**The Effects of Anthropogenic Traffic Noise on Auditory and Endocrine Function in the Blacktail Shiner, *Cyprinella venusta***

by

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Abstract

Acoustic signals are a vital component of important behaviors such as courtship, foraging, parental care, and aggression. Acoustic communication is a modality utilized by a plethora of animals, including fishes. Recent work demonstrates that many freshwater fishes are soniferous and produce context specific vocalizations. Despite the high volume of studies documenting the behavioral significance of sound production in fishes, few papers examine the physiological outcome of acoustic communication networks. This dissertation will be the first to investigate the acoustic modulation of gonadal hormones in both male and female freshwater stream cyprinids belonging to the genus *Cyprinella*. Waterborne levels of estradiol and Prostaglandin F2-alpha will be measured in female Blacktail Shiners (*Cyprinella venusta*) in response to courtship signals. Waterborne levels of 11-ketotestosterone will be measured in male *C. venusta* in response to agonistic signals. The subsequent chapters of this dissertation will examine an often-ignored pollutant on freshwater stream fishes: anthropogenic traffic noise. Current studies show that noise from bridge crossings propagates significantly into freshwater streams. The second chapter of this dissertation will be the first to examine *C. venusta* auditory sensitivity and stress responsiveness when exposed to ecologically relevant levels of bridge traffic. The final chapter will be an amalgamation of the previous two chapters. Waterborne gonadal hormones will be measured in male and female *C. venusta* when exposed to acoustic signals masked with traffic noise. Collectively, these studies will provide insight into the physiology of acoustic signaling and how it is disrupted by anthropogenic noise pollution in stream fishes.

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## Introduction

Animal communication networks comprise multisensory signals, and many taxa communicate via acoustic signals (Zelick et al. 1999). Acoustic signals accompany critical behaviors such as courtship and agonistic displays (Moser-Purdy et al. 2017; Crocker-Buta, S.P. and Leary, C.J. 2018). Recent literature shows that freshwater fishes produce context dependent acoustic signals, similar to other well studied vocal animals (Phillips and Johnston 2008; Holt and Johnston 2014; Goll et al. 2017). Currently, there are approximately 800 soniferous fish species, and the majority of these species belong to the superorder Ostariophysi. Ostariophysans possess ancillary hearing adaptations that result in increased auditory sensitivity relative to other fishes; they are also an excellent model for investigating acoustic communication in fishes (Nelson 2006).

Current research has meticulously examined the behavioral significance of sound production in fishes. Males in the genus *Cyprinella*, for example, produce female directed vocalizations during courtship and spawning behaviors (Phillips and Johnston 2008; Holt and Johnston 2014). It is suggested that male vocalizations promote spawning behaviors in females – potentially synchronizing gamete release during spawning (Amorim et al. 2003). Few studies have addressed the endocrinological aspect of these behavioral interactions; however, several papers documented the modulation of androgens in male plainfin midshipmen exposed to conspecific signals (Remage-Healey and Bass 2005). To date, no research has addressed the hormonal changes of female fish in response to male acoustic cues.

One challenge faced by nearly all vocal animals is anthropogenic noise. As humans continue to urbanize the natural world, noise pollution will become more prevalent. In many instances, there is significant spectral overlap between anthropogenic noise and acoustic signals,

thereby masking communication between conspecifics (Warren et al. 2006). Masking has been shown to diminish parental care in avian models and delay maturity in orthopterans (Lucass et al. 2016; Gurule-Small and Tinghitella 2019). Urbanized animal populations exposed to chronic levels of noise experience reduced auditory sensitivity and elevated levels of cortisol (Neo et al. 2104; Shannon et al. 2014). While several species, including some fishes, have demonstrated phenotypic plasticity by changing vocalization frequencies, there are often costs on a prolonged timescale (Schroeder et al. 2012; Holt and Johnston 2014).

To better understand acoustic signaling in fishes, and how it may be disrupted by anthropogenic noise, several studies using the Blacktail Shiner (*Cyprinella venusta*) will be conducted. The acoustic behavior of *C. venusta* has already been described. Males produce two distinct vocalizations during the mating season. Growls are directed toward females during courtship and spawning. Knocks are produced while chasing other males away from breeding territories (Holt and Johnston 2014). *C. venusta* is also commonly found in streams impacted by anthropogenic noise; particularly by traffic noise propagating from bridge crossings (Holt and Johnston 2015).

The objectives of this dissertation are threefold. The first chapter will focus on the acoustic modulation of reproductive hormones in *C. venusta* when exposed to acoustic signals. Waterborne levels of estradiol ($E_2$) and prostaglandin $F2_\alpha$ ($PGF2_\alpha$) will be measured in females exposed to growl signals. Both of these hormones are crucial during courtship and spawning behaviors (). Waterborne 11-ketotestosterone (11KT) will be measured in males exposed to knock signals. Chapter two will investigate the effects of traffic noise on auditory sensitivity and cortisol in this species. The third chapter will measure $E_2$ in females and 11KT in males exposed

to acoustic signals masked by traffic noise. Collectively, these studies will provide novel insight into fish communication networks.

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# Chapter 1

## Acoustic Modulation of Reproductive Hormones in the Blacktail Shiner (*Cyprinella venusta*), a Soniferous Cyprinid


### Abstract

Animal communication networks consist of multisensory signals and often include an acoustic component. A large number of teleost fishes, including the blacktail shiner (*Cyprinella venusta*), produce specific vocalizations during courtship and agonistic behaviors. To investigate the role of acoustic signaling in breeding soniferous fishes in a physiological context, we conducted a series of playback trials. We measured waterborne levels of estradiol ($E_2$) and prostaglandin $F2_\alpha$ ($PGF2_\alpha$) in gravid females exposed to courtship calls, and levels of 11-ketotestosterone (11KT) in males exposed to agonistic calls. Hormone levels were quantified using enzyme-linked immunosorbent assays (ELISA) and analyzed using mixed effects linear models. Gravid females exhibited a substantial drop in $E_2$ and an increase in $PGF2_\alpha$ during courtship calls. Males exhibited a small, but statistically insignificant increase in 11KT during agonistic playback. Our research suggests that growls contribute to ovulatory and spawning behaviors in female *C. venusta*. Future work should consider the role of multimodal signals in teleost communication.


### Introduction

Animal communication networks frequently consist of signals sent via multiple sensory channels, and for many species, there is an acoustic component. Auditory signals transmit information that influences the behavioral response of the receivers, including: mate choice, foraging, territoriality, and parental care (Higham and Hebets 2013). These behavioral shifts are accompanied by fluctuations of numerous physiological parameters, including hormones. (Lynch and Wilczynksi, 2005; Adreani et al., 2018). In vocal territorial species, males increase

countersinging in response to vocalizations from neighboring males, coinciding with increased levels of circulating androgens (Goll et al. 2017; Moser-Purdy et al. 2017). Within the context of courtship, acoustic signals inform potential mates about the location and quality of the sender (Amorim et al. 2015; Knörnschild et al. 2017).

At a physiological level, these vocalizations have the capacity to moderate the gonadal hormones. In two species anurans, for example, females exhibit elevated levels of estradiol ($E_2$) after exposure to male chorus playback (Lynch and Wilczynksi, 2005; Crocker-Buta and Leary, 2018). The acoustic modulation of reproductive hormones is suggested to synchronize male and female mating behaviors, thus increasing the probability of successful fertilization, especially in species utilizing external fertilization (Lynch and Wilczynksi, 2005). Conducting similar studies with a broader array of taxa will provide valuable insight into the significance of acoustic signaling in animal communication networks.

The relationship between acoustic signaling and hormone modulation in teleost fishes is not well understood. Taxonomically, teleosts comprise nearly one third of all living vertebrates. Thus far, there are over 800 soniferous species (Friedman, 2010; Fine and Parmentier, 2015). Fish produce context specific acoustic signals that facilitate reproductive and agonistic interactions (Vasconcelos et al., 2012; Verzijden et al., 2010; Simões et al., 2008; Amorim and Neves, 2008). Meticulous work has identified the behavioral context of sound production in fishes, yet comparatively few papers address the hormonal response of the receiver. Existing research on the acoustic modulation of hormones in fishes skews toward the male response of a limited number of species (Remage-Healey and Bass, 2005). Furthermore, these species do not represent the full diversity of life history strategies exhibited by soniferous teleosts. Measuring

the hormonal response of male and female receivers in response to acoustic stimuli provides novel insight into teleost behavior and physiology.

In male teleosts, 11-ketotestosterone (11KT) is the primary androgen. This hormone is responsible for the development of sexually dimorphic traits (Borg, 1994). During agonistic interactions, 11KT levels fluctuate rapidly (Remage-Healey and Bass, 2005; Maruska, 2014). In females, $E_2$ promotes oocyte vitellogenesis (Joy and Chaube, 2015). During ovulation, oocytes also stimulate the production of prostaglandin $F2_\alpha$ ($PGF2_\alpha$). $PGF2_\alpha$ belongs to a class of lipid compounds that function similarly to hormones; in female teleosts, it acts on the brain, inducing reproductive physiology and behaviors (Sorenson et al., 1995). Released into the water via urine, $PGF2_\alpha$ functions as a potent pheromone, stimulating male courtship behaviors and endocrine responses (Kidd et al., 2012). To our knowledge, no studies have explored the acoustic modulation of $E_2$ and $PGF2_\alpha$ in female teleosts.

To investigate the hormonal outcome from acoustic signaling in a novel teleost species, we measured the change in waterborne hormones in male and female blacktail shiners (*Cyprinella venusta*) during acoustic playback trials. Our primary interest was to monitor acute fluctuations within individual fish; thus we implemented a repeated measures design. In teleosts, hormones diffuse passively across the gills, and waterborne levels of hormone reflect the levels circulating within the individual (Friesen et al., 2012; Ellis et al., 2005). For small-bodied fishes, this is an invaluable technique allowing repeated measures when other methods would be lethal. Fish in the genus *Cyprinella* are ideal models for investigating acoustic communication in fishes; all species in this genus are soniferous (Phillips and Johnston 2009). Sound production and associated behaviors in *C. venusta* have already been documented. Males produce two distinct signals during the mating season: knocks and growls. *C. venusta* spawn in the crevices of

submerged vegetation and bedrock in freshwater streams; dominant males will defend these crevices from intruders, including other males. Knocks are directed toward intruders and correlated with male aggressive behaviors. We exposed mature male *C. venusta* to knock playback and measured changes in 11KT. Growls are produced during both courtship and spawning behaviors (Holt and Johnston, 2014). We hypothesize that growls aid to synchronize mating behaviors in male and female *C. venusta*. Thus, we measured changes in $E_2$ and $PGF2_{\alpha}$in gravid females exposed to growl playback.

**Materials**

**Fish Collection and Maintenance:**

We collected *C. venusta* from Chewacla Creek (32.539166ºN; -85.4966763ºW) in Lee County, Alabama using a 3 m seine net. Sexually receptive males were identified by the presence of secondary sexual characteristics: yellow fins, white fin tips, and breeding tubercles. Potentially gravid females were identified by the presence of a distended abdomen and a clear absence of male traits. Subjects were transported to the Fish Biodiversity Lab at Auburn University, Alabama in coolers containing creek water; aerators were placed in each cooler during transit. Fish were housed in established 75 L aerated tanks containing substrate and java ferns at low densities (< 10 fish $tank^{-1}$). We fed fish bloodworms (*Chironomid spp.*) ad libitum daily. Fish were maintained on a 14:10 light to dark cycle to simulate a seasonally appropriate photoperiod. Water temperatures were maintained between 21 and 23ºC, corresponding to creek temperatures. The Institutional Animal Use and Care Committee at Auburn University approved all experimental procedures (permit number 2017 – 3079).

**Acoustic Playback Calibration:**

The acoustic signals used for this study were previously recorded as part of another project describing sound production in *C. venusta* (see Holt and Johnston, 2014). We selected a subset of the cleanest growl and knock signals to reduce signal degradation during playback (fig. 4). The sound pressure levels (SPL) of each signal were calibrated in a 200 L glass test tank filled with 65 L of water. To ensure growl and knock signals were calibrated to appropriate sound levels in this test tank, we placed a hydrophone (Hi-tech HTI-96-MIN, sensitivity- 164.4 re 1V/µPa, frequency response: 0.002–30 kHz) inside a 700 ml glass collection dish containing 450 ml of water. This hydrophone was connected to a Marantz digital recorder. The collection dish and hydrophone were placed in the 200 L tank 5.5 cm away from an underwater speaker (UW – 30, Universal Sound Inc., Oklahoma City, OK). We played growls and knocks from a Mac Book Pro using Raven Pro 1.4 software (Cornell University, Ithaca, NY) connected to an amplifier. Signals emitted from the underwater speaker were recorded with the hydrophone. We analyzed the recorded signals using Raven 1.4 software to determine the appropriate playback volume. For a complete description of the methods used to calibrate acoustic signals see Crovo et al. 2015. Growls were calibrated to an SPL of approximately 80 dB, and nocks were calibrated to100 dB. These SPL levels reflect the values recorded from male *C. venusta* in the field (Holt and Johnston, 2014). Naturally, conducting bioacoustics research under laboratory conditions has challenges. Glass tank walls alter the resonance and reverberation of acoustic signals. For our experimental design, the minimum resonant frequency that would create signal distortion was 2, 230 Hz. This frequency is beyond the auditory sensitivity of our model species (Akamatsu 2002; Holt and Johnston 2014).

**Acoustic Playback and Hormone Collection**

We tested fish between 5 and 7am during the months of May and July 2016 – 2017. This test period is ecologically relevant because *C. venusta* spawns most actively during the predawn hours from early to mid-summer. To minimize handling stress before each trial, fish were quickly captured with a clean aquarium net and immediately placed into a small opaque bucket containing aged water. We transported the bucket to the test room and placed the fish into a clean 700 ml glass collection dish containing 450 ml of water; this dish was placed 5.5 cm away from the underwater speaker. Collection dishes were large enough to allow fish free movement. The water used for hormone collection was taken from a designated tank maintained at housing conditions; this tank never contained fish. The tank and filter components were cleaned with 100% ethanol and distilled water and allowed to air dry prior to conducting the study. The time between initial capture and placing the fish into the test tank did not exceed 60 seconds. Furthermore, test subjects for the day were collected from separate tanks to eliminate stress from repeated capture attempts. No additional activities were conducted in the lab during the test period. We measured waterborne levels of $E_2$ ($n$ = 12), $PGF_{2\alpha}$ ($n$ = 6) and 11KT ($n$ = 9) for this study. Glassware and nets were washed with 100 % ethanol and allowed to dry between uses. Fish were tested within one week of collection.

Subjects were given two consecutive treatments. Males and females were first administered a baseline trial consisting of 30 minutes of silence. Acoustic stimuli were not played from the speaker during this period. While there were background levels of noise inherently present in the test room; the sound levels were below the hearing threshold of *C. venusta* (Crovo et al., 2015). After the baseline trial, fish were immediately transferred to a new collection dish containing clean water and returned to the 200 L tank. Male subjects received a knock playback; females received a growl playback. Each acoustic segment was approximately

10 seconds in duration and was followed by a 10 second period of silence; this was looped for 30 minutes. A subset of male fish were exposed to recordings substituting white noise calibrated to 100 dB for comparison. We were unable to expose female *C. venusta* to white noise due to our limited number of individuals. After testing, fish were euthanized with an overdose of MS-222 (Tricaine methanesulfonate). Females were dissected to determine the stage of oocyte maturation. We only included females with mature ovaries in subsequent analyses.

At the end of each trial, we filtered raw water samples through cellulose filters (Whatman Filter Papers, Grade 2) to remove any coarse particulates. Water samples were then filtered through C18 cartridges (Sep-pak, Waters Technology Corporation, Milford, MA) primed with 4ml of 100% ethanol followed by 4ml of distilled water. Cartridges were covered with parafilm on both ends and stored at -83 ºC until extraction.

**Hormone Extraction and Analysis:**

Prior to extraction, we thawed the cartridges at room temperature for 30 minutes. We extracted hormones from cartridges (Sep-pak, Waters Technology Corporation, Milford, MA) using an extraction manifold (Waters Technology Corporation, Milford, MA) connected to a Rocker 300 vacuum pump (Rocker Scientific Co., Ltd.). Cartridges were first washed with two 2 ml aliquots of ultrapure water. Hormones were extracted using two 2 ml aliquots of ethyl acetate. Samples were placed in a dry bath and evaporated under a stream of nitrogen gas. All of the hormone samples were analyzed using ELISA kits (Cayman Chemical, Ann Arbor, MI). The dried residues were reconstituted with 500 µl ELISA buffer.

Serial dilutions of pooled *C. venusta* samples were completed for each hormone tested to determine the appropriate dilution factor. Additionally, each kit was validated for use with this species by achieving parallelism between serially diluted samples of pooled *C. venusta* hormones

and the standard curve provided with the kit. The intra assay variation for the ELISA plates were: 1.0% for 11KT; 14.2% for $PGF2_{\alpha}$; 7.6% and 8.5% for $E_2$. The inter assay variation for the $E_2$ plates was 11.7%. Inter assay variations for the 11KT and $PGF2_{\alpha}$ plates were not calculated because all of the samples were analyzed on a single plate. We only considered levels of free, unconjugated hormones in this study. All samples were run in duplicate.

**Results**

We used RStudio to complete all statistical tests (RStudio Team, 2015). Data was tested for normality and homoscedasticity using the Shapiro-Wilk and Bartlett's test. We did not find a significant interaction between size and hormone release ($p_{11KT} = 0.51$; $p_{E2} = 0.10$; $p_{PGF2\alpha} = 0.136$). A linear mixed-effects model was used to analyze changes in hormone levels to account for the nested data structure using the R package 'lme4' (Bates, 2015). Mean $E_2$ levels ($n = 12$) decreased significantly from 219.6 to 194.5 pg/g fish during the growl playback ($p= 0.035$; $F_{1, 11} = 5.75$). Mean levels of $PGF2_a$ ($n = 6$) increased significantly from 388.3 to 593.6 pg/g fish during the growl playback ($p = 0.018$; $F_{1, 5} = 11.83$). Male 11KT ($n = 9$) levels increased slightly during the knock playback; however, this change was not statistically significant ($p = 0.23$; $F_{1, 8}= 1.61$). The mean 11KT values were 323.4 and 352.2 pg/g fish for the control and knock trials.

**Discussion**

This study is the first to document rapid acoustic modulation of male and female reproductive hormones in a freshwater stream fish. Gravid females exhibited a significant decrease in $E_2$ levels after receiving the growl playback. During oogenesis, $E_2$ levels are elevated in early to mid-vitellogenesis and decline once yolk deposition is completed (Munakata & Kobayashi et al 2010). A decline in $E_2$ during ovulation and spawning is documented in several fishes (Matsuyama et al., 1988; Joy and Chaube, 2015; Pham and Nguyen 2019). The

physiological relevance of reduced post-ovulatory and spawning $E_2$ levels may reflect the steroidogenic shift from inhibiting $E_2$ to stimulating maturation inducing steroid 17$\alpha$, 20$\beta$ dihydroxyprogesterone (Joy and Chaube, 2015). The observed decrease of *C. venusta* $E_2$ levels in response to courtship signals is also consistent with results we collected from soniferous darters in the subgenus *Catonotus* (Speares, 2012).

$PGF2_\alpha$ stimulates labor and maternal behavior in mammals (Widoski and Curtis, 1989). Our results, combined with contemporary literature, suggests $PGF2_\alpha$ has a similar function in teleosts by synchronizing egg release with the appropriate spawning behaviors (Juntti et al., 2016). In recently ovulated goldfish (*Carassius auratus*), which are also in the family Cyprinidae, high concentrations of $PGF2_\alpha$ and its metabolite, 15-keto-prostaglandin F2α (15K-PGF2α), are released in the urine (Appelt and Sorensen, 1999). These compounds act as pheromones in male *C. auratus* and stimulate mating behavior (Stacey and Kyle, 1983). Coordinating male and female gamete release would certainly increases the probability of successful fertilization in this species. Collectively, our research suggests growl acoustic signals contribute to inducing spawning behavior in *C. venusta*. Growls may encourage short term endocrine responses in gravid females; however, multimodal communication including chemical visual cues may be required to fully stimulate sexual behaviors (Delgadillo et al., 2012; Field et al. 2018). In situ*, C. venusta* signals are produced in close proximity to the intended receiver with associated visual behaviors (Holt and Johnston, 2014). Contrary to other soniferous teleosts, such as plainfin midshipmen, *C. venusta* do not produce advertisement calls that propagate long distances (Brantley and Bass, 1994).

While male *C. venusta* showed a modest spike in 11KT during knock playbacks; this differential was not statistically significant. Subject responses exhibited high individual

variation. Similar to growls, knocks are produced in close proximity and attenuate beyond approximately 50 cm (Holt and Johnston, 2015). Current evidence suggests in agonistic contexts, male fish have a stronger response when presented with multimodal rather than unimodal stimuli (Chabrolles et al., 2017; Escobar-Camacho and Carleton, 2015). Given the costs of sustaining elevated levels of androgens, it may be maladaptive to consistently mount aggressive responses with a unimodal signal (Zohdy et al., 2017; Wingfield et al., 2001).

Our study supports the importance of acoustic signaling in *Cyprinella* social behavior; however, it is important to note that we focused on the immediate individual response to a unimodal signal. This work provides a discrete glimpse of hormonal activity within individuals. Future work should examine the role of multimodal stimuli on hormone modulation over longer periods of time in this genus. In a broader context, our results highlight the need to consider species with contrasting life histories to fully appreciate communication in fishes.

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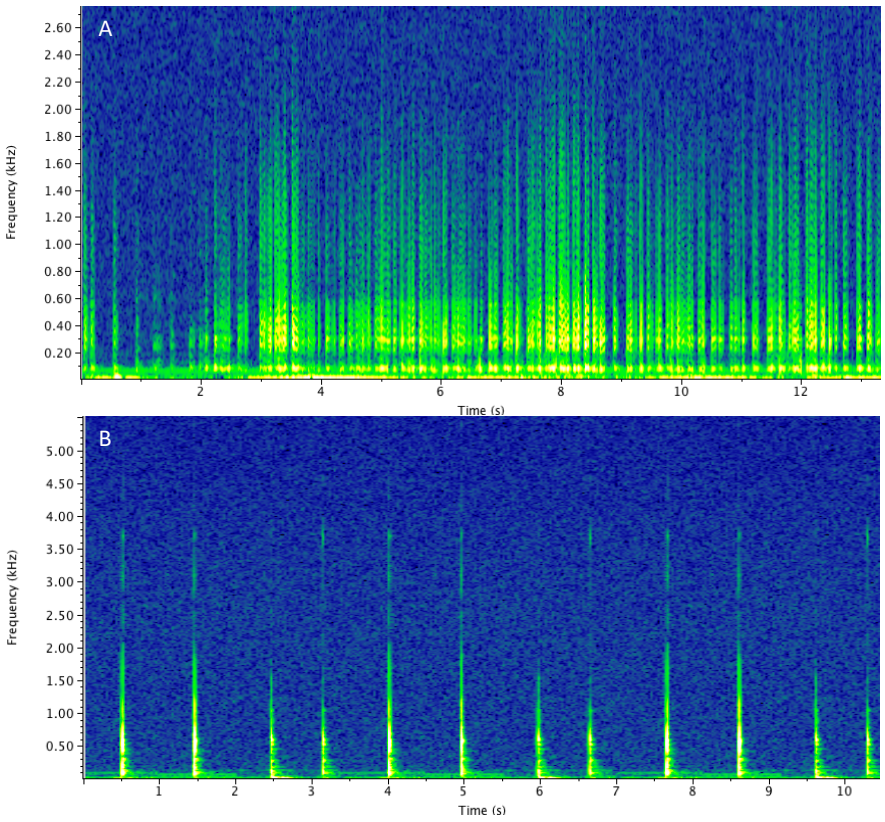


**Fig. 2-1:** Spectrograms of male *C. venusta* Growl and Knock Signals. The courtship growl signal is represented in A; the agonistic knock signal is represented in B.

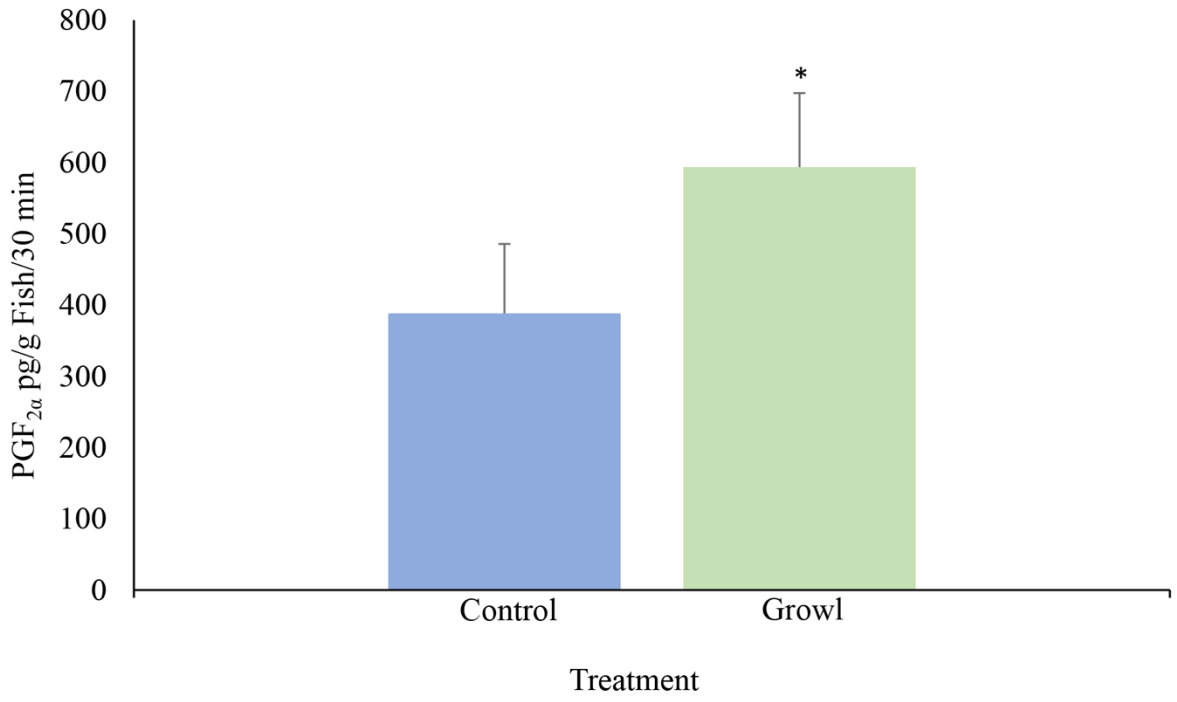


**Fig. 2-2:** Effect of Treatment on PGF2α Levels in Female *C. venusta*. $PGF_{2\alpha}$ levels decreased in females (n = 6) during growl playbacks relative to the control treatment. Data are represented as mean ± SE. An * denotes an alpha criterion of <0.05

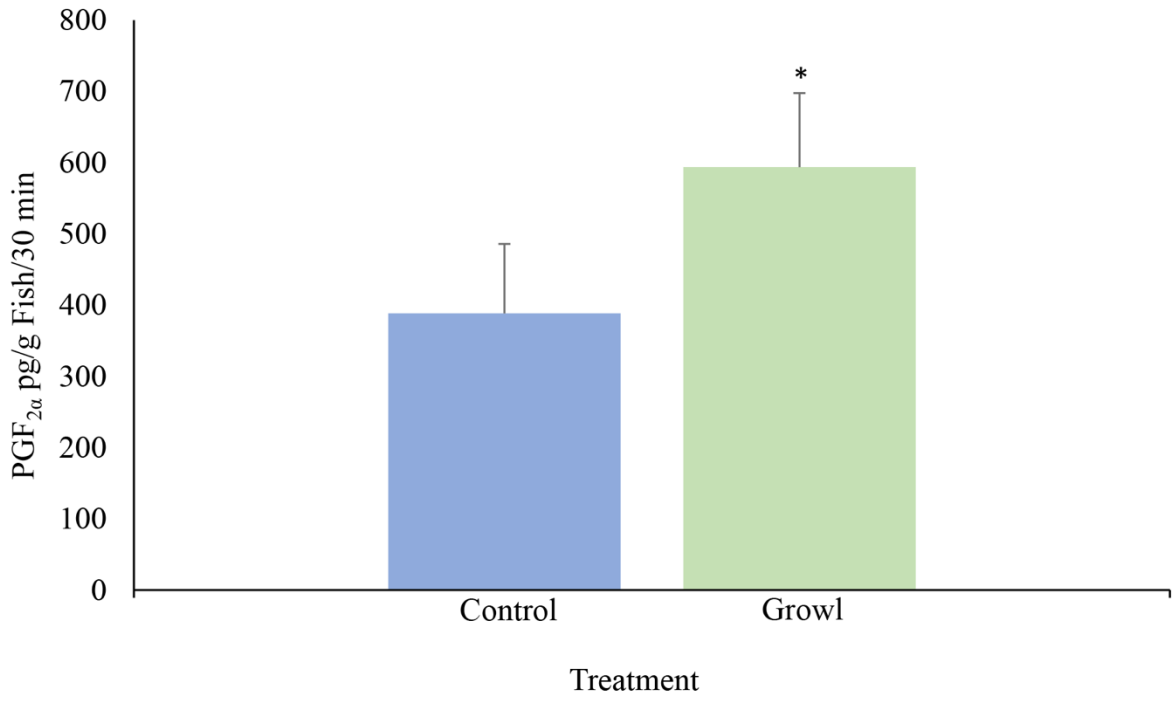


**Fig. 2-3:** Effect of Treatment on Estradiol Levels in Female *C. venusta*. Estradiol levels decreased in females (n = 12) exposed to growl playbacks relative to the control treatment. Data are represented as mean ± SE. An * denotes an alpha criterion of <0.05

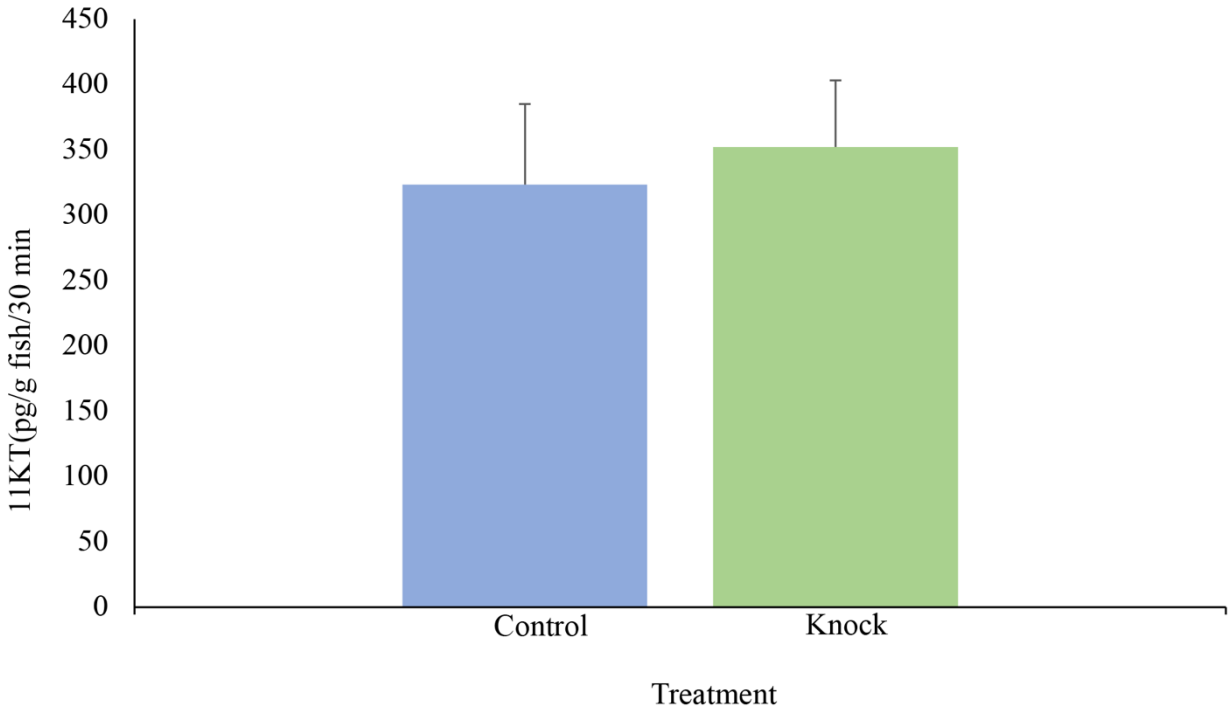


**Fig. 2-4:** 11KT levels increased slightly during knock playbacks relative to the control treatment; this change was not statistically significant. Data are represented as mean ± SE

# Chapter 2

## Stress and Auditory Responses of the Otophysan Fish, *Cyprinella venusta*, to Road Traffic Noise


### Abstract

Noise pollution from anthropogenic sources is an increasingly problematic challenge faced by many taxa, including fishes. Recent studies demonstrate that road traffic noise propagates effectively from bridge crossings into surrounding freshwater ecosystems; yet, its effect on the stress response and auditory function of freshwater stream fishes is unexamined. The blacktail shiner (*Cyprinella venusta*) was used as a model to investigate the degree to which traffic noise impacts stress and hearing in exposed fishes. Fish were exposed to an underwater recording of traffic noise played at approximately 140 dB re 1 μPa. Waterborne cortisol samples were collected and quantified using enzyme immunoassay (EIA). Auditory thresholds were assessed in control and traffic exposed groups by measuring auditory evoked potentials (AEPs). After acute exposure to traffic noise, fish exhibited a significant elevation in cortisol levels. Individuals exposed to 2 hours of traffic noise playback had elevated hearing thresholds at 300 and 400 Hz, corresponding to the most sensitive bandwidth for this species.


### Introduction

Acoustic signaling is an important mode of communication for numerous taxa, including fishes (Amorim et al. 2013; Holt and Johnston 2014). At present, sound production is documented in over 800 species of fish representing 109 families (Kasumyan 2000). In many fishes, sound production accompanies numerous crucial behaviors including: courtship, spawning, agonistic interactions, and competitive feeding (Phillips and Johnston 2008; Amorim and Hawkins 2000; Hawkins and Amorim 2000). Many species utilizing acoustic cues, including

those in the series Otophysi, also possess ancillary hearing adaptations to detect these signals. Otophysan fishes constitute approximately 64 % of all known freshwater fishes (Nelson 2006).

Currently, there is a growing volume of literature documenting the deleterious effects of anthropogenic noise on acoustic communication in the natural world; although one study with birds found no effect of road noise on stress hormones (Wysocki et al. 2006; Schroeder et al. 2012; McIntyre et al. 2014). Noise generated from human-related activities, such as transportation networks, commercial shipping, seismic exploration, resource extraction, and urbanization presents a unique challenge to organisms in both terrestrial and aquatic environments (Vasconcelos et al. 2007; Francis et al. 2011; Kaiser et al. 2015). Anthropogenic noise often has frequencies similar to those utilized by vertebrates that communicate acoustically, effectively masking communication networks (Francies et al. 2011; Holt and Johnston 2015). Furthermore, in contrast to natural sources of ambient noise in the environment, the comparatively recent presence of anthropogenic noise from an expanding human population has not provided adequate time for acoustic signal evolution. In addition to disrupting communication networks, chronic anthropogenic noise has detrimental effects on the physiology and behavior of numerous exposed species (Blickley et al. 2012; Neo et al. 2104; Shannon et al. 2014).

In fishes, noise exposure compromises fitness related behaviors, such as foraging and antipredator responses (Simpson et al. 2015; Purser and Radford 2011). Exposed fishes also experience changes in auditory and neuroendocrine function. Several studies examined the effect of boat noise on the auditory thresholds of fishes (Scholick and Yan 2002; Codarin et al. 2009; Caiger et al. 2012; Caro et al. 2014). For soniferous otophysans, decreased auditory sensitivity is particularly harmful because it may reduce the capacity to detect acoustic signals from

conspecifics. Hearing loss for these species would be deleterious during the mating season as acoustic signals are a crucial component of courtship (Holt and Johnston 2014; Phillips and Johnston 2008). Limited auditory function may be further compounded by the masking effects of traffic noise (Holt and Johnston 2015). Consequently, otophysans are especially vulnerable to the growing omnipresence of anthropogenic noise. In conjunction with a shift in auditory thresholds, boat noise elicits an increase in the stress hormone, cortisol, in several freshwater fishes (Wysocki et al. 2006).

While noise generated from aquatic activities pose a clear threat to fishes, research demonstrates that road traffic noise from bridge crossings propagates a significant distance into freshwater streams (Holt and Johnston 2015). The effect of this noise on the stress response and auditory thresholds of exposed freshwater fishes is unexplored. As road networks continue to expand globally, it is imperative to understand how otophysan fishes, which dominate freshwater systems, are affected by present traffic noise levels (Carol et al. 2014; Watts et al. 2007). To elucidate the effects of road traffic noise, we designed a series of manipulative experiments to measure changes in cortisol levels and auditory thresholds of a model soniferous otophysan, the blacktail shiner (*Cyprinella venusta*), during exposure to an underwater recording of road traffic noise.

## Methods

### Fish Collection and Maintenance

We collected *C. venusta* from Little Uchee Creek in Moffits Mill located in Lee County, Alabama (32. 549244 °N; -85.278513 °W) using 10 ft seine nets. The permit to collect *C. venusta* at this location was issued by the Alabama Department of Conservation and Natural Resources (ACDNR). Immediately after collection, fish were transported to the Fish Biodiversity

Lab in coolers containing creek water. An air stone was also provided for each cooler. Fish were housed in 20 L aquaria with gravel substrate and allowed to acclimate 24 hours prior to testing. Fish were maintained at ambient light and temperature conditions. Fish were fed bloodworm (*Chironomid sp.*) larvae daily. The methods used for this project were approved by the Animal Care and Use Committee at Auburn University (protocol number 2012 – 2016: 2333).

**Traffic Noise Acquisition**

The road traffic noise used for the stress and auditory experiments was recorded at a beam bridge crossing in Macon County, Alabama in March 2010 as part of a traffic noise propagation study. A hydrophone (Hi-tech HTI-96-MIN, sensitivity –164.4 re 1V/µPa, frequency response: 0.002 – 30 kHz) connected to a digital recorder (Marantz PMD 661) sampling at a rate of 44.1 kHz was used to record traffic. The hydrophone was positioned 8 cm off the stream bed and 3 m downstream from the bridge piling. The hydrophone was attached to the end of a PVC pipe. This pipe was secured between two submerged sandbags; the sandbags were placed downstream from the hydrophone. Traffic was recorded for several minutes. The methods used to record the traffic noise used in this study are fully described in Holt and Johnston (2015).

**Waterborne Cortisol Collection**

Waterborne cortisol samples were collected between 4 and 6:30 am during the months of February and March 2014. This test period was chosen because noise from passing vehicles could be detected in the test room, and there was minimal car traffic on the road next to the lab building at this time. Individual test fish (n = 7) were placed into a rectangular 700 mL glass collection dish containing 450 mL of dechlorinated water. Once in the collection dish, fish were partially submerged in a 200 L aquarium and positioned 6.5 cm away from an underwater

speaker (UW – 30, Universal Sound Inc., Oklahoma City, OK). The collection dishes were large enough so that fish could move freely to mitigate confinement stress. Each fish received the control and noise treatment on separate days; the treatment order was randomized. To control for diel fluctuations in cortisol levels, fish were tested at the same time of day for both treatments.

The control treatment consisted of a 30 min period of silence. During this treatment, nothing was played from the underwater speaker; the speaker was not connected to the playback system. The traffic treatment consisted of a traffic recording looped for 30 minutes; this recording was played at a volume of approximately 140 dB re 1 µPa. To determine the playback volume, a hydrophone was placed in a collection dish that was positioned 6.5 cm away from the underwater speaker. This hydrophone was connected to a digital recorder (Marantz PMD 661; sampling rate 44.1 kHz). The traffic recording was presented from a Dell laptop using Raven Pro 1.4 software (Cornell University, Ithaca, NY). The laptop was connected to a SLA1 studio amplifier (Applied Research and Technologies); the underwater speaker in the 200 L test aquaria was also connected to this amplifier. The traffic playback file recorded on the Marantz digital recorder was analyzed in Raven Pro 1.4 (Cornell University, Ithaca, NY). The peak dB levels were measured in 2 s intervals and averaged to estimate the approximate volume.

At the end of each trial, water samples were filtered immediately using primed C-18 cartridges (Sep-pak, Waters Technology Corporation, Milford, MA). Cartridges were stored at -80 °C prior to running assays. Free cortisol was eluted from cartridges with two 2 mL washes of ethyl acetate and evaporated under nitrogen gas. The dried residues were resuspended in enzyme immunoassay (EIA) buffer. All samples were diluted (1:200) prior to being loaded into the plate. The plated was incubated and developed in accordance with the Cortisol EIA kit instructions (Cayman Chemical, Ann Arbor, MI). The cortisol kit was validated for use with *C. venusta* by

achieving parallelism between the standard curve provided with the kit and a serially diluted sample of pooled *C. venusta* cortisol.

**Measuring Auditory Thresholds**

The experimental design to assess the effect of traffic noise on auditory sensitivity was similar to the design used for cortisol collection. Individuals were exposed to the same traffic recording looped for a period of 2 hours at 140 dB re 1 μPa (n = 5). Individuals in the control treatment were exposed to a period of silence for 2 hours; no sounds were played from the speaker during the control treatment (n = 5). These trials were conducted in the same tank used for the cortisol collections. As these tests consisted of a longer exposure period, individuals were placed in a 10 L square tank. The distance between the edge of tank and the underwater speaker was 7 cm. Power spectra illustrating the spectral characteristics of the traffic playback and the ambient noise from the control treatment are presented in figure 1. It is true that the glass walls of aquaria cause reverberation of the acoustic stimulus. The minimum resonant frequency that would create distortion in the experimental design we used was 6, 372 Hz, which is well above the hearing threshold of *C. venusta* [27].

Additionally, particle acceleration of the traffic noise was measured as the difference in RMS pressure between two hydrophones (a = 0.195). Acceleration was calculated using the following formula: $a = -((p_1 - p_2 / d) / \rho))$. In this formula, $p_1 - p_2$ is the pressure difference between the two hydrophones; d is the distance between the hydrophones; ρ is the density of freshwater (997.1 kg/m$^3$). Particle acceleration measures from three orthogonal axes were combined into a single value using the equation: $a=\sqrt{x^2 + y^2 + z^2}$ , where x, y, and z are the three axes. The degree of particle acceleration (m s$^{-2}$) associated with the frequency tones presented during the AEP trials was calculated for each dB level using the same method [28].

The two recording hydrophones were placed 3 cm apart in the AEP test chamber; the midpoint between the hydrophones corresponded to the location of the fish's head during a trial. Electrical noise from the underwater speaker prevented the calculation of particle accelerations below 95 dB. Consequently, the particle accelerations of tones between 80 and 100 dB were extrapolated using data from 105 to 150 dB. These methods are fully outlined in Holt and Johnston (2011).

Auditory sensitivity after each treatment was determined by measuring auditory evoked potentials (AEPs). Auditory thresholds were measured at 100, 200, 300, 400, 600, 800, and 1,000 Hz; these frequencies are within the hearing range of *C. venusta*. Each frequency was presented to individual fish from 70 – 155 dB in 5 dB increments, and 250 responses were averaged for each presentation. These tones were 10 ms in duration with a rise and fall time of 2 ms. The frequency tones were generated using Sig Gen software and hardware (Tucker Davis Technologies, Gainesville, FL) and played through an underwater speaker (UW – 30, Universal Sound Inc., Oklahoma City, OK) located in the auditory test chamber (Tremetrics AR 9S Audiometric Booth). The test tank inside the chamber was a 79 cm section of PVC pipe that was capped at both ends with a 16.5 × 52 cm hole located in the top of the pipe. Water was filled to a depth of 23 cm. The underwater speaker was suspended 10 cm below the water's surface and 39 cm away from the left side of the tank.

Prior to testing, frequency tones were calibrated using a hydrophone (Hi-Tech HTI-96-MIN, sensitivity -164.4 re 1V/µPa) and a GW GOS-6xxG dual trace oscilloscope. During calibration, the hydrophone was positioned where the recording electrode on the fish's head would be located during a trial. For each tone, the peak voltage readings from the oscilloscope were converted to dB re 1 µPa, and these values were used to compile a normalization file. The normalization file was used to make adjustments to ensure that the sound pressure levels were

correct. The corrected frequency tones were played to the fish using BioSig software and hardware (Tucker Davis Technologies, Gainesville, FL).

After each treatment, test fish were wrapped in gauze and restrained in a clay bed at a depth of 11 cm and 8 cm away from the underwater speaker (UW-30, Universal Sound Inc., Oklahoma City, OK). Auditory thresholds were measured as the potential difference between electrodes (Rochester Electro-Medical, Inc., Tampa, FL) inserted subcutaneously above the brainstem and in the caudal peduncle. A grounding electrode was inserted into the clay bed. Electrodes were insulated with fingernail polish except at the tip inserted in the fish. Electrodes fed into a Medusa RP2.1 pre-amplifier connecting to a RA16 base station processor feeding to the BioSig software (Tucker Davis Technologies, Gainesville, FL). Auditory traces were generated using BioSig software. Tones were also presented in opposite phases (90° and 270°), and the traces were averaged to cancel stimulus artifacts. Auditory thresholds were determined visually as the lowest sound level to elicit an AEP response. Audiograms generated from visually assessing thresholds are similar to audiograms produced using statistical methods (Schrode et al. 2014).

**Results**

Waterborne cortisol levels for control and traffic treatments were compared using a one-way repeated ANOVA. Auditory thresholds were compared via a repeated one-way ANOVA on frequency and treatment. Data were tested for normality using the Kolmogorov-Smirnov test. An alpha criterion of 0.05 was used for all tests. All statistics were completed using SPSS version 22 (IBM SPSS Corporation, Chicago, IL).

Cortisol levels were significantly elevated after traffic noise exposure ($n = 7$, $F_{1, 6} = 6.546$, $p = 0.043$). Individuals released $8.2 \pm 2.3$ ng $g^{-1}$ 30 $min^{-1}$ when exposed to traffic noise and $5.7 \pm$

1.1 ng $g^{-1}$ 30 $min^{-1}$ when exposed to the control treatment (Fig. 2). These results equate to approximately a 44.0 % change in the cortisol production of exposed *C. venusta*. The intra-assay coefficient of variation was 2.3 %. The inter-assay coefficient of variation was not calculated because all of the samples were analyzed on one plate.

**Discussion**

The results in this study are consistent with the literature documenting the effects of anthropogenic noise on stress and hearing; however, this is the first study to investigate the effects of terrestrial traffic noise on an otophysan, freshwater stream fish (Wysocki et al. 2006; Kaiser et al. 2015; Scholick and Yan 2002). The elevation in *C. venusta* cortisol during acute traffic noise exposure was likely influenced by a combination of the sound pressure level, temporal variability, and acoustic structure of road traffic. The noise generated from road traffic fluctuates with respect to frequency and amplitude; noise levels also vacillate throughout the day, making it an irregular stressor. Intermittent acoustic stimuli, such as traffic noise, are suggested to have more prolonged effects on the stress response than constant stimuli (Blickley et al. 2012; Neo et al. 2014; Shannon et al. 2014). Similar cortisol elevations were observed in fishes exposed to recordings of boat noise (Wysocki et al. 2006). Our study investigated the acute effects of traffic exposure on the stress response of *C. venusta*; whether this species acclimatizes to traffic noise in the environment over time remains to be determined.

Auditory threshold shifts were greatest at 300 and 400 Hz – where *C. venusta* hearing was most sensitive. These results are of biological significance as the agonistic and courtship signals of this species have similar frequencies (Holt and Johnston 2015). A potential mechanism explaining the observed threshold shifts is sensory hair cell damage from traffic noise exposure. Several studies show a correlation between noise exposure and the apoptosis of hair cells in

fishes (Smith et al. 2006; Hastings 2010). It is noteworthy to recognize that fishes continue to add hair cells to auditory epithelia throughout ontogeny and have the capacity to regenerate lost cells after acoustic trauma (Smith et al. 2006; Higgs et al. 2001). The time required to regain pre-exposure auditory thresholds, however, is dependent upon the intensity and duration of the noise (Smith et al. 2006). In a study by Smith et al (2004), goldfish, *Carassius auratus*, exposed to 170 dB re 1μPa white noise for 24 hours experienced significant, albeit not complete, recovery after 18 days.

A similar study exposed *C. auratus* to 158 dB re 1μPa white noise for 24 hours; individuals recovered auditory thresholds after 3 days (Amoser and Ladich 2003) We did not establish the time required for *C. venusta* to regain auditory thresholds after traffic noise exposure; however, even a temporary period of reduced auditory function could be detrimental for vital behaviors such as conspecific communication and predator detection. Previous work demonstrates that *C. venusta* increases its signal amplitude under noisy conditions (Holt and Johnston 2014). It is unknown if this behavioral plasticity is sustainable for long periods. Future work should investigate auditory shifts and recovery periods for a series of exposure levels to further understand the ecological effects of traffic noise in the environment.

There are caveats to investigating the impacts of traffic noise on fishes under laboratory conditions. The traffic stimulus presented to the fish in aquaria is not completely identical to the noise experienced by fish in the environment. Distortions of the stimulus would occur at higher frequencies that are well beyond the documented auditory thresholds for *C. venusta*; the playback used in this study was a reliable representation of traffic noise in the environment.

While some fish have the opportunity to alter habitat use to avoid anthropogenic noise, the movement of fishes inhabiting small streams is restricted, including species of *Cyprinella*

(Johnston 2000; Jacobsen et al. 2014). Indeed, traffic noise propagated from a bridge can disrupt the natural soundscape of a stream up to a distance of over 12, 000 m (Holt and Johnston 2015). Watersheds in urbanized regions may, therefore, provide few refuges from noise. As a result, populations of this species unable to avoid traffic noise may have degraded hearing for extended periods. Morning commuter traffic would be the most problematic as this species spawns early in the day (Warren et al. 2006). As traffic noise is predominantly low frequency, it effectively masks the acoustic signals of *C. venusta* (Holt and Johnston 2015). Masking, combined with decreased auditory sensitivity and elevated glucocorticoids, could have negative fitness consequences for *C. venusta* and other soniferous otophysans exposed to heavy traffic levels in the environment.

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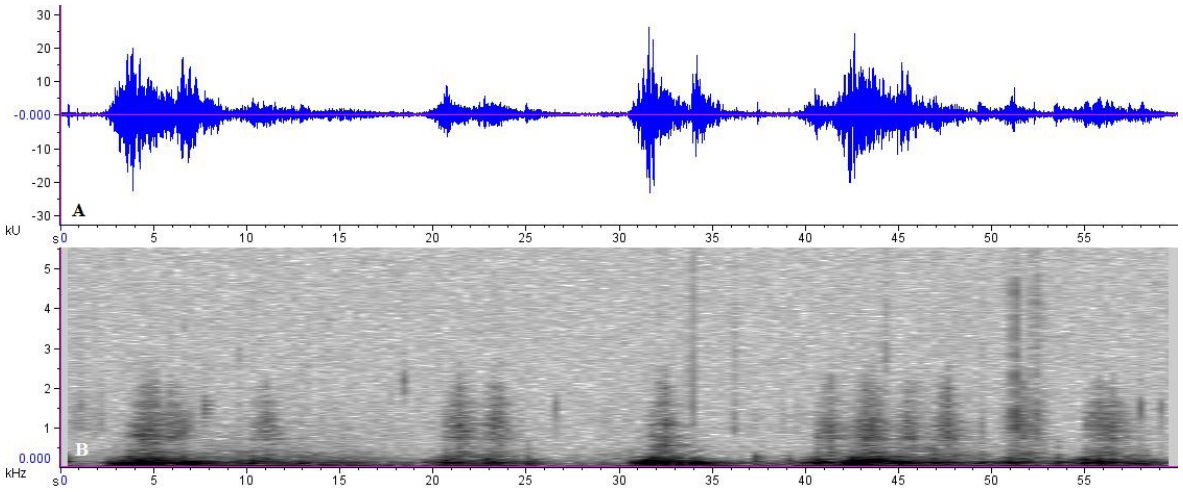


**Fig. 1-1:** (A) Oscillogram of traffic noise used in study. (B) Sonogram of traffic noise (1.95 Hz resolution).

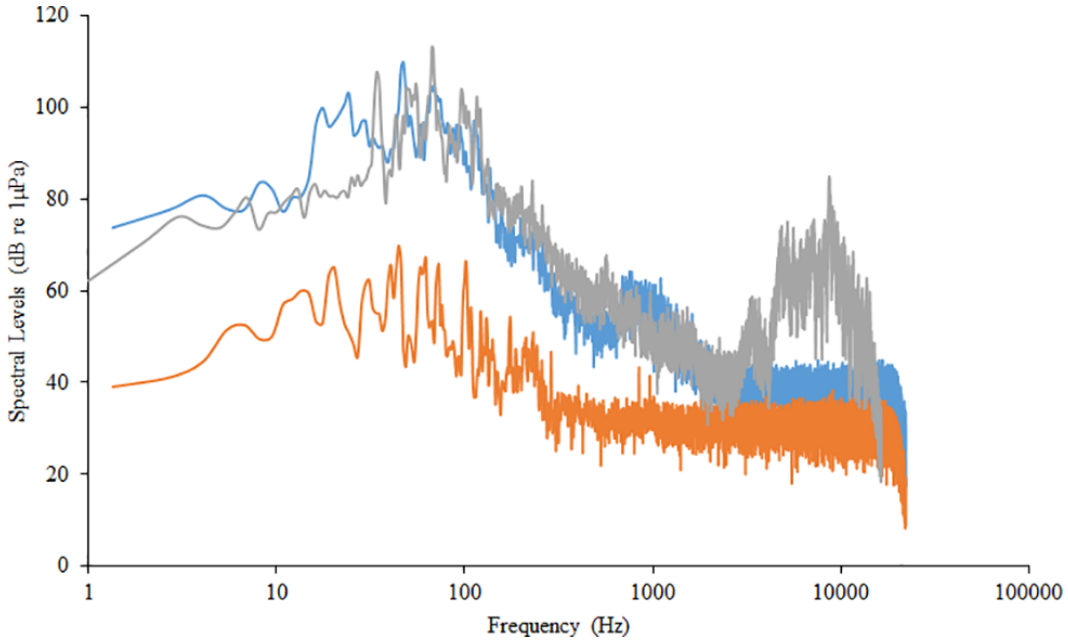


**Fig. 1-2:** Power spectra for traffic playback (gray line) and control treatments (orange line). The power spectrum of the original traffic recording is provided for reference (blue line). Spectra were created with a resolution of 1.35 Hz.

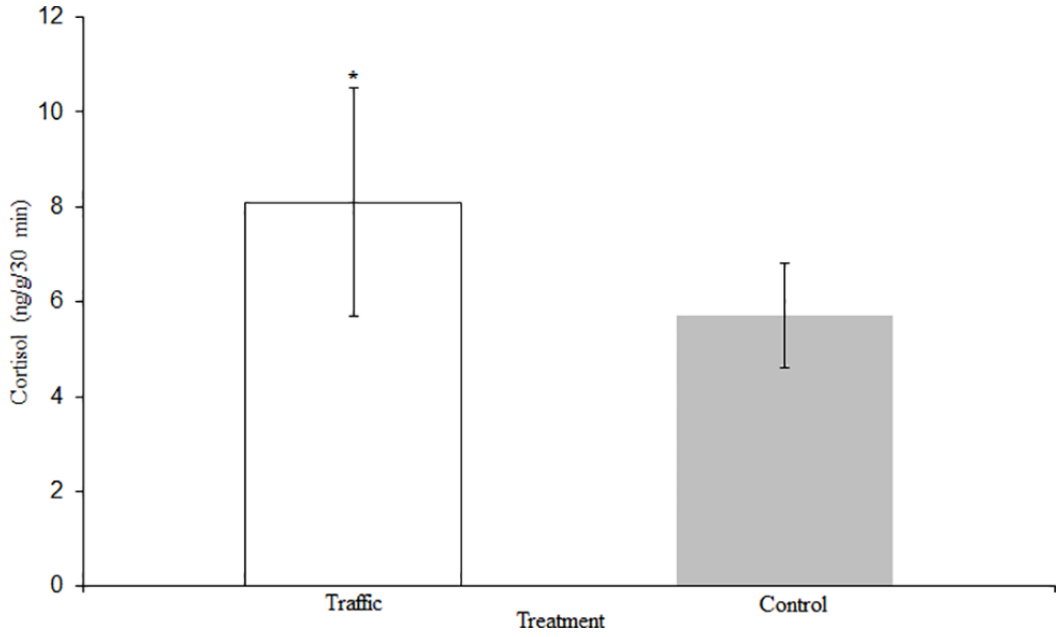


**Fig. 1-3:** Cortisol levels for *C.venusta* exposed to the control and traffic treatments presented as the mean ± SE ng $g^{-1}$ $min^{-1}$. * denotes $p < 0.05$

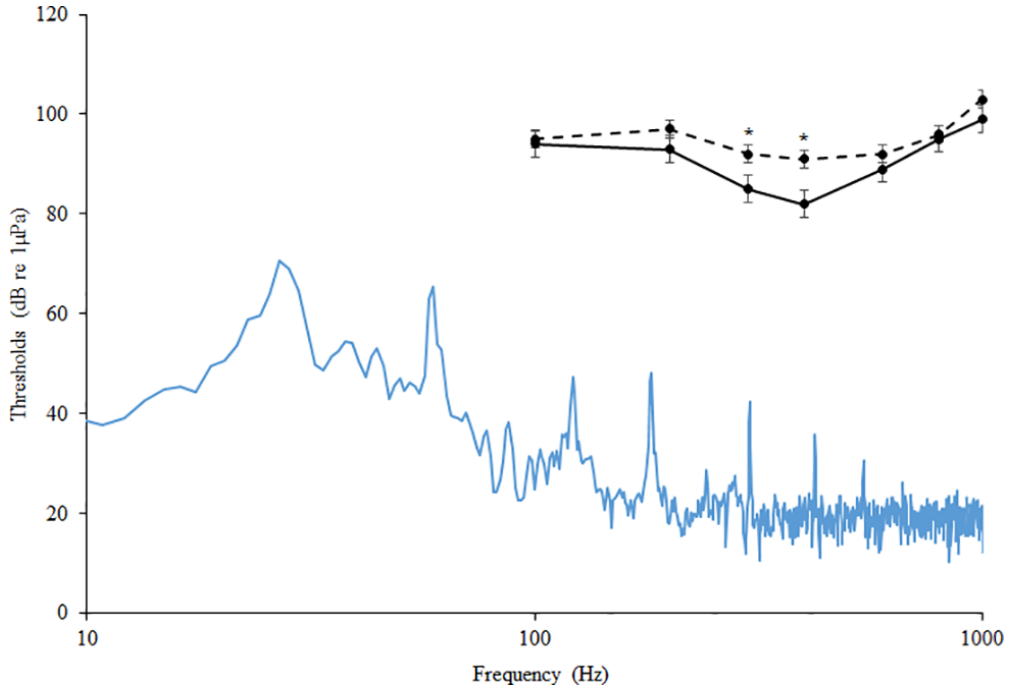


**Fig. 1-4:** Auditory hearing thresholds for *C. venusta* exposed to control treatment (solid line) and traffic treatment (dashed line) presented as the mean SPL ± SE dB re 1 µPa. * denotes $p < 0.05$. The power spectrum of the background noise in the test chamber is represented by the blue line.

## Chapter 3

## The Effects of Masking on Acoustic Modulation of Hormones in the Blacktail Shiner, *Cyprinella venusta*


### Abstract

Anthropogenic noise pollution has multimodal effects on animal communication networks. In environments affected by noise, acoustic signals are often masked between conspecifics. While masking is recognized in terrestrial and marine ecosystems, it is not studied extensively in freshwater stream biota. A large proportion of freshwater fishes communicate acoustically, and these signals aid to modulate key hormones during the mating season. To better understand the physiological consequences of masking in soniferous fishes, we conducted a series of masked playback trials using the Blacktail Shiner (*Cyprinella venusta)*. Males produce specific acoustic signals during courtship and aggressive behaviors. We measured waterborne levels of estradiol in females exposed to masked courtship signals and waterborne levels of 11-ketotestosterone in males exposed to masked agonistic signals. Males and females exposed to the masked playback did not exhibit statistically significant changes in hormone levels; however, these results may have biological significance. Females exposed to unmasked playback experience a significant


### Introduction

Anthropogenic noise disrupts animal communication networks at multiple levels of organization. Noise pollution reduces the ability of animals to detect acoustic signals by damaging auditory sensitivity, masking, and eliciting a stress response (Halfwerk and Slabbekoorn 2015; Nichols et al. 2015; Crovo et al. 2015). These consequences create obstacles for communication between conspecifics, other crucial behaviors, including foraging and predator avoidance are also disrupted (Ferrari et al. 2018; Evans et al. 2018). Despite these

challenges, many taxa demonstrate degrees of plasticity in response to noise pollution (Nichols et al. 2015). Urbanized bird populations, for example, increase song frequencies to limit spectral overlap from noise pollution (Halfwerk et al. 2011). Some species will increase the volume of their signal (Holt and Johnston 2014).

Much of the work investigating the deleterious effects of noise pollution focuses on terrestrial and marine ecosystems (Halfwerk and Slabbekoorn 2015; Nichols et al. 2015). Freshwater systems, however, are particularly sensitive to anthropogenic disturbances, and fishes are acutely vulnerable (Carrizo et al. 2017). Approximately 64% of freshwater fishes belong to the superorder Ostariophysi (Nelson 2006). Ostariophysan fishes possess greater auditory sensitivity relative to other species. Furthermore, a large proportion of Ostariophysans are soniferous, making them susceptible to noise pollution (Kasumyan 2000). Contrary to other taxa, fishes inhabiting freshwater streams display limited movement and may not be able to avoid noisy habitats (Johnston 2000).

Fishes in the genus *Cyprinella* are idea models for investigating the costs of noise pollution in a freshwater stream environment. Preliminary work on the Blacktail Shiner (*Cyprinella venusta*) revealed this species increases the amplitude of its signals under noisy condtions (Holt and Johnston 2014). Exposure to traffic noise propagated from bridge crossings results in elevated hearing thresholds, cortisol levels, and signal masking (Crovo et al. 2015; Holt and Johnston 2016). Our objective for this study was to examine the effects of masking within the context of reproductive physiology. Male *C. venusta* produces two distinct vocalizations during the breeding season: knocks and growls. Dominant males are highly territorial and chase intruders from spawning sites; knocks are correlated with agonistic displays. Growls are directed toward females during courtship and spawning behaviors (Holt and Johnston 2014). Playback

studies suggest knocks and growls contribute to modulating reproductive hormones in this species. We measured waterborne levels of 11-ketotestosterone in males and estradiol in females in response to masked and unmasked signals.

## Methods

### Masked Acoustic Playback Calibration:

The acoustic signals used for this study were previously recorded as part of another project describing sound production and traffic noise in *C. venusta* (Holt and Johnston, 2014). We selected a subset of the cleanest growl and knock signals to reduce signal degradation during playback (fig. 4). The traffic noise was recorded 3 m downstream from a bridge crossing in Macon County, Alabama (Holt and Johnston 2015). Masked playbacks were created using Audacity software. A 30 second segment of traffic noise was overlaid in growl and knock signals. The sound pressure levels (SPL) of masked signals were calibrated in a 200 L glass test tank filled with 65 L of water. To ensure the signals were calibrated to relevant sound levels in this test tank, we placed a hydrophone (Hi-tech HTI-96-MIN, sensitivity- 164.4 re 1V/µPa, frequency response: 0.002–30 kHz) inside a 700 ml glass collection dish containing 450 ml of water. This hydrophone was connected to a Marantz digital recorder. The collection dish and hydrophone were placed in the 200 L tank 5.5 cm away from an underwater speaker (UW – 30, Universal Sound Inc., Oklahoma City, OK). We played masked growls and knocks from a Mac Book Pro using Raven Pro 1.4 software (Cornell University, Ithaca, NY) connected to an amplifier. Signals emitted from the underwater speaker were recorded with the hydrophone. We analyzed the recorded signals using Raven 1.4 software to determine the appropriate playback volume. Masked growls were calibrated to an SPL of approximately 80 dB, and masked knocks

were calibrated to100 dB. These SPL levels reflect the values recorded from male *C. venusta* in the field (Holt and Johnston, 2014).

**Masked Acoustic Playback and Hormone Collection**

We tested fish between 5 and 7am during the months of May and July 2016 – 2017. This test period is ecologically relevant because *C. venusta* spawns most actively during the predawn hours from early to mid-summer. To minimize handling stress before each trial, fish were quickly captured with a clean aquarium net and immediately placed into a small opaque bucket containing aged water. We transported the bucket to the test room and placed the fish into a clean 700 ml glass collection dish containing 450 ml of water; this dish was placed 5.5 cm away from the underwater speaker. Collection dishes were large enough to allow fish free movement. The water used for hormone collection was taken from a designated tank maintained at housing conditions; this tank never contained fish.

The tank and filter components were cleaned with 100% ethanol and distilled water and allowed to air dry prior to conducting the study. The time between initial capture and placing the fish into the test tank did not exceed 60 seconds. Furthermore, test subjects for the day were collected from separate tanks to eliminate stress from repeated capture attempts. No additional activities were conducted in the lab during the test period. We measured waterborne levels of $E_2$ ($n$ = 7) and 11KT ($n$ = 6) for this study. Glassware and nets were washed with 100 % ethanol and allowed to dry between uses. Fish were tested within one week of collection.

Subjects were given two consecutive treatments. Males and females were first administered a baseline trial consisting of 30 minutes of silence. Acoustic stimuli were not played from the speaker during this period. While there were background levels of noise inherently present in the test room; the sound levels were below the hearing threshold of *C.*

*venusta* (Crovo et al., 2015). After the baseline trial, fish were immediately transferred to a new collection dish containing clean water and returned to the 200 L tank. Male subjects received a masked knock playback; females received a masked growl playback. After testing, fish were euthanized with an overdose of MS-222 (Tricaine methanesulfonate). Females were dissected to determine the stage of oocyte maturation. We only included females with mature ovaries in subsequent analyses.

At the end of each trial, we filtered raw water samples through cellulose filters (Whatman Filter Papers, Grade 2) to remove any coarse particulates. Water samples were then filtered through C18 cartridges (Sep-pak, Waters Technology Corporation, Milford, MA) primed with 4ml of 100% ethanol followed by 4ml of distilled water. Cartridges were covered with parafilm on both ends and stored at -83 ºC until extraction.

**Hormone Extraction and Analysis:**

Prior to extraction, we thawed the cartridges at room temperature for 30 minutes. We extracted hormones from cartridges (Sep-pak, Waters Technology Corporation, Milford, MA) using an extraction manifold (Waters Technology Corporation, Milford, MA) connected to a Rocker 300 vacuum pump (Rocker Scientific Co., Ltd.). Cartridges were first washed with two 2 ml aliquots of ultrapure water. Hormones were extracted using two 2 ml aliquots of ethyl acetate. Samples were placed in a dry bath and evaporated under a stream of nitrogen gas. All of the hormone samples were analyzed using ELISA kits (Cayman Chemical, Ann Arbor, MI). The dried residues were reconstituted in ELISA buffer. We only considered levels of free, unconjugated hormones in this study. All samples were run in duplicate.

**Results**

RStudio was used to complete all statistical tests (RStudio Team, 2015). Data was tested for normality and homoscedasticity using the Shapiro-Wilk and Bartlett's test. A linear mixed-effects model was used to analyze changes in hormone levels to account for the nested data structure using the R package 'lme4' (Bates, 2015). Mean $E_2$ levels ($n$ = 7) decreased slightly, but insignificantly, from 217.0 to 205.1 pg/g fish during the masked growl playback ($p$= 0.39; $F_{1, 7}$ = 0.83). Male 11KT ($n$ = 6) levels increased slightly during the masked knock playback; however, this change was not statistically significant ($p$ = 0.33; $F_{1, 6}$= 1.15). The mean 11KT values were 173.6 and 196.2 pg/g fish for the control and masked trials.

**Discussion:**

Freshwater stream fishes are often overlooked with respect to conservation efforts. Yet current evidence shows that they are equally vulnerable to noise pollution relative to marine and terrestrial taxa (Holt and Johnston 2015; Holt and Johnston 2015; Crovo et al. 2015). Lower frequency components of traffic noise propagate up to 1200 m from a bridge crossing, and these frequencies overlap with growl signals (Holt and Johnston 2015). The spectral overlap reflects the observed results obtained from female *C. venusta* exposed to a masked growl playback. Previous work on this species suggests that growl signals help to promote reproductive behaviors by decreasing levels of circulating $E_2$. In teleosts, a decline in $E_2$ correlates with ovulatory and spawning behaviors (Matsuyama et al., 1988; Joy and Chaube, 2015; Pham and Nguyen 2019). *C. venusta* populations inhabiting particularly noisy environments may therefore have limited reproductive success during the mating season.

Male *C. venusta* did not experience a significant shift in 11KT levels in response to masked knocks; however, the role of knocks in male agonistic interactions remains unclear.

Unmasked knocks did not modulate levels of 11KT significantly in earlier playback studies. It is possible that *C. venusta* is similar to other species in that males require multimodal signals to mount an aggressive response (Chabrolles et al. 2017). Future work should consider visual and chemical signals with masked and unmasked acoustic communication.

While this study demonstrates the masking effects of traffic noise, it is important to recognize that there are multimodal effects on communication networks including reduced auditory sensitivity and increased signal amplitudes (Holt and Johnston 2015; Crovo et al. 2015). Under natural conditions, it is likely these effects are compounded, particularly in environments where noise is chronic. Furthermore, the data obtained from females highlight the need to acknowledge the effect of noise pollution with respect to conservation and fish communication networks.

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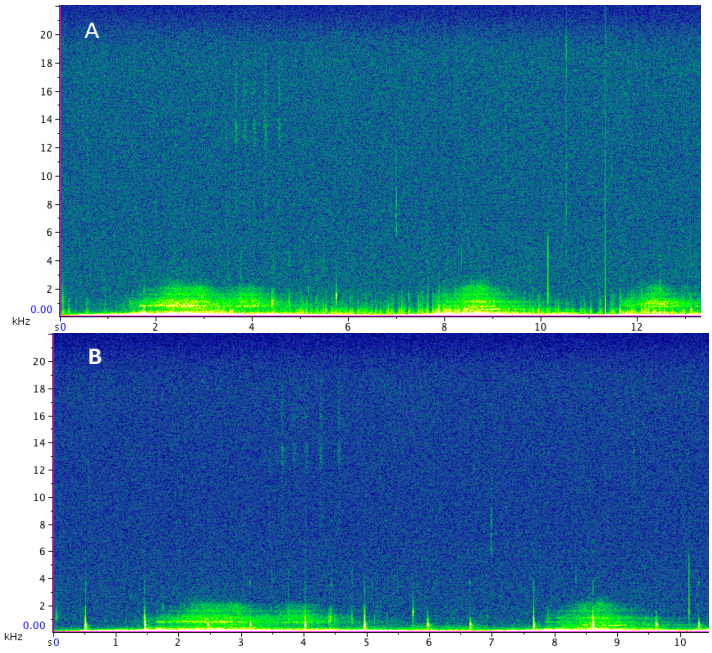


**Fig. 3-1:** Spectrograms of masked male *C. venusta* Growl and Knock Signals. The masked courtship growl signal is represented in A; the masked agonistic knock signal is represented in B.

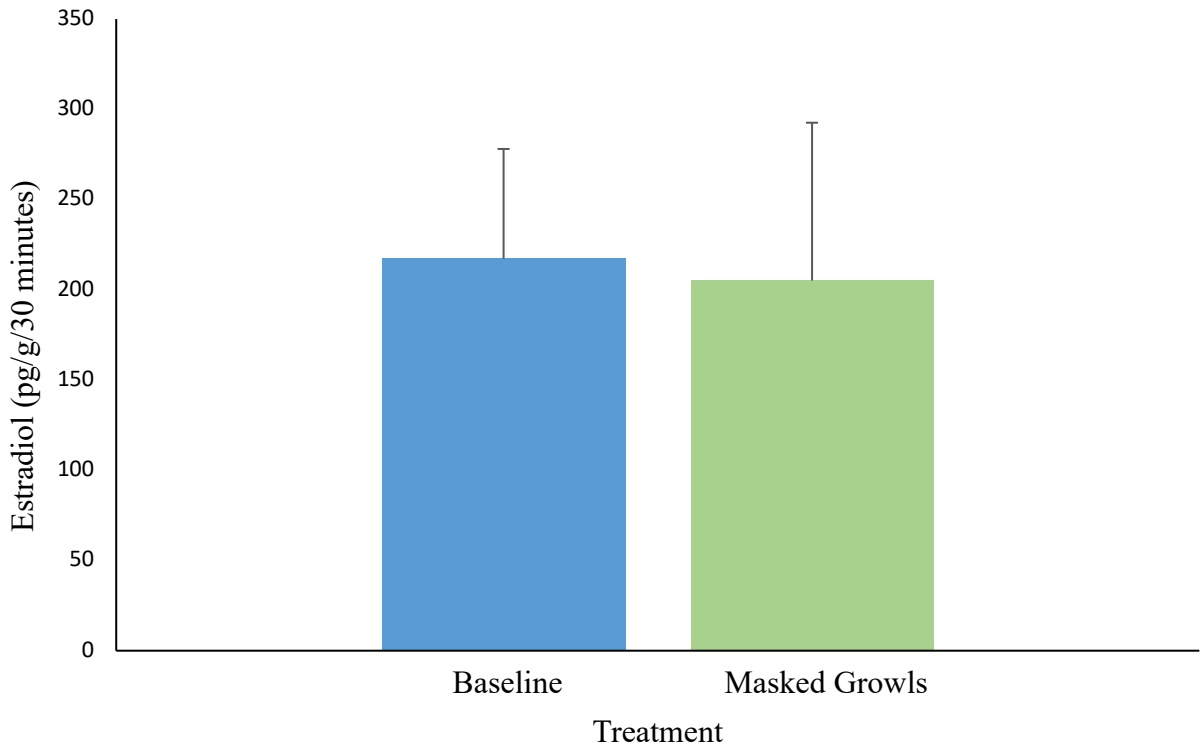


**Fig. 2-3:** Estradiol levels in female *C. venusta* exposed to baseline and masked growl playback. Data are represented as mean ± SE

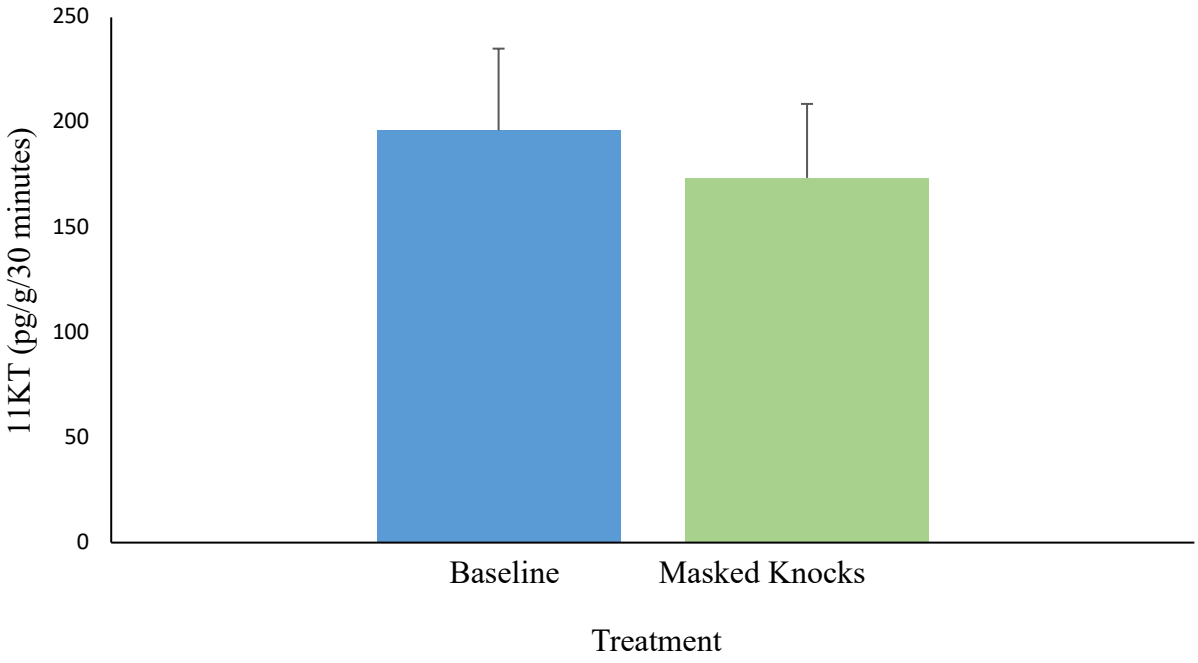


**Fig. 3-3:** 11KT levels in male *C. venusta* exposed to baseline and masked knock playback. Data are represented as mean ± SE