual variation to within-signal variation.

RESULTS

Acoustic repertoire

Male *Cyprinella galactura* produced complex acoustic signals consisting of 1-3 call types arranged in any combination in a given signal. These call types are called knocks, short knocks and pulses, which are usually arranged in organized trains called bursts. All call types were relatively low frequency and non-harmonic. All call types could occur singly or in trains and could occur in any combination together.

Knocks had a mean dominant frequency of 91.8Hz (n=318) and a mean duration of 166ms (n=487) for agonistic signals, and a mean dominant frequency of 103.9Hz (n=73) and mean duration of 155ms (n=46) for courtship signals. Short knocks had a mean dominant frequency of 79.0Hz (n=142) and a mean duration of 69ms (n=327) for agonistic signals, and a mean dominant frequency of 73.8Hz (n=15) and 72ms (n=20) for courtship signals. Pulses had a mean dominant frequency of 53.8 (n=294) and a mean duration of 0.027ms (n=207) for agonistic signals, and a mean dominant frequency of 75.4Hz (n=113) and a mean duration of 0.035ms (n=133) for courtship signals. However, pulses were usually grouped into a burst which is an organized train of pulses. Bursts had a mean duration of 321ms (n=489) for agonistic signals and a mean duration of 508ms (n=133) for courtship signals. Bursts could be divided into two types: aggressive growls and courtship purrs. These varied by associated context and were characterized by unique pulse parameters.

Differences among call types were tested with single-factor ANOVA. All courtship call types were significantly different from one another in both duration (F=24.218, p=0.000) and dominant frequency (F=18.338, p=0.000) and all agonistic call types were significantly different in both duration (F=229.430, p=0.000) and dominant frequency (F=142.269, p=0.000). All call types were used for all contexts however, some call types were more prevalent in aggressive contexts and some call types were more prevalent in courtship contexts.

Level 1 - Within-signal variation

Ninety-one individual agonistic lateral display (n=44) and courtship (n=47) signals were analyzed. Coefficients of variation were calculated for all possible signal parameters which for individual signals include: burst duration, burst interval, pulse duration, and pulse interval (Table 3-1). Coefficients of variation ranged from 13.0% to 105.0%. Pulse signal parameters had the smallest coefficients of variation (13.0% - 15.2%) and were the most stereotyped properties at the within-signal level. Courtship pulse duration had a mean CV of 13.0% and pulse interval had a mean CV of 14.6%. Agonistic pulse duration had a mean CV of 13.8% and pulse interval had a mean CV of 15.2%. Burst duration and interval were the most variable properties (89.9% - 105.0%) at the within-signal level. Courtship burst duration had a mean CV of 91.8% and courtship burst interval had a mean CV of 100.1%. Agonistic burst duration had a mean CV of 89.9% and agonistic burst interval had a mean CV of 105.0%. Variability between courtship and agonistic parameters within a signal was similar and no particular context was more variable.

Level 2 – Within-individual variation

Twenty-four males within populations were compared to see if acoustic differences existed at the individual level (Table 3-2). Agonistic signals (16 males-404 calls) and courtship signals (8 males-139 calls) were analyzed separately. Coefficients of variation were calculated for the following signal parameters: total call duration, knock duration, knock interval, short knock duration, short knock interval, burst duration, burst interval, pulse duration, pulse interval, pulse rate, burst rate, knock dominant frequency, short knock dominant frequency and burst dominant frequency. Coefficients of variation ranged from 11.6% to 82.5%. Parameters that were the most stereotyped (11.6% - 38.8%) include temporal parameters: knock duration (C-21.7%, A-28.9%), short knock duration (C-11.6%, A-18.2%), pulse duration (C-20.1%, A-18.7%), pulse interval (C-20.6%, A-27.0%), pulse rate (C-21.6%, A-29.2%), and spectral parameters: knock dominant frequency (C-29.6%, A-38.8%), short knock dominant frequency (C-30.7%, A-27.7%), and burst dominant frequency (C-22.4%, A-19.6%). Parameters that were the most variable (48.5% - 82.5%) include: total call duration (C-74.5%, A-80.2%), knock interval (C-82.5%, A-74.2%), short knock interval (C-68.2%, A-80.9%), burst interval (C-56.8%, A-77.5%), burst rate (C-56.1%, A-53.6%), and burst duration (C-48.5%, A-48.8%).

Variability between courtship and agonistic parameters within individuals was similar; however, agonistic signals were more variable in 9 of the 14 parameters. Short knock interval and burst interval contained the largest difference in variation between the contexts.

Level 3 – Within-population variation

Thirty-two males from the four populations were examined for within-population variation (Nottely River, n=7; Lick Creek, n=11; Crooked Creek, n=5; Piney Fork, n=9). However, to evaluate the effect of body size on signal variation at the within-population level, Pearson correlations of standard length with signal parameters were conducted (Table 3-3). Standard length was not significantly correlated with any signal parameter in the Nottely River population. Standard length was significantly positively correlated with agonistic burst duration in the Lick Creek (R=0.378, p=0.012, n=43) and Piney Fork (R=0.428, p=0.016, n=31) populations. Standard length was also significantly positively correlated with courtship burst duration in the Crooked Creek (R=0.510, p=0.000, n=50) and Piney Fork (R=0.412, p=0.030, n=28) populations. Standard length was significantly correlated with agonistic burst dominant frequency in the Lick Creek (R=0.538, p=0.021, n=18) and Crooked Creek (R=0.331, p=0.001, n=92) populations, and courtship burst dominant frequency in the Lick Creek (R=0.539, p=0.021, n=18) and Piney Fork (R=-0.458, p=0.019, n=26) populations. All of these correlations with dominant frequency were positive with the exception of the Piney Fork population. Standard length was significantly positively correlated with agonistic single pulse interval in the crooked creek population (R=0.283, p=0.006, n=94). Standard length was significantly positively correlated with courtship pulse rate in the Crooked Creek population (R=0.528, p=0.000, n=50) and negatively correlated with courtship pulse rate in the Piney Fork population (R=-0.635, p=0.000, n=28). Standard length was significantly positively correlated with courtship pulse duration and pulse interval in the Crooked Creek (Pulse dur: R=0.550, p=0.000, n=50; Pulse intv: R=0.525, p=0.000, n=50) and Piney Fork (Pulse dur: R=0.668, p=0.000, n=28; Pulse intv: R=0.648, p=0.000, n=28) populations. In addition, in Piney Fork standard length was significantly correlated for agonistic parameters: short knock dominant frequency (R=0.540, p=0.008, n=23), burst rate (R=-0.741, p=0.000, n=31), and single pulse duration (R=0.737, p=0.000, n=34), and for courtship parameters: total call duration (R=-0.636, p=0.000, n=37), burst rate (R=-0.521, p=0.004, n=28), and single pulse duration (0.673, p=0.000, n=24).

Although body size did account for a small amount of variation in some signal parameters, there was very little effect on the significant differences between individuals before and after size adjustments (Table 3-3). However, necessary signal parameters were regressed against body size and adjusted parameters were used in analysis. Overall, significant individual differences were not prevalent in most populations (Table 3-4). Males from the Nottely River population were significantly different for burst rate (F= 8.543, p=0.000) and nearly significant for pulse duration (F=4.607, p=0.005) and pulse interval (F=4.099, p=0.009) in the agonistic context. Males from the Lick Creek population were significantly different for pulse duration (F=8.543, p=0.000), pulse interval (F=7.408, p=0.000), and single pulse duration (F=6.122, p=0.002) for the agonistic context. Males from Lick Creek were also nearly significant for pulse duration (F=5.933, p=0.005). Males from the Crooked Creek population were not significantly different from one another for any one parameter. However, males from the Piney Fork population were significantly different from one another in agonistic parameters: total call duration (F=7.713, p=0.000), single pulse duration (F=16.794, p=0.000), and burst rate (F=14.888, p=0.000), and courtship parameters: total call duration (F=19.170, p=0.000), pulse duration (F=16.297, p=0.000), pulse interval (F=14.809, p=0.001), single pulse duration (F=13.745, p=0.001) and pulse rate (F=12.953, p=0.001).

Multiple comparison tests from ANOVA among individuals reveal which males were significantly different. In the Nottely River population, male 5 (103.29mm) was significantly different from male 1 (93.3mm) and from male 2 (108.44) for agonistic burst rate. In the Lick Creek population, male 2 (88.16mm) was significantly different from male 1 (105.21mm), male 6 (82.39mm), and male 8 (91.59mm) for agonistic pulse duration, pulse interval, and single pulse duration. In the Piney Fork population, male 1 (105.19mm) was significantly different from male 2 (102.81mm), male 3 (101.3mm), and male 9 (105.19mm) for agonistic total call duration. Male 1 was significantly different from male 2 and male 9 for agonistic single pulse duration and from male 9 for agonistic pulse rate. Male 5 (84.4mm) and male 6 (83.5mm) were significantly different for courtship total call duration, pulse duration, pulse interval, single pulse duration and pulse rate.

Coefficients of variation were calculated for the following signal parameters: total call duration, knock duration, knock interval, short knock duration, short knock interval, burst duration, burst interval, pulse duration, pulse interval, pulse rate, burst rate, knock dominant frequency, short knock dominant frequency, and burst dominant frequency (Table 3-5). Coefficients of variation ranged from 36.0% to 132.5%. Parameters that were the most stereotyped (36.9%-61.0%) included spectral parameters: knock dominant frequency (C-41.6%, A-57.5%), short knock dominant frequency (C-36.9, A-61.0%) and burst dominant frequency (C-54.4%, A-55.6%). Parameters that were the most variable (79.8%-132.5%) included: total call duration (C-79.8%, A-132.5%) and burst interval (C-92.0%, A-119.0%). Variability between courtship and agonistic signal parameters was compared and agonistic parameters were more variable in 13 of the 14 parameters, with total call duration, knock duration, short knock interval, burst duration, burst interval, pulse duration, pulse interval, pulse rate, burst rate, knock dominant frequency, and short knock dominant frequency, having coefficients of variation at least 10% greater than the courtship coefficients of variation.

Level 4 – Within-species variation

Four populations of *Cyprinella galactura* were examined for within species (among population) variation. Results from ANOVA reveal significant differences among populations in mean knock duration, mean pulse duration, mean pulse interval, and mean single pulse duration for agonistic and courtship contexts (Table 3-6). Agonistic (F=22.035, p=0.000) and courtship (F=12.012, p=0.000) mean knock duration were significantly different among the four populations. Agonistic (F=76.816, p=0.000) and courtship (F=21.472, p=0.000) mean pulse duration was also found to be significantly different among the four populations. Agonistic (F=17.002, p=0.000) and courtship (F=22.156, p=0.000) mean pulse interval were significantly different among the four populations. Agonistic (F=89.708, p=0.000) and courtship (F=22.976, p=0.000) mean single pulse duration were also significantly different among the populations.

Agonistic multiple comparison tests found all of the above parameters to be significantly different for all populations except for Nottely River (Appalachians) and Piney Fork (Ozarks). Lick Creek (Appalachians) and Crooked Creek (Ozarks) pulse duration and pulse interval were also not significantly different from one another. Courtship multiple comparison tests revealed fewer significant differences among the populations. However, populations from the Ozarks (Crooked Creek and Piney Fork) and populations from the Appalachians (Nottely River and Lick Creek) were not significantly different from one another for burst dominant frequency, burst secondary frequency, and knock duration. Appalachian populations were not significantly different from one another for pulse duration, pulse interval, burst duration, and single pulse duration.

Multivariate analysis of variance (MANOVA) was used to determine if population differences existed given multiple signal parameters considered together (Table 3-6). Only the parameters deemed significant from ANOVA were used in MANOVA. Parameters used included: burst dominant frequency, knock duration, pulse rate, pulse duration, pulse interval, burst duration, and single pulse duration. Populations were found to be significantly different for both contexts.

Coefficients of variation were calculated for the following signal parameters: total call duration, knock duration, knock interval, short knock duration, short knock interval, burst duration, burst interval, pulse duration, pulse interval, pulse rate, burst rate, knock dominant frequency, short knock dominant frequency and burst dominant frequency (Table 3-7). Coefficients of variation ranged from 62.4% to 191.2%. In general, spectral properties such as short knock dominant frequency (C-77.7%, A-86.7%) and burst dominant frequency (C-77.1%, A-73.2%), and temporal parameters such as knock duration (C-73.0, A-80.9) and short knock duration (C-73.1, A-74.6%) were the least variable. Also, courtship pulse parameters pulse duration (C-62.7%), pulse interval (C-62.4%) and pulse rate (C-69.3%) were the least variable. Parameters that were the most variable include total call duration (C-120.6%, A-191.2%), knock interval (C-176.4%, A-103.1%), burst duration (C-91.0%, A-127.8%) and burst interval (C-104.4%, A-152.0%). Variability between courtship and agonistic parameters was compared, and agonistic parameters were more variable in 10 of the 14 parameters. Agonistic parameters total call duration, burst duration, burst interval, pulse duration, pulse interval, pulse rate, and burst rate had coefficients of variation at least 10% greater than courtship parameters.

Level 5 – Among-species variation

Four species of *Cyprinella* were analyzed for among-species variation. Species used for comparison with *C. galactura* included: *C. gibbsi*, *C. trichroistia*, and *C. callisema*. A total of 478 signals (334 agonistic: 48-*C. trichriostia*, 53-*C. callisema*, 233-*C. galactura*; 144 courtship: 72-*C. gibbsi*, 24-*C. trichriostia*, 12-*C. callisema*, 36-*C. galactura*) were analyzed. *Cyprinella gibbsi* rarely produced sounds during agonistic encounters and no aggressive calls were recorded. The call characteristics of courtship and agonistic signals of the four species are summarized in Table 3-8. Differences in acoustic parameters were tested with single-factor ANOVA. Significant differences were found between the species in all acoustic parameters for both contexts.

For agonistic signals, two parameters were found to be significantly different in all four species: dominant frequency (F=63.381, p=0.000) and pulse rate (F=153.977, p=0.000). Total call duration was also found to be significantly different (F=11.976, p=0.000), but post hoc tests reveal that total call duration was not significantly different between *C. callisema* and *C. galactura*. Burst duration (F=12.166, p=0.000), burst rate (F=21.350, p=0.000), pulse duration (F=66.389, p=0.000), and pulse interval (F=70.452, p=0.000) were all found to be significantly different among the species. However, for all of these parameters, *C. gibbsi* and *C. trichriostia* were not found to be significantly different from one another in the multiple comparisons.

For courtship signals, post hoc tests reveal that *C. gibbsi* and *C. trichriostia* were not significantly different from one another in any of the parameters, including dominant frequency and pulse rate. In addition, *C. trichroistia* and *C. galactura* were not significantly different from one another in dominant frequency. This may have been due to the distinct frequency differences in *C. galactura* between agonistic and courtship signals. Also, *C. callisema* and *C. galactura* were not significantly different in total call duration. *Cyprinella callisema*, *C. gibbsi* and *C. trichroistia*, were not significantly different from one another in burst duration or burst rate.

Coefficients of variation were calculated for the following signal parameters: total call duration, burst duration, burst interval, pulse duration, pulse interval, pulse rate, burst rate, and burst dominant frequency (Table 3-9). Knocks and short knocks CVs were not calculated as not all species produced these call types. Coefficients of variation ranged from 31.7% to 146.6%. Spectral parameter burst dominant frequency (C-48.8%, A-43.8%) and pulse parameters pulse duration (C-80.3%, A-51.7%), pulse interval (C-77.9%, A-67.0%) and pulse rate (C-31.7%, A-70.2%) were the least variable among the species. Burst interval (C-133.9%, A-146.6%), total call duration (C-120.7%, A-91.8%) and burst duration (C-120.7%, A-91.8%) were the most variable. When comparing variability between courtship and agonistic parameters at the among species level no particular context was found to be more variable than the other across the majority of parameters.

Trends in variability

Variability was compared for courtship (Table 3-10) and agonistic (Table 3-11) parameters across the 5 levels of analysis. In both contexts the same trend was apparent, variation for most parameters decreased from the within-species level to the within-signal level. These parameters included: total call duration, knock duration, knock interval, short knock duration, pulse duration, pulse interval, pulse rate, burst rate, knock dominant frequency, short knock dominant frequency, and burst dominant frequency. However, some parameters were more variable at the lower levels of analysis, within individuals and within a signal.

Variation at one level was more easily compared to the next level by calculating ratios between the two levels (Table 3-12). Within-population (among individual) variation was compared to within-individual variation by calculating the ratio between the within-population and within-individual CVs. If the value was less than one, variation increased at the within-individual level. For courtship signals, 2 out of the 14 signal parameters had an increase in variability at the within-individual level, short knock interval (0.85) and burst duration (0.97). For agonistic signals, no signal parameter was more variable at the within-individual level. In addition for those parameters that could be measured within a signal, ratios were calculated between within-individual and within-signal level. Likewise, if the value is greater than one, variation decreased at the within-signal level, and if the value is less than one variation increased at the within-signal level. For courtship and agonistic signals, burst duration (C-0.53, A-0.54) and burst interval (C-0.57, A-0.74) had an increase in variation at the within a signal level.

When comparing among-species variation to within-species variation, many parameters were found to be slightly less variable among the species (ratios <1). These included agonistic parameters: total call duration (0.60), burst duration (0.72), burst interval (0.97), pulse duration (0.50), pulse interval (0.64), pulse rate (0.86), burst rate (0.59) and burst dominant frequency (0.60), and courtship parameters: pulse rate (0.46), burst rate (0.56) and burst dominant frequency (0.63).

DISCUSSION

This study was the second to examine acoustic signal variation in fishes and the first to examine variation at five levels of analysis: within a signal, within individuals, within a population (among males), within a species (among populations), and among species (within a genus). Signal variation was found to be present at all levels with certain parameters consistently less variable than others.

Gerhardt (1991) defined static properties as those signal parameters with a coefficient of variation less than 5% and dynamic properties as those with a coefficient of variation greater than 15%. These values could not be used in *Cyprinella* as overall variability was much greater and ranged from 13.0% to 176.4%. However, rather than a set value to determine if static and dynamic properties exist, examination of trends in variability across set levels of analysis is effective and universal across taxa.

Several trends were evident in *Cyprinella galactura* in terms of signal variability. Temporal pulse parameters pulse duration, pulse rate, and pulse interval were found to be highly stereotyped and decreased in variability from the within-species level to the within-signal level. Spectral parameters knock dominant frequency, short knock dominant frequency, and burst dominant frequency were also found to be highly stereotyped with a decrease in variability from the within-species to the within-signal level. These parameters could be considered static properties. Another set of temporal parameters, short knock interval, burst duration, and burst interval were found to be quite variable, especially at the within-signal and within-individual level. These parameters could be considered dynamic properties.

Johnson (2001) looked at signal variation in the longear sunfish, *Lepomis megalotis*, and found significant differences in call parameters at every level of analysis. She found that temporal parameters were less variable and therefore more highly constrained than spectral parameters, with the exception of pulse rate and call duration. The trend was similar in *C. galactura* with a few notable differences. Spectral parameters were less variable than most other parameters except for those temporal pulse parameters that may be morphologically constrained. Actually, there were two classes of temporal parameters, those most likely constrained by morphology and less variable, and those most likely controlled by physiology or behavior and more variable.

Even though they were not more constrained than pulse parameters, spectral parameters in *C. galactura* fell towards the more constrained end of the spectrum. Spectral parameters are least variable in frogs and toads (Bee et al., 2001; Giacoma and Castellano, 2001) as they were highly coupled to their mechanism of sound production, where as the typically more variable temporal pulse parameters are controlled by behavior. These spectral static properties in anurans generally are those signal parameters highly coupled to morphology (Gerhardt, 1991; Giacoma and Castellano, 2001).

Although the mechanism of sound production is unknown in *Cyprinella*, low frequency pulsed signals (bursts) are often produced by contraction of muscles associated with the gas bladder (Demski et al., 1973; Fine et al., 1977). The highly stereotyped pulse parameters in *Cyprinella* most likely coincide with this believed mechanism of sound production. Spectral properties, while not as dependant on morphology as in anurans, still have some morphological ties in fishes (perhaps body size of the male or the related size of the swim bladder), which may explain why they are still relatively stereotyped.

Dynamic properties are generally those signal parameters which are controlled by behavior or physiology (Gerhardt, 1991; Giacoma and Castellano, 2001). This may be the case with the gross temporal parameters in male *C. galactura* signals, as burst duration or total call duration may have more to do with male motivation or energetics. Calling is known to be costly in anurans. Wells and Taigen (1986) found that acoustic signaling in gray tree frogs, *Hyla versicolor*, can be energetically demanding. Taigen and Wells (1985) also found that rates of oxygen consumption are higher during episodes of calling than during episodes of maximum locomotion. Ryan (1988) reviewed calling energetics in several species of frogs and insects and found that even though calling can be energetically demanding, females may actually prefer components of signals which represent an expenditure of energy as a measure of male quality. This coincides with the Zahavi handicap principle (Zahavi, 1991) in which costs must be imposed on the sender for a signal to be honest. These highly variable temporal parameters may be an honest indicator of male quality as males that can call for long periods of time may be more fit as they can expend more energy.

Results from *Lepomis* (Johnson, 2001) may vary from *Cyprinella*, because *Lepomis* signals are not as complex. For example, the variable temporal parameter pulse duration in *Lepomis* is probably equivalent to the *Cyprinella* knock duration. Johnson (2001) found pulse rate to be unimportant in *Lepomis*, not being significantly different at any level of analysis, even though it is known to be very important in anurans (Giacoma and Castellano, 2001). However, sunfish pulse rate is probably equivalent to a

Cyprinella knock rate (# knocks/total call duration) in a train of knocks. This is quite different from the highly structured *Cyprinella* burst pulse rate which is a very stereotyped fine-scale temporal structure that may contain important species information.

Variable parameters burst duration and burst interval may be important in individual recognition, such as in female mate choice and among neighboring males. However, the fact that burst duration is more variable both within-individuals and within-signals in courtship signals as opposed to being only more variable within-signals in agonistic signals may lean toward a possible role in female mate choice. Perhaps males that can call for a long time, varying the length of the pulse bursts, are more attractive to females. Burst duration was found to be significantly different in courtship among the populations of *C. galactura*. Sexual selection can act on signal properties not used in species identification. This may cause signal divergence (as seen in burst duration), but not result in speciation. This was found to be the case in populations of the planthopper, *Nilaparvata bakeri* (Claridge and Morgan, 1993).

When variability was compared between agonistic and courtship contexts, agonistic signals were found to be much more variable than courtship signals both within a species and within populations. However, within individuals and within a signal, variation between the contexts was similar. Perhaps agonistic signals are more general within a species and within populations as they are used with heterospecifics as well as conspecifics. At the within-individual and within-signal level, agonistic calls would not need to be as general, or courtship calls may be equally variable as they need to convey more information at these levels.

Although body size may play a role in explaining some signal variation, the effect was not large in this particular study. There were no consistent relationships between any one signal parameter and body size. Dominant frequency is negatively correlated with body size in frogs (Castellano et al., 1999), birds (Tubaro and Mahler, 1997) and fish (Myrberg et al., 1993). However, dominant frequency in *C. galactura* was only negatively correlated in one context from one population. Body size certainly does not account for the majority of the variation found in *C. galactura*. Some acoustic signal parameters of *C. galactura*, even after body size adjustment, still exhibited significant differences between males, and even males that were the same body size had significantly different signals.

Bee (2004) found that North American bullfrogs, *Rana catesbeiana*, could discriminate individuals based on individual variation in advertisement calls. He found that spectral and fine-temporal parameters contained less within-individual variation compared to gross temporal properties and that they contributed most statistically towards discriminating among males. This is similar to findings in *C. galactura*. Spectral properties and fine temporal properties such as pulse duration, pulse interval and pulse rate were less variable than other properties within males. Also, these were the same properties that contributed to differences between the males.

Not only does individual variation occur in *C. galactura*, but some populations exhibited more variation than others. The Piney Fork population exhibited the most individual differences in both agonistic and courtship signals. One possible explanation for this could be environmental differences in habitat or community structure. For example, if nesting territories were limited (either by the number of males competing or the number of suitable crevices), individual identification may have been more necessary as males might have needed to know a neighbor from an aggressive intruder. Both birds (Hyman and Hughes, 2006) and anurans (Bee and Gerhardt, 2001a,c) have been known to distinguish neighbors from non-neighbors by their acoustic signals. Individual signal variation would also be important in locations where several species of *Cyprinella* are sympatric. In fact, the Piney Fork population is sympatric with *Cyprinella whipplei*. Likewise, if nests were limited, females would have more at stake in selecting a potentially fit mate.

This study is the first to characterize variation differences in acoustic parameters in the genus *Cyprinella*. It is certainly possible that a typical *C. galactura* signal is more complex than initially thought, having multiple parameters that may evolve at different rates, and encode different types of information, such as species identification, individual identification, and mate quality. This study is a first and necessary step in understanding the processes that influence signal evolution in this species. This work will pave the way for future studies which may include playbacks to determine if females prefer certain signal components over others and if males can distinguish different males (such as neighbors versus non-neighbors).

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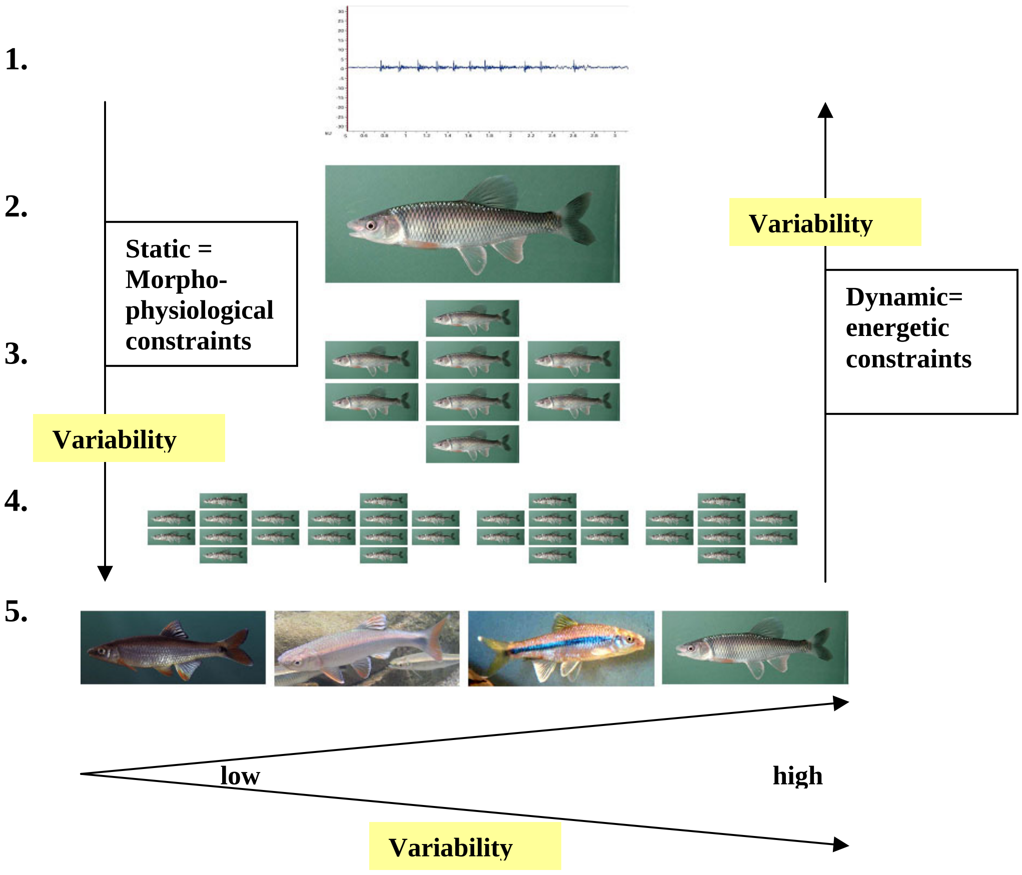

Fig. 3-1. Levels of variation studied in *Cyprinella galactura*. 1=within a signal, 2=within a male, 3=within a population, 4=within a species, 5=among species. Static and dynamic signal variability is depicted.

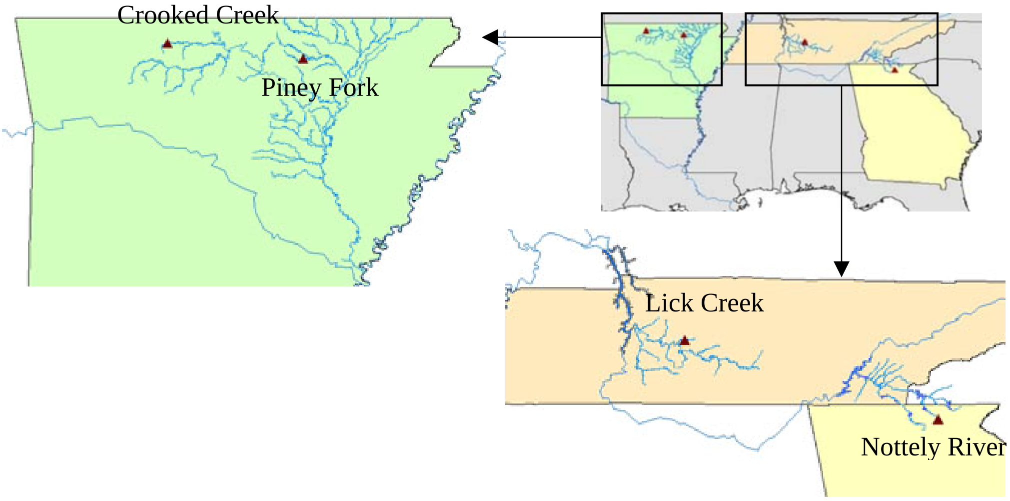

Fig. 3-2. The study populations of *Cyprinella galactura* from the Ozark mountains (Crooked Creek - White River drainage, Piney Fork - Black River drainage) and the Appalachian mountains (Lick Creek - Duck River drainage, Nottely River -Tennessee river drainage).

TABLE 3-1. COEFFICIENTS OF VARIATION (%) FOR COURTSHIP AND AGONISTIC PARAMETERS WITHIN A SIGNAL FOR MALE *Cyprinella galactura*. Rank in order from most variable (1) to least variable (4) is listed within each context. The most variable parameter between courtship and agonistic contexts (>10% difference) is in bold.

Level 1 - Within a signal

	Rank	Courtship CV (%)	n	Rank	Agonistic CV (%)	n
Burst duration	2	91.8 ± 31.0	47	2	89.9 ± 28.6	44
Burst interval	1	100.1 ± 59.0	47	1	105.0 ± 44.8	44
Pulse duration	4	13.0 ± 5.8	47	4	13.8 ± 6.0	44
Pulse interval	3	14.6 ± 6.4	47	3	15.2 ± 6.2	44

TABLE 3-2. COEFFICIENTS OF VARIATION (%) FOR COURTSHIP AND AGONISTIC PARAMETERS WITHIN INDIVIDUAL *Cyprinella galactura*. Rank in order from most variable (1) to least variable (14) is listed within each context. The most variable parameter between courtship and agonistic contexts (>10% difference) is in bold.

Level 2 - Within individuals

	Rank CV (%)	Courtship	n	Rank CV (%)	Agonistic	n
Total call duration	2	74.5 ± 11.3	8	2	80.2 ± 20.7	16
Knock duration	10	21.7 ± 4.1	6	9	28.9 ± 12.0	11
Knock interval	1	82.5 ± 41.2	6	4	74.2 ± 17.7	9
Short knock duration	14	11.6 ± 6.4	4	14	18.2 ± 11.9	9
Short knock interval	3	68.2 ± 46.6	3	1	**80.9 ± 31.1**	9
Burst duration	6	48.5 ± 12.3	8	6	48.8 ± 16.5	16
Burst interval	4	56.8 ± 12.4	8	3	**77.5 ± 36.7**	16
Pulse duration	13	20.1 ± 9.4	8	13	18.7 ± 6.5	16
Pulse interval	12	20.6 ± 8.7	8	11	27.0 ± 24.9	16
Pulse rate	11	21.6 ± 9.0	8	8	29.2 ± 32.1	15
Burst rate	5	56.1 ± 19.1	8	5	53.6 ± 23.7	16
Knock dom. freq.	8	29.6 ± 14.5	4	7	38.8 ± 14.5	8
Short knock dom. freq.	7	30.7 ± 11.4	3	10	27.7 ± 10.5	7
Burst dominant frequency	9	22.4 ± 7.3	7	12	19.6 ± 14.2	12

TABLE 3-3. RESULTS FROM ANOVA BETWEEN INDIVIDUAL MALES IN POPULATIONS BEFORE AND AFTER BODY SIZE ADJUSTMENT. Only parameters in which standard length had a significant correlation are included. Asterisks designate changes in significance after adjustment. Significant p-values $\alpha = 0.004$ based on a Bonferroni correction. Abbreviations for populations include: LC = Lick Creek, CC = Crooked Creek, PF = Piney Fork.

	Pop	Before size adjustment		After size adjustment	
		F	**p**	**F**	**p**
Agonistic					
Burst duration	LC	3.572	0.010	3.572	0.025
	PF	5.715	0.008	3.928	0.000
Pulse duration	PF	3.644	0.004	3.644	0.039*
Pulse interval	PF	6.868	0.004	4.361	0.022*
Single pulse duration	PF	19.710	0.000	16.794	0.000
Single pulse interval	CC	5.947	0.001	4.368	0.006*
Pulse rate	PF	7.379	0.003	5.426	0.010*
Burst rate	PF	19.015	0.000	14.888	0.000
Short knock dominant frequency	PF	8.363	0.002	6.976	0.005*
Burst dominant frequency	LC	0.538	0.069	1.920	0.181
	CC	3.809	0.013	2.778	0.046
Courtship					
Total call duration	PF	23.721	0.000	19.170	0.000
Burst duration	CC	16.863	0.000	6.337	0.015*
	PF	5.304	0.030	2.574	0.121
Pulse duration	CC	20.842	0.000	9.365	0.004
	PF	20.986	0.000	16.297	0.000
Pulse interval	CC	18.290	0.000	8.054	0.007*
	PF	18.782	0.000	14.809	0.001
Single pulse duration	LC	9.524	0.095	2.641	0.275
	PF	18.179	0.000	13.745	0.001
Pulse rate	CC	18.575	0.000	8.054	0.007*
	PF	17.602	0.000	12.953	0.001
Burst rate	PF	17.602	0.000	12.952	0.001
Burst dominant frequency	LC	3.075	0.076	1.233	0.319
	PF	6.363	0.019	3.309	0.081
Burst secondary frequency	CC	29.482	0.000	16.025	0.000

TABLE 3-4. RESULTS OF ONE-WAY ANOVA OF WITHIN-POPULATION ACOUSTIC PARAMETERS. Significant p-values (α = 0.004 based on a Bonferroni correction) in bold. Crooked Creek courtship population was excluded with only 2 males (1 with only 3 signals) producing courtship calls. Contexts are abbreviated: A=agonistic, C=courtship.

Sites:	Nottely River		Lick Creek		Crooked Creek		Piney Fork	
	A	C	A	C	A	C	A	C
Total call duration	0.076	0.302	0.085	0.353	0.035	---	**0.000**	**0.000**
Average knock duration	0.559	0.459	0.648	0.196	0.108	---	0.783	0.234
Short knock duration	---	---	0.329	---	---	---	0.140	0.141
Burst duration	0.053	0.420	0.010	0.067	0.028	---	0.008	0.030
Pulse duration	0.005	0.511	**0.000**	0.005	0.997	---	0.039	**0.000**
Pulse interval	0.009	0.600	**0.000**	0.928	0.948	---	0.022	**0.001**
Single pulse duration	0.288	0.079	**0.002**	0.005	0.168	---	**0.000**	**0.001**
Pulse rate	0.028	0.510	0.858	0.208	0.540	---	0.010	**0.001**
Burst rate	**0.000**	0.231	0.599	0.162	0.198	---	**0.000**	0.052
Knock dominant frequency	0.363	0.538	0.286	0.483	0.411	---	0.408	0.626
Short knock dominant frequency	---	---	0.566	---	---	---	0.005	0.072
Burst dominant frequency	0.352	0.061	0.069	0.076	0.013	---	0.029	0.019
MANOVA	0.451	0.091	0.155	0.532	0.693	---	0.873	0.702

TABLE 3-5. COEFFICIENTS OF VARIATION (%) FOR COURTSHIP AND AGONISTIC PARAMETERS WITHIN POPULATIONS OF *Cyprinella galactura* (n=4). Rank in order from most variable (1) to least variable (14) is listed within each context. The most variable parameter between courtship and agonistic contexts (>10% difference) is in bold.

Level 3 – Within a population	Rank	Courtship CV (%)	Rank	Agonistic CV (%)
Total call duration	1	79.8 ± 49.0	1	1**32.5 ± 44.3**
Knock duration	13	41.3 ± 25.0	9	**71.0 ± 26.3**
Knock interval	3	84.7 ± 34.2	5	92.8 ± 18.9
Short knock duration	10	47.1 ± 41.1	14	36.0 ± 23.7
Short knock interval	7	57.6 ± 66.6	4	**95.5 ± 40.0**
Burst duration	5	47.1 ± 38.3	7	**74.2 ± 8.3**
Burst interval	2	92.0 ± 24.6	2	**119.0 ± 42.2**
Pulse duration	8	43.4 ± 34.0	10	**70.8 ± 7.7**
Pulse interval	9	43.5 ± 34.1	6	**76.4 ± 8.2**
Pulse rate	11	42.7 ± 33.5	8	**74.1 ± 13.7**
Burst rate	4	66.1 ± 42.1	3	**101.1 ± 29.5**
Knock dom. freq.	12	41.6 ± 14.5	12	**57.5 ± 26.1**
Short knock dom. freq.	14	36.9 ± 3.2	11	**61.0 ± 20.7**
Burst dominant frequency	6	54.4 ± 41.7	13	55.6 ± 6.8

TABLE 3-6. RESULTS OF ONE-WAY ANOVA OF *Cyprinella galactura* COURTSHIP AND AGONISTIC ACOUSTIC PARAMETERS. Significant p-values for ANOVA ($\alpha = 0.004$ based on a Bonferroni correction) and MANOVA ($\alpha = 0.05$) in bold. MANOVA analysis only includes the following variables: burst dominant frequency, knock duration, pulse rate, pulse duration, pulse interval, burst duration, and single pulse duration.

Parameters	**Courtship**	**Agonistic**
Total call duration	0.363	0.019
Average knock duration	**0.000**	**0.000**
Short knock duration	**0.001**	**0.000**
Burst duration	**0.000**	**0.000**
Pulse duration	**0.000**	**0.000**
Pulse interval	**0.000**	**0.000**
Single pulse duration	**0.000**	**0.000**
Pulse rate	**0.000**	**0.000**
Burst rate	0.055	**0.000**
Knock dominant frequency	0.015	0.302
Short knock dominant frequency	0.185	0.389
Burst dominant frequency	**0.000**	0.326
MANOVA	**0.003**	**0.000**

TABLE 3-7. COEFFICIENTS OF VARIATION (%) FOR COURTSHIP AND AGONISTIC PARAMETERS WITHIN THE SPECIES *Cyprinella galactura*. Rank in order from most variable (1) to least variable (14) is listed within each context. The most variable parameter between courtship and agonistic contexts (>10% difference) is in bold.

Level 4 - Within a species	Rank	Courtship CV (%)	n	Rank	Agonistic CV (%)	n
Total call duration	2	120.6	150	1	**191.2**	256
Knock duration	11	73.0	45	11	80.9	81
Knock interval	1	**176.4**	37	8	103.1	64
Short knock duration	10	73.1	20	12	74.6	83
Short knock interval	3	106.2	15	5	105.6	76
Burst duration	7	91.0	133	3	**127.8**	208
Burst interval	4	104.4	129	2	**152.0**	195
Pulse duration	12	62.7	133	6	**104.5**	208
Pulse interval	13	62.4	133	7	**104.3**	208
Pulse rate	14	69.3	132	10	**81.3**	208
Burst rate	6	101.3	133	4	**113.9**	208
Knock dom. freq.	5	**101.8**	34	14	71.4	52
Short knock dom. freq.	8	77.7	12	9	86.7	52
Burst dominant frequency	9	77.1	104	13	73.2	157

TABLE 3-8. AGONISTIC (A) AND COURTSHIP (C) CALL MEASUREMENT SUMMARY FOR *Cyprinella* SPECIES. Mean values with standard deviations, between species F tests. Paired-samples t-tests were used to test for differences between context within species. Only significant results are displayed.

Parameters		*C. gibbsi*	*C. trichroistia*	*C. callisema*	*C. galactura*	
Dominant frequency (Hz)	A	---	78.444 ± 3.043 (n=24)	65.667 ± 8.253 (n=53)	51.346 ± 13.634 (n=58)	F=63.381, p=0.000
	C	84.394 ± 4.634 (n=72)	79.124 ± 5.166 (n=48)	63.418 ± 7.896 (n=12)	74.459 ± 26.716 (n=31)	F=11.976, p=0.000
Total call duration	A	---	6.964 ± 18.491 (n=24)	3.477 ± 3.560 (n=53)	3.337 ± 4.693 (n=233)	F=3.241, p=0.040
	C	2.248 ± 2.201 (n=72)	2.310 ± 2.680 (n=48)	4.607 ± 4.498 (n=12)	5.500 ± 4.617 (n=36)	F=10.494, p=0.000
Burst duration	A	---	0.191 ± 0.128 (n=24)	0.193 ± 0.087 (n=44)	0.453 ± 0.425 (n=99)	F=12.166, p=0.000
	C	0.241 ± 0.100 (n=72)	0.299 ± 0.193 (n=48)	0.320 ± 0.301 (n=12)	0.498 ± 0.241 (n=31)	F=14.728, p=0.000
Burst rate	A	---	2.827 ± 2.133 (n=24)	2.383 ± 1.680 (n=44)	1.155 ± 0.864 (n=95)	F=21.350, p=0.000
	C	2.883 ± 0.689 (n=72)	2.639 ± 1.386 (n=48)	2.274 ± 1.855 (n=12)	1.432 ± 0.565 (n=31)	F=14.667, p=0.000
Pulse duration	A	---	0.016 ± 0.001 (n=24)	0.021 ± 0.005 (n=44)	0.035 ± 0.011 (n=95)	F=66.389, p=0.000
	C	0.017 ± 0.002 (n=72)	0.019 ± 0.004 (n=48)	0.023 ± 0.004 (n=12)	0.028 ± 0.006 (n=31)	F=73.417, p=0.000
Pulse interval	A	---	0.016 ± 0.002 (n=24)	0.020 ± 0.004 (n=44)	0.034 ± 0.011 (n=95)	F=70.452, p=0.000
	C	0.018 ± 0.002 (n=72)	0.019 ± 0.003 (n=48)	0.022 ± 0.005 (n=12)	0.028 ± 0.005 (n=31)	F=67.613, p=0.000
Pulse rate	A	---	35.855 ± 4.798 (n=24)	28.563 ± 4.837 (n=44)	17.728 ± 5.255 (n=95)	F=153.977, p=0.000
	C	32.309 ± 3.116 (n=72)	31.045 ± 7.695 (n=48)	25.830 ± 4.554 (n=12)	20.105 ± 4.580 (n=31)	F=42.499, p=0.000

TABLE 3-9. COEFFICIENTS OF VARIATION (%) FOR COURTSHIP AND AGONISTIC PARAMETERS AMONG SPECIES OF *Cyprinella* (*C. galactura*, *C. trichroistia*, *C. gibbsi*, and *C. callisema*). Rank in order from most variable (1) to least variable (8) is listed within each context. The most variable parameter between courtship and agonistic contexts (>10% difference) is in bold.

Level 5 - Among species	Rank	Courtship CV (%)	n	Rank	Agonistic CV (%)	n
Total call duration	2	**124.2**	282	2	114.0	303
Burst duration	3	**120.7**	265	3	91.8	246
Burst interval	1	133.9	254	1	**146.6**	239
Pulse duration	4	**80.3**	262	7	51.7	246
Pulse interval	5	**77.9**	261	5/6	67.0	246
Pulse rate	8	31.7	264	4	**70.2**	246
Burst rate	6	56.9	265	5/6	**67.0**	246
Burst dominant frequency	7	48.8	236	8	43.8	202

Table 3-10. Courtship Coefficients of variation of *C. galactura* acoustic properties at different levels of analysis. Number examined in parenthesis. Among species analysis includes: *C. galactura*, *C. callisema*, *C. trichroistia*, and *C. gibbsi*. Abbreviated levels of analysis: AS = among species, WS = within species, WP = within population, WI = within individual, WC = within call.

	Coefficients of Variation (CV)				
Levels examined:	5	4	3	2	1
	AS	WS	WP	WI	WC
Parameters:					
Total call duration	124.2	120.6	79.8 ± 49.0	74.5 ± 11.3	---
	(282)	(150)	(4)	(8)	
Knock duration	---	73.0	41.3 ± 25.0	21.7 ± 4.1	---
		(45)	(4)	(6)	
Knock interval	---	176.4	84.7 ± 34.2	82.5 ± 41.2	---
		(37)	(4)	(6)	
Short knock duration	---	73.1	47.1 ± 41.1	11.6 ± 6.4	---
		(20)	(4)	(4)	
Short knock interval	---	106.2	57.6 ± 66.6	68.2 ± 46.6	---
		(15)	(4)	(3)	
Burst duration	120.7	91.0	47.1 ± 38.3	48.5 ± 12.3	91.8 ± 31.0
	(265)	(133)	(4)	(8)	(47)
Burst interval	133.9	104.4	92.0 ± 24.6	56.8 ± 12.4	100.1 ± 59.0
	(254)	(129)	(4)	(8)	(47)
Pulse duration	80.3	62.7	43.4 ± 34.0	20.1 ± 9.4	13.0 ± 5.8
	(262)	(133)	(4)	(8)	(47)
Pulse interval	77.9	62.4	43.5 ± 34.1	20.6 ± 8.7	14.6 ± 6.4
	(261)	(133)	(4)	(8)	(47)
Pulse rate	31.7	69.3	42.7 ± 33.5	21.6 ± 9.0	---
	(264)	(132)	(4)	(8)	
Burst rate	56.9	101.3	66.1 ± 42.1	56.1 ± 19.1	---
	(265)	(133)	(4)	(8)	
Knock dom freq	---	101.8	41.6 ± 14.5	29.6 ± 14.5	---
		(34)	(4)	(4)	
Short knock dom freq	---	77.7	36.9 ± 3.2	30.7 ± 11.4	---
		(12)		(7)	
Burst dom freq	48.8	77.1	54.4 ± 41.7	22.4 ± 7.3	---
	(236)	(104)	(4)	(7)	

TABLE 3-11. AGONISTIC COEFFICIENTS OF VARIATION OF *C. galactura* ACOUSTIC PROPERTIES AT DIFFERENT LEVELS OF ANALYSIS. Number examined in parenthesis. Among species analysis includes: *C. galactura*, *C. callisema*, *C. trichroistia*, and *C. gibbsi*. Abbreviated levels of analysis: AS = among species, WS = within species, WP = within population, WI = within individual, WC = within call.

	Coefficients of Variation (CV)				
Levels examined:	5	4	3	2	1
	AS	WS	WP	WI	WC
Parameters:					
Total call duration	114.0 (303)	191.2 (258)	132.5 ± 44.3 (4)	80.2 ± 20.7 (16)	---
Knock duration	---	80.9 (81)	71.0 ± 26.3 (4)	28.9 ± 12.0 (11)	---
Knock interval	---	103.1 (64)	92.8 ± 18.9 (4)	74.2 ± 17.7 (9)	---
Short knock duration	---	74.6 (83)	36.0 ± 23.7 (4)	18.2 ± 11.9 (9)	---
Short knock interval	---	105.6 (76)	95.5 ± 40.0 (4)	80.9 ± 31.1 (9)	---
Burst duration	91.8 (246)	127.8 (208)	74.2 ± 8.3 (4)	48.8 ± 16.5 (16)	89.9 ± 28.6 (44)
Burst interval	146.6 (239)	152.0 (195)	119.0 ± 42.2 (4)	77.5 ± 36.7 (16)	105.0 ± 44.8 (44)
Pulse duration	51.7 (246)	104.5 (208)	70.8 ± 7.7 (4)	18.7 ± 6.5 (16)	13.8 ± 6.0 (44)
Pulse interval	67.0 (246)	104.3 (208)	76.4 ± 8.2 (4)	27.0 ± 24.9 (16)	15.2 ± 6.2 (44)
Pulse rate	70.2 (246)	81.3 (208)	74.1 ± 13.7 (4)	29.2 ± 32.1 (15)	---
Burst rate	67.0 (246)	113.9 (208)	101.1 ± 29.5 (4)	53.6 ± 23.7 (16)	---
Knock dom freq	---	71.4 (52)	57.5 ± 26.1 (4)	38.8 ± 14.5 (8)	---
Short knock dom freq	---	86.7 (12)	61.0 ± 20.7 (4)	27.7 ± 10.5 (7)	---
Burst dom freq	43.8 (202)	73.2 (157)	55.6 ± 6.8 (4)	19.6 ± 14.2 (12)	

TABLE 3-12. RATIOS BETWEEN COEFFICIENTS OF VARIATION (CV) OF COURTSHIP AND AGONISTIC *C. galactura* ACOUSTIC PROPERTIES. AS/WS = among-species CV/within-species CV, WS/WP = within-species CV/within-population CV, WP/WI = within-population CV/within-individual CV, WI/WC = within-individual CV/within-call CV. Values less than one, which indicate an increase in variability from one level to the next, are in bold.

	Courtship				Agonistic			
	AS/WS	WS/WP	WP/WI	WI/WC	AS/WS	WS/WP	WP/WI	WI/WC
Parameters:								
Total call duration	1.03	1.51	1.07	---	**0.60**	1.44	1.65	---
Knock duration	---	1.77	1.90	---	---	1.14	2.46	---
Knock interval	---	2.08	1.03	---	---	1.11	1.25	---
Short knock duration	---	1.55	4.06	---	---	2.07	1.98	---
Short knock interval	---	1.84	**0.85**	---	---	1.11	1.18	---
Burst duration	1.33	1.93	**0.97**	**0.53**	**0.72**	1.72	1.52	**0.54**
Burst interval	1.32	1.14	1.62	**0.57**	**0.97**	1.28	1.54	**0.74**
Pulse duration	1.28	1.45	2.16	1.55	**0.50**	1.48	3.79	1.36
Pulse interval	1.25	1.44	2.10	1.41	**0.64**	1.37	2.83	1.78
Pulse rate	**0.46**	1.62	1.98	---	**0.86**	1.10	2.54	---
Burst rate	**0.56**	1.53	1.18	---	**0.59**	1.13	1.89	---
Knock dom freq	---	2.45	1.41	---	---	1.24	1.48	---
Short knock dom freq	---	2.11	1.20	---	---	1.42	2.20	---
Burst dom freq	**0.63**	1.42	2.43	---	**0.60**	1.32	2.84	---

CONCLUSIONS

This study was the first to examine acoustic signal variation in Cyprinids, using disjunct Central Highland species, *Cyprinella galactura*, as a model. Although signals were quite complex, the species-specific call types and their associated signal parameters provided an excellent model for examining the evolution of acoustic communication in freshwater fishes. Before a detailed analysis of signal divergence in this species could be achieved, a description of sound production and associated behavior was essential. *Cyprinella galactura* were found to produce three call types: knocks, short knocks, and pulses. Each signal could contain gross-scale and fine-scale temporal structure, and signals could be monophasic, diphasic or triphasic.

Male *C. galactura* produced sounds during both courtship and agonistic contexts which allowed comparisons between the contexts to occur at all levels of analysis. This gave further insight as to how courtship versus agonistic signals may evolve in this species. These sounds were found to be most frequent during low and moderate level displays, and decrease during the highest level of motivation under both contexts. Agonistic signals are significantly more variable than those produced during courtship, and the two contexts can be distinguished on the basis of temporal and spectral parameters. In addition, larger males and territory owners won the majority of contests for spawning sites.

Significant amounts of geographic signal divergence were found between the four populations of *Cyprinella galactura*. As in anurans (Smith et al., 2003), and other fishes (Johnson, 2001; Parmentier et al., 2005), pulse signal parameters: pulse rate, pulse duration, pulse interval, and single pulse duration were the most important parameters in separating the populations. Courtship signals were found to be less variable and more stereotyped than agonistic signals. A hypothesized method of signal divergence by geographic isolation is proposed for courtship signals, as adjacent populations were found to be more similar to one another than nonadjacent populations. This may be due to the importance of courtship signals in species identification/mate recognition. Signal divergence in agonistic signals seems to be much more random with adjacent populations clustering with nonadjacent populations. Hypothesized methods of divergence could be genetic drift, or unknown differences in the environment.

Acoustic signals do not necessarily evolve together as one unit, and different parameters may be under different types of selection pressures, and evolve at different rates. Trends were examined in signal parameter variability and signal properties were categorized by their coefficients of variation at different levels of analysis (Gerhardt, 1991). This provided insight as to how signal parameters may be evolving in *C. galactura*. The more stereotyped, and fine-scale acoustic pulse parameters were found to decrease in variability from the species level to within-a-signal level. These properties are static, and may be used in species recognition. The more variable, and gross-scale acoustic parameters, burst duration and burst interval, were found to increase in variability at the individual and within-a-signal level. These properties are dynamic, and may be used in mate choice and may possibly indicate male quality as males that can call for long periods of time may be more fit as they can expend more energy. Ryan (1988) reviewed calling energetics in several species of frogs and insects and found that even though calling can be energetically demanding, females may actually prefer components of signals which represent an expenditure of energy as a measure of male quality.

This study is a first step in understanding the processes that influence signal evolution in this species. This work will pave the way and aid in the design of future studies in this species, and in the genus *Cyprinella*. These studies may include the more elaborate playback systems necessary to examine the role of acoustic signaling and the signal components in female choice, species identification, and the identification of neighbor vs. non-neighboring territorial males.

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APPENDICES

APPENDIX A.

MEANS AND STANDARD DEVIATIONS (NUMBER OF SIGNALS ANALYSED IN PARENTHESES) OF *Cyprinella galactura* SIGNAL PARAMETERS BY CONTEXT.

	Contexts:				
Parameters:	SA	MFC	MMC	LD	Courtship
knock dom freq	76.624 ± 33.615 (n=45)	92.005 ± 36.303 (n=87)	88.260 ± 39.833 (n=94)	91.429 ± 37.172 (n=52)	93.031 ± 48.999 (n=34)
knock sec freq	261.428 ± 65.423 (n=44)	292.289 ± 90.267 (n=85)	280.387 ± 106.223 (n=93)	267.192 ± 80.319 (n=52)	258.347 ± 74.843 (n=34)
short knock dom freq	71.904 ± 22.947 (n=28)	71.303 ± 29.591 (n=42)	79.878 ± 22.641 (n=34)	78.509 ± 27.602 (n=52)	69.425 ± 27.829 (n=12)
short knock sec freq	260.871 ± 47.600 (n=28)	258.501 ± 62.952 (n=42)	255.260 ± 80.647 (n=34)	244.070 ± 55.455 (n=51)	226.517 ± 42.000 (n=12)
burst dom freq	54.385 ± 20.315 (n=26)	53.304 ± 13.992 (n=48)	52.210 ± 16.410 (n=50)	54.070 ± 16.547 (n=157)	71.423 ± 21.933 (n=104)
burst sec freq	201.594 ± 67.188 (n=26)	202.057 ± 52.547 (n=48)	247.482 ± 98.566 (n=50)	203.556 ± 65.125 (n=157)	247.893 ± 79.059 (n=104)
total call duration	3.317 ± 4.868 (n=167)	2.110 ± 2.830 (n=220)	3.055 ± 3.620 (n=287)	6.702 ± 6.919 (n=257)	6.390 ± 5.371 (n=150)
avg num knocks	1.800 ± 1.486 (n=82)	1.670 ± 1.212 (n=126)	2.220 ± 1.730 (n=186)	2.100 ± 1.655 (n=81)	1.670 ± 1.187 (n=45)
knock duration	0.172 ± 0.062 (n=82)	0.160 ± 0.067 (n=126)	0.175 ± 0.061 (n=186)	0.154 ± 0.056 (n=81)	0.155 ± 0.054 (n=45)
knock interval	0.727 ± 0.701 (n=45)	1.001 ± 0.888 (n=65)	0.966 ± 1.113 (n=119)	0.722 ± 0.710 (n=64)	1.000 ± 1.154 (n=37)
avg num short knocks	3.090 ± 2.952 (n=70)	2.200 ± 2.174 (n=82)	2.930 ± 2.430 (n=89)	5.100 ± 6.339 (n=83)	2.000 ± 1.376 (n=20)
short knock duration	0.069 ± 0.011 (n=70)	0.070 ± 0.014 (n=82)	0.069 ± 0.015 (n=89)	0.068 ± 0.015 (n=83)	0.072 ± 0.011 (n=20)
short knock interval	0.570 ± 0.670 (n=54)	0.639 ± 0.584 (n=58)	0.654 ± 0.850 (n=63)	0.388 ± 0.443 (n=76)	0.476 ± 0.657 (n=15)

Appendix A. Continued.

	SA	MFC	MMC	LD	Courtship
num bursts	7.820 ± 80037 (n=60)	3.790 ± 3.732 (n=77)	4.780 ± 4.418 (n=102)	10.060 ± 9.275 (n=207)	7.990 ± 6.098 (n=133)
burst duration	0.212 ± 0.119 (n=60)	0.342 ± 0.269 (n=77)	0.343 ± 0.333 (n=102)	0.336 ± 0.270 (n=207)	0.508 ± 0.443 (n=133)
burst interval	0.309 ± 0.565 (n=60)	0.521 ± 0.894 (n=68)	0.328 ± 0.326 (n=91)	0.194 ± 0.270 (n=194)	0.297 ± 0.304 (n=129)
burst rate	1.832 ± 1.905 (n=60)	1.338 ± 1.166 (n=77)	1.322 ± 0.886 (n=102)	1.643 ± 1.022 (n=207)	1.357 ± 0.864 (n=133)
num pul/burst	4.170 ± 2.153 (n=60)	6.627 ± 5.949 (n=77)	6.987 ± 6.404 (n=102)	7.093 ± 4.792 (n=207)	8.609 ± 7.083 (n=133)
pulse duration	0.031 ± 0.011 (n=60)	0.032 ± 0.012 (n=77)	0.030 ± 0.010 (n=102)	0.027 ± 0.010 (n=207)	0.035 ± 0.014 (n=133)
pulse interval	0.030 ± 0.011 (n=60)	0.031 ± 0.012 (n=77)	0.031 ± 0.012 (n=102)	0.028 ± 0.015 (n=207)	0.034 ± 0.013 (n=133)
pulse rate	21.552 ± 6.445 (n=60)	21.873 ± 11.577 (n=77)	20.919 ± 7.388 (n=102)	24.036 ± 15.553 (n=207)	17.605 ± 4.700 (n=132)
avg num single pulses	6.230 ± 5.255 (n=65)	3.300 ± 2.702 (n=60)	5.550 ± 6.516 (n=67)	10.210 ± 9.767 (n=171)	6.340 ± 7.136 (n=95)
single pulse duration	0.033 ± 0.010 (n=65)	0.034 ± 0.010 (n=60)	0.036 ± 0.010 (n=67)	0.029 ± 0.011 (n=171)	0.036 ± 0.011 (n=95)
single pulse interval	0.275 ± 0.268 (n=61)	0.340 ± 0.287 (n=52)	0.455 ± 0.922 (n=66)	0.231 ± 0.215 (n=170)	0.289 ± 0.361 (n=91)

APPENDIX B.

MEANS AND STANDARD DEVIATIONS (NUMBER OF SIGNALS ANALYSED IN PARENTHESES) OF *Cyprinella galactura* POPULATION SIGNAL PARAMETERS BY CONTEXT.

Nottely River

	Contexts:				
Parameters	SA	MFC	MMC	LD	Courtship
knock dom freq	67.375 ± 22.288 (n=10)	95.006 ± 37.471 (n=31)	88.480 ± 38.358 (n=35)	90.325 ± 42.563 (n=6)	102.686 ± 46.533 (n=7)
knock sec freq	224.944 ± 63.318 (n=9)	286.377 ± 77.271 (n=29)	247.989 ± 62.979 (n=34)	211.883 ± 71.663 (n=6)	300.786 ± 49.293 (n=7)
short knock dom freq	71.217 ± 14.932 (n=6)	67.445 ± 19.895 (n=6)	74.340 ± 11.812 (n=6)	69.600 ± 11.728 (n=6)	57.850 ± 23.264 (n=2)
short knock sec freq	249.700 ± 63.274 (n=6)	275.283 ± 37.020 (n=6)	209.420 ± 46.407 (n=6)	269.050 ± 62.366 (n=6)	240.650 ± 55.084 (n=2)
burst dom freq	---	55.107 ± 16.854 (n=15)	46.643 ± 10.883 (n=15)	49.619 ± 13.956 (n=20)	55.529 ± 15.986 (n=17)
burst sec freq	---	213.275 ± 33.884 (n=15)	223.913 ± 58.675 (n=15)	175.946 ± 68.897 (n=20)	206.879 ± 115.61 (n=17)
total call duration	1.471 ± 2.639 (n=36)	2.083 ± 3.055 (n=71)	3.110 ± 3.824 (n=88)	7.758 ± 7.044 (n=40)	5.636 ± 4.610 (n=35)
avg num knocks	1.890 ± 1.771 (n=28)	1.690 ± 1.412 (n=54)	2.190 ± 1.401 (n=74)	1.76 ± 1.393 (n=17)	2.00 ± 2.049 (n=11)
knock duration	0.174 ± 0.053 (n=28)	0.177 ± 0.072 (n=54)	0.195 ± 0.065 (n=74)	0.168 ± 0.0420 (n=17)	0.203 ± 0.063 (n=11)
knock interval	0.950 ± 0.919 (n=10)	1.067 ± 1.022 (n=28)	1.160 ± 1.424 (n=45)	0.551 ± 0.436 (n=15)	0.671 ± 0.548 (n=8)
avg num short knocks	1.860 ± 0.900 (n=7)	1.860 ± 1.215 (n=7)	4.600 ± 2.881 (n=5)	9.670 ± 5.922 (n=6)	1.000 ± 0.000 (n=2)
short knock duration	0.065 ± 0.010 (n=7)	0.072 ± 0.010 (n=7)	0.073 ± 0.011 (n=5)	0.072 ± 0.006 (n=6)	0.072 ± 0.015 (n=2)
short knock interval	1.401 ± 1.553 (n=5)	0.310 ± 0.172 (n=4)	0.264 ± 0.197 (n=5)	0.298 ± 0.170 (n=6)	0.026 (n=1)

APPENDIX B. CONTINUED.

Parameters	SA	MFC	MMC	LD	Courtship
num bursts	2.00 (n=1)	3.560 ± 4.246 (n=27)	3.940 ± 3.967 (n=32)	7.910 ± 6.307 (n=34)	7.26 ± 5.05 (n=31)
burst duration	0.183 (n=1)	0.375 ± 0.290 (n=27)	0.428 ± 0.515 (n=32)	0.565 ± 0.438 (n=34)	0.498 ± 0.241 (n=31)
burst interval	0.685 (n=1)	0.458 ± 0.710 (n=22)	0.434 ± 0.394 (n=25)	0.351 ± 0.357 (n=25)	0.249 ± 0.256 (n=30)
burst rate	1.010 (n=1)	1.373 ± 1.218 (n=27)	1.070 ± 0.864 (n=32)	1.058 ± 0.444 (n=34)	1.432 ± 0.565 (n=31)
num pul/burst	2.50 (n=1)	6.872 ± 4.722 (n=27)	7.131 ± 7.559 (n=32)	8.919 ± 6.136 (n=34)	10.200 ± 5.458 (n=31)
pulse duration	0.046 (n=1)	0.032 ± 0.013 (n=27)	0.341 ± 0.009 (n=32)	0.037 ± 0.010 (n=34)	0.028 ± 0.005 (n=31)
pulse interval	0.045 (n=1)	0.031 ± 0.012 (n=27)	0.034 ± 0.010 (n=32)	0.037 ± 0.009 (n=34)	0.028 ± 0.005 (n=31)
pulse rate	13.661 (n=1)	20.262 ± 7.344 (n=27)	17.360 ± 4.054 (n=32)	16.015 ± 3.291 (n=34)	20.105 ± 4.580 (n=31)
avg num single pulses	3.50 ± 2.121 (n=2)	2.700 ± 1.780 (n=20)	4.920 ± 6.776 (n=25)	7.060 ± 6.675 (n=17)	2.700 ± 1.809 (n=20)
single pulse duration	0.045 ± 0.007 (n=9)	0.039 ± 0.011 (n=20)	0.041 ± 0.011 (n=25)	0.040 ± 0.009 (n=17)	0.029 ± 0.005 (n=20)
single pulse interval	0.439 ± 0.093 (n=2)	0.453 ± 0.352 (n=18)	0.560 ± 1.007 (n=24)	0.293 ± 0.191 (n=17)	0.329 ± 0.312 (n=18)

Appendix B. Continued.

Lick Creek

	Contexts:				
Parameters	SA	MFC	MMC	LD	Courtship
knock dom freq	131.317 ± 80.681 (n=9)	93.986 ± 26.151 (n=7)	88.406 ± 37.455 (n=16)	100.077 ± 31.958 (n=11)	108.275 ± 24.296 (n=4)
knock sec freq	239.628 ± 98.374 (n=9)	326.236 ± 64.233 (n=7)	324.684 ± 124.200 (n=16)	296.646 ± 80.971 (n=11)	333.250 ± 93.756 (n=4)
short knock dom freq	46.450 ± 4.879 (n=2)	85.940 ± 34.793 (n=5)	75.867 ± 26.518 (n=3)	72.782 ± 26.243 (n=11)	42.300 ± 0.000 (n=2)
short knock sec freq	284.850 ± 20.718 (n=2)	263.420 ± 24.341 (n=5)	300.033 ± 72.111 (n=3)	249.400 ± 36.833 (n=10)	232.300 ± 0.000 (n=2)
burst dom freq	70.550 ± 44.373 (n=7)	51.050 ± 14.940 (n=6)	57.689 ± 22.548 (n=9)	50.917 ± 15.834 (n=18)	88.611 ± 20.332 (n=18)
burst sec freq	179.286 ± 79.523 (n=7)	222.742 ± 40.855 (n=6)	356.233 ± 164.892 (n=9)	188.636 ± 74.213 (n=18)	252.808 ± 50.786 (n=18)
total call duration	5.871 ± 6.444 (n=65)	2.744 ± 3.096 (n=35)	3.343 ± 4.022 (n=75)	8.055 ± 7.796 (n=52)	8.028 ± 6.487 (n=25)
avg num knocks	2.000 ± 1.668 (n=24)	1.790 ± 1.528 (n=14)	2.270 ± 2.304 (n=48)	2.650 ± 2.498 (n=20)	1.200 ± 0.447 (n=5)
knock duration	0.206 ± 0.072 (n=24)	0.229 ± 0.068 (n=14)	0.201 ± 0.046 (n=48)	0.207 ± 0.062 (n=20)	0.198 ± 0.047 (n=5)
knock interval	0.598 ± 0.434 (n=18)	0.720 ± 0.763 (n=7)	0.862 ± 0.692 (n=30)	0.699 ± 0.530 (n=15)	1.010 ± 1.262 (n=4)
avg num short knocks	3.820 ± 3.529 (n=34)	1.830 ± 1.150 (n=18)	2.960 ± 2.742 (n=28)	3.590 ± 2.874 (n=17)	2.330 ± 1.528 (n=3)
short knock duration	0.070 ± 0.012 (n=34)	0.072 ± 0.014 (n=18)	0.073 ± 0.017 (n=28)	0.077 ± 0.019 (n=17)	0.061 ± 0.004 (n=3)
short knock interval	0.506 ± 0.509 (n=29)	0.859 ± 0.606 (n=12)	0.755 ± 1.128 (n=21)	0.461 ± 0.493 (n=15)	0.446 ± 0.404 (n=3)
num bursts	9.130 ± 9.011 (n=38)	4.300 ± 3.585 (n=20)	4.450 ± 3.674 (n=38)	12.210 ± 11.636 (n=43)	6.670 ± 4.167 (n=24)
burst duration	0.204 ± 0.092 (n=38)	0.343 ± 0.286 (n=20)	0.284 ± 0.192 (n=38)	0.376 ± 0.244 (n=43)	1.036 ± 0.722 (n=24)

APPENDIX B. CONTINUED.

Parameters	SA	MFC	MMC	LD	Courtship
burst interval	0.403 ± 0.686 (n=38)	0.339 ± 0.332 (n=20)	0.325 ± 0.328 (n=34)	0.296 ± 0.419 (n=42)	0.267 ± 0.217 (n=22)
burst rate	1.659 ± 2.058 (n=38)	1.569 ± 1.087 (n=20)	1.418 ± 0.870 (n=38)	1.593 ± 1.109 (n=43)	1.019 ± 0.684 (n=24)
num pul/burst	3.995 ± 1.586 (n=38)	8.484 ± 9.604 (n=20)	5.735 ± 4.164 (n=38)	8.995 ± 5.772 (n=43)	16.586 ± 10.694 (n=24)
pulse duration	0.031 ± 0.009 (n=38)	0.027 ± 0.005 (n=20)	0.031 ± 0.009 (n=38)	0.025 ± 0.008 (n=43)	0.033 ± 0.008 (n=24)
pulse interval	0.030 ± 0.009 (n=38)	0.026 ± 0.005 (n=20)	0.031 ± 0.009 (n=38)	0.029 ± 0.031 (n=43)	0.033 ± 0.008 (n=24)
pulse rate	21.044 ± 6.294 (n=38)	23.164 ± 6.738 (n=20)	20.089 ± 5.289 (n=38)	28.236 ± 30.438 (n=43)	16.610 ± 3.365 (n=24)
avg num single pulses	6.570 ± 6.181 (n=42)	5.440 ± 4.096 (n=9)	5.300 ± 7.093 (n=23)	5.150 ± 4.849 (n=27)	1.600 ± 0.894 (n=5)
single pulse duration	0.035 ± 0.008 (n=42)	0.028 ± 0.008 (n=9)	0.032 ± 0.009 (n=23)	0.028 ± 0.010 (n=27)	0.036 ± 0.008 (n=5)
single pulse interval	0.338 ± 0.297 (n=39)	0.191 ± 0.155 (n=9)	0.509 ± 1.121 (n=23)	0.288 ± 0.265 (n=27)	0.403 ± 0.344 (n=5)

APPENDIX B. CONTINUED.

Crooked Creek

Parameters	Contexts: SA	MFC	MMC	LD	Courtship
knock dom freq	107.383 ± 54.698 (n=6)	90.883 ± 40.084 (n=35)	89.129 ± 48.686 (n=21)	83.041 ± 40.665 (n=27)	113.689 ± 58.055 (n=13)
knock sec freq	250.000 ± 116.269 (n=6)	292.611 ± 109.017 (n=35)	331.938 ± 147.632 (n=21)	262.844 ± 79.478 (n=27)	243.177 ± 62.881 (n=13)
short knock dom freq	83.100 (n=1)	69.021 ± 38.868 (n=14)	78.157 ± 21.761 (n=14)	74.000 ± 33.382 (n=12)	63.167 ± 21.415 (n=3)
short knock sec freq	245.300 (n=1)	232.186 ± 78.348 (n=14)	276.179 ± 103.032 (n=14)	231.100 ± 48.189 (n=12)	176.500 ± 49.830 (n=3)
burst dom freq	52.519 ± 13.241 (n=16)	51.313 ± 12.624 (n=16)	54.442 ± 18.267 (n=18)	54.595 ± 15.560 (n=92)	67.452 ± 19.808 (n=40)
burst sec freq	202.619 ± 73.191 (n=16)	178.047 ± 49.979 (n=16)	225.006 ± 63.619 (n=18)	212.077 ± 64.078 (n=92)	231.298 ± 54.387 (n=40)
total call duration	2.459 ± 2.889 (n=20)	1.780 ± 2.515 (n=74)	2.713 ± 3.244 (n=76)	5.142 ± 3.794 (n=110)	6.668 ± 5.585 (n=50)
avg num knocks	1.170 ± 0.408 (n=6)	1.540 ± 0.822 (n=39)	2.070 ± 1.624 (n=29)	1.870 ± 0.957 (n=31)	1.400 ± 0.507 (n=15)
knock duration	0.094 ± 0.004 (n=6)	0.115 ± 0.037 (n=39)	0.113 ± 0.030 (n=29)	0.111 ± 0.020 (n=31)	0.115 ± 0.018 (n=15)
knock interval	0.474 ± 0.346 (n=4)	0.939 ± 0.796 (n=19)	0.704 ± 0.755 (n=21)	0.909 ± 0.967 (n=25)	1.488 ± 1.671 (n=11)
avg num short knocks	1.000 (n=1)	1.470 ± 0.730 (n=30)	2.140 ± 1.865 (n=35)	2.190 ± 1.388 (n=27)	1.330 ± 0.577 (n=3)
short knock duration	0.071 (n=1)	0.0612 ± 0.014 (n=30)	0.064 ± 0.014 (n=35)	0.057 ± 0.014 (n=27)	0.058 ± 0.007 (n=3)
short knock interval	---	0.691 ± 0.750 (n=17)	0.726 ± 0.778 (n=19)	0.380 ± 0.566 (n=25)	0.157 ± 0.045 (n=2)
num bursts	6.880 ± 5.875 (n=16)	4.000 ± 3.830 (n=22)	6.440 ± 3.568 (n=18)	9.330 ± 6.595 (n=100)	9.260 ± 6.729 (n=47)
burst duration	0.198 ± 0.146 (n=16)	0.219 ± 0.140 (n=22)	0.350 ± 0.205 (n=18)	0.229 ± 0.139 (n=100)	0.291 ± 0.133 (n=47)

Appendix B. Continued.

Parameters	SA	MFC	MMC	LD	Courtship
burst interval	0.095 ± 0.074 (n=16)	0.723 ± 1.350 (n=19)	0.221 ± 0.200 (n=18)	0.123 ± 0.139 (n=98)	0.276 ± 0.214 (n=47)
burst rate	2.609 ± 1.645 (n=16)	1.389 ± 1.297 (n=22)	1.740 ± 0.946 (n=18)	2.006 ± 1.017 (n=100)	1.561 ± 1.039 (n=47)
num pul/burst	4.744 ± 3.190 (n=16)	4.756 ± 2.869 (n=22)	11.355 ± 8.347 (n=18)	6.107 ± 3.763 (n=100)	5.179 ± 2.037 (n=47)
pulse duration	0.024 ± 0.002 (n=16)	0.027 ± 0.006 (n=22)	0.019 ± 0.005 (n=18)	0.022 ± 0.004 (n=100)	0.032 ± 0.005 (n=47)
pulse interval	0.023 ± 0.002 (n=16)	0.026 ± 0.006 (n=22)	0.018 ± 0.004 (n=18)	0.023 ± 0.017 (n=100)	0.031 ± 0.005 (n=47)
pulse rate	25.427 ± 3.655 (n=16)	22.646 ± 5.122 (n=22)	31.158 ± 7.530 (n=18)	27.461 ± 6.209 (n=100)	18.607 ± 3.343 (n=47)
avg num single pulses	5.800 ± 3.121 (n=15)	3.080 ± 2.515 (n=25)	4.800 ± 6.340 (n=5)	11.160 ± 9.210 (n=94)	6.500 ± 6.873 (n=46)
single pulse duration	0.022 ± 0.003 (n=15)	0.029 ± 0.006 (n=25)	0.027 ± 0.005 (n=5)	0.022 ± 0.004 (n=94)	0.033 ± 0.005 (n=46)
single pulse interval	0.104 ± 0.084 (n=15)	0.365 ± 0.258 (n=19)	0.145 ± 0.083 (n=5)	0.201 ± 0.187 (n=94)	0.246 ± 0.316 (n=45)

APPENDIX B. CONTINUED.

Piney Fork

	Contexts:				
Parameters	SA	MFC	MMC	LD	Courtship
knock dom freq	64.965 ± 23.051 (n=20)	87.175 ± 30.139 (n=14)	86.977 ± 37.178 (n=22)	108.675 ± 21.030 (n=8)	53.320 ± 11.175 (n=10)
knock sec freq	273.530 ± 50.631 (n=20)	286.757 ± 77.914 (n=14)	249.032 ± 67.390 (n=22)	282.850 ± 78.521 (n=8)	218.400 ± 68.680 (n=10)
short knock dom freq	83.053 ± 23.922 (n=19)	70.238 ± 22.621 (n=17)	85.196 ± 27.326 (n=12)	85.924 ± 27.532 (n=23)	88.660 ± 28.956 (n=5)
short knock sec freq	262.695 ± 46.263 (n=19)	272.803 ± 60.455 (n=17)	238.763 ± 53.684 (n=12)	242.002 ± 64.109 (n=23)	248.560 ± 15.835 (n=5)
burst dom freq	58.600 ± 24.951 (n=3)	54.973 ± 12.426 (n=11)	51.463 ± 11.762 (n=8)	57.680 ± 21.273 (n=27)	74.568 ± 22.031 (n=26)
burst sec freq	216.200 ± 75.359 (n=3)	210.400 ± 73.103 (n=11)	219.900 ± 40.673 (n=8)	204.917 ± 54.844 (n=27)	277.150 ± 63.392 (n=26)
total call duration	1.525 ± 1.912 (n=46)	2.211 ± 2.730 (n=40)	3.047 ± 3.180 (n=48)	7.753 ± 9.708 (n=56)	5.999 ± 4.930 (n=37)
avg num knocks	1.670 ± 1.049 (n=24)	1.840 ± 1.068 (n=19)	2.340 ± 1.589 (n=35)	2.230 ± 1.641 (n=13)	1.860 ± 0.949 (n=14)
knock duration	0.154 ± 0.045 (n=24)	0.154 ± 0.314 (n=19)	0.151 ± 0.045 (n=35)	0.158 ± 0.042 (n=13)	0.146 ± 0.034 (n=14)
knock interval	0.811 ± 0.881 (n=13)	1.159 ± 0.795 (n=11)	0.962 ± 1.132 (n=23)	0.525 ± 0.359 (n=9)	0.800 ± 0.838 (n=14)
avg num short knocks	2.570 ± 2.316 (n=28)	3.330 ± 3.293 (n=27)	3.810 ± 2.379 (n=21)	7.420 ± 8.599 (n=33)	2.250 ± 1.545 (n=12)
short knock duration	0.070 ± 0.010 (n=28)	0.077 ± 0.011 (n=27)	0.074 ± 0.013 (n=21)	0.071 ± 0.009 (n=33)	0.078 ± 0.006 (n=12)
short knock interval	0.454 ± 0.400 (n=20)	0.550 ± 0.459 (n=25)	0.567 ± 0.646 (n=18)	0.376 ± 0.339 (n=30)	0.608 ± 0.803 (n=9)
num bursts	2.000 ± 1.225 (n=5)	2.750 ± 1.753 (n=8)	5.500 ± 7.251 (n=14)	11.840 ± 14.038 (n=31)	8.390 ± 7.289 (n=28)
burst duration	0.325 ± 0.182 (n=5)	0.564 ± 0.289 (n=8)	0.299 ± 0.199 (n=14)	0.373 ± 0.201 (n=31)	0.419 ± 0.280 (n=28)

APPENDIX B. CONTINUED.

Parameters	SA	MFC	MMC	LD	Courtship
burst interval	0.212 ± 0.167 (n=5)	0.691 ± 1.030 (n=7)	0.284 ± 0.290 (n=14)	0.150 ± 0.099 (n=30)	0.434 ± 0.482 (n=27)
burst rate	0.825 ± 0.181 (n=5)	0.504 ± 0.217 (n=8)	1.100 ± 0.714 (n=14)	1.186 ± 0.878 (n=31)	1.204 ± 0.919 (n=28)
num pul/burst	4.000 ± 2.151 (n=5)	6.298 ± 2.300 (n=8)	4.438 ± 1.972 (n=14)	5.530 ± 2.911 (n=31)	4.905 ± 2.350 (n=28)
pulse duration	0.047 ± 0.018 (n=5)	0.054 ± 0.014 (n=8)	0.035 ± 0.012 (n=14)	0.040 ± 0.010 (n=31)	0.050 ± 0.023 (n=28)
pulse interval	0.048 ± 0.020 (n=5)	0.054 ± 0.013 (n=8)	0.038 ± 0.019 (n=14)	0.039 ± 0.009 (n=31)	0.049 ± 0.021 (n=28)
pulse rate	14.591 ± 7.581 (n=5)	21.953 ± 32.193 (n=8)	18.144 ± 6.668 (n=14)	15.682 ± 4.226 (n=31)	13.089 ± 3.915 (n=28)
avg num single pulses	5.830 ± 2.714 (n=6)	3.000 ± 2.608 (n=6)	7.360 ± 5.358 (n=14)	13.120 ± 13.232 (n=34)	10.040 ± 9.062 (n=24)
single pulse duration	0.045 ± 0.011 (n=6)	0.046 ± 0.008 (n=6)	0.035 ± 0.008 (n=14)	0.042 ± 0.009 (n=34)	0.048 ± 0.015 (n=24)
single pulse interval	0.236 ± 0.228 (n=5)	0.150 ± 0.084 (n=6)	0.297 ± 0.484 (n=14)	0.238 ± 0.246 (n=33)	0.317 ± 0.477 (n=23)