Acoustic communication, Reproduction and Hormone Modulation in the *Catonotus* darters

by

Patricia Speares Noel

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Approved by

Dr. Carol Johnston, Chair, Associate Professor of Fisheries and Allied Aquaculture
Dr. Geoff Hill, Associate Professor of Biological Sciences
Dr. Mary Mendonça, Associate Professor of Biological Sciences
Dr. Lawrence Page, Associate Professor of Zoology

Abstract

Acoustic communication is an essential part of life for a variety of living beings. Acoustic communication is known to be essential to reproduction in mammals, birds, amphibians, insects and fish. Recently there have been many papers published that bring to light the extensive acoustic repertoire of freshwater fishes. However, studies of how this method of communication is used with the environment and the details of the physiological responses to this communication modality are somewhat less common. This dissertation will attempt to answer these questions using two species of darters in the sub-genus *Catonotus*. This will be the first documentation of vocalizations in *Etheostoma neopterum* and the first to make a direct comparison of the temporal and spectral components of vocalizations recorded in the laboratory and those recorded in the field. In addition, the hearing sensitivities of these fish and the acoustics of their freshwater stream environment will be examined. This dissertation will also be the first documentation of allopaternal care and the costs of reproduction in male *E. neopterum*. Finally this dissertation will examine the physiological endocrine response of male and female *E. crossopterum* to playbacks of male and female vocalizations. This will be done by using a non-invasive protocol which isolates free estradiol, testosterone, cortisol and 11-ketotestoerone from the holding water of fishes. These collections will be done before and after exposure to conspecific courtship or aggressive vocalizations. These investigates will help to form a clearer picture of how acoustics are used in concert with reproductive behaviors in *Catonotus* darters.

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Chapter 1: Introduction

Acoustic communication is a modality that is common in many different taxonomic groups including fishes (Ladich 2004; Amorim 2006). Recently there have been many studies documenting acoustic vocalizations made by freshwater fishes (Ladich 1989; Lugli et al 1995; Johnston and Vives 2003; Johnston and Phillips 2003; Phillips and Johnston 2008; Kierl and Johnston 2010). However, far fewer studies looked at other aspects of acoustic communication. How these signals travel in their environment, the hearing abilities of the organism and how these signals are interpreted into physiological responses are all essential concepts when examining sound production and reproductive behaviors.

Knowing the physiological response of individuals to social signals, such as acoustic communication, can help researchers determine the ultimate function of the vocalizations. It is well known that environmental cues, such as photoperiod, help individual organisms time reproduction to the appropriate season. This timing allows these organisms to be able to ensure that conditions, such as food availability and temperature, will be adequate for both the organism and its offspring. Social cues can also ensure that individuals become reproductively active in the presence of other conspecifics, thus ensuring synchronization for reproduction. Examples of social cues include chemical signaling, which is common in fish and well studied in goldfish (*Carassius auratus*) (Kobayashi 2002). Females of this species release urinary prostaglandins to signal readiness to spawn. Females can increase the frequency with which they release this signal in the presence of a receptive male and males have been shown to be attracted to this signal (Applet and Sorensen 2007). Urinary output of the hormone 11-ketotestosterone has also been shown to increase in 5 species of African cichlids, as a result of the presence of another male (Hirschenhauser et al. 2008). The visual presence of another male has been shown to be enough to cause a change in steroid hormone levels in the Siamese fighting fish (*Betta splendens*) (Dzieweczynski et al 2006).

Acoustic communication has been well documented to act as a social stimulus and cause hormone modulation. Exposure to male vocalizations has been shown to cause elevations in hormone levels in frogs (Burmeister and Wilczynski 2000; Lynch and Wilczynski 2006) birds, (Cheng et al 1998; Maney et al 2007) and fish (Remage-Healey and Bass 2005; Remage-Healey et al 2006).

Studies have implicated the hypothalamic-pituitary-gonadal axis (HPG) as being responsible for regulating the physiological response to social stimuli such as acoustic vocalizations (Balthazart 2007). In particular, neurons containing gonadotropin-releasing hormone (GnRH) in the hypothalamus have been shown to be responsive to social stimulus. A primary example of this is the African cichlid (*Haplochromis burtoni*), which exhibits size variation in the neurons located in the preoptic area (POA) of the hypothalamus (Greenwood and Fernald 2004). Territorial males exhibit larger neurons than non-territorial males. Researchers were able cause a change in the soma size of these neurons, simply by rearranging social groups, making non-territorial males become territorial and vice versa. Males that exhibited an increase in neuron size also exhibited an increase in gonad somatic index (GSI) (Francis et al 1993). It is believed that the GnRH within in these neurons is released, which causes the release of LH, which in turn regulates sex hormones and gonad growth.

Similar mechanisms have also been implicated in regulation of the hormonal response to conspecific vocalizations. One study found an increase in the number of GnRHir neurons and an increase in androgens in male green treefrogs (*Hyla cinerea*) exposed to conspecific male choruses (Burmeister and Wilczynski 2005). Single cell recordings in the ring dove (*Streptopelia risoria*) found that neurons in the POA and the anterior hypothalamus responded to female ‘coo’ vocalizations. This study also found both excitatory and inhibitory units that responded to the vocalizations. These recordings also coincided with a change in LH concentration taken from the pituitary veins (Cheng et al 1998).

Changes in secretion of LH result in changes of sex hormones such as estradiol, testosterone, progesterone and, in fishes, 11-ketotestosterone. Many studies looking at the response of the endocrine system to social signaling find these hormones are modulated by exposure to these signals (Oliveira et al 1996; Burmeister and Wilczynski 2000).

Darters of the subgenus *Catonotus* represent a system for which acoustic vocalizations have been documented (Johnston and Johnson 2000; Speares et al 2010; Speares and Johnston 2011), however the use of these signals as social cues has never been investigated. Furthermore, although there have been studies of reproductive behaviors (Page 1985) in some of these species, there are still many things that are unknown for many of the individuals of this group. Therefore the fringe darter (*Etheostoma crossopterum*) and the lollypop darter (*Etheostoma neopterum*) from within this subgenus were selected to be the subjects of this dissertation.

Male *Catonotus* darters set up nests under flat rocks and court females to lay eggs on the flat ceiling of their shelter. The females lay their eggs in a single layer and multiple females may spawn in a single nest. Once females have laid their eggs, the male is left to guard the eggs until they hatch. Egg guarding may be energetically costly as males will vigorously defend their nests and eggs (Knouft and Page 2004), they will also care for eggs by cleaning (Knouft et al 2003) and fanning them. Behaviors exhibited by other freshwater species, such as allopaternal care and filial cannibalism, have been shown to lessen the energetic demand of nest guarding in males. The reproductive ecology of the darters such as *E. neopterum* makes this species a prime candidate for these behaviors but to date it is unknown if these behaviors occur in this species.

Males in the subgenus *Catonotus* are the only group in the genus *Etheostoma* which have been shown to be vocal. Darters produce low frequency (less than 200 Hz) vocalizations, which form three distinct call types: knocks, purrs and drums (Johnston and Johnson 2000). All three of these vocalizations are used in aggressive and courtship contexts. Previous studies have shown that shallow water, which is the preferred habitat of the darters in this study, is not a favorable environment to using low frequency vocalizations. Studies involving freshwater gobies who produce similar vocalizations to darters (Lugli and Fine 2003; 2007) as well as theoretical calculations (Mann 2006) show that these signals should only be able to propagate across small distances, if at all. The ambient noise within a stream environment can also be extremely variable according to microhabitat.

This dissertation will provide a more detailed understanding of reproductive behaviors in *Catonotus* darters, by examining reproductive behaviors, sound production and the physiological response of these darters to conspecific vocalizations. The objectives of this dissertation are to 1.) Document the use of acoustic signaling in the lollypop darter, *E. neopterum* and determine how these signals are used for communication in their natural environment. 2.) Assess the effect of male traits, such as condition and size, on female reproductive choice and determine if there are possible costs of these traits 3.) Document the effect of inter- nest distance nest selection, reproductive behaviors and conspecific interactions in a natural field environment. 4.) Investigate the hormonal response of male and female darters to playbacks of male aggressive and courtship sounds.

The first part of this dissertation will be devoted to describing the acoustic vocalizations of *E. neopterum*. Although vocalizations have been documented in other closely related species of darters, this will be the first documentation of sound production in this species. This chapter will be the first to compare spectral and temporal characteristics of darter vocalizations recorded in the laboratory with those recorded in a stream environment. Measurements of sound propagation and ambient noise recordings will also be conducted in a stream with a naturally occurring population of *E. neopterum.* These measurements will assess how these signals are being used in this natural environment. For example, if propagation distance is limited, as predicted, then this indicates that these vocalizations are most likely not used as advertisement signals and function as short distance signals. The auditory brainstem response (ABR) technique will also be used to describe the hearing sensitivities of *E. neopterum,* this will be the first investigation of this type in darters.

The next two chapters of this dissertation are devoted to studies on the reproductive behavior of *Etheostoma neopterum* conducted in the field environment. Since male darters guard nests and provide parental care to eggs, females may chose males to spawn with based on traits that would make the most of their reproductive investment. For example in the Johnny darter (*Etheostoma nigrum*) females chose males that aggressively courted them because these males would also aggressively defend eggs (Grant and Colgan 1983). This chapter will investigate the effect of male standard length and condition on reproductive success in *E. neopterum* using artificial nest tiles. The reproductive costs associated with these male traits will also be discussed here.

The second field chapter investigates the effect of inter-nest distance on nest selection and possible reproductive behaviors such as allopaternal care and filial cannibalism. These behaviors have been seen in closely related species of darters (Constanz 1985; Bandoli 2002; Stiver and Alonzo 2010) but this would be the first documentation of these behaviors in *E. neopterum.* These behaviors are suspected to allow males to improve their overall reproductive success. Investigating the effect of inter-nest distance on nest choice, is also particularly relevant to this species, because it is currently unknown if *Catonotus* darters nest within the propagation distance of conspecific vocalizations.

The final chapter of this dissertation investigates the possibility of acoustic vocalizations in darters serving as social cues to promote hormone modulation in both male and female darters. If darter vocalizations have a short propagation distance, as suggested by theoretical studies (Mann 2006), then this system may represent a novel example of acoustic vocalizations serving as social cues. The majority of research on hormone modulation caused by exposure to vocalizations involves signals that can be heard for long distances or are used as advertisement signals. If the function of darter vocalizations is for short distance communication, then these vocalizations could cause a different hormonal response than was shown in previous studies (Remage-Healey and Bass 2005).

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Chapter 2

Sound production, hearing and the acoustic environment of *Etheostoma neopterum*, the lollypop darter

In order for signals to be successful they must be able to be sent and clearly received by individuals. The successful reception of acoustic signals in aquatic environments can depend greatly on the environment in which they are sent, but also on the physiological abilities of the organism. Male darters in the sub-genus *Catonotus* have been shown to produce vocalizations during aggressive and courtship interactions. However, these vocalizations are low frequency (below 200 Hz) and therefore should not propagate in their natural shallow water environment. This has led to questions about the function of these signals. The hearing ability of these darters is also currently uninvestigated. This paper will be the first to compare darter vocalizations recorded from both the laboratory and the field. This paper also investigates the propagation distance of these sounds in their natural habitat as well as the ambient noise spectra near naturally-occurring nests. Finally, this paper will investigate the hearing abilities of *E. neopterum* using the auditory brainstem response (ABR) technique. These data will help form a clearer picture on how this fish uses acoustic signaling as well as set up methodology for future vocalization recordings and studies.

Introduction

Acoustic communication is widely used in many organisms including fishes (for a review see Ladich 2004; Amorim 2006). In recent years there have been many studies documenting sound production in freshwater fishes (Ladich 1988; Lugli et al 1995; Johnston and Vives 2003; Johnston and Buchanan 2007), however, there are still many species whose acoustic abilities are unknown. Even less is known about how these signals are used in their natural environment.

Acoustic signals can be constrained by the physiological abilities of the intended receiver and the environmental conditions around them. Acoustic signals in aquatic environments face larger constraints than those used in terrestrial environments (Forest et al 1993). Shallow (1-2 m) freshwater streams have been found to foster limited propagation for low frequency vocalizations (Mann 2006; Lugli and Fine 2003; 2007). In addition, shallow streams may have high levels of ambient noise due to the presence of small waterfalls and riffles. The hearing ability of the fish within the stream may also be a physiological constraint for these signals. Fish may have hearing abilities that are most sensitive to the dominant frequency of the environment around them or have hearing sensitivities that match the dominant frequency of their own vocalizations (Ladich and Yan 1998; Johnston et al. 2008; Holt and Johnston 2011). The hearing ability of fishes, therefore, may hinder the detection of conspecific signaling through masking or it may enhance detection of these signals.

Etheostoma neopterum is a small benthic species of fish that is found in a limited distribution in northern Alabama and Tennessee. Although closely related species of darters have been documented to produce low frequency vocalizations (Johnston and Johnson 2000), the vocal abilities of *E. neopterum* are undocumented. There has also never been an investigation into the hearing abilities of any darters in the sub genus *Catonotus*. These fish are found in shallow water (less than 1 m) where propagation of acoustic communication signals should be extremely limited (Mann 2006).

Although there have been many studies to date on the acoustic vocalizations of darters, all have taken place in the lab environment. Theoretically, the acoustic signals of the *Catonotus* darters recorded in the lab should maintain spectral and temporal integrity if recorded close to the sounds source (Akamatsu et al 2002). However, sounds recorded in the laboratory have never been directly compared to sounds recorded in the fish's natural environment.

This objective of this study is to determine if *E. neopterum* produces acoustic vocalizations and if there are any differences in the spectral and temporal characteristics of these vocalizations when they are recorded in the laboratory or the field environment. In addition, this study uses the auditory brainstem response technique (ABR) to determine if *E. neopterum* can detect frequencies that are representative of conspecific vocalizations. Ambient noise recordings and the measurement of the propagation distance of these sounds will also be used to gain a better understanding of how these vocalizations are used in their natural environment.

Methods

Field Site Locality and Fish Collection

All fish were collected from the Last Butler Creek system in Wayne County Tennessee (35.07097, -87.645724). All field recordings were done at this location, downstream from the collection site. Collections and field recordings were never done in the same part of the stream. At this site there was a variety of substrates; however, most *E. neopterum* males were found in areas with little to no flow that had a silty substrate with large rocks.

Collections took place between the months of March-May in 2009-2011. Fish were caught using a 10 foot kick seine net. Both males and females were collected and placed into coolers and transported to the Fish Biodiversity laboratory at Auburn University which was about 5 hours away. The coolers were filled with water from the creek and stress-coat water treatment was added. After arriving at the laboratory, fish were allowed to acclimate to the temperature of the laboratory.

The fish were moved to the holding tanks once the water in the coolers, warmed to within 3 degrees (3-12 hours) of the water in the holding tanks. Males were identified by their larger size and the presence of egg mimics on their dorsal fin. Large males were housed alone and smaller males were held with one other male. Females were separated into visibly gravid and non-gravid females. These two groups were housed separately with 3-5 females per tank.

Holding tanks were 76 L glass aquaria which contained small gravel as substrate, carbon filters and an air-stone. These tanks contained 1-3 shelters composed of either natural rocks taken from the field site or ceramic tiles. The shelters were propped up at one end to allow fish to enter and exit easily. Fish were fed frozen blood worms daily at approximately 9 am. The temperature of the tanks was kept between 19 – 20°C and these fish were exposed to natural light through windows. Fish remained in the same tank until they were used for recording trials or auditory brainstem response (ABR) testing. Once fish were used for one of these trials they were placed into a different tank. Some fish that were used for vocalization trials were also used for hearing trials, however, once fish were used for hearing trials they were not used for vocalization trials.

Field Behavior and Vocalization Recordings

Field recordings took place March –May of 2010 and 2011. Field behaviors and vocalizations were monitored and recorded using both video and audio recording methodologies. A black and white underwater camera that fed into a monitor located on the stream bank (Aqua-Vu ZT 60, Nature Vision Inc., Brainerd, MN) was used to visually monitor and record behaviors. Videos were recorded and saved in AVI format and were later viewed and analyzed using Windows Media Player Software. These videos allowed the observer to distinguish between aggressive and courtship vocalizations. Notes on behaviors and interactions were taken by the observer in real time, and these observations were later supplemented with video analysis. All sounds produced in concert with behaviors directed at other males were considered to be aggressive and sounds directed towards females were considered to be courtship vocalizations. For this study vocalizations made during both courtship and spawning were considered courtship vocalizations.

Equipment used to record vocalizations in the field was also used for all other field recordings. Sounds were recorded using an HTI hydrophone (Hi-Tech, HTI-96-

MIN; sensitivity: 164.4 re. 1 v/µPa) connected to a Marantz handheld recorder (PMD661 Handheld SD Recorder). Headphones connected to the Marantz recorder allowed the observer to acoustically monitor these recordings. Sounds were recorded in wave format files (.wav) which were exactly 1 minute in duration. The recording equipment was set to automatically assign a consecutive number to each file so that they could be precisely matched to the clock located on the video files. These signals were later downloaded onto an HP laptop and analyzed using Raven Pro 1.3 software (Cornell University, Ithaca, New York).

All field recordings were done at nests where males were already present and actively guarding eggs. The recording equipment was placed so that the nest was slightly propped up by the camera and the hydrophone was then placed beside the camera. If the male swam away from his nest while the equipment was being placed, the observers waited for the male to return (usually within 10 minutes) and then began recording.

While waiting for the male to acclimate to the recording equipment, gravid females and males were collected from downstream of the recording site and placed into separate 5 gallon buckets with an air-stone. After the subject male resumed normal activity, (i.e. egg fanning, looking outside nest for intruders) a single male or female was placed in front of the nest. This was done to facilitate sound production and interactions with the subject male. Recording continued for one hour, if the interactions lasted that long. However, if the non-resident fish immediately swam away from the nest, or if 20 minutes passed and no interactions occurred, then another male or female was introduced.

Vocalization analysis

Identical analysis was preformed for vocalizations recorded in the field and in the laboratory. All analysis was done using Raven Pro 1.3. These vocalizations were classified as drums, knocks, and purrs similar to what has been documented in other species of *Catonotus* darters (Johnston and Johnson 2000). Knocks were classified as broadband signals that contained only one pulse. The duration and dominant frequency of these signals were recorded. Purrs were identified as broadband signals that contained more than one pulse in rapid succession (Fig. 2-1). Total duration and dominant frequency were also recorded for these signals. In addition, the duration of each pulse and the average inter-pulse duration of each pulse were recorded. These characteristics were averaged per call and reported as the average pulse duration and average inter-pulse interval for each purr vocalization. The number of pulses in each call was not used in the analysis because it was found to be correlated with the total duration of the purrs. The last vocalization type observed was a drum. These were tonal vocalizations that contained multiple harmonic bands. The total duration along with the number of inflection points, and the fundamental frequency were recorded for each drum vocalization (Fig. 2-2). An inflection point was determined to be when the slope of the call changed direction as observed on the spectrogram (i.e. when the call appeared to go from an ‘up sweep’ to a ‘down sweep’) (Hanning window, FFT 1981, bandwidth 32 Hz). All durations were calculated using waveforms. Frequencies were determined using power spectrums (Drums and purrs: Hanning Window, FFT 10000, bandwidth 6.34; knocks: Hanning Window, FFT 1000, bandwidth 6.34 Hz). All of the characteristics above were reported as the mean characteristic per male.

Propagation

Propagation distance of an *E. neopterum* vocalization was examined by playing a previously recorded vocalization through an underwater speaker (UW-30, University Sound, Oklahoma City, OK). The speaker hung so that it was completely submerged and approximately 2 cm off the bottom of the stream. A meter stick was placed on the bottom of the stream and used to measure distance from the speaker. Since *E. neopterum* nests were found in areas of the stream with both silty and small cobble substrates, propagations were measured at both substrate types. At each site measured there was a naturally occurring *E. neopterum* nest within 30 cm of the speaker placement.

A previously recorded drum vocalization was used to measure propagation. The sound level of the playback was calibrated in the laboratory using Raven Pro 1.3 and a GW GOS-6xxG dual trace oscilloscope. The vocalization was played at a pre-calculated sound pressure level of 80 dB rel 1µPa at the sound source. This is similar to sound levels recorded from other closely related species of darters (Speares et al. 2010). Recordings were made in 5 cm increments up to 70 cm from the speaker. The drum was used because the tonal structure and duration of this vocalization made it easier to distinguish from the background noise compared to the other two types of vocalizations. The propagation distance of the sound was determined to be the farthest point away from the speaker where the drum vocalization could still be identified on the waveform.

Ambient Noise

The ambient noise of the environment was evaluated using methodologies adapted from previously published work (Lugli and Fine 2003; Speares et al 2010). One minute recordings were performed at 10 artificial nest sites, which were readily used by male *E. neopterum* in another study. Three 700 ms samples were randomly chosen from each of these recordings. The power spectrum function in Raven Pro 1.3 was then used to calculate the pressure density spectra for each sample (Hanning window, 16384 FFT, 2.7 Hz). The pressure density spectra were reported in 30 Hz intervals between 0 and 1020 Hz. These values were converted from an arbitrary dB level into dB rel 1µPa using a previously calculated calibration coefficient that took into consideration the gain of both the hydrophone and Marantz recorder. A total of 30 measurements (10 sites, 3 measurements each) were averaged to find a representative pressure density spectrum for the stream habitat. Standard deviations were also calculated at each point.

Laboratory Recording Protocol

Laboratory recordings of vocalizations were done in a 76 L glass tank with a small amount of sand for substrate. These tanks contained a single shelter made from a flat rock that was propped up on one end. This tank also contained a small carbon filter and an air-stone. Both the air-stone and the filter were turned off prior to recording. In an effort to cut out as much background noise as possible the spectrogram was examined for electrical noises (approximately 60 or 120 Hz) prior to recording. If any electrical noise was found, items were unplugged if possible.

A Bruel and Kjaer 8103 hydrophone (sensitivity 27.1 µV/Pa), Brüel and Kjaer type 2635 charge amplifier was used to record vocalizations in the laboratory. The hydrophone was placed within 5 cm of the shelter. Raven Pro 1.3 was used to record the sounds. These sounds were recorded into time marked files and analyzed at a later date.

All interactions between two male darters were considered to be antagonistic interactions and all interactions between the subject male and a female were considered to be courtship interactions. All analysis of sounds was done using Raven Pro 1.3 using the identical protocol used to analyze the field vocalizations.

All recordings were started just before sunrise (around 5:30 am) between the months of March –June. A single male was placed into the recording tank the night before the recording session and allowed to acclimate to the tank environment overnight. The next morning a new male and/or gravid female was introduced into the tank. Recordings began immediately after the new fish was introduced; however, interactions usually began within 5 minutes of the introduction. If 20 minutes passed and interactions did not occur then the non-resident fish was removed and replaced by a new fish. All interactions were either observed by a researcher who took notes on the interactions of the fish, or the interactions were taped using a Sony handi-cam.

ABR recording protocol

Brainstem recordings were obtained from both males and females using a set up nearly identical to previously reported studies (Holt and Johnston 2011). A piece of PVC piping (73 cm, capped at both ends) with a hole in the top to access the fish, was used for the presentation of the signal. An underwater speaker (UW-30, University Sound Inc, Oklahoma City, USA) was submerged to a depth of 10 cm (at the center of the speaker). The subject fish was placed so that the head of the fish was submerged 11 cm underwater and was 8 cm from the center of the underwater speaker. The subject was secured to a clay-covered platform. The fish was kept in place by a cloth mesh material that was secured to the platform with dissecting pins.

The methodology used to present signals and obtain thresholds can be found in detail in Wright et al (2005), so it will only be briefly described here. Three electrodes were used to capture the physiological response of the fish to the sounds presented (Rochester Electro-Medical, Inc., Tampa FL). The recording electrode was placed on the dorsal side of the fish at the midpoint between the opercula. The reference electrode was placed in the nose area of the fish and the grounding electrode was placed into the clay.

The signal used was a pure-tone generated using hardware and software from Tucker Davis Technologies (TDT Gainesville, Florida). Tones were generated and calibrated using Sig Gen Software, and presented using BioSig Software. Tones were calibrated by using a Bruel and Kjaer 8103 hydrophone (sensitivity 27.1 µV/Pa), Brüel and Kjaer type 2635 charge amplifier, and a GW GOS-6xxG dual trace Oscilloscope. Voltage readings off of the oscilloscope were transformed into dB rel 1µPa and adjustments were made to the sounds using a normalization file to make the sound pressure reading accurate. During calibration the hydrophone was placed where the fish's head would be during the trial.

Pure tone signals were played in 5 db step intervals from 65 – 150 db. The frequencies tested were 100, 200, 300, 400, 600, 800, 1000 and 2000 Hz. Once a threshold response was observed the sounds were presented for 15 dB above threshold. The frequencies were presented from lowest to highest frequency, except for 400 Hz, which was tested at the beginning, middle and end of the experiment. This frequency was used to determine if the fish's response changed throughout the trial. If the fish moved and an electrode became disconnected, then the trial started over from the beginning. Thresholds were assessed visually as the dB level at which the evoked potential was visible above the background noise levels of the tank. This visual trace assessment is commonly used (Kenyon et al. 1998, Higgs et al. 2001) and does not differ from more-quantitative methods (Mann et al. 2001).

Particle acceleration vectors were calculated for each level of dB and frequency. This was done by measuring the pressure gradient between two identical HTI hydrophones. The hydrophones were placed exactly 3 cm apart with the midpoint between the hydrophones being the exact placement of the fish's head in the ABR tank. Simultaneous recordings were used to obtain waveforms that were then subtracted and the RMS voltage was obtained. The distance between the hydrophones and the density of freshwater (997.8 ms^{-1}) were used to calculate particle acceleration ($ms\text{-}^{2}$) from RMS voltage. These measurements were taken in the 3 orthogonal axes. The square root of the sum of the squares of each axis was found for final particle acceleration at every point.

Statistics

Statistical tests and descriptive statistics (i.e. mean, standard deviation) were calculated using IBM SPSS statistics (Version 19, IBM Corporation, Armonk, New York). Analysis of covariance was used to test for statistical differences in vocalization characteristics between laboratory and field vocalizations and also between contexts. Male standard length (SL) was used as a covariate in this analysis since SL contributes to variation in sound production in many species of fishes (De Jong et al 2007; Amorim and Neves

2008). Analysis of variance was also used to assess data from the ABR experiment. This analysis was used to determine if there was a frequency at which there was a statistically different auditory threshold. Prior to analysis all data were determined to have homogeneous variances as determined by a Levine's test and normality was assessed using a Shapiro-Wilk test.

Results

This study found that there was no statistical difference in call characteristics between the laboratory and field vocalizations. There was also no difference between the vocalizations made under courtship and aggressive contexts (Table 1-1).

Knocks recorded in the laboratory were not significantly different in dominant frequency 135.1 (± SD 20.97) or total duration 0.06 (±0.021) from knocks recorded in the field (dominant frequency=112.5±16.25; total duration=0.041±0.011) (frequency: df=1, F=3.510, p=0.088; duration: df=1, F=3.312, p=0.096). Drums recorded in the laboratory had a mean fundamental frequency of 173.3 Hz (±28.45), a mean duration of 0.47 seconds (±0.167) and on average they contained 2.10 (±1.48) inflection points. This was not statistically different from drums recorded in the field, which had a mean fundamental frequency of 156.73 Hz (±26.90) (df=1, F=2.468, p=0.137) and average duration of 0.57 seconds (±0.252) (df=1, F=0.417, p=0.528) and an average of 2.4 (±1.19) inflection points (df=1, F=3.385, p=0.086). Finally purrs recorded in the laboratory had a mean frequency of 112.3 Hz (±25.31) a mean total duration of 0.36 seconds (±0.231) and pulses with an average duration of 0.07 seconds (±0.090) and a mean inter-pulse interval of 0.06 seconds (±0.017). This was not different from the purrs recorded in the field, which had a dominant frequency of 87.7 Hz (±6.27) (df=1, F=1.531, p=0.242) a mean total duration of 0.58 seconds (±0.382) (df=1, F=2.777, p=0.124); the mean duration of these pulses was 0.03 seconds (±0.007) (df=1, F=1.678, p=0.222) and the average inter-pulse interval was 0.040 seconds (±0.014) (df=1, F=1.41, p=0.259).

Knocks made during aggressive interactions had an average duration of 0.05 seconds (±0.02) and an average dominant frequency of 124.0 Hz (±17.25). This was not significantly different from knocks made during courtship interactions, which lasted an average of 0.5 seconds (±0.22) (df=1, F=0.052, p=0.824) and had an average frequency of 126.5 Hz (±26.7) (df=1, F=0.204, p=0.660). Purrs made during aggressive and courtship contexts were not significantly different with respect to duration (courtship: 0.46 s ±0.31, aggression: 0.46 s ±0.34; df=1, F=0.028, p=0.871), dominant frequency (courtship: 102.7 Hz ± 27.16, aggression: 103.5 Hz ±22.63; F=0.001, p=0.975), average pulse duration (courtship: 0.073 s ± 0.10; aggression: 0.04 s ±0.01; df=1, F=0.029, p=0.382), and average inter-pulse interval (courtship:0.04 s ± 0.013; aggression: 0.06 s ±0.019; df=1, F=1.671, p=0.223). Drum vocalizations made in aggressive and courtship interactions were not significantly different with respect to duration (courtship: 0.45 seconds ±0.31; aggression 0.46 ±0.33; df=1, F=1.228, p=0.285), fundamental frequency (courtship: 173.5 Hz ±32.78; aggression 158.20 ±22.10; df=1, F=0.934, p=0.349) and the number of inflections points (courtship: 2.4 ±1.6, aggression: 2.0±0.94; df=1, F=1.542, p=0.233).

Propagation

The propagation of *E. neopterum* drums was limited. In both the rocky and silty substrate the sound was able to propagate 55 cm before it was undetectable from background noise in the waveform. Both sites had a similar pattern of propagation as well, with neither site appearing to be partially better or worse for retaining the sound (Fig. 2-3). In each habitat the sounds lost over 30 dB rel 1µPas of energy over 50 cm.

Ambient Noise

The overall ambient noise spectrum of the *E. neopterum* habitat was fairly quiet (Fig. 2-4). The maximum noise level was found at 30 Hz where the noise level was 80.9 dB. The noise level fell off drastically after this point. The noise level remained relatively constant after 150 Hz. The lowest sound level appeared to occur around 240 Hz, however, this value is not drastically lower than any of the surrounding values.

Auditory Brainstem Response

The results of the ABR testing revealed significant differences in the level of auditory thresholds among frequencies (df=6, F=43.567, $p<0.001$). The frequencies of maximum hearing sensitivity of *E. neopterum* were 100-300 Hz. Post hoc tests revealed that there was no difference in the mean threshold for 100, 200 and 300 Hz ($p>0.10$ for all values) however, these frequencies were significantly lower than all the other frequencies ($p<0.05$ all values). The average hearing threshold at 400 Hz was significantly higher than 100-300 Hz (100 Hz p=0.001, 200 Hz and 300Hz p=0.024), however it was also significantly lower than the mean frequencies of 600, 800 and 1000 Hz (600 Hz p=0.001, 800 and 1000 Hz $p<0.001$). There was no significant difference between the thresholds found for 600 Hz, 800 Hz and 1000 Hz (p>0.10 for all values) (Fig. 2-5). No physiological responses were found for 2000 Hz; therefore this frequency was not included in the analysis of *E. neopterum* vocalizations.

Discussion

This study provides the first evidence of sound production by male *E. neopterum.* These males made 3 distinct call types, the knock, the drum and the purr in both aggressive and courtship contexts. All three of these call types, were previously found to be made by other species of darters (Johnston and Johnson 2000, Speares and Johnston 2011).

Overall, the vocalizations recorded from *E. neopterum* males were similar to those recorded in other closely related species. The average fundamental frequency of an *E. neopterum* courtship drum in this study was 102.7 Hz. This is similar to what has been seen in other species of *Catonotus* darters, including, *E. oophylax* (99.1 Hz) (Speares and Johnston 2011) *E. nigripinne* (129.5 Hz) and *E. crossopterum* (76.6 Hz) (Johnston and Johnston 2000). This differs, however, from the fundamental frequency of the drum vocalization of *E. flabellare* which has a higher frequency (308 Hz) (Speares et al 2010). Another difference between this and previous studies is that, *E. neopterum* vocalizations did not vary between aggressive and courtship. A difference between contexts has been seen in many species of darters (Johnston and Johnson 2000; Speares and Johnston 2011).

This study found no difference in the spectral or temporal characteristics of vocalizations recorded in small a glass aquarium or in the field. This finding has major applications for aquatic bioacoustics. Recording in the laboratory setting can be preferential to recording in the field. Temperature, photoperiod and potential interactions can all be easily manipulated and standardized in the laboratory. However the acoustics of small glass tanks can be complicated and are not suitable for recording all sounds (Akamatsu et al 2002). Low frequency darter vocalizations did not appear to be significantly affected by the tank environment.

This finding that low frequency darter sounds may be recorded in the laboratory must be interpreted with caution. During this study it was imperative to cut down on background and electrical interference whenever possible. The filter and the air-stone were both turned off in the recording tank as well as any other tanks in the same room. The shape, size and material that a tank is made from will also greatly affect the resonance frequency of a tank (Akamatsu et al 2002). Therefore, researchers must also exercise caution when attempting to extrapolate these results.

Propagation of male vocalizations was found to be limited in the field. This confirms theoretical calculations of propagation distances in freshwater streams (Mann 2006). From these data we can assume that, if conspecific interactions are occurring then these fish are either exhibiting inter-nest visits, or they are living within the propagation distance of these sounds. It is likely that both of these options are occurring. Female nest visits were observed although not formally documented throughout the 2010 and 2011 field seasons. Recent studies have also found that male *E. neopterum* will nest at nesting tiles placed at distances as close as 10 cm from each other (Speares Noel and Johnston *In prep*). Since these males are nesting within the propagation distance of their own vocalizations, there is an opportunity for a communication network to occur in this species. For example, males that vocalize to nearby females could face a risk of these signals being perceived by unintended receivers such as neighboring males.

The ambient noise of a stream inhabited by *E. neopterum* was louder at lower frequencies (under 100 Hz) but the sound level fell dramatically at higher frequencies. Although there was no clear ‘quiet window’ as seen in other studies (Lugli and Fine 2003; Speares et al. 2010), the mean fundamental or dominant frequency of all three types of *E. neopterum* vocalizations fell within the quiet area of the ambient noise spectrum. There was not much high frequency ambient noise observed in this habitat. This could be due to there being little water movement and bubbles, in the low flow environment that *E neopterum* prefers. These sounds have been documented to be higher in frequency (Updegraff and Anderson 1991). This finding supports the evidence that these vocalizations can be used in this environment with little or no masking occurring from the ambient noise of the environment.

The frequencies of maximum hearing sensitivity for both pressure measurements and particle acceleration measurements were found to be 100 – 300 Hz. This range encompasses the dominant or fundamental frequency of all three vocalization types of *E. neopterum.* Although it is debated whether *E. neopterum* is able to hear the pressure component of vocalizations, these measurements were reported to be consistent with other studies. Some groups of fish have hearing specializations such as Webberian ossicles (Von Frisc 1938) that allow them to have a lower hearing threshold over a greater frequency range. To our knowledge, darters of the genus *Etheostoma* do not have any hearing specializations. The absence of such a structure is supported by the shape of the audiogram found in this study, which is similar to that of other fish with no specialized hearing structures (Amoser et al 2004; Holt and Johnston 2011).

This paper finds that the acoustic environment and the physiological abilities of *E. neopterum* enable these fish to effectively use this communication modality. Although the propagation distance of these signals may be limited, these signals may still be traveling far enough to reach other conspecific nests. The ambient noise spectrum and hearing abilities of *E. neopterum* support the use of acoustic communication. Further research should be done to examine the purpose of close range acoustic communication in this species. However, the current research has given us a clearer picture about how these signals are used in their environment.

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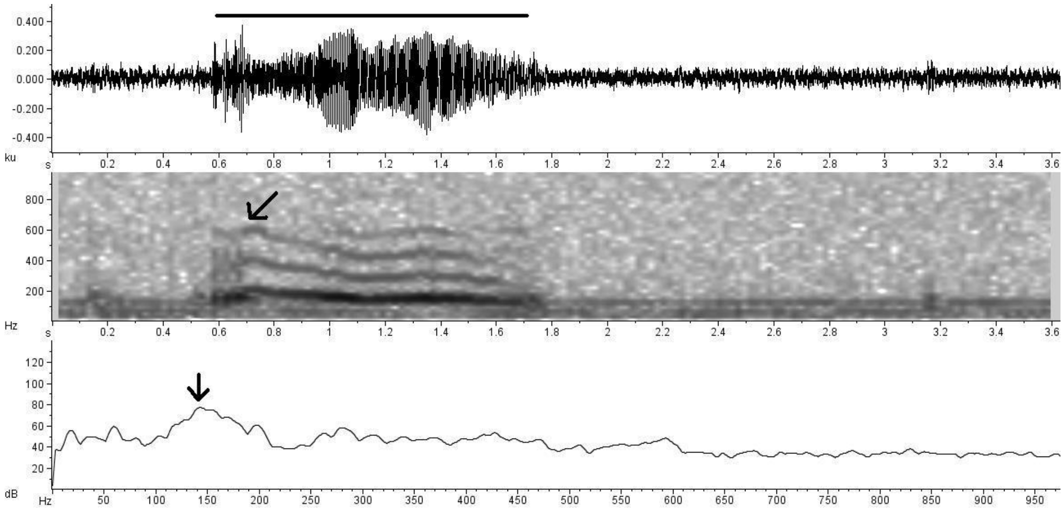

Fig. 2-1: A representative sample of a drum vocalization. The black line in the waveform indicates duration, the black arrow on the spectrogram indicated an example inflection point and the arrow on the power spectrum indicates fundamental frequency

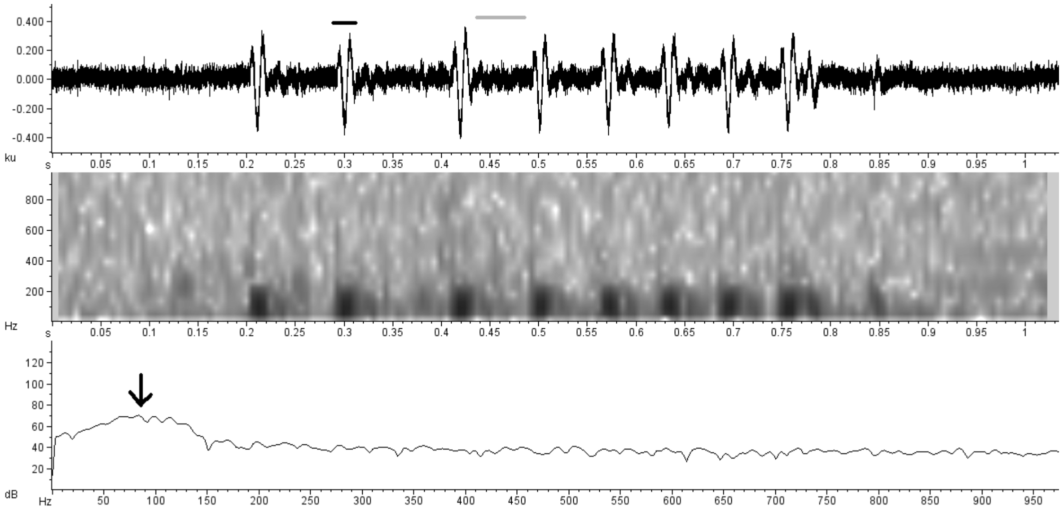

Fig 2-2: A representative sample of a purr vocalization made by a male *Etheostoma neopterum*. The black bar on the waveform indicates pulse duration, the gray bar on the waveform indicates how the inter-pulse duration was calculated. The black arrow on the power spectrum indicates the dominant frequency.

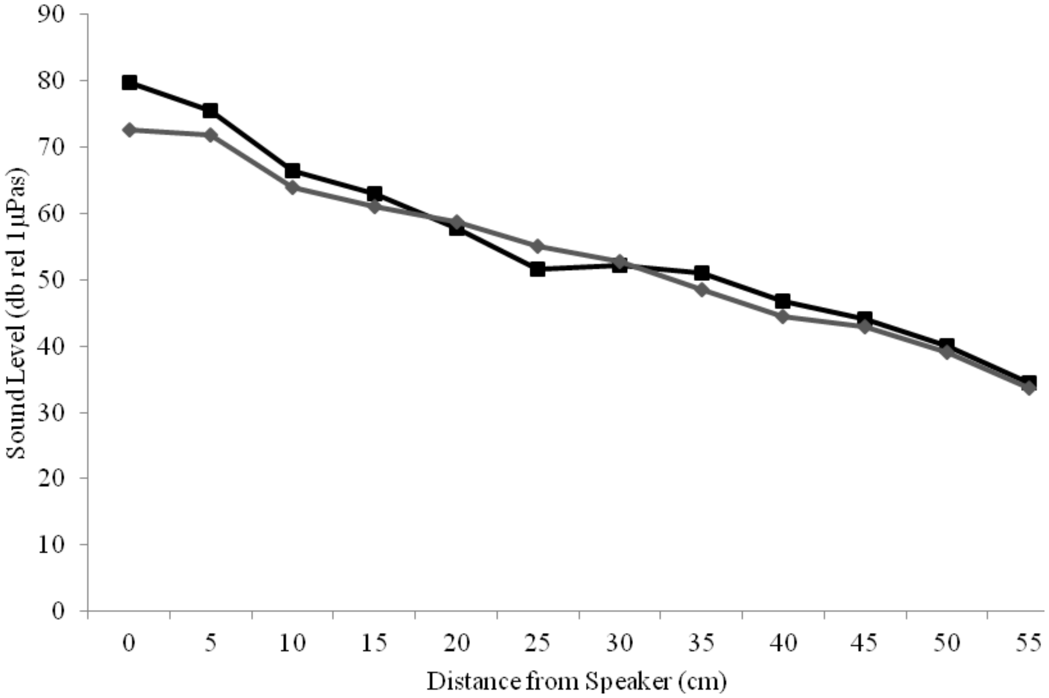

Fig 2-3: Propagation of drum vocalization of a male *Etheostoma neopterum*. The gray line represents a site with a silty substrate and the black line represents a rocky substrate. Both sites showed a dramatic drop in sound pressure level over 55 cm. Recordings were done for 65 cm however, after 55 cm the sound could no longer be detected above the background noise.

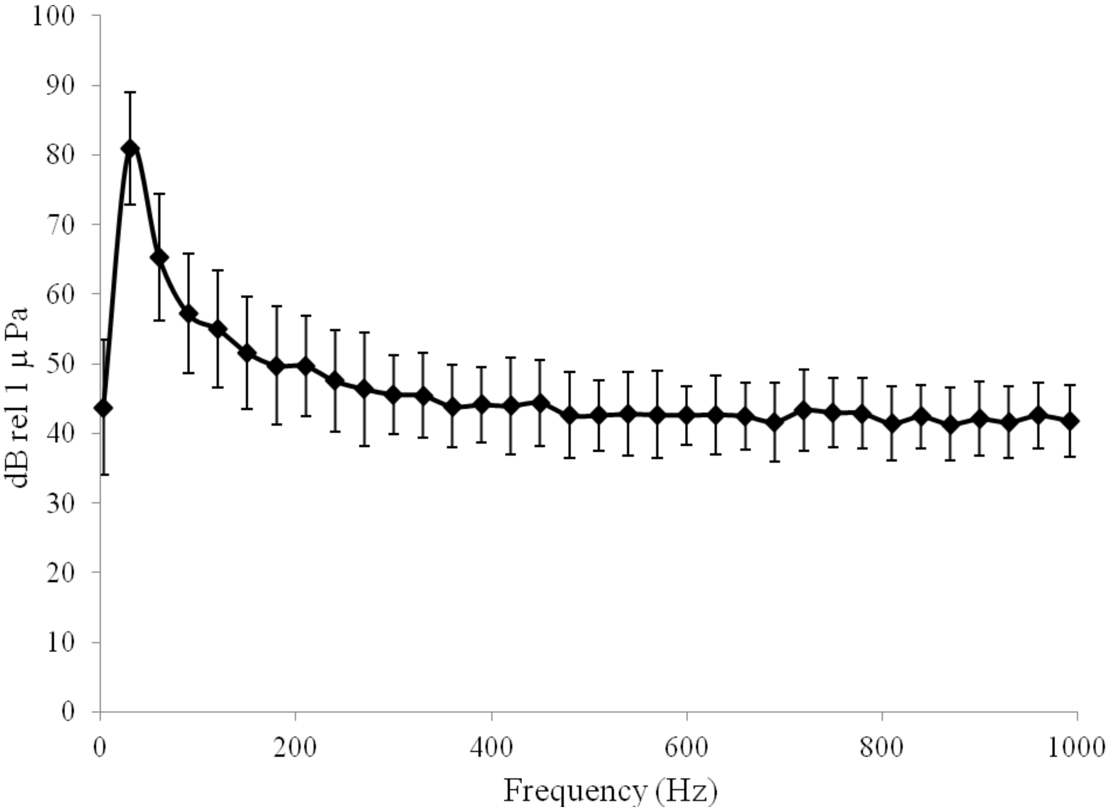

Fig. 2-4: The ambient noise spectrum of the habitat of *Etheostoma neopterum*. Ten active nest sites were averaged, bars represent the standard deviation of the noise level at each site.

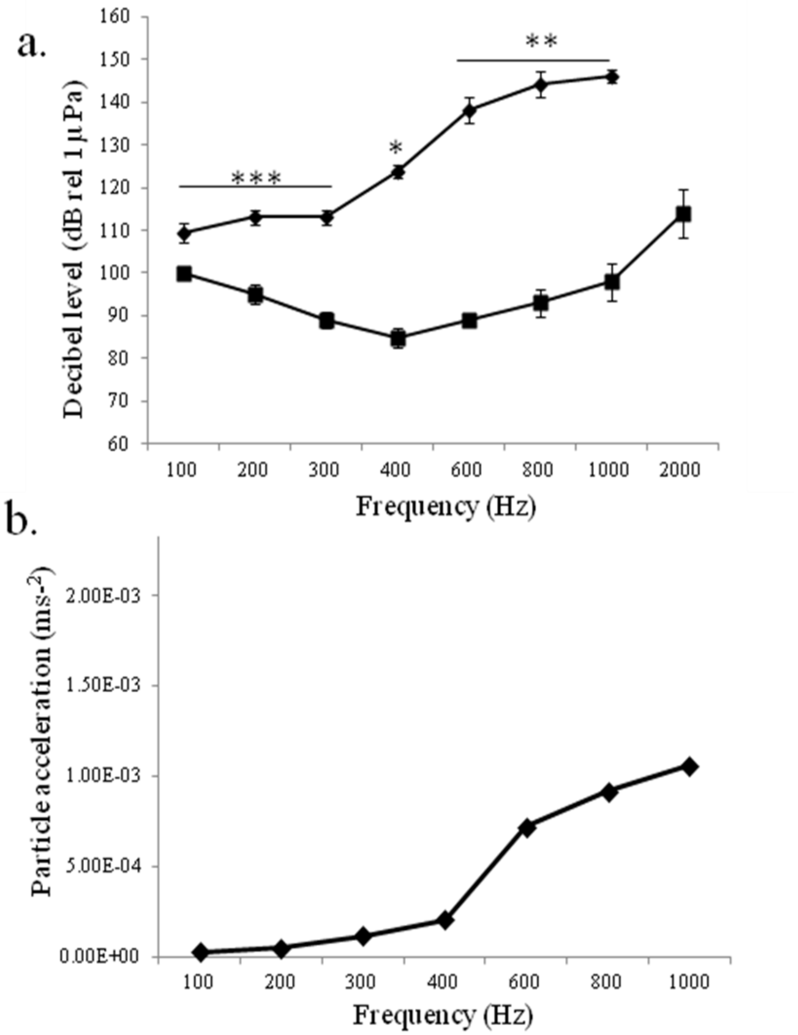

Fig. 2-5: a.) An audiogram of the hearing thresholds of *Etheostoma neopterum* (Solid diamonds) with an audiogram of a gold fish for comparison (Solid squares). *Etheostoma neopterum* has a significantly lower threshold at 100, 200 and 300 Hz. The threshold at 400 Hz was significantly higher than the lowest frequencies but lower than the higher frequencies. The thresholds at 600 Hz, 800 Hz, and 1000 Hz was significantly higher than all of the lower frequencies .**b.)** An audiogram of the particle acceleration thresholds of *E. neopterum.* Particle acceleration values were found by calculating the pressure difference between 2 hydrophones for each frequency and 5 db pressure level examined.

Table 1: Vocalization characteristics measured in different contexts and different environments. Numbers indicate mean values ± 1 standard deviation numbers in parenthesizes are the number of males sounds were collected from. F and p valuse from multivariate ANOVA

	Courtship Mean	Aggression Mean	F	P	Field Mean	Lab Mean	F	P
Purrs								
Duration (s)	0.46 ±0.314 (8)	0.46 ±0.338 (8)	0.028	0.871	0.58 ±0.382 (7)	0.36 ±0.231 (9)	2.777	0.124
Frequency (Hz)	100.2 ±30.41 (8)	104.0 ±18.86 (8)	0.001	0.975	89.1 ±17.18 (7)	112.3 ±25.31 (9)	1.531	0.242
Pulse duration(s)	0.07 ±0.098 (8)	0.04 ±0.013 (8)	0.029	0.382	0.03 ±0.006 (7)	0.08 ±0.089 (9)	1.678	0.222
Inter-pulse Interval (s)	0.04 ±0.013 (8)	0.06 ±0.019 (8)	1.671	0.223	0.04 ±0.014 (7)	0.06 ±0.017 (9)	1.418	0.259
Pulse Frequency (Hz)	102.7 ±27.16 (8)	103.5 ±22.63 (8)	0.101	0.757	87.7 ±6.27 (7)	112.3 ±25.31 (9)	3.006	0.111
Knocks								
Duration(s)	0.05 ±0.22 (8)	0.05 ±0.18 (8)	0.052	0.824	0.4 ±0.01 (7)	0.6 ±0.02 (9)	3.312	0.096
Frequency (Hz)	126.5 ±26.73 (8)	124.0 ±17.25 (8)	0.204	0.660	112.5 ±16.25 (7)	135.1 ±20.97 (9)	3.510	0.088
Drums								
Duration (s)	0.451 ±0.18 (10)	0.577 ±0.230 (10)	1.228	0.285	0.566 ±26.90 (9)	0.47 ±0.167 (11)	0.417	0.528
Frequency (Hz)	173.5 ±32.78 (10)	158.2 ±22.10 (10)	0.934	0.349	156.73 ±26.90 (9)	173.3 ±28.45 (11)	2.468	0.137
Number of points	2.48 ±1.64 (10)	19.84 ±0.942 (10)	1.542	0.233	2.40 ±1.186 (9)	2.10 ±1.480 (11)	3.385	0.086

Chapter 3

Allopaternal Care, Egg Eating and Inter-nest Distance of Nests in *Etheostoma neopterum*

Allopaternal care and filial cannibalism are two strategies that male teleost fish use to increase their overall reproductive success. It is unknown however, how inter-nest distances and population densities affect these and other conspecific interactions in a small stream environment. This field study is the first to document both of these behaviors in the Lollypop Darter, *Etheostoma neopterum.* This study also uses artificial nest tiles placed in close (10 cm apart) and far (100 cm apart) configurations in order to determine the effect of inter-nest distance on conspecific interactions. Nest distances in the field did not affect the prevalence of these two behaviors, but can have an effect on overall nest success. It was found that males do not have a preference between close and far nest sites. However, males that used tiles in the close configurations were more likely to spawn with a female, based on the presence of eggs in their shelter. Overall, there was no difference in the number tiles used or the number of successful nests in the close or far configurations.

Introduction

Many species rely on multiple modalities to assess conspecifics for both courtship and antagonistic interactions. In small stream habitats, visibility may be limited due to water movement or turbidity. Acoustic communication also may have a limited range of effectiveness in this environment due to the low distance sounds propagate in shallow waters (Mann 2006). Species that seek to use these visual or acoustic modalities for assessment may favor living in close proximity to other individuals. Small inter-nest distances however, can have positive and negative consequences, and nesting density has been a popular topic in the literature for a variety of taxonomic groups (Fishes; Goldschmidt et al. 1992; Neff et al. 2004; Anurans; Ryan et al. 1981; Birds; Best 1978; Butler and Trivelpiece 1981; Major and Kendal 1996)

The frequency of conspecific encounters is affected by inter-nest distance. This may result in both positive and negative interactions. Longear Sunfish (*Lepomis megalotis*) and Bluegill (*L. macrochirus*) are two fishes in the family Centrarchidae, in which individuals may either nest in colonies or as solitary nesters. In both of these species, there is an increase in cuckoldry when individuals are in a colony compared to those which nest farther apart (Gross 1991; Jennings and Phillips 1992; Neff 2001). Male sticklebacks which nest close together also exhibit a higher rate of sneak fertilizations than those that are farther apart (Goldschmidt et al. 1992).

Egg predators and filial cannibalism are other common threats to eggs in freshwater ecosystems (Smith and Reay 1991; Manica 2002) and these factors may be affected by inter-nest distance. Filial cannibalism is when a male eats his own eggs in order to gain nutrients needed to continue the reproductive season, without leaving the nest vulnerable to other predators (Rohwer 1978). Filial cannibalism has been documented in darters and other species of fishes (Lindstrom and Sargent 1997; Bandoli 2002; Neff 2003). Smaller inter-nest distances may cause an increase in the frequency of antagonistic interactions, such as encounters with conspecifics and other egg predators, making nest guarding more energetically costly for males. Therefore, smaller inter-nest distances could increase the occurrence of egg eating by egg predators and also indirectly cause an increase in filial cannibalism, however this is currently unstudied.

Closer nests may also have the positive effect of increasing the probability of successful mating encounters. Small inter-nest distances may attract more females who seek to assess multiple mating partners with a minimal amount of effort and exposure to predators. This was found to be true for the coral reef fish *Abudefduf abdominalis* where females were shown to prefer to mate with males in larger colonies (Tyler 1995). Jennings and Phillips (1992) found that, while female long ear sunfishes did not show a preference for clumped nest sites, these closer nests did allow for subordinate males to have more breeding opportunities. Small inter-nests distances may be especially useful to species that use acoustic signals for courtship and mate attraction because these signals will travel very small distances in shallow freshwater environments. Females that seek to acoustically assess multiple males at one time should therefore prefer nests grouped close together.

Opportunities for encountering or attracting allopaternal males may also increase with a decrease in inter-nest distance. Alloparental care is care provided to unrelated offspring (See Wisenden 1999). A common form of allopaternal care in fishes is when males care for nests which contain unrelated eggs and achieve an increase in mating success (Stiver and Alonzo 2010) due to a female preference for males with eggs already in their nest. Allopaternal care has been found in multiple species of freshwater fishes where males provide parental care to eggs (Constanz 1985; Sargent 1989; Johnston 2000) however, it is currently unknown if nest density plays a role in the frequency of this behavior.

The objectives of this study are to investigate the effects of inter-nest distance on nest selection, male success, and the presence of allopaternal care and egg eating in the darter *Etheostoma neopterum.* Male darters set up nests and provide parental care in the form of nest guarding, egg fanning, and egg cleaning (Knouft et al. 2003; Knouft and Page 2004). These males also use low frequency vocalizations for courtship and aggressive interactions (Speares Noel and Johnston *In prep*). Therefore it is predicted that this species will utilize artificial nest tiles, within the theoretical propagation distance of their sounds (< 1 m). Furthermore, we predict that males that use nests located closer together may have more conspecific interactions leading to more instances of allopaternal care and egg eating, if these behaviors are present in this species.

Methods

Field observations of *E. neopterum* and habitat manipulations were done in the Last Butler Creek system near Iron City, Wayne County, Tennessee (35.07097, -87.645724). This study began on 15 March 2011. At this time the water temperature was 11°C and males were found under large rocks and in breeding coloration. Forty five *E. neopterum* were collected at this time by kick seining into a 10 foot seine net. Each male was marked with a unique set of colors using the visible implant elastomer system (VIE, Northwest Marine Technology Inc., Shaw Island Washington, USA). All markings were done on the dorsal side of the males so that if they were recaptured, markings could be easily interpreted without removing the male from the water. Marking took less than one minute and fish did not need to be anesthetized. Markings included lines on to the right or left side of the first or second dorsal fin or before the dorsal fin and various combinations of these patterns. Although the suitability of VIE markings, in regards to survivability, and mark retention was not investigated in this study, this methodology has been found to be highly retained and safe in another species of darters (Weston and Johnson 2008). Length (standard length-SL mm) and weight (g) were taken for each individual. In this species non-reproductive males look identical to non-gravid females; therefore males were identified by their breeding coloration and by the presence of egg mimics on their dorsal fin.

Artificial shelters were placed in the stream on the first day of the study. Males of this species readily used these tiles as shelters in pilot studies during previous reproductive seasons. The shelters were composed of 14 X 14 cm tiles propped up to a height of 2.5 cm. Each nest was identified by a number on the top and bottom of the shelter. To assess the effect of inter-nest distance on shelter choice, nest success and other behaviors four tiles were placed in a square with the opening of each tile facing inward (Fig. 3-1). This study design included 2 treatments. The in the “close” treatment all four tiles were placed 10 cm a part in each direction, the “far” treatments were set up so that the distance between nests was 100 cm. Each treatment was replicated 5 times within the same stream. All nests were placed in areas where males had been collected earlier in the day and all of these locations had a silty substrate and all large stones present around the tiles were removed. Water depth and flow were reported for each tile site. All groups of tiles were placed at a minimum distance of 3 meters from each other. This distance was chosen because male *E. neopterum* use vocal communication during the breeding season (Speares Noel and Johnston *In prep).* These vocalizations have a low dominant frequency and therefore, theoretically, these vocalizations should propagate less than 0.5 m in the shallow stream habitat (Mann 2006). A total of 3 meters between treatments should assure that there is no acoustic communication between the groups of tiles. However, acoustic communication could be possible between tiles in the close treatment.

Throughout the season tiles were lifted and inverted to check for the presence of males and eggs. Prior to checking the tile, a circular metal cage was lowered over the tile, in order to trap any fish present. If the male present under the nest was previously marked then the color pattern was recorded without removing the male from the water. Unmarked males were captured with a small aquarium net and marked with their own unique color combination. At this time length and weight were also recorded. Measurements were only taken once for each fish in order to minimize capture stress and the amount of time that a male was separated from his nest. If eggs were present in the nest, while a male was being marked the nest was removed and placed into a separate 5 gallon bucket with stream water and an air-stone. These males were separated from their nests for 5 – 15 minutes. After the male and his nest were returned to the stream, the males readily returned under the tiles. If spawning was occurring when a nest was lifted, then the nest was returned immediately and rechecked about an hour later. All tiles with eggs were photographed with an Olympus underwater camera (Stylus 720SW, Olympus America

Inc) and the eggs were later counted using Microsoft paint software. All tiles were checked between 9 and 11 am.

Egg cannibalism

Egg cannibalism was determined by counting the number of eggs present in photographs of the nest tiles throughout the season. If differences in the number of eggs present in a nest were detected among observation dates then the photographs were compared to determine which eggs were eaten. Eggs were classified as eaten if they completely disappeared before they were mature. Eggs were classified as hatched, if the eggs were previously very developed (eyes and fry movement visible to the researcher) and small parts of the eggs were left behind. Egg cannibalism was not investigated in tiles after eggs started to hatch.

Condition Index

Data collected for all marked males included weight and standard length. These data were used to calculate Fulton's condition index (Ricker 1975, also see Nash et al. 2006) for each fish. This index was chosen because it is a commonly used and accepted condition indicator in the field of aquaculture (Sutton et al. 2000) and has also been used in previous reproductive studies (Neff et al. 2004). It was calculated for each male using the following formula:

$$K = \frac{Weight(g)}{[Standard\ Length\ (mm)]3}$$

This index was used, along with SL to assess the males found at the nest tiles throughout the season. To minimize handling stress to the males, measurements were only taken the first time that the male was encountered. This was the first day that he set up residency under a tile and/or gained eggs. Therefore this will be referred to this as the ‘initial condition’ of the male.

Statistics

All statistics were conducted using PASW 18 statistics package (IBM SPSS Corporation, Chicago Illinois, USA). Comparisons between close and far tiles groups were done using chi square test and a Fisher’s exact test. Differences between nest guarding males and allopaternal males were determined using Mann Whitney statistical tests. Spearman’s Rho correlations were conducted to assess the relationship between the number or percentage of eggs eaten and the SL or initial condition of the guarding male.

Results

Current velocities for shelters with males ranged from 0.0 ms^{-1} to 0.1 ms^{-1} with an average flow of 0.024 ms^{-1} (± SD 0.023 ms^{-1}). Males occupied shelters that were at depths ranging from 90 mm to 620 mm and the average depth was 226 mm (±103.03 mm). The water temperature ranged from 11° C to 16° C from March until May of 2011.

Recapture Rate

In total 94 individuals were marked over the course of the study and 31 individuals were recaptured (33.6% recapture rate). The majority of these recaptures (29 out of 31) were found under nest tiles or natural nests. Two marked individuals were observed swimming between nests but did not seem to be associated with any particular cavity. Of the 29 individuals who were caught multiple times, 8 were never caught at the same nest twice. However, of these 8 only 3 males stayed within the same group of 4 tiles. The majority of the recaptured fish exhibited site fidelity as they were caught at the same nest tile at least twice.

Nest site selection

The nest tiles were used by 30 different males throughout the reproductive season. Of these 30 males, 17 selected tiles in the far treatment and 13 males used tiles from the close treatment. The number of close and far tiles that male *E. neopterum* were found under did not differ from random (χ^2=0.533, df=1, p>0.20). Of the 30 tiles where males were found, 19 contained eggs at one point in the observation period. All 13 of the close tiles that were used by males had eggs at least once during the study and the males at 6 out of the 17 nests chosen in the far treatment successfully obtained eggs. Males who chose the close nests were more likely to have eggs under their nest than males which chose the far nests (p<0.001, Fisher's exact test). However, successful hatching of at least one clutch was only able to be confirmed in 10 nests (4 far and 6 close). Therefore, males who chose the close nests were not more likely to successfully hatch at least one clutch of eggs than males who chose far nests (p=0.255, Fisher's exact test) (Fig. 3-2).

Allopaternal care

This study did find substantial evidence of allopaternal care occurring in *E. neopterum*. Of the 10 successful nests examined, 6 instances were found where the original guarding male was replaced by another male.

The appearance of allopaternal males was associated with the disappearance of eggs, but never more than 12.8% of the total eggs present in the nest. These eggs were assumed to have been eaten. This is in contrast to the unsuccessful nests where the presence of another male (not considered an allopaternal male) coincided with the disappearance of 100 % of the eggs which were also assumed to be eaten by the new male. At one nest the allopaternal male was present early in the incubation period of the eggs (days 2-5), this was the only case however, where the original male returned for two days and then left again. After the original male left, the same allopaternal male returned to the nest. For quantitative purposes, both instances of the same allopaternal male showing up at the same nest were counted as a single allopaternal event. In all other allopaternal events the allopaternal males were present in the later part of the egg incubation (day 7 or later) and stayed at the nest until the eggs hatched. Four of the allopaternal males were present in the close configurations and 2 allopaternal males were present in a far configuration. The distribution of allopaternal individuals between close and far nest was not significantly different from random ($\chi2=0.667$, $p>0.10$).

The mean SL for parental males (66.8±5.93) was statistically higher than that of the allopaternal males (60.2±2.63) (U=2.000, p=0.016). The mean initial condition index of the parental males (1.97±0.373) in successful nests was also higher than the allopaternal males (1.67±0.292), however these results were not statistically significant (Mann-Whitney U=8.00, p=0.247) (Fig. 3-3).

In all but one of these allopaternal events more eggs were found in the nest after the guarding male deserted the nest and the allopaternal male appeared. Therefore in almost all of the cases of allopaternal care, the new males were able to successfully court females and gain their own clutches.

Egg predation and filial cannibalism

Egg predation appeared many times throughout the season. In all but one of the unsuccessful nests 100% of the eggs disappeared and were assumed to be eaten by the guarding male or other egg predators. Filial cannibalism was very common in the successful nest tiles as well. Eggs disappeared and were assumed to have been eaten by the guarding male in 8 out of 10 of the successful nests. On average parental males ate a total of 53.0 eggs, and the average percentage of eggs eaten by parental males was 15.6% of the total eggs in the nest. Allopaternal males ate an average of 34.7 eggs per nest and the average percentage of total eggs that were eaten by allopaternal males was 7.9%. Although on average allopaternal males ate less eggs and a smaller percentage of the eggs, these differences were not statistically significant (percent eaten: U=15.0, p=0.865; number of eggs eaten; U=15.0, p=0.865) (Fig. 3-4 a and b). Parental males at nests in the far treatment ate more eggs on average (75.8 eggs and 34.3%) than parental males at the close nests (32.1 eggs and 7.3%). However, these differences were also not significant (number of eggs eaten: U=5.0, p=0.486; percent of eggs eaten: U=3.50, p=0.200) (Fig. 3-4 c and d). Neither the number of eggs eaten at a nest nor the percentage of eggs eaten at a nest were correlated to the SL of the guarding male (Percent eaten: ρ=-0.410, p=0.314; number of eggs eaten ρ=0.030, p=0.943). These were also not related to the initial condition of the male at the nest (Percent eaten ρ=0.359, p=0.382; Number of eggs eaten ρ=0.265, p=0.526) (Fig. 3-5).

Nests in which all of the eggs were eaten had an average of 198.1 eggs prior to being eaten and nests where at least one clutch of eggs successfully hatched had an average of 420.4 eggs present. There was a significant difference between these groups (U=12.0, p=0.006).

Discussion

This study found that inter-nest distance did not have an effect on male shelter choice. However, since males at closer nests were more likely to gain eggs than those at far nests inter-nest distance may affect a male's ability to obtain eggs from females. This study is also the first to provide evidence of both allopaternal care and filial cannibalism in *E. neopterum.* It was also determined that inter-nest distance did not have an effect on either of these behaviors.

Allopaternal care most likely occurs in this species through nest abandonment, which has been shown in other species of darters (Constantz 1985). Allopaternal males in the current study were significantly smaller than the males that they replaced. Other studies in of teleost fishes have found that male size can be related to their ability to hold a shelter (Lindstrom 1992; Stammler and Corkum 2005). Therefore, it is unlikely that the smaller allopaternal males were able to displace the larger parental males and these larger males probably deserted the nest on their own.

Allopaternal males were found at 60% of the successful nests that we observed over the course of this study. This is a higher rate of allopaternal care than seen in previous studies of *Etheostoma olmstedi* (DeWoody et al. 2000 – documented allopaternal males at 19% of sites). However it is still lower than was reported in other studies of *E. olmstedi* (Stiver and Alonzo 2010 – stated over 80% of nests had allopaternal males).

This study found that egg eating and possibly filial cannibalism is occurring in *E. neopterum*. Inter-nest distance was not found to have an effect on the number or percentage of eggs eaten from a nest. There was also no correlation found between the number of eggs or the percentage of eggs eaten and the SL or initial condition of the males. One possible explanation for the variation in number of eggs eaten per nest could be a variation in overall energy expenditure by males. Males which expended more energy, or had a decrease in condition may have eaten more eggs. We were not able to address this possible variation in our study because condition was only measured once in order to minimize capture stress to the animals. It is possible that some fish experienced changes in condition throughout the season that were not detected in our study. Nests that had a smaller number of eggs were more likely to be completely eaten. This finding supports other studies that have found that males will only defend a nest if the reward in reproductive success is higher than the cost of guarding the nest (Lindstrom 1998).

A surprising finding in this study is that there was no statistical difference between the amount of eggs eaten by the parental males and the allopaternal males. It was expected that guarding males would eat less eggs to avoid lowering their overall reproductive success. Allopaternal males are assumed to be unrelated to the eggs present in the nest when they arrive and therefore could possibly not exhibit the same restraint.

Allopaternal males may not have eaten more eggs because of a female preference for males with eggs in their nests. It is likely that this female preference exists in this species because the males of this species develop egg mimics during the reproductive season. A female preference for males with eggs is thought to be the driving force for this phenomena as well as allopaternal care (Rohwer 1978; Page and Swofford 1984; Knapp and Sargent 1989). This preference also makes eggs a valuable commodity for allopaternal males who seek to attract females. Even though the eggs in their nest are unrelated to the allopaternal males they are still highly valuable. This may account for why allopaternal males were not found to eat more eggs than the paternal males.

Male *E. neopterum* that used tiles from the close treatment were more likely to have eggs than males who used tiles in the far treatments. This could be due to females preferring areas where there is a high density of nests. This may allow them to assess many nests and potential spawning partners, using the smallest amount of energy. Traveling farther to assess multiple nests and males would require more energy expenditure and also more exposure to predators and other dangers.

In addition to being able to visually assess males in the close treatments, females may also be able to acoustically assess males. Like other darters in the Spottail group of the sub-genus *Catonotus*, (Johnston and Johnson 2000) male *E. neopterum* are vocal during courtship, spawning and aggressive interactions (Speares Noel and Johnston *In prep*). Since the nests in the close treatments are within the theoretical propagation distance of *E. neopterum* vocalizations (Mann 2006; Speares Noel and Johnston *In prep*) females located near tiles in the close treatment should be able to acoustically assess multiple males that may be present simultaneously. Nests in the far treatment however, may be acoustically isolated from one another. In many species of fish, including darters (Speares and Johnston 2011), characteristics of vocalizations can be indicative of male size and therefore vocalizations could be used as an assessment metric by both males and females.

Although males that used tiles in the close treatments were more likely to have eggs, they were not more likely to hatch successful clutches when compared to males in the far tiles. This may be because close tiles are more attractive to nest predators. In sticklebacks closer nests were found to increase the incidence of ‘egg raiding behavior’ (Goldschmidt et al. 1992). However, the current study did not find any difference between the amount of eggs eaten between the ‘close’ and ‘far’ treatments. Nests that are located together may also be more susceptible to communicable diseases and fungus.

These data suggest an interesting trade off for males, where being closer to other nests may increase positive conspecific interactions (i.e. access to females and allopaternal males), as well as other negative factors (i.e. exposure to predators and diseases). Male and female preferences for nest distance, the presence of allopaternal care and egg cannibalism are all important life history traits which have been shown in other species of fish but never in *E. neopterum*, and rarely in a natural field setting.

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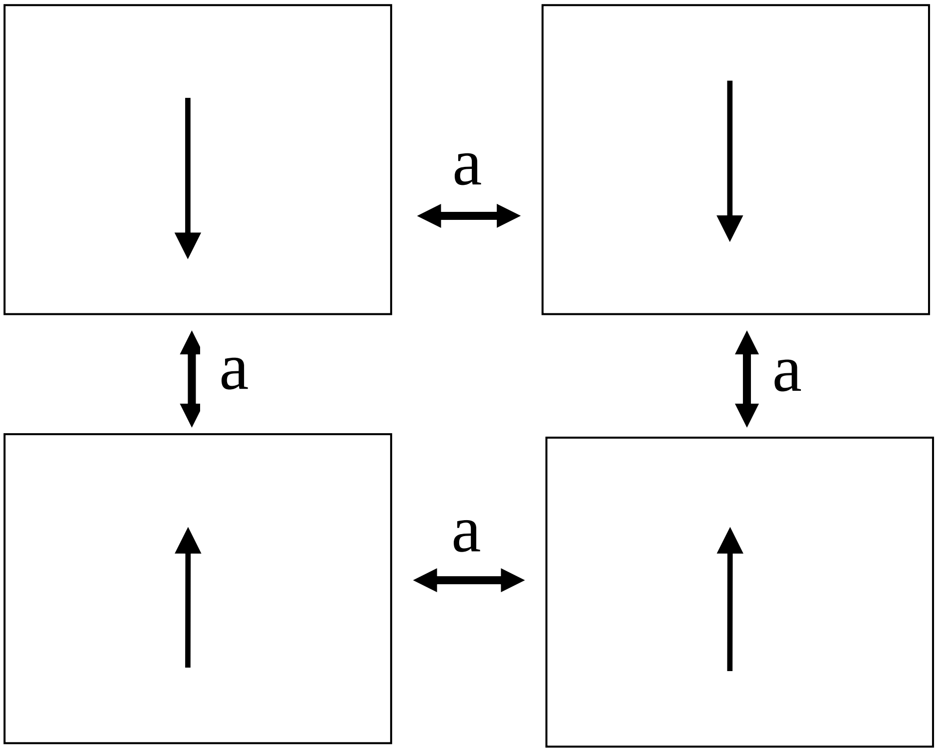

Fig. 3-1: Diagram representing the arrangement of the nest tiles. Arrows indicate the direction of the opening of the tiles. Distance ‘a’ was 10 cm in the close treatment and 100 cm in the ‘far’ treatment.

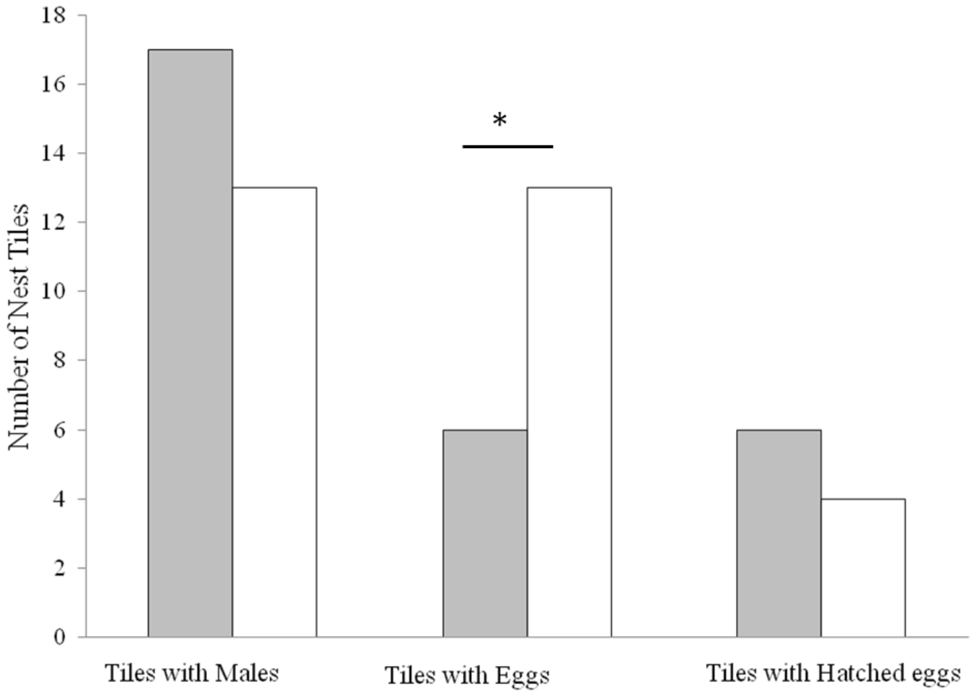

Fig. 3-2: Comparing the number of tiles where there was a male was present, eggs present and where eggs were successfully hatched. The gray bars represent tiles in the far treatment and the white bars represent tiles in the close treatment. * represents a significant difference using a Fishers Exact test ($p < 0.001$).

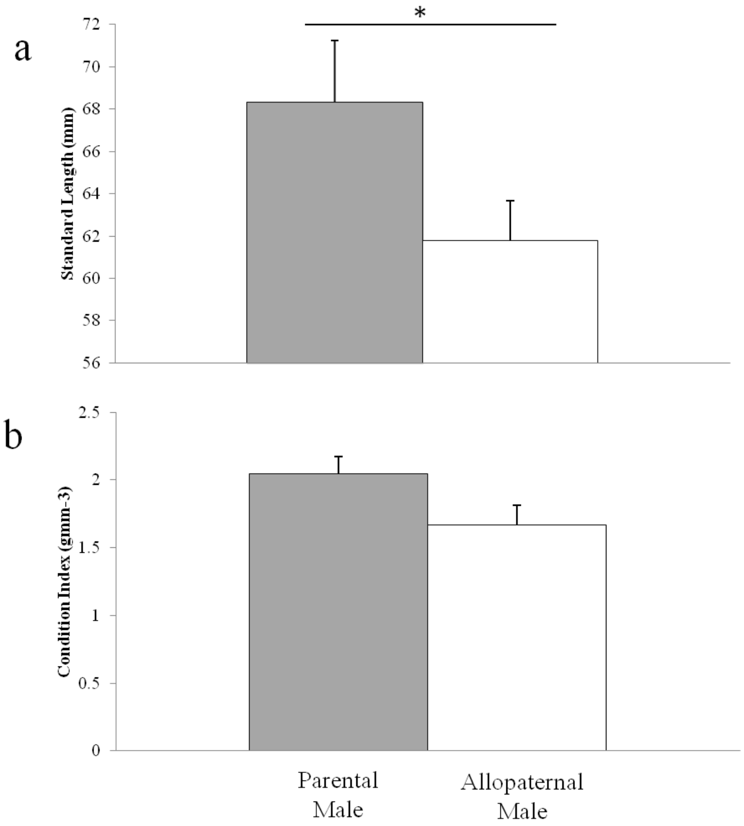

Fig. 3-3: Graphical representation of the **a.** standard length and **b.** initial condition index of males guarding nests (gray bars) and the allopaternal males (white bars) at nests were allopaternal care was found.* represents a significant difference between the standard length of guarding males and allopaternal males (U = 2.00, p = 0.016).

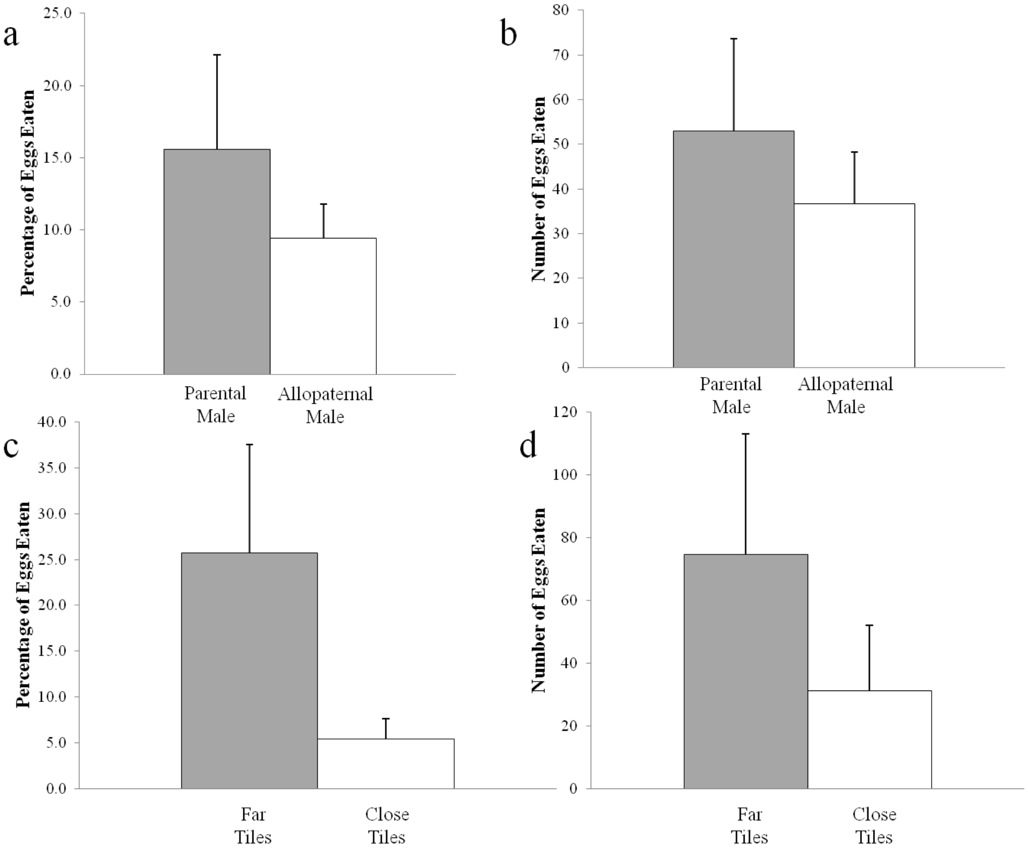

Fig.3- 4: Graphical representation of egg eating in *Etheostoma neopterum.* There was no statistical difference between the (a.) percentage of eggs eaten or (b.) the number of eggs eaten between parental males (gray) and allopaternal males (white). There was also no difference between the (c.) percentage of eggs eaten or the (d.) number of eggs eaten in the far (gray) or close (white) treatments.

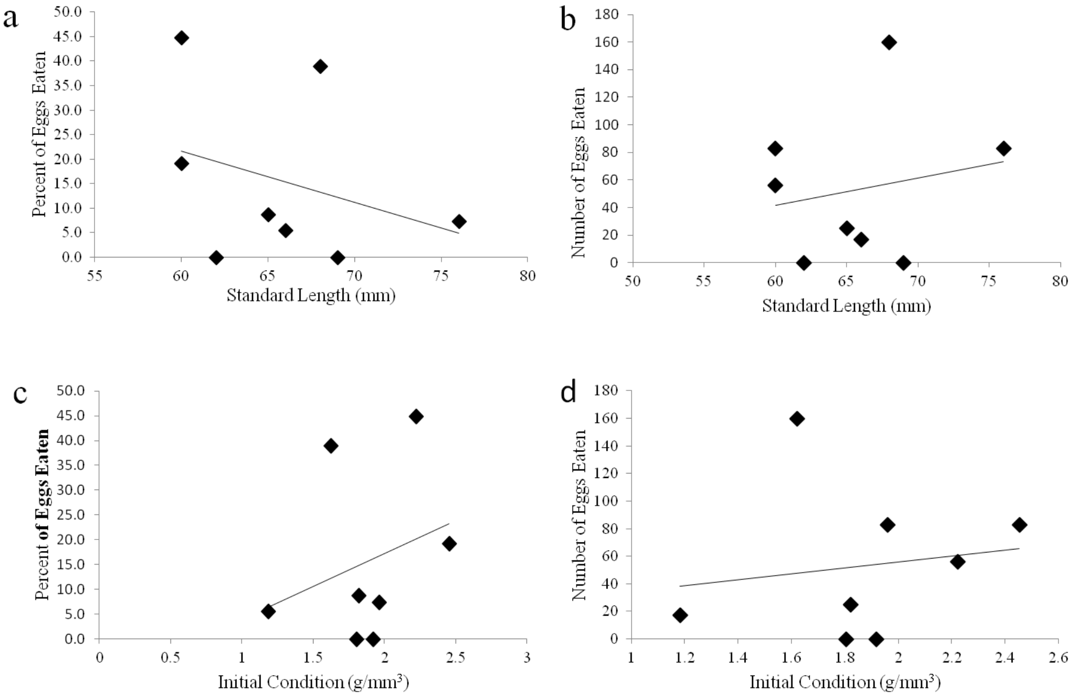

Fig 3-5: The relationship between (a) the percentage of eggs eaten in a nest and the SL of the parental male (b) the number of eggs and the SL of the parental male (c) the percentage of eggs eaten and the initial condition of the parental male and (d) the number of eggs eaten and the initial condition of the male. None of these factors were found to be correlated by a Spearman’s correlation test.

Chapter 4

Costs of Reproduction in *Etheostoma neopterum*: A field study

In order to be successful an organism must balance the costs of future reproduction with the need for current reproductive efforts. This study examines the dynamics and the costs of reproduction in the lollypop darter (*Etheostoma neopterum*). Males in a naturally occurring population of darters readily used artificial nest tiles. Males were marked, and the condition and size of the males were recorded. Eggs left on the tiles by females were also tracked throughout the reproductive season. A significant correlation was found between the size of the male (standard length, mm) and the number of eggs present in his nest. Successful males were also significantly larger than unsuccessful and non-parental males. In addition, average male condition and average male length of the population was found to decrease over the observation period. This suggests that the reproductive season is energetically expensive to males and this could possibly cause a high mortality rate among nest guarding males in this species.

Introduction

In all organisms reproduction involves two subsets of costs, those paid for current survival and those paid for producing future generations (Stearns 1989). These costs become even more apparent when parental care is present. When parental care is strictly preformed by the male, females may choose their mates based on specific male characteristics in order to maximize the benefits of their reproductive investment. Paternal care, however, requires a male to balance the energy needed to care for their offspring (current reproductive investment) with maintaining resources for future reproductive investments (Williams 1966). Therefore, males may adopt a strategy that allows them to take advantage of female preferences while also planning energetically for future spawning opportunities.

Male parental care is common throughout teleost fishes (Gross and Sargent 1985). In these systems females may chose a mate based on characteristics which represent a male's ability to protect a shelter and eggs from intruders and nest predators. Nest guarding and caring for eggs can be energetically expensive; therefore male condition may be an important aspect of female choice. Males in better condition may be able to defend nests and for longer periods of time. Female choice based on male condition can be seen in female sticklebacks (*Gasterosteus aculeatus*). These females are well known for their preference for redder males (Semler 1971). This red coloration has been shown to be correlated to male condition (Milinski and Bakker 1990).

Male size may also be a trait females use to assess potential mates. Size has been shown to be related to a male's ability to keep a shelter (Bisizza and Marconato 1988;

Stammler and Corkum 2005), and in some species females have been shown to have a preference for larger males (Lindström 1988). Males may be able to maximize their reproductive success by maintaining multiple traits such as large size and better condition. For example, both male length and condition was found to be correlated to male success in the angel blennies (Hastings 1988). However, these traits may also be costly for males to maintain.

The lollypop darter (*Etheostoma neopterum*) is a species in the subgenus *Catonotus*, and persists in small streams in northern Alabama and Tennessee. Like other *Catonotus*, *E. neopterum* males maintain shelters under flat rocks, where they will court multiple females to lay eggs on the underside of the shelter (Page 1985). The females then leave the eggs and the males provide parental care until the eggs have hatched. This site fidelity of males to their nests while the eggs are developing makes them easy to mark and track. In addition, this species is an ideal candidate for field study because of their high population density and the ease of observing them due to their shallow, clear water habitat.

The objective of this study is to determine the effect of male size and condition on reproductive success in the lollypop darter and determine if there are costs associated with these traits. We used male size (measured by standard length –SL) and condition as metrics of male quality and the change in these values in the population as a measure of male reproductive costs. The number of eggs and number of days at the nests was used as a measure of mating success. Our predictions are that larger males will be more reproductively successful and be able to gain more eggs. In addition we expect to see a decrease in male condition at the population level throughout the observation period.

Methods

This field study was conducted on a naturally occurring population of *E. neopterum* in the Last Butler Creek System outside of Iron City, Tennessee (35.07097, -87.645724). This study began on March 15, 2011 and concluded on May 16, 2011 which coincided with the peak of the reproductive season.

An initial group of 45 individuals was collected on the first day of the study, using a 10 foot kick seine net. Of these 45 individuals 34 were identified as males by the presence of egg mimics on their dorsal fin. The other 11 individuals could not be positively identified as males or female, but were marked in case they would develop egg mimics later in the study. These males were collected by kick seining throughout the entire study site, in an effort to collect as many males as possible. Each male was marked with a unique color pattern on their dorsal side using the visible implant elastomer system (VIE, Northwest Marine Technology Inc., Shaw Island Washington, USA). Marks were made on the dorsal side of the fish so that they could be seen from above the water. Weight (g) and standard length (SL-mm) of each male was recorded after they were marked. These data were used to calculate Fulton's condition index (Ricker, 1975; also see Nash et al. 2006) for each fish. This was calculated using the following formula:

$$K = \frac{Weight(g)}{[Standard\ Length\ (mm)]3}$$

Since the fish were removed from the water to be weighed, this was assumed to be an extremely stressful event. It was feared that repeated removal of males from their nest would cause these males to abandon their nests. To minimize handling stress to the males, this index was only calculated once, when the fish was first caught and marked. Therefore, for the rest of this study this will be referred to as the “initial condition” of the males.

Artificial nest tiles composed of 14 X 14 cm tiles were used in this study as shelters, which males readily used and defended. Fifty nest tiles were placed in the stream on the first day of the study, in areas where males had been collected. Throughout the study period the tiles were checked for the presence of eggs and males. Nests were checked 3-5 times per week. If a male was present under the nest, he was identified by his colored markings and the eggs present in the nest were photographed for later analysis, using an Olympus underwater camera. If the male present under the nest was not marked, then the fish was caught with an aquarium net, marked, measured and then returned to his nest. If a male was unmarked and his shelter contained eggs, then his shelter was also removed from the stream and placed in a 5 gallon bucket with an air-stone, until the male was ready to be returned to the stream. These males were separated from their nests for 5-15 minutes, however when replaced into the stream, males readily returned to their shelters.

At the conclusion of the study all nest tiles were recollected. At this time the entire section of the stream included in this study was collected with a kick seine net. In total 53 *E. neopterum* were caught (28 males, 25 females). Length and weight were recorded for all individuals but since female data was not collected throughout the study period only male data was used in further analysis. The purpose of this end-of-the-year collection was to determine how many marked males could be recollected at the end of the study period and to investigate whether the average condition and/or average size of the males varied when compared to the beginning of the season.

Male classification

Males found under nest tiles throughout the study were placed into one of four categories. Males found under nest tiles but never found with eggs (therefore never chosen by females), were labeled as 'non parental males'. Males that were found under tiles, guarding eggs, but whose eggs never successfully hatched were classified as 'unsuccessful parental males'. Males found under nests where at least one clutch was determined to have successfully hatched were classified as 'successful parental males'. Finally, males that were found under nests where another male was previously found with eggs were classified as allopaternal males. In order to be an allopaternal male, these males also could not have eaten more than half of the eggs present from the previous male. This current study did not address allopaternal males, therefore data from these males were not included in the following analysis. If an allopaternal male was found to be present in a nest, then only egg data collected prior to the arrival of the allopaternal male was included in this study.

Eggs were determined to have successfully hatched if they disappeared and were advanced in development when they were last observed. Eggs were considered advanced in development, if the fry could be seen moving inside of the eggs. If no fry were visible when the eggs disappeared then these eggs were considered eaten.

Statistics

Statistical tests were performed using PASW 18 statistics package (IBM SPSS Corporation, Chicago Illinois, USA) (Mean, standard error, comparison of means, and correlations). Analysis included both parametric and non-parametric methodologies. Multiple Mann-Whitney statistical tests, with a bonferroni correction were used to compare the successful parental males, non-successful parental males and non parental males. The alpha value calculated for this comparison using a bonferroni correction was 0.017.

Results

Behavioral and Nesting Ecology

Male *E. neopterum* were found to maintain shelters by burrowing into the silty substrate around nests. This allowed an occupied nest to be identified by one or two tunnels going in and out of the tile. Males were also found to have cleared area under a tile so that when the structure was lifted, there was a concave area where the tile had been. These indentations could have been an intentional attempt to make more space or a by-product of fanning the eggs as has been seen in other species of darter (Knouft et al. 2003). Eggs found in nests were arranged in a similar manner to that already published for this and other species of *Catonotus* darters (Page 1985). Females laid eggs in a single layer, most clutches were directly next to one another but occasionally there would be spaces between clutches. Males were found to have multiple clutches of different ages. On average 2.5 concurrently occurring clutches could be identified per nest. Older eggs could be easily distinguished from younger eggs by the presence of eyes and movement of fry that were visible to the naked eye.

The average time from the eggs being laid until hatching was 8.2 days at an average temperature of 13.5 °C. Only successful nests for which the exact date of egg laying and hatching could be identified were used for this calculation.

Nest turnover

Parental males at successful nests spent an average of 14.1 days at their nests. The average successful nest had eggs present for 16.4 days. The difference between these values is because allopaternal males extended the amount of time that a nest was active with eggs, past the presence of the paternal male. The unsuccessful parental males were present at nests for an average of 4.3 days; however the average amount of time that eggs were present in an unsuccessful male's nest was 3.2 days. The average time spent at a tile by a non-parental male was 3.4 days. There was a statistical difference between number of days spent at the nest for the successful and non-successful parental males (U=7.0, p=0.004), the non-successful and non parental males (U=35.0, p=0.008) and the successful males and the non-parental males (U=7.0, p<0.001) (Fig. 4-1a). There was also a statistical difference between the number of days that eggs were present in the nests of the successful and non-successful parental males (U=5.0, p=0.001) (Fig. 4-1b).

Male Size and Egg Number

A Spearman's rho correlation was used to examine the relationship between the number of eggs in a nest and the size (SL) or initial condition of the guarding male present at that nest. The maximum number of eggs found in the nest was determined by counting the number of eggs on the photographs. Two males in this study were able to gain eggs at multiple tiles. In each case, the males started a nest, then an allopaternal male was found at the first nest, and the parental male was found at a new nest with eggs. For these males the sum of the maximum number of eggs present in each nest, prior to the appearance of the allopaternal males, was used for this correlation. Data from both successful parental and non-successful parental males were used in this correlation. Spearman's rho test revealed a significant correlation between male size and the maximum number of eggs present in that male's nest (ρ=0.613, p=0.012) (Fig. 4-2a). There was not a significant correlation however, between the maximum number of eggs in a male's nest and the male's initial condition (ρ=0.059, p=0.829) (Fig. 4-2b).

Condition Index and SL Comparisons-beginning of study period vs. end

Initial condition index was calculated for 34 males at the beginning of the observation period and 28 males at the end of the study. The condition index of males was statistically higher for males measured at the beginning of the study (2.13±SD 0.454) than at the end (1.59±0.264) (U=112.00, p<0.001) (Fig. 4-3a). Males collected at the end of the study were also significantly smaller (54.7 mm±5.49) than those collected at the beginning (59.52±5.247) (U=257.00, p=0.002) (Fig. 4-3b). Despite the fact that the final collection of the study period covered the entire study site, none of the males that were marked throughout the study were found during the final collection.

Condition and SL comparisons- successful vs. unsuccessful males

In total 10 successful males, 9 non-successful males and 20 non-parental males were tracked throughout this study. There was no significant difference between the initial condition index for the successful parental males (2.03±0.324), the unsuccessful parental males (1.94±0.219) or the non-parental males (1.99±0.572) (Successful versus non-successful U=29.50, p=0.541; successful versus non-parental U=84.00, p=0.657; non-parental versus non-successful u=83.00, p=0.981) (Fig. 4-4a). Non parental males had a mean SL of 57.1 mm (±6.97) which was significantly smaller than the mean SL of successful males (66.1 mm±6.29) (Mann Whitney U=36.00, p=0.007). There was also a marginally significant difference between the mean SL of the unsuccessful parental males (55.12 mm±9.57) and the successful parental males (Mann Whitney U=10.50, p=0.011). However, there was no difference between unsuccessful parental males and the non-parental males (U=75.0, p=0.684) (Fig. 4-4b).

Discussion

This study found that larger *E. neopterum* males had higher reproductive success than smaller males. Male SL was found to be significantly positively correlated to the number of eggs present in the male's nest, and males which guarded successful nests were significantly larger in size than unsuccessful parental males, and non-parental males. This finding is consistent with many other studies, in which females prefer medium- to large-sized males (Keenleyside et al. 1985; Bissaza and Marconato 1988). Studies have also shown that male size is related to the ability of males to hold shelters (Stammler and Corkum 2005) and protect eggs. Therefore larger males may be successful because they are not only able to obtain eggs through a females preference for their size but are also better able to protect their eggs and shelter until the eggs have successfully hatched.

This study found a significant decrease in the average male condition from the beginning of the study period until the end. This suggests that the reproductive season is energetically costly to male *E. neopterum*. This energetic cost associated with parental care has been shown to occur in other freshwater species (Steinhart et al. 2004). Male darters in the subgenus *Catonotus* provide their eggs with parental care, in the form of guarding, fanning and cleaning (Knouft et al. 2003; Knouft and Page 2004). In addition, if a male leaves his nest for even a short period of time, then egg predators will quickly enter the nest and begin to eat the eggs (Speares and Johnston, *personal observation*). This lack of feeding time and a constant need for energy to guard eggs is likely the cause of the weight loss seen in the population during the study period.

At the end of the study we were not able to recapture any males that were previously marked at nest tiles earlier in the season. Currently it is unknown if male *E. neopterum* are able to spawn for multiple seasons, or if there is a high mortality rate for guarding males. The inability to locate any of the previously marked males at the end of the study period, however, suggests a high mortality rate for nesting males during the breeding season.

The idea of a high mortality rate for larger nest guarding males is also supported by the findings that males found at the end of the study period where significantly smaller in SL than those collected at the beginning of the observation period. It is reasonable to assume higher energetic costs for larger nest guarding males than smaller non-successful and non-parental males. As previously discussed the number of eggs present in a male's nest was found to be positively correlated to male size. Other studies have shown that the amount of parental care performed increased with the number of eggs present in a nest (Sargent 1988). If this holds true for darters then larger males with more eggs may also have to exert more paternal care resulting in a higher energetic demand. Our study also found that successful parental males spent more consecutive days at their shelters than non-successful males. Therefore these larger males may be losing valuable foraging time, which smaller males without nests may still be able to afford. Overall, these results suggest that males are putting more energy into reproductive effort than into current survival.

If there is a high energetic cost for guarding males as these data suggest, this may help to explain the presence of allopaternal care in darters. Allopaternal males have been well studied in the tessellated darter (*Etheostoma olmstedi*) (Constanz 1985; DeWoody et al. 2000; Stiver and Alonzo 2010). The presence of allopaternal males allows paternal males to leave their nests and eggs, in order to gain additional spawning sites within the same reproductive season. Recent research has also found that allopaternal males occur in *E. neopterum* as well, and that these males often care for eggs but consume a small percentage of the eggs present in the nest (Speares Noel and Johnston *In prep*). If the paternal males are not likely to survive for another reproductive season then the opportunity to gain additional nest sites is extremely valuable. This could be worth the cost of losing a small percentage of their eggs to nest predation by allopaternal males, if the allopaternal males could ensure the survival of the majority of the eggs.

An interesting finding of the current study is that there is no statistical difference between the SL or initial condition index of males that were never chosen by females (represented in this study by non-parental males) and males that were chosen by females but were unsuccessful (represented by the un-successful parental males). However, the successful males were statistically different from both of these groups. This may mean that size is indeed an important component for successfully being able to guard eggs until they hatch but not necessarily for being chosen by a female. Since females did appear to spawn with some of the smaller males, there may be other factors that are important to female choice besides size. For example in guppies, (*Poecilia reticulata*) dominance, coloration and courtship intensity were all found to be correlated to male mating success (Kodric-Brown 1993). In *Catonotus* darters, males court females, using vocalizations (Johnston and Johnson 2000) but also behaviors such as female chases and leading the female into the nest. Future studies in this species should examine if length or intensity of courtship display in *E. neopterum* could be correlated to male success or the number of eggs deposited in their shelter.

Parental care can be summarized into three distinct costs including adult survival costs, mating costs and future reproductive costs (Gross and Sargent 1985). This study has given us insight into how *E. neopterum* deals with these costs and how this may vary with size. Knowing more about these reproductive behaviors is key to promoting the survival of this species. These behaviors should be taken into consideration when planning for the future of this species.

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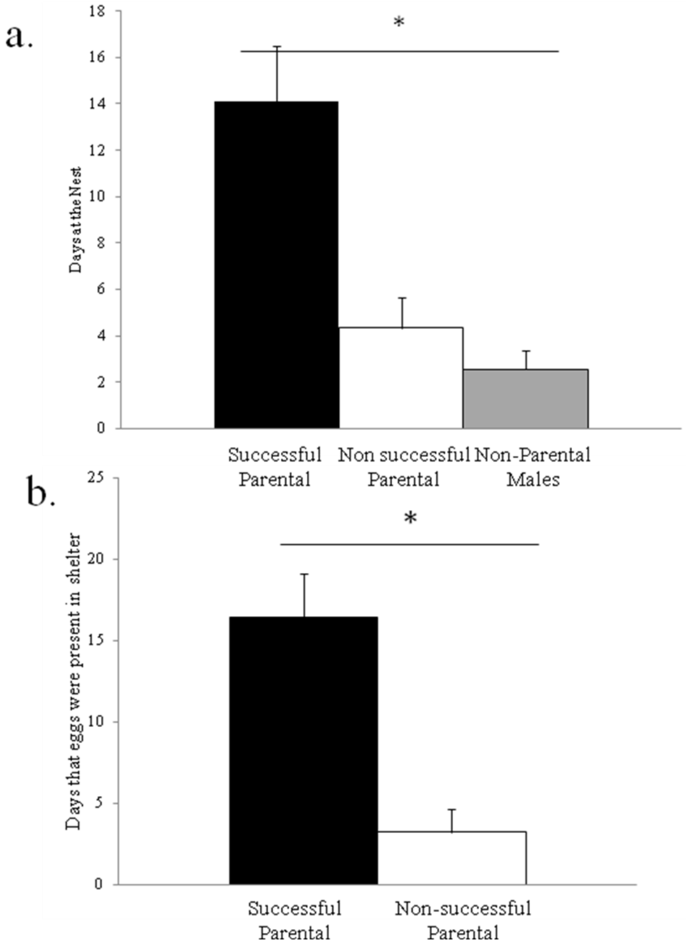

Fig.4-1: a. The number of days spent at a nest by successful males (black bar), non-successful parental males (white bar) and non-parental males (gray bar). The (*) indicates that all three groups are statistically different based on multiple Mann Whitney Tests with a bonferroni correction (α = 0.017). **b.** The number of days that eggs were present in the nests of successful males (black bar) and non-successful males (white bar). Eggs were present for significantly more days in the successful males than in the non-successful males.

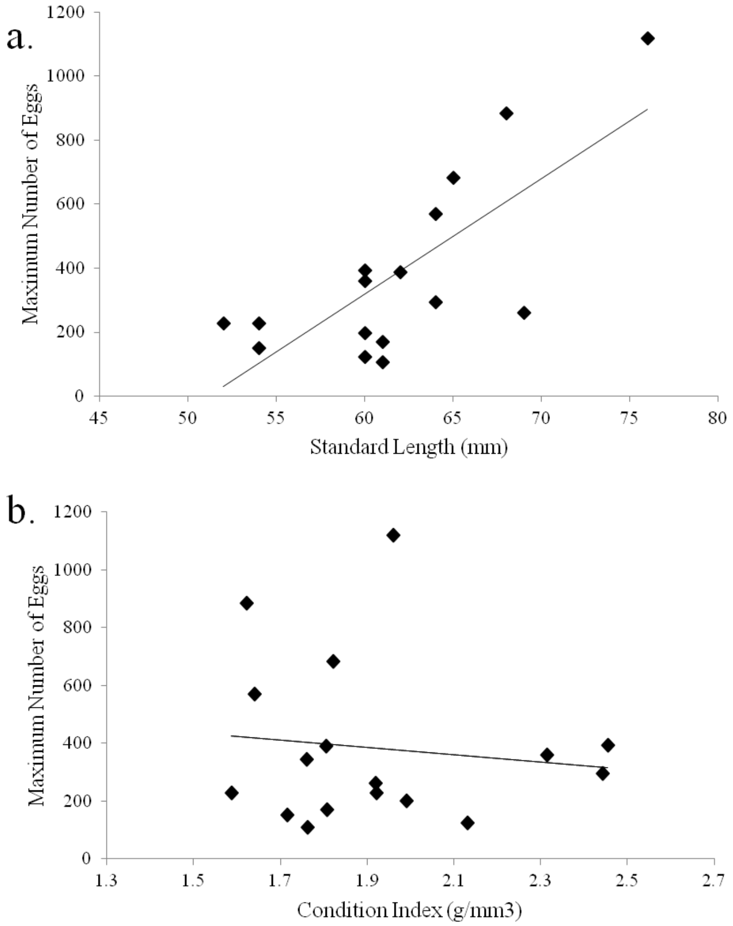

Fig. 4-2: a. The relationship between male standard length (mm) and the maximum number of eggs found in a male's nest. These two factors are significantly correlated (ρ = 0.613, p =0.012). **b.** The relationship between initial condition of the male and the maximum number of eggs in his nest. There no significant relationship between these factors according to a Spearman's rho correlation test (ρ= 0.059, p = 0.829).

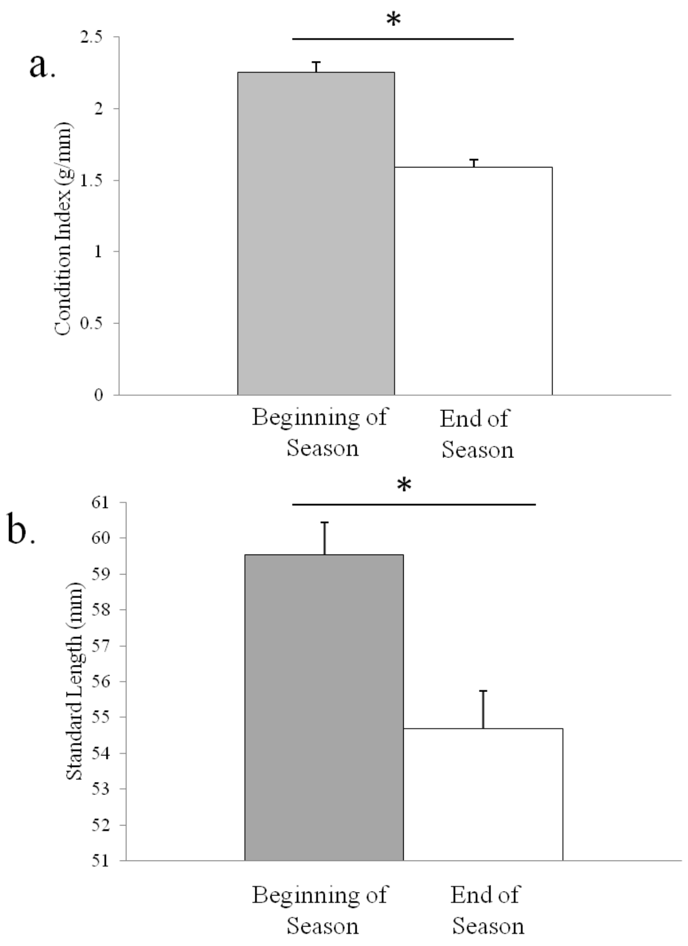

Fig. 4-3: A graphical representation of the initial condition **a.** and the standard length **b.** of males caught at the beginning of the study period (Collected March 15, N= 34, represented by the gray bars) and the end of the study period (Collected May 16, N=28, represented by the white bar). (*) indicates significant difference between groups. (Condition Index: U = 112.00, p < 0.001; Standard length U = 257.00, p = 0.002).

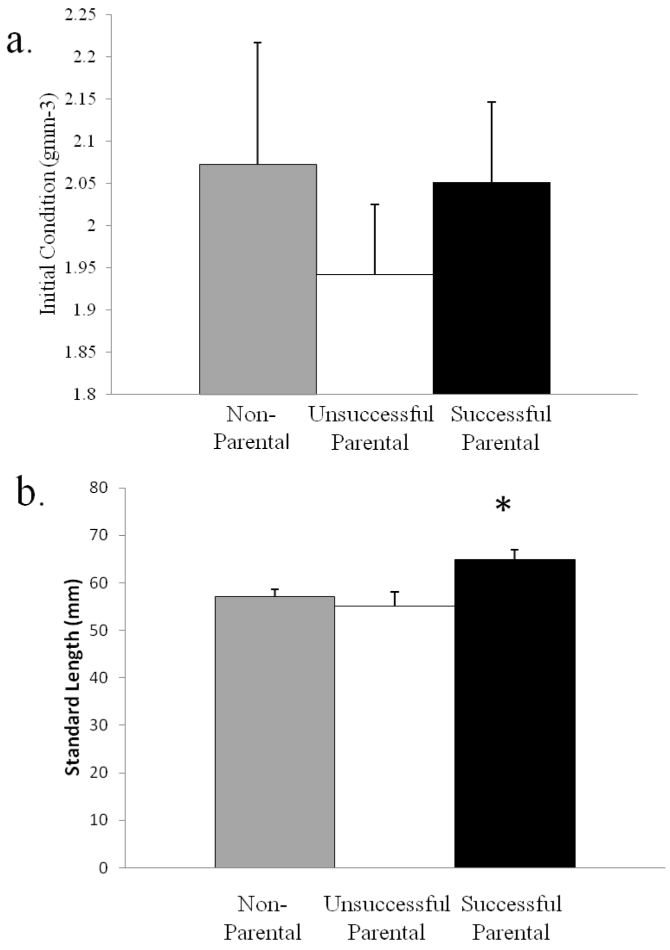

Fig. 4-4: Graphical representation of differences in **a.** initial condition and **b.** standard length of males with different degrees of reproductive success. The gray bars represent males which were found under artificial nest tiles but never had eggs (non-parental males). The white bar represents males which had eggs under artificial nest tiles but did not raise eggs to hatching (non-successful parental males). Black bar represents males guarding nests which had at least one successfully hatched clutch. The (*) represents the significant difference between the standard length of the successful males from the other

two groups. (non-parental: U = 36.00, p = 0.007; non-successful parental U = 10.5, p = 0.011). The alpha value for this comparison after a bonferroni correction is 0.017.

Chapter 5

Acoustic vocalizations as social cues: Hormone modulation in response to vocalization playbacks

Many studies to date have looked at the relationship between social signaling and the endocrine system. The majority of these studies include signals that are used for long-distance communication or sounds that have the ability to be carried far from the sound's source. Acoustic communication in darters, such as the fringe darter (*Etheostoma crossopterum*), represents a system where signals are constrained to very short distances due to habitat characteristics and signal structure. In this study we examine the effect of hearing male aggressive and courtship vocalizations on the hormone levels of male and female *E. crossopterum.* Levels of testosterone, 11-ketotestosterone, cortisol and estradiol were investigated. Males showed a significant decrease in testosterone and 11-ketotestosterone levels and females exhibited a significant decrease in estradiol levels after exposure to conspecific courtship vocalizations. Since this communication modality is used for close interactions, this study suggests that male vocalizations have an inhibitory effect on androgens in non-territory holder males because these males seek to avoid aggressive encounters with courting males. The decrease in estradiol in females may signal a social priming

mechanism, which prepares females to ovulate. This investigation into social signaling gives us insight into the ultimate functions of sound production by darters.

Introduction

Recently many studies have investigated the relationship between the endocrine system and social signaling (Bzoska and Obert 1980; Wingfield and Wada 1989; Francis et al 1993). Recognizing and understanding physiological responses to social signals allows researchers to have deeper insight into the ultimate functions of these signals. Many of these studies include signals that are used for advertisements, such as chemical cues, or broadcast vocalizations (Solis and Pena 1997; Burmeister and Wilczynski 2000; 2005; Remage-Healey and Bass 2005). These signals have the potential to travel long distances in short periods of time. However, much less research has involved signals that are used solely for communication over short distances. Signals designed for short distances may or may not cause the same physiological responses as signals that are intended to travel long distances.

Acoustic cues in particular have been shown to cause modulations in hormones related to both stress and reproduction. Remage-Healy and Bass (2006) conducted playbacks in the natural environment of the midshipman. This study found that guarding males responded to playbacks with an increase in 11-ketotestosterone and cortisol, as well as an increase in calling behavior. 11-ketotestosterone is the main androgen in fish that is involved in reproduction (Borg 1994) and courtship behaviors, such as sound production (Fine et al. 1982).

Testosterone and 11-Ketotestosterone were found to be correlated with courtship rate and territoriality in the tilapia *Oreochromis mossambicus* (Oliveira et al 1996). In midshipman cortisol, which is a stress hormone, was also found to increase in response to predator vocalizations (Remage-Healey and Bass 2006).

Studies have also shown a strong interaction between female reproductive status and exposure to conspecific vocalizations. Maney et al. (2007) found a rapid elevation in female levels of LH and Erg-1 expression in the mediobasal hypothalamus in response to playbacks of male vocalizations in birds. Conspecific male vocalizations also affect female reproductive status in anurans, such as the midwife toad (*Alytes muletensis*) (Lea et al. 2001). Gravid, female midwife toads that were exposed to male vocalizations had eggs that remained ready to be fertilized by a male for a longer period of time. In contrast, females exposed to heterospecific vocalizations or silence reabsorbed their eggs (Lea et al. 2001). To our knowledge there have been no studies looking at the effect of conspecific vocalizations on female reproductive status in teleost fishes. However, given the effects seen in other taxonomic groups and the ability of conspecific vocalizations to modulate hormones in males, this avenue deserves further investigation. In particular, investigations into levels of testosterone and estradiol are warranted as changes in these have been shown to signal final oocyte maturation and ovulation in teleosts (Kagawa et al. 1983; Peter and Yu 1997).

Acoustic signaling by darters represents a different system than many of the previously mentioned studies because, in this system, signals have very short propagation distances (Speares Noel and Johnston *In prep*). Vocalizations made by darters in the subgenus *Catonotus* are low frequency (below 200 Hz) (Johnston and Johnson 2000; Speares and Johnston 2011). In addition, many of these species inhabit streams that are less than 1 meter in depth. Theoretically, these two factors should make it impossible for signals to propagate (Mann 2006) and actual in-field measurements have found that these signals can travel a maximum of 50 cm before being indistinguishable from background noise (Speares Noel and Johnston *in prep*). This extremely limited propagation means that receivers of these signals may be in very close proximity to the signaling male.

The objective of this study is to look at the physiological response of both male and female darters to playbacks of male darter vocalizations. Levels of testosterone, estradiol, cortisol and 11-ketotestorone (in males only) will be monitored in fish exposed to silence and playbacks of conspecific vocalizations. Testosterone, estradiol and 11-ketotesterone were chosen since they are all associated with reproduction or aggression in teleosts. Cortisol was included in this study to determine if there was a stress response associated with the signal presentation or hormone collection methodologies. The purpose of this study is to clarify the overall function of short-distance acoustic communication in a small stream environment.

Methods

Fish collection and housing

Etheostoma crossopterum were collected from Cowpen Creek, (Lauderdale County, Alabama, USA, 34.966163 N, -87.551775 W) in 2010 and St. Florian Branch of Shoal Creek (Lauderdale County, Alabama, USA, 34.87347 N, -87.60239 W) in 2011.

Collections were made by kick seining into a 10 foot net. All fish were placed into coolers containing creek water and transported to Auburn University by car. All fish were allowed to acclimate overnight in the coolers in the lab until the water was approximately within 3 degrees of the tank temperatures. To facilitate this process frozen water bottles were added to the tanks to lower the temperature of the tanks.

Fish were housed in 76 L tanks with a small cobble substrate and multiple flat rocks to use as shelters. All tanks were equipped with an air-stone and a filter. Males were housed individually in tanks and gravid females were housed with two to four individuals per tank. Water temperature ranged from 19-21°C and fish were given access to natural light through windows as well as a artificial period of 14L:10D. All water samples were collected from fish within 14 days of being taken from the field.

Water Collection

Hormone collection was performed by extracting free hormones from holding water. The water was processed using methodologies previously documented in the literature (Earley et al 2006, Wong et al 2008). Fish were placed into small 700 mL glass collection tanks that measured approximately 6 cm W X 12cm L X 6cm D. This small tank was filled with water from a baseline tank which was held in the exact same conditions as the fish holding tanks, however the baseline tank never had fish in it. The small collection tank was then covered with a glass cover and placed directly in front of an underwater speaker, placed in a 280 L tank. The collection tank was placed 5 cm from an underwater speaker (UW-30, Universal Sound Inc) connected to a car amplifier which in turn was connected to a dell lap top computer. The fish was then exposed to one of two trials. The control trial was composed of 30 minutes of silence. During the control trial the speaker was unplugged from the amplifier to ensure that the speaker was completely silent. The treatment trial consisted of a 15 minute playback period which contained either an *E. crossopterum* courtship or aggressive vocalization.

After the first trial the fish was removed from the collection tank using a small aquarium net and placed into an identical small collection tank filled with new baseline water. The second collection tank was replaced in front of the speaker and the fish received the second treatment. Generally, the control trial was run first followed by the treatment trial. In order to rule out any treatment effect of order, a random selection of 2 courtship trials and 2 aggressive trials were run with the treatment trial first and the control trial second. The results of these trials showed the same trends as the rest of the trials.

All water collection trials were conducted between 5:00 and 7:00 am during the months of March thru May 2010 and 2011. These trials were timed so that the first collection coincided with sunrise. At the time of the water collections there were no other activities going on in the laboratory. All glassware used was washed with 2 washes of 100% ethanol, followed by 2 washes with distilled water. Glassware was then left to air dry for 24 hours before being reused.

The courtship and aggressive signals used in this study were prerecorded in the lab from a male *Etheostoma crossopterum* (Fig. 5-1). For details in this recording procedure see Johnson and Johnston (2000). These signals were selected because they were very clean signals, and they exhibited traits that made them better suited for transferring through the glass collection tank. For example it was found that vocalizations that were played back through glass did not exhibit frequency modulation clearly; therefore the signals chosen were relatively flat signals that retained spectral characteristics such as frequency well. Frequency has been shown to be correlated to size in many species of fish (Lobel and Mann 1995; Amorim and Neves 2007) including darters (Speares and Johnston 2011). Sounds were played using Windows Media Player. The sounds were played at a volume that was recorded to be approximately 90 dB from the inside of the collection tank. The playback sounds consisted of the courtship or aggressive vocalization, followed by 10 seconds of silence. This track was looped to play continuously for the first 15 minutes of the treatment trial.

Only data collected from males and gravid females were included in the analysis. Male *E. crossopterum* were identified by secondary sexual characteristics including their second dorsal fin coloration, dark head, large size and general territorial behaviors. Gravid females were identified as smaller, lighter in coloration with an enlarged belly. After hormone collection, the gravid females were placed into a tank with nest-guarding males. Some females spawned with these males, however, the females which did not spawn, were sacrificed and dissected to confirm their sex. The ovaries of the dissected females were classified as “mature” “ripening” or ‘ripe” (Heins and Baker 1993). Dissections were also used to confirm the sex of smaller males. Only females that were considered “ripe” were included in this analysis.

Hormone extraction

Hormones were collected from water by drawing 400 mL of holding water through C-18 cartridges (Sep-Pak, Waters Technology Corporation, Milford, Massachusetts) using a 24 port vacuum manifold (VWR, International CITY) . All water was processed at 15 units. All cartridges were primed with two 2 mL washes of 100% methanol and washed after water collection with two 2 mL washes of distilled water. Cartridges were frozen at -80 C for storage prior to assays.

Free hormones were extracted from the cartridges using two, 2 mL washes with ethyl acetate. Following this extraction, two, 2 mL washes with 100 % methanol were added to clean test tubes to elucidate out conjugated steroids. Conjugated steroids were not addressed in this study. Samples were dried using a nitrogen manifold and re-dissolved in 400 mL assay buffer. Prepackaged Enzyme Immunoassay (EIA) kits (Cayman Chemical, Ann Arbor, Michigan) were used to quantify hormone concentrations. Kits were run in accordance with the directions provided with the kit. Levels of testosterone, estradiol, and cortisol were investigated in both male and gravid female samples and 11-keto-testosterone levels were investigated in samples from males. Plates were read at 410 nm using a plate spectrometer.

After the samples were run two samples were excluded as outliers, one sample was from a female that jumped out of the collection tank before the courtship treatment and the other was from a male that was suspected to be sick, due to his lack of reaction to the net after the trials. Since there was a wide variation in size of individuals involved in this study, especially for males, the samples were presented as pg/g. Intra-assay variation and inter-assay variation was assessed by using a pooled darter sample in the first and last duplicate wells of the plates. The average inter-assay and intra-assay coefficient of variation were calculated to be 21.36% and 9.69% respectively.

The kits were validated to be used with darter samples, using a pooled sample comprised of 100µL samples taken from 15 different darter samples. Parallelism was achieved in which the standard curve provided with the kit did not differ from a serial dilution of the pooled sample for each of the hormones tested (11-Ketotestosterone: t=0.0086, p=0.9933; Cortisol: t=0.0086, p=0.9923; Estradiol: t=0.009, p=0.9928; Testosterone: t=0.278, p=0.7857).

Plasma validation

Many studies have already shown that the free steroid hormones that are collected from water using this methodology are representative of levels of hormones present in the plasma of the subject fish (Ellis et al. 2004; Wong et al. 2008). However, since this protocol has never been used on a species of darter, an additional objective of this study was to validate this methodology in this species. To assess the relationship between plasma and water collection methods, ten female and 10 male *E. crossopterum* darters were placed into 400 mL distilled water for 1 hour. After the hour, plasma was collected from each fish through the caudal vein of the fish. The plasma samples kept at -80 C until they were run using the same protocol that was used for the water samples. A Pearson’s correlation was done to investigate the relationship between the level of hormone present in the plasma and water hormone samples taken from each fish.

Statistics

Multiple, repeated measures analysis of variance were conducted to determine the difference between the levels of hormones collected during the control and treatment trials. Each hormone, sex and treatment was examined separately. An additional analysis was conducted to assess the effect of time spent in the laboratory environment on cortisol level, however, it was found that there was no significant relationship between baseline cortisol levels and the number of days spent in the lab (*r*=-0.259, p=0.232). Therefore these data were not included as a covariate in this analysis.

Results

Courtship Playback Vocalizations

Playbacks of male courtship sounds caused significant decrease in the level of testosterone (N = 10, df=1, F=9.937, p=0.012) (Fig. 5-2) and 11-ketotestosterone (N=6, df=1, F=6.721, p=0.049) (Fig. 5-3) in male darters. The courtship playbacks also caused a significant decrease in the levels of estradiol detected in samples from female darters (N=9, df=1, F=9.731, p=0.014) (Fig. 5-4). Although there appeared to be a drop in testosterone levels of female *E. crossopterum* when they were exposed to courtship sounds this difference was not significant (N=12, df=1, F=2.641, p=0.132). The courtship playback also did not cause a significant difference in levels of estradiol (N=10, df=1, F=1.428, p=0.263) in males or levels of cortisol in either sex (Males: N=5, df= 1, F=0.141, p=0.726; Females: N=7, df=1, F=0.446, p=0.529).

Aggressive Playback Vocalization

Aggressive playback vocalizations caused a marginally significant change in levels of estradiol in female darters (N=7, df=1, F=4.662, p=0.074). However, females who were exposed to aggressive vocalizations did not show a difference in the levels of cortisol (N=7, df=1, F=0.516, p=0.500) or testosterone (N=8, df=1, F=0.019, p=0.894). Males exposed to aggressive vocalizations did not exhibit significant differences in levels of testosterone (N=8, df=1, F=0.196, p=0.671), estradiol (N=6, df=1, F=1.172, p=0.328) 11-ketotestosterone (N=3, df=1, F=1.204, p=0.387), or cortisol (N=5, df=1, F=0.067, p=0.808) when compared to control levels.

Plasma and Water correlations

A Pearson's correlation test was used to determine if there was a significant correlation between levels of hormones found in the plasma and water samples taken from the same individuals. For all hormones measured there was a significant positive relationship between these two measurements (11 Kt: *r*=0.526, p=0.032; Cortisol: *r*=0.741, p=0.004; Estradiol: *r*=0.524, p=0.045; Testosterone: *r*=0.580, p=0.009). These correlations indicate that the water collected from the fish is significantly correlated to the level of hormones present in the plasma of these animals.

Discussion

This study looked at the hormonal response of female and male darters to conspecific vocalizations. Both males and females in this study showed a significant decrease in their respective sex hormones.

Males exhibited a significant decrease in both testosterone and 11-KT after exposure to conspecific courtship vocalizations. 11-Ketotestosterone is considered in many species to be the dominant androgen involved in teleost reproduction (Borg 1991). In the bluegill (*Lepomis macrochirus*) parental males show a marked increase in 11-KT during the spawning period of the reproductive season (Kindler et al 1989). This hormone has also been found to be associated with male coloration in both salmon (Idler et al 1961) and the stoplight parrotfish (*Sparisoma viride*) (Cardwell and Liley 1991). While testosterone is not the main androgen in teleost males, there is evidence that it is essential to reproductive functions such spermatogenesis. A decrease in both of these androgens could indicate a decrease in these reproductive traits in males.

Since these darter vocalizations are used in close contexts (< 1 m), hearing a conspecific vocalization would indicate the presence of another courting male in the immediate area. Male darters, such as *E. crossopterum* readily defend their nests and eggs from intruders (Knouft and Page 2004). Males will aggressively chase other males away from nests and females. Often these interactions will result in injury to one or both of the individuals. The males in the current study were also not territory holders. Some males were presumed to have territories in the field but they were removed from these territories and placed into tanks and moved again the morning of the study. Males who are not defending a territory may seek to avoid interacting with spawning males and thus avoid injuries. This decrease in androgens could indicate an effort to avoid confrontation with the nest guarding male nearby.

Another intriguing possible implication of decreasing 11-ketotestosterone in the current study has to do with male alternative reproductive tactics. Fish species that exhibit alternative male reproductive tactics are characterized by having one nest holding male (or type I male) who establishes a territory and courts females (Gross and Charnov 1980; Brantley and Bass 1994). There is also a smaller male type (or type II male) which sneaks fertilizations from the nest guarding male. Often these smaller males will look or act like females to avoid detection and the aggression of the larger nest guarding male. Recent studies have shown that the nest guarding males have an increased level of 11-KT compared to the smaller alternative morph males (Brantley et al 1993; Kindler et al 1989, Oliveira et al 2001a;b). In the peacock blenny younger, smaller males sneak into nests to gain fertilizations by mimicking female courtship behaviors and coloration. It has been shown in this species that 11-KT inhibits this ‘female-like’ courtship behavior and coloration in sneaker males (Oliveira et al. 2001a).

If these alternative male reproductive strategies exist in *E. crossopterum* the decrease in 11-KT, seen in this study could indicate that males are switching from male courtship behavior to more ‘female-like’ courtship behavior in order to gain access to nests and females for fertilizations. This is most likely to be occurring in smaller males and not necessarily larger nest holding males. The reproductive ecology of darters is very similar to other teleost systems which use these tactics. Male *E. crossopterum* also have a large variation in male size, which is common when alternative reproductive morphs strategies are present. The time course at which such a change occurs should also be investigated.   Although confirming this occurring in *E. crossopterum* would need a lot of further investigation, this is an intriguing area for future research.

This study was also the first to show conspecific male vocalization can cause changes in hormone levels in females. Gravid females had a significant decrease in the sex hormone, estradiol after exposure to male courtship vocalizations. In fish, a drop in both estradiol and testosterone can be seen prior to ovulation (Kagawa et al 1983; Kime 1993; Peter and Yu 1997). This could indicate that male vocalizations act as a priming mechanism which prepares a gravid female to spawn. Future studies in this area should include an investigation into the change in progesterone levels in female fish responding to vocalization playbacks. The idea of an ovulation priming mechanism is not novel in fish (Kobayashi et al. 2002), however, if this is what is occurring here, this would be the first acoustic example of this in fishes.

The ability of male vocalizations to act as an ovulation priming mechanism may be supported by the reproductive behaviors of males during spawning. Often during a spawning bout there are non-spawning, gravid females present under or in close proximately to the nesting cavity (Field and laboratory *personal observations*). Despite a risk of egg predation by non-spawning females, spawning males generally tolerate these females and do not chase them off. The male is also frequently vocalizing while spawning (Johnston and Johnson 2000) despite risks, such as attracting other males and/or predators. This may imply that the males are receiving some benefit from vocalizing and from females being present under the nest. If a male could prime a female for ovulation it may increase the chance that that female would spawn with him and increase his overall reproductive success.

Further research is needed to confirm that vocalizations could be used as a priming mechanism in females. It would be interesting to include in these studies an investigation into progesterone levels of these females. Progesterone levels in female salmon spike after the drop in estradiol and testosterone is observed prior to ovulation (Kime 1993).

An alternative theory for why these females are exhibiting a decrease in estradiol is that, similar to the males in this study, the females perceive the male courtship vocalizations as a threat. Although males are generally tolerant of a female presence under the nest, male aggression towards non-spawning females is not non-existent.

Playbacks of aggressive vocalizations did not cause a change in hormone levels in males or females. This lack of response could be due to an inability to interpret the signal, or the subjects may be disregarding the signal. One reason that these individuals may be disregarding an aggressive signal may be a lack of a visual presence of other males. *Etheostoma crossopterum* spawn under nest cavities where it is often hard to see the spawning pair. This may be why visual signals are not as necessary to obtain a physiological response to courtship and spawning signals but visual cues may be essential to interpreting aggressive signals as a threat.

During this study there was never a significant difference found between cortisol levels during the control and treatment trials. This could be a result of there not being an intense stress response to this collection methodology. Other studies have shown that convict cichlids (*Amatitlania nirgrofasciata*) have an initial stress response to being confined to a beaker, however this response leveled off after 3-4 exposures to confinement (Wong et al 2008). Since no significant difference was found between trials in the current study we can assume that there was no increase in stress response associated with the presentation of the sounds. Therefore, the differences this study found in reproductive hormones are not the result of an interaction between the stress and reproductive hormones.

Due to the constraints of their shallow water environment darters use acoustic communication solely for the purposes of close range interactions. This study has found that the physiological response of individuals to these vocalizations reflects this ultimate function of these signals. These responses are different than those seen in other studies involving long range communication. This study was the first to show that female teleost fish have a hormonal response to conspecific sounds. This study also found that male darter vocalizations had an inhibitory effect on the androgen levels of nearby conspecific males. This response is most likely due to these males responding to the close and immanent presence of a spawning, territorial male. These signals therefore could serve a dual purpose of priming females for reproduction while deterring nearby males from entering their territory. This study highlights the importance of considering communication as not only an interaction between two individuals but as a network in which there can be many unintended receivers.

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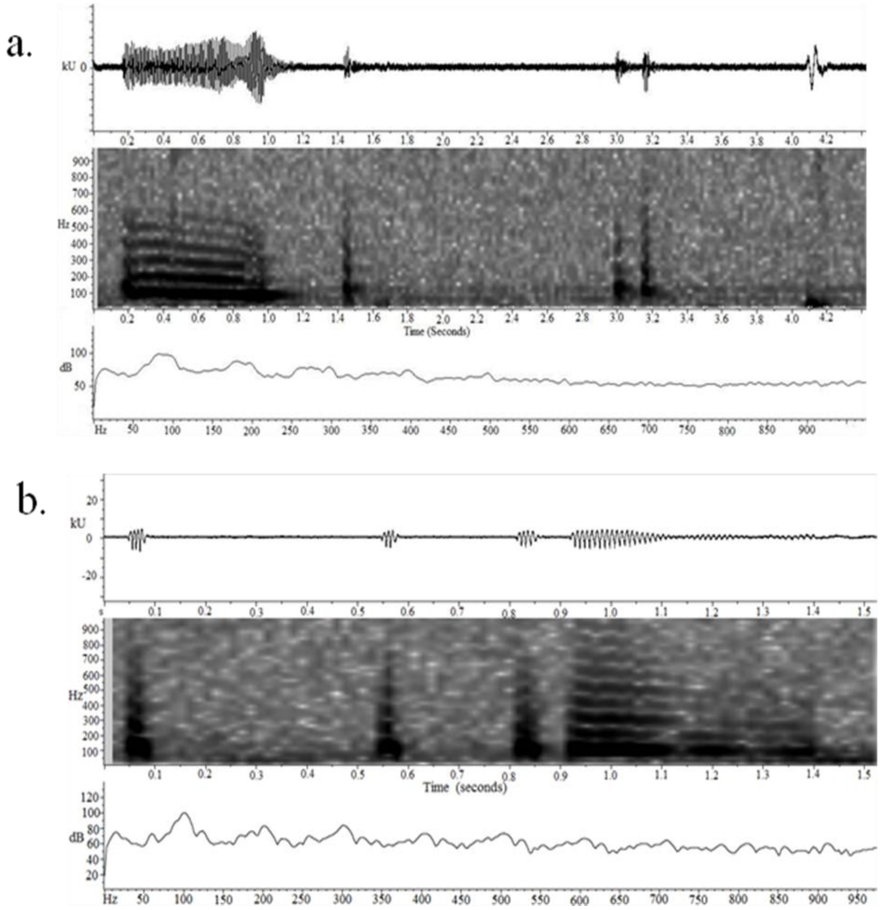

Fig. 5-1: The waveform, spectrogram and power spectrum of the a. courtship and b. aggressive vocalizations used for the playback experiments. Each contained a drum vocalizations and a series of knocks (Spectrogram: Hanning window, FFT=1981, bandwidth 32 Hz; Power spectra: Hanning window, FFT=10000, bandwidth 6.34 Hz)

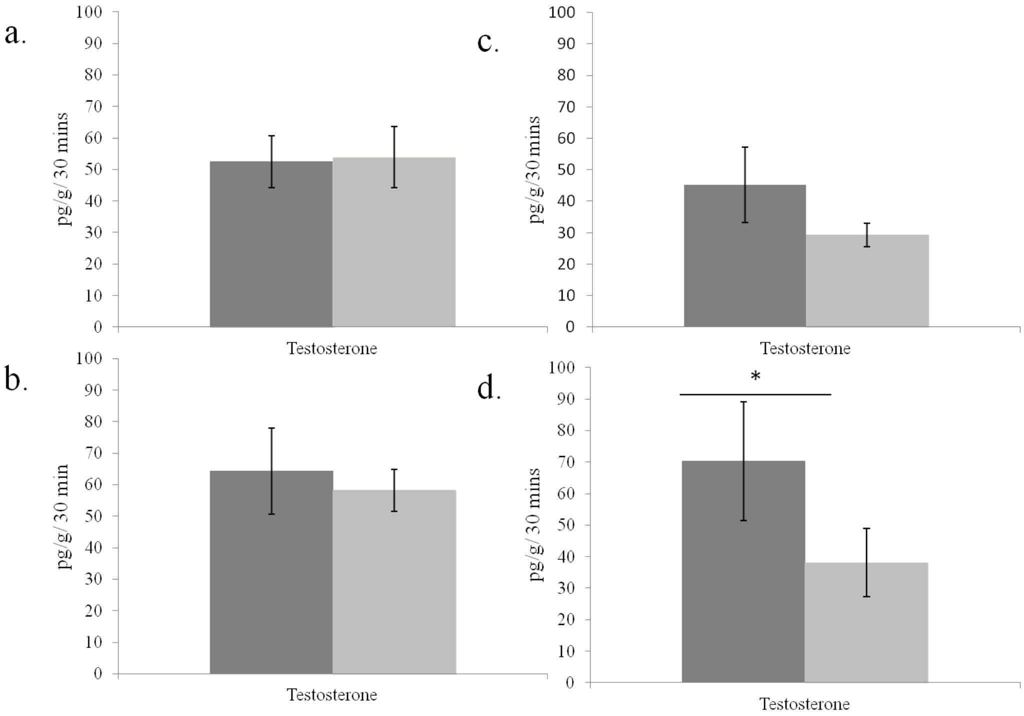

Fig. 5-2: The change in hormone levels of testosterone for female *Etheostoma crossopterum* to (a) aggressive vocalizations and (b) courtship vocalizations. There was no significant difference between the levels of testosterone for males exposed to control silence and aggressive vocalizations (c) however, there was a difference between the control silence and the exposure to courtship vocalizations.

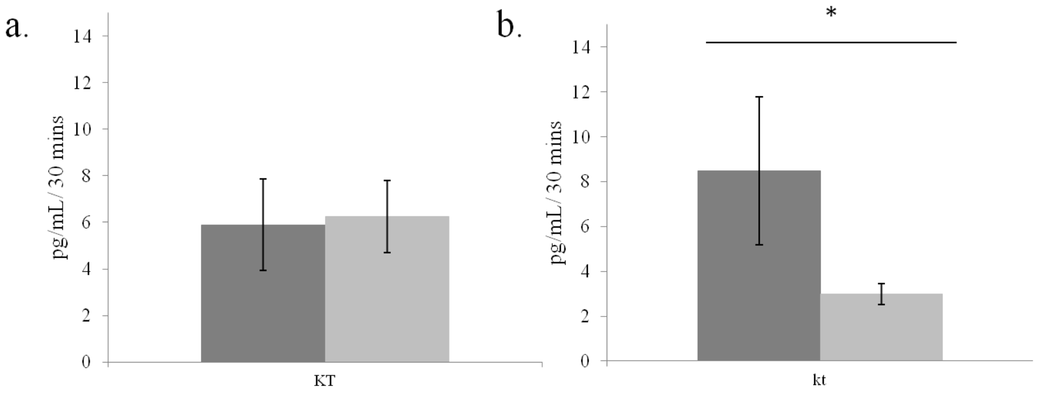

Fig 5-3: The levels of 11-ketotesosterone of male fish during controlled silence and (a) aggressive vocalizations and (b) courtship vocalizations.

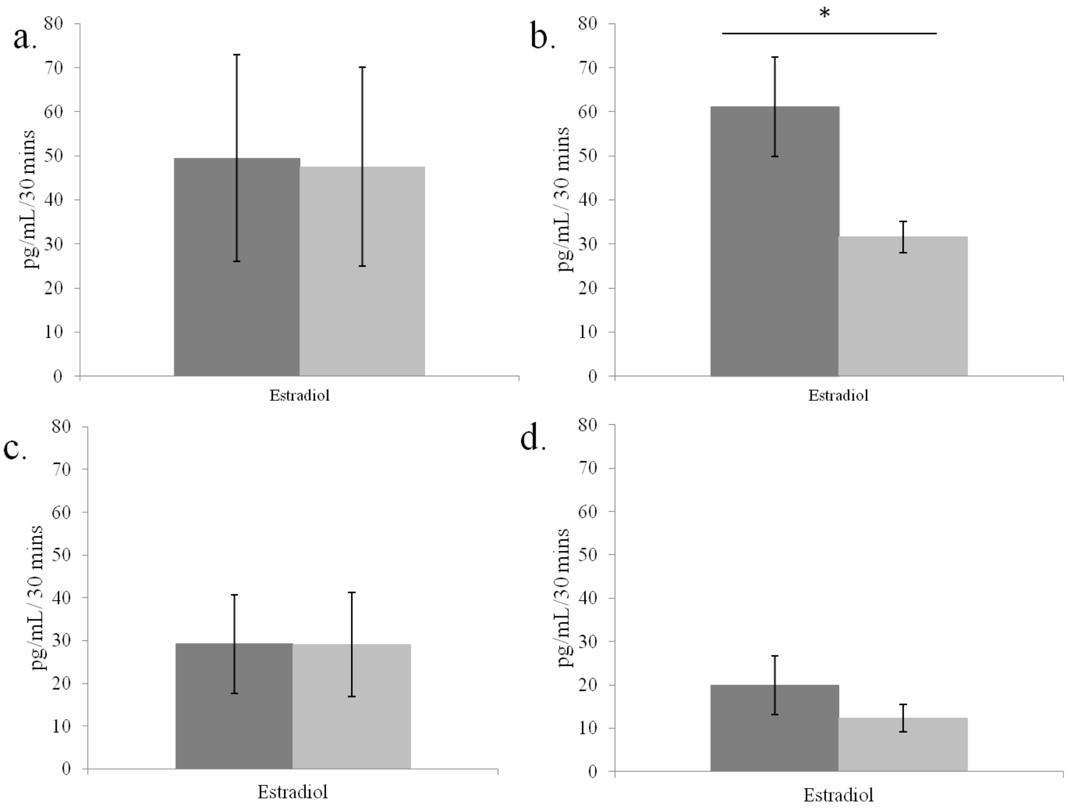

Fig. 5-4: The response of gravid females to (a) aggressive vocalizations and (b) courtship vocalizations. Females had significantly lower levels of estradiol after exposure to courtship vocalizations then to the control silence. The response of males to (c) aggressive playbacks and (d) courtship playbacks.

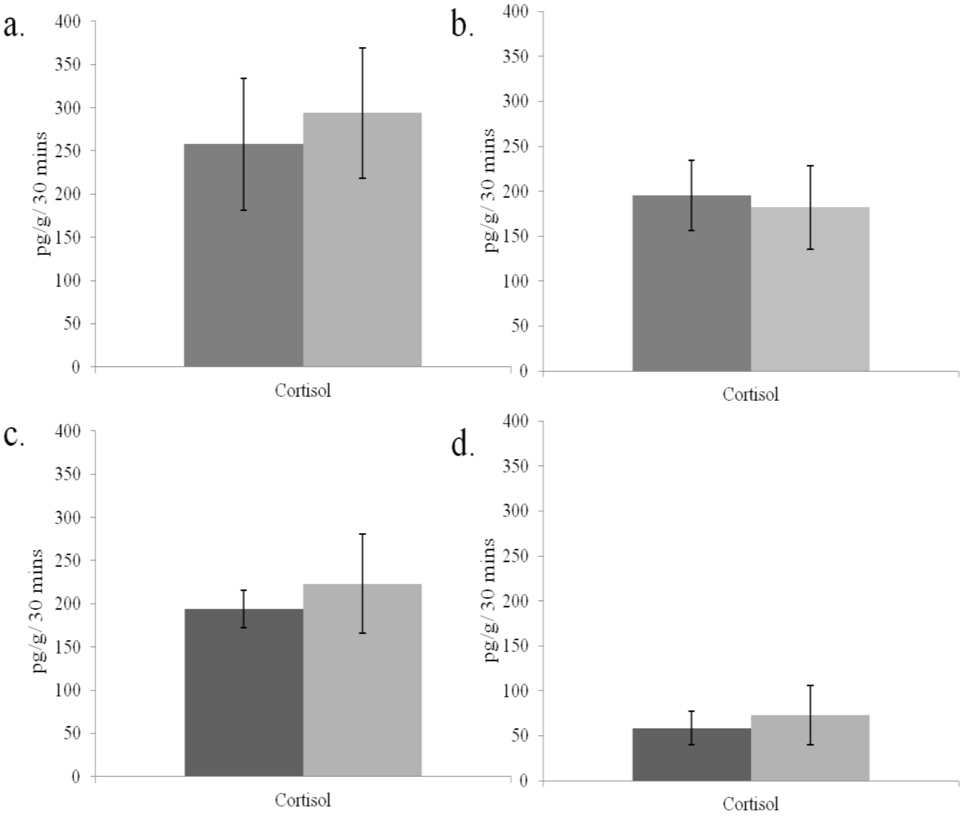

Fig 5-5: There was no significant difference between the cortisol levels of fish when they were exposed to a control silence or courtship vocalization for gravid females (a) or males (d). There was also no difference between silence and aggressive vocalizations for gravid females (b) or males (c).

Conclusions

This paper looked at reproductive behaviors and acoustic communication in the fringe darter (*Etheostoma crossopterum*) and the lollypop darter (*Etheostoma neopterum*). Previous studies had shown that acoustic communication was used by *E. crossopterum* (Johnston and Johnson 2000) however this study was the first documentation of sound production in *E. neopterum.* Prior to this study it was also unknown how or if these vocalizations were being used in the field because it has been documented that shallow streams are not an optimal environment for acoustic communication (Forrest et al 1993). This study found that the slow moving habitat preferred by *E. neopterum* has an overall low amount of ambient noise, including the range of the dominant frequency of *E. neopterum* vocalizations. The hearing frequencies of maximum sensitivity in *E. neopterum* were also found to coincide with the dominant frequency of *E. neopterum* vocalizations. As predicted by theoretical studies (Mann 2006) the propagation distance of male darter vocalizations was limited to less than 1 meter. Despite this limitation these vocalizations are likely to be used for short distance communication in darters. The perception of these signals appears to be aided by the hearing sensitivity and the ambient noise environment of these darters.

This paper also used field studies to further understand both inter and intra-sexual interactions in *E. neopterum.* This study found evidence that females may prefer to spawn with larger males based on the number of eggs found in artificial nests. Over the course of a reproductive season there was a decrease in the average condition and size found for a random selection of male individuals in the *Etheostoma neopterum* population. This could be an indication of a high energetic demand on nest guarding males over the reproductive season, and possibly a higher mortality rate among larger males. This high energetic demand in males supports to use of behaviors such as allopaternal care and filial cannibalism, which were also documented to occur in *E. neopterum* for the first time in this study. The presence of allopaternal care is especially interesting in this species, because previously all interactions between males were considered to be aggressive interactions. However, the relationship between an allopaternal male and a guarding parental male represents a mutuality positive relationship.

Finally this paper is the first documentation of conspecific vocalizations causing hormone modulation in darters. This study found that both males and female *E. crossopterum* exhibited a decrease in their respective sex hormones. This is the opposite of what has been seen in previous studies (Remage Healey and Bass 2005; Maney et al 2007). However, darter vocalizations may be a novel signaling environments compared to previous studies because these signals are meant for close distance communication and not advertisement. The decrease in androgens (11-ketotestosterone and testosterone) seen in males may be a result of the subject males not being territory holders and therefore seeking to avoid confrontation. The decrease in estradiol seen in females could be the result of acoustic vocalizations being a social cue to promote ovulation, however, further research into this finding is needed.

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