Physiological and Behavioral Impacts of Railway Noise on Stream Fishes

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
May 4, 2019

Keywords: bioacoustics, anthropogenic noise,
conservation, stress, railroad

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Abstract

While the expansion of anthropogenic noise studies in aquatic habitats has produced

conservation-based results for marine organisms, little attention has been paid to the potential

impacts of anthropogenic noise on freshwater stream fishes. Recent work showed that Blacktail

Shiner (*Cyprinella* venusta) exhibited multiple responses to road noise, however, community-

wide effects of anthropogenic noise pollution have not yet been investigated. By examining six

metrics of stress on four ecologically and evolutionarily disparate species of stream fishes

(*Campostoma oligolepis* Largescale stoneroller; *Etheostoma rufilineatum*, Redline Darter;

Cyprinella venusta; and *Lepomis macrochirus*, Bluegill Sunfish), these laboratory experiments

aimed to describe the potential impacts of anthropogenic noise on these understudied organisms.

Each species included represents a unique combination of hearing sensitivity and water column

position. The anthropogenic noise source used throughout this research consisted of an

underwater audio recording of a train crossing a stream via a beam-style bridge. Physiological

and behavioral metrics were compared across the presence and absence of noise playback. In

addition, measurements of water-borne cortisol were collected from Blacktail Shiner exposed to

rail-noise presented in variable temporal arrangements to further elucidate dynamics of the

primary stress response in this species. Noise playback had no statistically significant effect on

blood glucose or water-borne cortisol levels, however, repeatedly decreased concentrations of

water-borne cortisol observed in Blacktail Shiner implies potential inhibition of a

neuroendocrine stress response. The presence of noise produced significant changes in

ventilation rate, total distance swam, average swimming velocity, and the elicitation of a startle response in a portion of the species observed. Effects of noise were observed in certain species contrary to what would be hypothesized based on their theoretical hearing sensitivity. These results demonstrate that predicting susceptibility to this type of stressor cannot be accomplished by simply considering hearing ability or water column position. More importantly, we show that anthropogenic noise can disrupt a variety of behavioral and physiological processes that potentially serve as proxies for fitness-impacts and should be considered an environmental stressor and a driver of habitat degradation to the affected species and potentially communities.

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INTRODUCTION

As global transportation increases, it is estimated that by 2050, road and railway lengths will expand by 60% (Dulac, 2013), with current US road miles already approximating total stream and river miles (Riitters and Wickham, 2003). The expansion of transportation infrastructure is a known contributor of light and chemical pollution, the creation of movement barriers and habitat modification (Ree *et al.*, 2015), but a relatively understudied aspect of this ubiquitous impact is its role in the production of anthropogenic noise. Anthropogenic noise can be defined as any human generated-sounds that may be detrimental to an organism (Slabbekoorn *et al.*, 2010). Outside of the civilian road; artificial waterways, trails, railroads and utility easements are all potential sources of noise. Research on terrestrial organisms exposed to noise has shown a reduction in available habitat (Schaub *et al.*, 2008; Blickley *et al.*, 2012a; Ware *et al.*, 2015), alterations of communication signals (Vargas-Salinas *et al.*, 2014; Templeton *et al.*, 2016) and signs of physiological stress (Blickley *et al.*, 2012b; Tennessen *et al.*, 2014; Green *et al.*, 2015). Stress, inhibition of signal reception, and reduction of reproductive or foraging habitat can have significant fitness consequences in these systems. The detriment caused by transportation-induced noise, however, is not limited to terrestrial habitats.

Work concerning anthropogenic noise and aquatic organisms has primarily focused on marine mammals with only a recent expansion to fishes (Cox *et al.*, 2016). However, acoustic perception is of great importance to spawning (Holt and Johnston, 2014), predator detection (Mann *et al.*, 1997), foraging (Holt and Johnston, 2011) and competitive interactions (Amorim and Hawkins, 2000) in a number of species. More importantly, noise-induced impacts relevant to these activities and more have been reported (Handegard *et al.*, 2003; Vasconcelos *et al.*, 2007; Purser and Radford, 2011; Sebastianutto *et al.*, 2011; Bruintjes and Radford, 2013; Simpson *et*

al., 2016). In addition to impacting critical behaviors, noise exposure has proved to affect a variety of physiological processes, most commonly observed through the increase of circulating glucocorticoids and energy substrates which are common indicators of a primary stress response (Wysocki *et al.*, 2006; Buscaino *et al.*, 2010; Celi *et al.*, 2016). Unfortunately, a deficit of research asking these questions in small stream fishes exists.

Freshwater fishes in lotic systems show commonalities with marine species in regard to the importance of acoustic perception and the impact noise has upon it. While anthropogenic noise in marine habitats is often caused by shipping vessels (Wysocki *et al.*, 2006), Navy-operated sonar systems (Halvorsen *et al.*, 2012) and pile driving (Mueller-Blenkle *et al.*, 2010), anthropogenic noise in streams is primarily generated by bridges crossings. While road bridges produce fairly constant noise, signals from railway bridges can be characterized by higher amplitude, shorter durations (Halfwerk *et al.*, 2011) and more sporadic occurrences. With this, the two sources can be thought of as chronic and acute stressors respectively, however, the importance of varying temporal characteristics or amplitude of stimuli in regard to eliciting a stress response is still unknown. Determining which species will be impacted by anthropogenic noise is also still unclear.

A lack of information regarding the anatomical and physiological characteristics predicting individual susceptibility to noise poses an inherent problem for managing this type of disturbance. Historically, fishes have been divided into hearing generalists and specialists, with the latter possessing anatomical adaptations that allow for detection of the pressure component of sound by bringing the inner ear into close proximity to a pressure-to-mechanical displacement converter such as an air bubble (Popper and Fay, 2011). One example of these adaptations is the presence of Weberian ossicles, a synapomorphy of the superorder Otophysi, which connect the

swim bladder (air bubble) to the inner ear via modified vertebrae. Species lacking an air bubble are theoretically not capable of detecting the pressure component of sound, and those without a specialization to connect the air bubble to the inner ear show diminished hearing sensitivity. Species lacking a specialization (Graham and Cooke, 2008), and even an air bubble (Johnston and Johnson, 2000) however, are still able to detect and be affected by the particle motion component of sound through the lateral line and superficial neuromasts. What further complicates predicting which species are at risk is the ability of certain benthic fishes to detect substrate vibrations (Janssen, 1990). This suggests that individuals in contact with the benthos may be affected by both the particle motion and vibratory components of noise. Research concerning this aspect of transportation-induced noise is absent from the literature.

Holt et als' (2014) study was one of the first to document the impacts of anthropogenic noise on small, freshwater stream fishes. This work showed that in the presence of road noise, Blacktail Shiner increased the amplitude of vocalizations in order to effectively transmit to conspecifics (i.e. The Lombard Effect) (Holt and Johnston, 2014). Research on Blacktail Shiner has also revealed that the presence of road noise lead to a significant increase in hearing thresholds and levels of the primary stress hormone cortisol (Crovo *et al.*, 2015). While these studies document clear detrimental impacts to this species, we believe the diversity of species studied must expand in order to better represent native fish communities. In line with a 2016 meta-analysis (Cox *et al.*, 2016), we also see the need for increased emphasis on the measurement of both behavioral and physiological disturbances when studying noise affecting fishes. While this integrative approach has been used to study the impacts of anthropogenic noise on fish (Buscaino *et al.*, 2010), work concerning multiple, sympatric stream species is rare

(Radford *et al.*, 2016). While assessing fitness impacts to a fish community is difficult, a necessary first step is to understand individual-level consequences.

In this study, we examined the physiological and behavioral effects of acoustic and vibratory pollution propagated by an active railway bridge on four ecologically and evolutionarily distinct species of sympatric stream fishes that differ in auditory anatomy and water column position. These data were gathered to better understand the impacts of noise on species that represent a common fish community and if anatomy or habitat use may infer species-specific susceptibility to anthropogenic noise. Through two manipulative laboratory experiments, six indices of disturbance were measured in Blacktail Shiner, Bluegill (*Lepomis macrochirus*), Largescale Stoneroller (*Campostoma oligolepis*) and Redline Darter (*Etheostoma rufilineatum*) in the presence and absence of an anthropogenic noise stimulus. The six indices of disturbance measured were blood glucose, water-borne cortisol, ventilation rate, total swimming distance, average swimming velocity and the elicitation of a startle response. Additional trials exposing Blacktail Shiner, a species previously observed to increase water-borne cortisol levels in the presence of road noise (Crovo *et al.*, 2015), to variable temporal presentations of the noise stimulus were conducted in order to elucidate the effect of the temporal arrangement of noise on the elicitation of a neuroendocrine stress response. We hypothesized that detrimental shifts in the indices measured would be most prevalent in the benthic Otophysan, Largescale Stoneroller and least prevalent in the pelagic "hearing generalist", Bluegill Sunfish due to their hearing sensitivity and location in the water column. In addition, we hypothesized that the most constant temporal presentation of noise stimulus would produce the largest increase in water-borne cortisol concentrations in Blacktail Shiner. Our results provide insight into the importance of understanding species-specific disturbance responses in stream fishes exposed anthropogenic

noise and the complexity of noise-induced stress responses. We also discuss potential

community-wide fitness implications further designating noise emanating from transportation

infrastructure as a significant and ubiquitous environmental stressor.

METHODS

Fish Collection and Husbandry

Each species studied represented a unique combination of general hearing proficiency (specialist or generalist) and water column position (pelagic or benthic) (Table 1). Blacktail Shiner, Largescale Stoneroller, Redline Darter, and Bluegill Sunfish fulfilled these combinations. Individuals were collected via seine between May and June 2018 from Chewacla Creek (Lee County, AL), Auburn University Fisheries Pond 15 (Lee County, AL), Middle Cypress Creek (Lauderdale County, AL) and Shoal Creek (Lauderdale County, AL) respectively. All animals were collected at a distance of at least 100 stream meters away from any bridge crossing to control for previous exposure.

Fishes were transported to the lab in aerated coolers containing stream water and housed in multiple, 68 L aquaria with identical vegetation, diet (*Chironomid spp.)* temperature (22°C), diel period (12:12) and ceramic cover tiles with the exception of Largescale Stoneroller which were housed at higher densities in one, 193 L aquarium. Fishes were given an acclimation period of at least 24 hours prior to testing. This project was covered by Auburn University animal protocol number 2017-2333.

Anthropogenic Noise Acquisition

The anthropogenic noise playback used throughout this research was recorded at a beam-style bridge crossing Red Creek (Macon County, Alabama) during the passing of a train. 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) was placed 7.3 m upstream of the center bridge pile and 8 cm above the stream bed in order to record the pressure component of the noise (water depth=18 cm). A geophone (PCB Piezotronics Model 608A11/030AC, sensitivity=10.4mV/g), which was routed through a signal conditioner set to 1x gain (PCB Piezotronics Model 480B21) and connected to a digital recorder (Tascam DR70MKII) was placed in the same position and used to record the substrate vibration component of the train passing. Field recordings were edited to only include the duration of a train crossing the stream (4 min 2 sec) and bound by a 3 second fade-in and fade-out (Figure 1). Rail-noise playbacks were then situated in the temporal arrangement desired for each experiment. Both pressure and vibratory components of playbacks were calibrated to reflect real-world levels by recording in-tank playbacks, using the same equipment and settings used to record rail noise in the field, and adjusting the playback level until it was equal to that observed in the field. In-tank levels were compared with field recordings in version 1.4 of Raven Pro software (Cornell University, Ithaca, NY). All editions to amplitude and temporal arrangement were done in version 2.2.1 of Audacity® recording and editing software (Mazzoni, 1999).

Physiological Experiment

Ventilation rate, blood glucose concentration and waterborne cortisol levels were independently collected from thirty individuals from each species in the presence (n=15) and absence (n=15) of the noise playback. Additional water-borne cortisol measurements were collected from Blacktail Shiner (n=7) exposed to rail-noise playbacks that differed in temporal arrangement from the multi-species trials. Fishes were tested individually in 700 mL glass collection dishes filled with 500 mL of dechlorinated tap water. Collection dishes were partly submerged in a 200 L aquarium and positioned 6.5 cm in front of a vertically oriented UW30 (Universal Sound Inc., Oklahoma City, OK) speaker connected to a SLA1 (Applied Research and Technologies) studio amplifier leading to a laptop. Unlike the behavioral experiment, this testing environment did not allow for the replication of substrate vibrations. All physiological experiments were conducted between 1700 and 2200 hours to control for diel variations in cortisol and glucose.

Individuals were placed into the collection dish and exposed to either a thirty-minute control period (silence) or a thirty-minute playback stimulus consisting of three, full train crossings equally interspersed with silence (triplicate playback) (Figure 2). During trials, collection dishes were topped with a thin mesh in order to prevent fish from escaping into the surrounding water. Ventilation rate was observed by recording individuals using a GoPro HERO4 positioned outside of the tank. The number of opercular beats were tallied over ten

seconds then expanded to beats per minute. Tallied opercular beats were measured immediately

after the onset of last two train crossing playbacks or where they would have occurred in the case

of control trials. These two values were then averaged. The first noise playback was omitted

from analysis in order to eliminate increased ventilation rates likely caused by transfer from

housing to test tank.

Immediately following the thirty-minute trial, individuals were transferred from the

testing environment to an anesthetic dish containing 75 mg/L of Tricaine methanesulfonate (MS

222) in order to measure blood glucose concentrations. After stage three anesthesia was reached,

defined as loss of equilibrium and cessation of locomotion (Schoettger and Julin, 1969), ~0.05

mL of blood was drawn using a heparinized, 32-gauge insulin syringe (MHC Medical Products,

LLC, Fairfield, OH) and placed on the test strip of an Accu-chek Compact Plus glucometer

(Roche Diagnostics Corp., Indianapolis, IN). Where relative measurements of blood glucose are

of interest, portable glucometers have proven to be an accurate and efficient alternative to other

methods (Beecham *et al.*, 2006). Blood glucose measurements were completed <5 minutes post-

trial.

The 500 mL of holding water was then filtered through a grade 2 Whatman paper filter

(GE Healthcare UK Limited, Buckinghamshire, ENG) then a primed (2 washes of DI water

followed by 2 washes of 100% Ethanol) C-18 cartridge (Sep-pak, Waters Technology

Corporation, Milford, MA). Cartridges were stored at -20°C prior to analysis. Free cortisol was

eluted from the cartridges with two, 2mL washes of 99.5% ethyl acetate followed by evaporation under nitrogen gas. Dried residues were resuspended in 500 µL of enzyme immunoassay (EIA) buffer and further diluted with EIA buffer to levels that allowed samples to fall within the standard curve of the cortisol ELISA kit used (Cayman Chemical, Ann Arbor, MI). The plate was incubated and developed in accordance with the Cortisol ELISA kit instructions. The cortisol kit was validated for use with all species by achieving parallelism between the kit standard curve and serially diluted samples taken from each species.

Additional trials testing the effect of temporal arrangement of rail-noise playbacks on water-borne cortisol concentrations were conducted using the same protocol as the multi-species experiment above. Individual *C. venusta* were exposed to either a 30-minute period of silence (n=7), a 30-minute period of continuous train noise (Figure 3) (n=7) or a single presentation of a train crossing followed by 25 minutes and 58 seconds of silence (Figure 4) (n=7).

<u>Behavioral Experiment</u>

Three metrics of swimming behavior were measured in fifteen individuals from each species in the presence and absence of noise playback. Swimming behavior was recorded from a GoPro HERO4 suspended 2.1 m above a 1,022 L mesocosm at sixty frames per second (fps). The experimental mesocosm included 15 cm of rinsed sand as substrate. Water depth was kept at a relatively shallow 23 cm to minimize potential distortion of results caused by vertical swimming. The noise stimulus was played from a laptop connected to a SLA1 studio amplifier

leading to two, UW-30 underwater speakers. One speaker was mounted vertically in the water

column on the far-right hand side of the mesocosm 1 cm off the substrate and the other buried

face up, 3 cm below the substrate directly in front of the vertically oriented speaker in order to

reproduce substrate vibrations. Both speakers played the same stimulus but were independently

amplified to better control the pressure and vibratory components of the playback. A

disconnected speaker was vertically mounted to the far-left side of the mesocosm in order to

avoid potential side bias.

Fishes were observed individually and given a fifteen-minute acclimation period in the

testing mesocosm. After acclimation, video recording was remotely engaged using the GoPro

mobile app. Behavioral trials consisted of a 4 minute and 2 second period of silence immediately

followed by the rail-noise playback of same length (Figure 5). Video recording was remotely

terminated at the end of the noise playback, resulting in an 8 min and 4 second testing period. All

behavioral trials were conducted between 1000 and 1400 hours.

Prior to movement analysis, behavioral trial videos were processed and converted into

image sequences (1 fps) using a command line script (www.github.com/rfriebertshauser/auburn-

fish-biodiversity). Fish position was tracked at each frame using the AnimalTracker plugin

(Gulyás *et al.*, 2016) of the open-source image analysis program Fiji (Schindelin *et al.*, 2012).

The sequential XY-coordinates produced from this tracking procedure were then used to

calculate total lineal distance swam (cm/trial) and average swimming velocity (cm/s/trial) for

control and noise exposure trials. To measure the elicitation of a startle response, swimming

velocity was compared across three, 10 second time periods surrounding the onset of noise

playback. The time periods included 10 seconds immediately before the onset of noise, 10

seconds during the initial fading-in of noise and 10 seconds of full-signal noise and were termed

"pre-noise", "fade-in", and "full-signal" respectively. (Figure 6).

<u>Statistical Analyses</u>

The effect of treatment (fixed factor with two levels) on blood glucose and ventilation

rate was tested with separate general linear models that each included species (fixed factor with

four levels), sex (fixed factor with two levels), total length (continuous fixed factor) and the

interactive effects of treatment by species. To analyze interaction contrasts not presented in the

standard table of coefficients for the physiological models above, a general linear hypothesis test

was used. Models using only sex, total length and treatment were then used to compare the effect

of noise within each species. Water-borne cortisol concentrations across treatments for each

species were compared using a non-parametric Kruskal-Wallis test due to non-normality of data.

The presence of species by treatment interactions were not tested due to stress induced shifts in

cortisol varying widely across taxa (Barton, 2002).

The effect of temporal arrangement (fixed factor with three levels) on water-borne

cortisol concentrations in *C. venusta* was tested using a non-parametric Dunn's Test adjusted for

multiple comparisons.

The effect of treatment (fixed factor with two levels) on lineal distance swam and average swimming velocity was tested with separate general linear models that each included species (fixed factor with four levels), sex (fixed factor with two levels), total length (continuous fixed factor) and the interactive effects of treatment by species. To test for the elicitation of a startle response, same model was used with the exception of treatment being replaced with time-period (fixed factor with three levels). To analyze interaction contrasts not presented in the standard table of coefficients for the behavioral models above, a general linear hypothesis test was used. Models using only sex, total length and treatment (or time-period) were then used to compare the effect of noise within each species. Behavioral models included fish ID as a random factor to account for pseudoreplication.

All statistical analyses were conducted using R version 3.3.4 (R Core Development Team, 2016) with an alpha criterion of 0.05. With the exception of water-borne cortisol, data are reported as effect size +/- standard error (SE). Cortisol data are reported as Δ median and interquartile range (IQR). Normality was observed visually from residual-fitted plots.

RESULTS

Physiological Metrics

Blood glucose levels were not significantly shifted by the presence of rail-noise playback in Blacktail Shiner, Largescale Stoneroller, Redline Darter or Bluegill Sunfish (p=0.46, 0.43, 0.13, & 0.25) (Figure 7).

Waterborne-cortisol concentrations were not significantly altered by the presence of noise playback in Blacktail Shiner, Largescale Stoneroller, Redline Darter, or Bluegill Sunfish (p=0.093, 0.071, 0.76, & 0.41) (Figure 8). However, the 2.43 (IQR=7.34-20.501 and 1.46 (IQR=7.46-12.54) ng/mL decrease in median cortisol concentrations exhibited by Blacktail Shiner and Largescale Stoneroller suggests biological significance.

Additional trials investigating temporal arrangement of rail-noise revealed no significant effect of continuous or single presentation noise playback on water-borne cortisol levels in Blacktail Shiner (p=0.58 & 0.46) (Figure 9). The 4.72 (IQR=2.77-6.31) and 5.49 (IQR=2.71-4.89) ng/mL decrease in water-borne cortisol concentrations however, demonstrates the repeatability of this unintuitive neuroendocrine response to a novel stressor.

The model used to test the effects of rail-noise treatment on ventilation rate across species produced significant interactions between species and treatment such that ventilation rate of Blacktail Shiner, Redline Darter, and Largescale Stoneroller shifted by 49.32 (+/-16.69 SE), 63.92 (+/-16.83 SE), and 69.22 (+/-16.82 SE) bpm (beats per minute) more in the presence of rail-noise compared to the same treatment in Largescale Stoneroller (p=0.0038, 0.00024, and 7.60e-05). Within-species analysis revealed that in the presence of noise playback Blacktail Shiner, Redline Darter, and Bluegill Sunfish increased ventilation rate by 35.017 (+/-9.67 SE), 53.38 (+/-12.29 SE), and 58.092 (+/-8.98 SE) bpm compared to control treatment (p=0.00012,

0.00019, and 7.48e-07) (Figure 10). This equates to a 20.96%, 36.94%, and 50.96% increase

relative to control trials. Largescale Stoneroller showed no statistically significant difference in

ventilation rate between treatment and control conditions.

<div align="center">Behavioral Metrics</div>

The model used to test the effects of rail-noise treatment on the total lineal distance swam

per trial across species produced significant interactions between species and treatment.

Significant interactions existed such that the total distance swam of Largescale Stoneroller,

Redline Darter, and Bluegill Sunfish shifted 1743.48 (+/-430.25 SE), 1444.3 (+/-430.25 SE), and

1197.8 (+/-430.25 SE) cm less in the presence of noise compared to the same treatment in

Blacktail Shiner (p=0.0002, 0.00079, and 0.0054). Within species analysis revealed that in the

presence of rail-noise playback, only Blacktail Shiner significantly decreased the total distance

swam per trial by 1459.29 (+/-456.35 SE) cm compared to control treatment (p=0.0064) (Figure

11). This equates to a 47.22% decrease relative to control trials.

Total lineal distance swam (cm measured each second) and average swimming velocity

(cm/1 second) were both calculated from 1 fps image sequences, therefore analysis of average

swimming velocity produced the same results as the analysis of total lineal distance swam simply

scaled to cm/s. The full model used to test the effects of rail-noise treatment on average

swimming velocity produced significant interactions such that the average swimming velocity of

Largescale Stoneroller, Redline Darter, and Bluegill Sunfish shifted 7.15 (+/-1.76 SE), 5.92 (+/-

1.76 SE), and 4.91 (+/-1.76 SE) cm/s less in the presence of noise compared to the same

treatment in Blacktail Shiner (p=0.0002, 0.00079, and 0.0054). Within species analysis revealed

that in the presence of noise playback, Blacktail Shiner decreased average swimming velocity by

<div align="center">15</div>

5.98 (+/-1.87 SE) cm/s compared to control treatment (p=0.0064) (Figure 12). This equates to a 47.22% decrease relative to control trials.

The full model used to test the elicitation of a startle response due to rail-noise treatment produced no statistically significant interactions between species and treatment. Within-species analysis revealed that during the full-amplitude time-period, Blacktail Shiner, Redline Darter, and Bluegill Sunfish significantly decreased swimming velocity by 2.90 (+/-0.87 SE), 3.24 (+/-0.82) and 1.81 (+/-0.74 SE) cm/s compared to the pre-noise time-period (p=0.0010, 0.0001, and 0.015).This equates to a 25.08%, 72.58%, and 26.43% decrease in swimming velocity relative to control trials. Blacktail Shiner and Redline Darter also significantly decreased swimming velocity by 2.70 (+/-0.87 SE) and 1.88 (+/-0.82 SE) cm/s during the full-amplitude time-period compared to the fade-in time-period (p=0.0021 and 0.023) (Figure 13). This equates to a 23.74% and 60.48% decrease in swimming velocity. No significant differences in swimming velocity between the pre-noise and fade-in time periods were observed. These reductions in swimming velocity suggest the elicitation of a startle response due to rail-noise playback. Results from both experiments are summarized in Table 2.

DISCUSSION

This study examined the physiological and behavioral responses of four species of stream fishes to anthropogenic noise playback in order to gain insight on species-specific disturbance profiles and how they may serve as indicators of individual and community-level fitness consequences. Our results show marked behavioral and physiological responses to noise playback but due to the species in which disturbances were elicited, our hypotheses were unsupported.

In the presence of noise playback, we observed no significant change in blood glucose concentrations in any of the four species. While these results are inconsistent with the literature (Wysocki *et al.*, 2007; Celi *et al.*, 2016; Vazzana *et al.*, 2017), it should be noted that the mobilization of glucose serves as a secondary stress response and is often observed over longer periods of time than our study observed. In addition, the caudal vasculature in these small fishes presented difficulty not only in acquiring the requisite volume but making sure the blood sample was not diluted by surrounding bodily fluids.

The presence of rail-noise induced no significant changes in water-borne cortisol concentrations among all four species observed. However, decreases in cortisol observed in Blacktail Shiner and Largescale Stoneroller and further substantiated by a decrease in Blacktail Shiner during additional trials suggests further complexity to this primary stress response than reported in most studies.

 Exposure to confinement, handling, ship noise and other stressors have consistently produced increased cortisol concentrations in fishes (Ellis *et al.*, 2004; Wysocki *et al.*, 2006; Fanouraki *et al.*, 2008; Kammerer *et al.*, 2010). More importantly, a recent study using an identical protocol as our physiological experiments observed Blacktail Shiner exhibiting

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significantly increased water-borne cortisol concentrations in the presence of traffic noise (Crovo *et al.*, 2015). We believe that this discrepancy in results may be due to either chronic or acute adaptations of these fishes' primary stress response to noise.

The higher amplitude rail-noise playback, relative to traffic noise, may have initiated an unusually fast negative-feedback response (i.e. acute adaptation). Cortisol is known to self-suppress its secretion by acting on certain levels of the hypothalamic-pituitary-interrenal (HPI) axis (Borski, 2000) and this rapid self-suppression has been observed in the literature (Bradford *et al.*, 1992; Young, 1995; Hinz and Hirschelmann, 2000; Medeiros and McDonald, 2012). While fishes exposed to silence (control treatment) were able to ostensibly mount and maintain a neuroendocrine response induced by the handling and confinement stress inherent to the testing environment, individuals exposed to this same stressor as well as noise-playback may have quickly secreted the requisite amount of cortisol to trigger a negative-feedback cascade resulting in the relatively lower hormone concentrations reported.

Adaptation to the noise profiles of home streams may also explain the observed decrease in cortisol in the presence of noise playback (chronic adaptation). The ambient noise inherent to lotic systems, especially in high flow areas, is far from silent (Amoser and Ladich, 2005). If Blacktail Shiner and Largescale Stoneroller have adapted to the acoustic scene of louder microhabitats (i.e. runs and riffles) then it is possible that the silence presented in the control trials may have been more novel, and thereby stressful, than the noise playback administered. This hypothesis could be tested by simply presenting a playback of ambient noise recorded from home streams in place of silence in control trials.

The observed increase in ventilation rate, in all species with the exception of Largescale Stoneroller, reflects findings in the literature concerning increased ventilation by noise (Nedelec

et al., 2016) and other stimuli (Gibson and Mathis, n.d.; Zhao *et al.*, 2017). The effect of noise was also significantly greater on Blacktail Shiner, Redline Darter, and Bluegill Sunfish compared to Largescale Stoneroller. If these fishes are indeed mounting a cortisol-induced negative feedback response, suggesting the lack of a physiological stress response, catecholamine hormones (i.e. epinephrine and norepinephrine) may still be produced via a separate pathway, the hypothalamic-sympathetic-chromaffin axis (HSC) (Conde-Sieira *et al.*, 2018), and lead to an increase in ventilation (Randall and Taylor, 1991).

A shift in swimming activity is a reaction to noise commonly seen in the literature(CITE SOME), however, the direction of change can vary and appears to species-specific (Kastelein *et al.*, 2007). In this study, only Blacktail Shiner decreased lineal distance swam and average swimming velocity. This effect was also significantly different than the effects observed in the other three species. Certain benthic species such as Redline Darter may present issues for observations of swimming parameters due to their fairly sedentary nature. However, we did observe the elicitation of a startle response in all species with the exception of Largescale Stoneroller. This short-term reduction of swimming activity represents an acute response to noise followed by habituation in Redline Darter and Bluegill Sunfish, supported by a lack of change in swimming activity over the entirety of their trials. While Redline Darter lacks a gas bladder, which suggests reduced sensitivity to the pressure component of sound, we believe that these individuals may have reacted to substrate vibrations presented in the testing tank which has been seen in other benthic species (Abboud and Coombs, 2000).

While Largescale Stoneroller is a member of the clade Otophysi and has been observed responding to acoustic stimuli (Holt and Johnston, 2011), this study observed no sign of stress in the presence of rail-noise playback. Similar, unexpected results have been observed in other

comparative, acoustics studies (Kastelein *et al.*, 2008). We believe, that our findings may be due to the juvenile status (lack of sexual maturity determined through post-mortem dissection) of Largescale Stoneroller used in our study. We were forced to use juvenile Largescale Stoneroller due to elevated mortality (associate with housing) in adults. Research has shown decreased auditory sensitivity in other juvenile Ostariophysans (Higgs *et al.*, 2002; Lechner *et al.*, 2011) therefore we propose this as one explanation for the lack of response in the young-of-the-year stonerollers used in this study. The mechanism behind this is likely under-ossification of the Weberian ossicles since time to full ossification is known to vary within the family Cypriniformes (Hoffmann and Britz, 2006). Future studies investigating the role of ontogeny on hearing in Largescale Stoneroller will be necessary to test this hypothesis. In addition to potential hearing insensitivity, the stress response has also been seen to vary across life-stages (Pottinger *et al.*, 1995) as well as within fishes from the same family (Pottinger, 2010).

<u>Indicators of Potential Fitness Consequences</u>

Behavioral and physiological performance can be linked to fitness through a network of relationships. While direct measurements of fitness were not taken in this study, we believe the disturbance responses observed may act as indicators of potential fitness consequences.

The neuroendocrine stress response has evolved to regulate individual homeostasis and those unable to mount this response may be unable to cope with demands brought on by environmental stressors (Romero, 2004). If cortisol secretion in Blacktail Shiner and Largescale Stoneroller is indeed inhibited, then downstream effects of cortisol needed to cope with acute stressors that are concurrent with rail noise would be halted as well. The observed increase in ventilation rate of Blacktail Shiner, Redline Darter, and Bluegill Sunfish is suggestive of an increase in metabolism (Millidine *et al.*, 2008) which can result in reallocation of energy away

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from growth (Barton and Iwama, 1991) to maintenance of the current stressor (Santos *et al.*, 2010). The impacts caused by this reallocation of energy may have even stronger implications to the fitness of developing or reproductive fishes. While the chronic reduction of swimming behavior in Blacktail Shiner and acute reduction in Largescale Stoneroller, Redline Darter, and Bluegill Sunfish suggests a temporary decrease in energy expenditure, alterations in motility may present multiple fitness consequences at the behavioral level such as decreased foraging (Voellmy *et al.*, 2014) or reproductive success. Blacktail Shiner are known to actively fight for access to nesting crevices (Heins, 1990). An overall reduction in motility could likely impair this critical behavior thereby compromising access to nesting sites. Unlike Blacktail Shiner, Redline Darter and Bluegill Sunfish both engage in parental care through nest guarding (Etnier and Starnes, 1993), which, in some species, requires diligent swimming to be upheld (Cooke *et al.*, 2002). Environmental stressors have also been seen to cause altered nesting behavior (Bruintjes and Radford, 2013) as well as decreased parental investment in young (Algera *et al.*, 2017) potentially impacting reproductive success. Furthermore, Bluegill Sunfishes' behavior of nesting in densely packed nest colonies (Boschung and Mayden, 2004) proposes fitness implications at an unusual scale. While measuring long-term impacts to reproduction and survival may be difficult, we believe the metrics of disturbance measured in this study may act as proxies for fitness consequences.

<u>Conservation Implications</u>

The 2018 Living Planet Report, prepared by the World Wide Fund for Nature, cites that 45% of the current abundance decline in freshwater fishes is attributable to habitat degradation and decline in Nearctic fishes. This fact combined with the predicted global addition of 335,000 km of rail lines by 2050 (Dulac, 2013) demonstrates the relevance and importance of this type of

research. Unlike previously studied traffic noise, the temporal structure of rail-noise, resulting in brief, high amplitude exposures may present an even more impactful and complex stressor. One tactic fishes may use to mitigate these stressors, which has been observed in marine species (Handegard *et al.*, 2003; Mueller-Blenkle *et al.*, 2010), is to simply avoid disturbed habitat (Schreck *et al.*, 2001). However, stream fishes, especially individuals restricted to specific microhabitats, may not have this option. The ability to alter habitat usage is further complicated by the fact that anthropogenic bridge noise has been predicted to impact natural soundscapes up to 12,000 meters from its source (Holt and Johnston, 2015). In addition to noise, this reduction in potential distribution inherently amplifies the impacts of other environmental stressors as well which are thought to act cumulatively upon the animals affected (Schreck, 2000). For example, increased water temperatures have been observed to force fishes to operate outside of their optimal ranges (Moffitt and Crawshaw, 1983; Farrell *et al.*, 2009), suggesting that the effects of climate change could potentially compound with the effects of noise we report in this study. Perhaps most importantly, it is thought that community disturbances such as trophic uncoupling could result from anthropogenic noise (Jacobsen *et al.*, 2014), thereby affecting systems at an community-wide level. These facts combined with our results on a diverse group of stream fishes, suggest that these consequences of noise could be felt throughout an entire community.

<div align="center">Conclusions</div>

Our results suggest that the relationship between taxa, water column position and susceptibility to noise are not clear and therefore, should be investigated from a mechanistic perspective. We believe that these results combined with the known diversity of hearing in fishes (Popper *et al.*, 2003) echoes the sentiments of a review on hearing capability (Popper and Fay, 2011) and that studying anthropogenic noise from the generalist/specialist vantage point may in

fact hamper research seeking conservation goals in this field. The behavioral and physiological impacts found in this study can serve as potential indicators of individual- and community-level fitness consequences in noise-exposed habitat. Future work should seek to find correlates between these indicators and measurements of survival and reproductive success (i.e. fitness) in freshwater fish communities at environmentally relevant spatial and temporal scales.

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Table 1. 2 x 2 matrix describing combinations of hearing sensitivity and water column position unique to each study species.

		Hearing Ability	
		Ostariophysan	Other
Water Column Zonation	Pelagic	*Cyprinella venusta*	*Lepomis macrochirus*
	Benthic	*Campostoma oligolepis*	*Etheostoma rufilineatum*

Table 2. Responses of behavioral and physiological metrics to rail-noise playback for each species studied. With the exception of water-borne cortisol, results are presented as effect size and p-value. Bold values indicate statistical significance (alpha criterion=0.05). Water-borne cortisol results are presented as Δ median and p-value due to non-normality.

Species		*C. venusta*	*C. oligolepis*	*E. rufilineatum*	*L. macrochirus*
Blood Glucose (mg/dl)	Effect Size	4.79741	3.7661	10.6269	-10.0818
	P-value	0.4642	0.4286	0.129	0.254
Water-borne Cortisol (ng/mL/30 min)	Δ median	-2.427	-1.464	-0.49	-0.778
	P-value	0.09298	0.07118	0.7557	0.4069
Ventilation Rate (bpm)	Effect Size	**35.0171**	-13.0684	**53.378**	**58.0916**
	P-value	**0.001248**	0.439	**0.00019**	**7.48E-07**
Total Lineal Distance Swam (cm/trial)	Effect Size	**-1459.291**	284.186	-15.0312	-261.4944
	P-value	**0.0064**	0.4265	0.8546	0.1844
Average Swimming Velocity (cm/s/trial)	Effect Size	**-5.980702**	1.1647	-0.061603	-1.0716983
	P-value	**0.0064**	0.4265	0.8546	0.1844
Startle Response (Pre-Noise to Full-Amplitude) (cm/s/10 sec)	Effect Size	**-2.895682**	-1.26897	**-3.247687**	**-1.812742**
	P-value	**0.001**	0.3739	**0.0001**	**0.015**
Startle Response (Fade-In to Full-Amplitude) (cm/s/10 sec)	Effect Size	**-2.693724**	-2.53053	**-1.877639**	1.129019
	P-value	**0.0021**	0.0766	**0.0231**	0.1289
Water-borne Cortisol (ng/mL/30 min) Continuous Presentation	Δ median	-4.717	NA	NA	NA
	P-value	0.57551	NA	NA	NA
Water-borne Cortisol (ng/mL/30 min) Single Presentation	Δ median	-5.489	NA	NA	NA
	P-value	0.464	NA	NA	NA

Figure 1. Waveform (a) and spectrogram (b) visualization of underwater recording of a train crossing a beam-style bridge used in behavioral and physiological experiments (4 min & 2 sec).
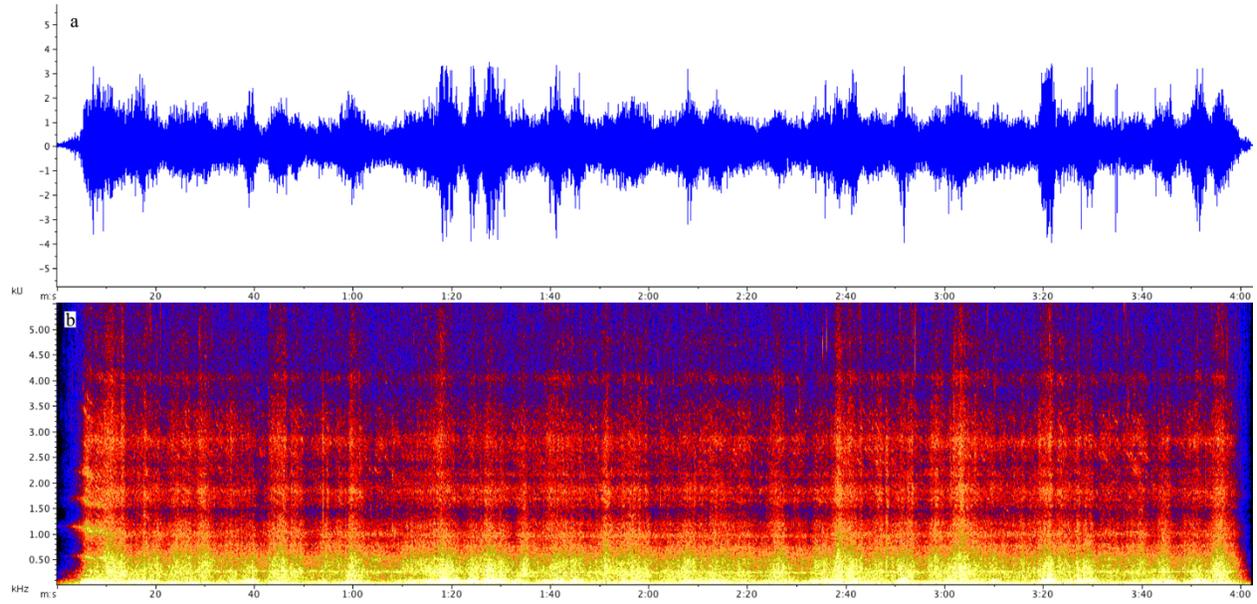
Figure 2. Waveform visualization of triplicate playback used in multi-species physiological trials (30 min).
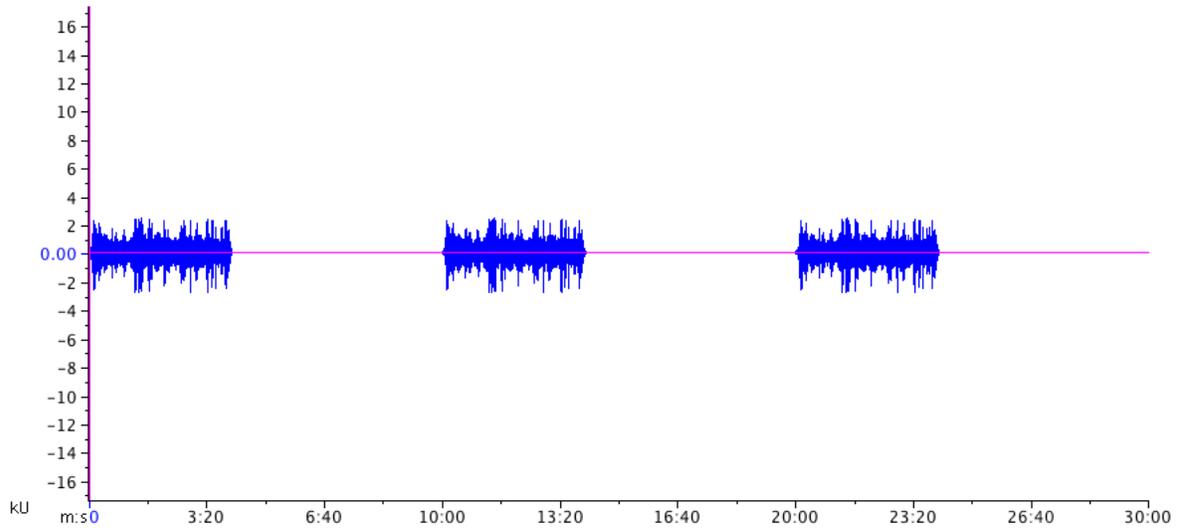
Figure 3. Waveform visualization of continuous playback used in trials investigating the effects of temporal arrangement of rail-noise on water-borne cortisol (30 min).
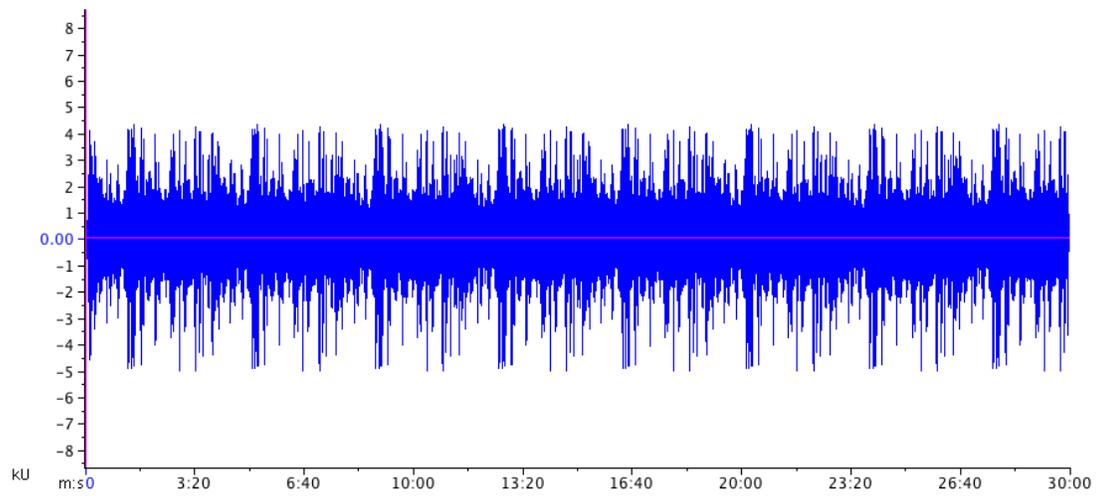
Figure 4. Waveform visualization of single playback used in trials investigating the effects of temporal arrangement of rail-noise on water-borne cortisol (30 min).
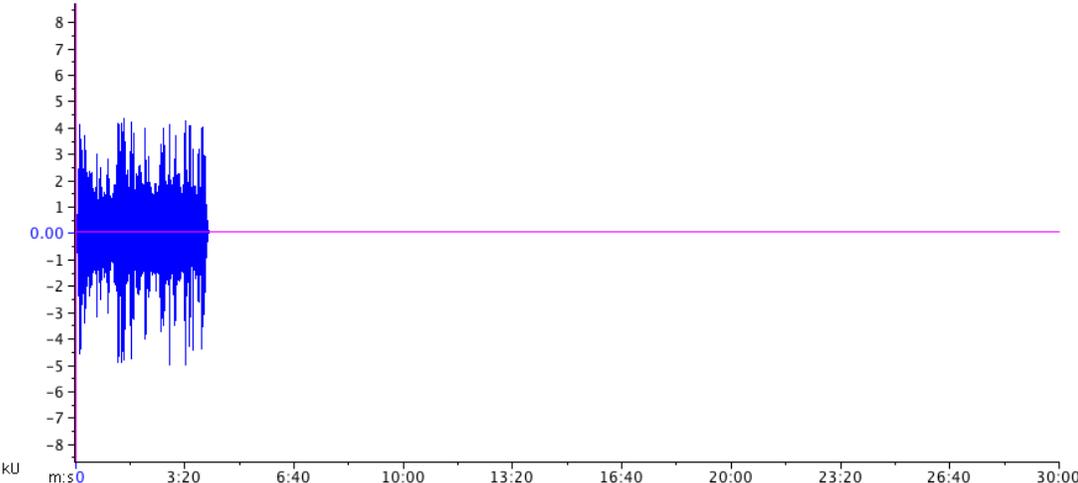
Figure 5. Waveform visualization of rail-noise playback used in behavioral trials (8 min & 4 sec).
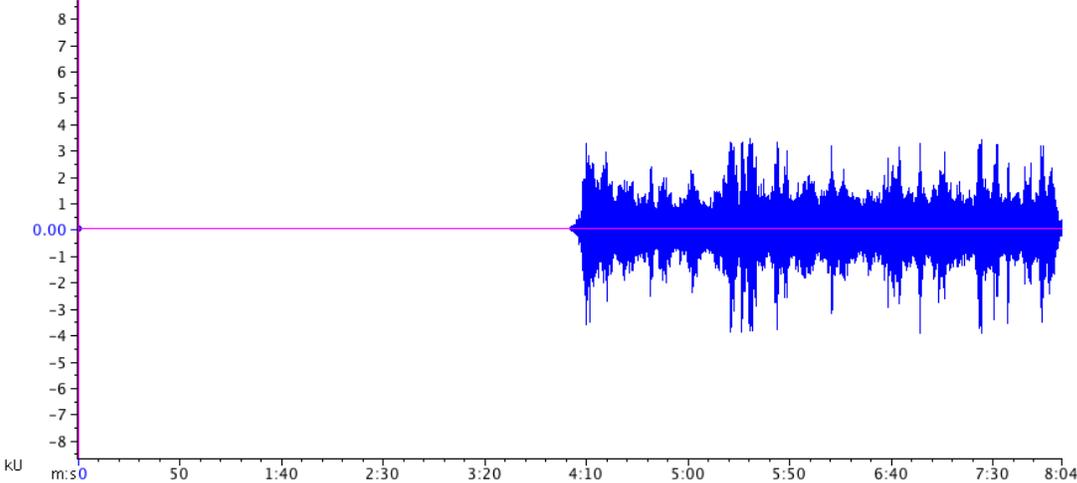
Figure 6. Waveform visualization of fine-scale time periods compared to measure the elicitation of a startle response. "*" denotes the onset of rail-noise playback.

| pre-noise (10 s) | fade-in (10 s) | full-signal (10 s) |
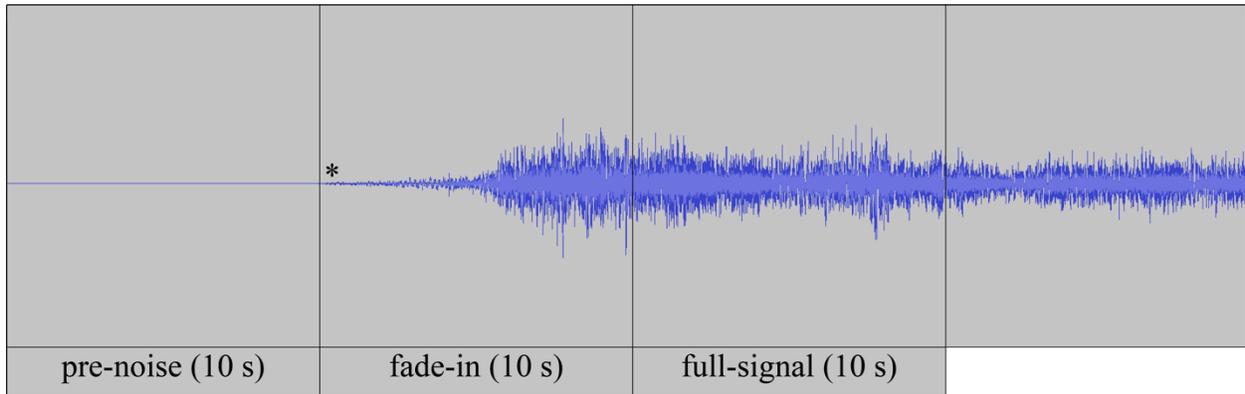
Figure 7. Bar graph comparing blood glucose concentrations (mg/dL) across control and rail-noise exposed individuals from each species. Presented as means ± SE. (n=15)
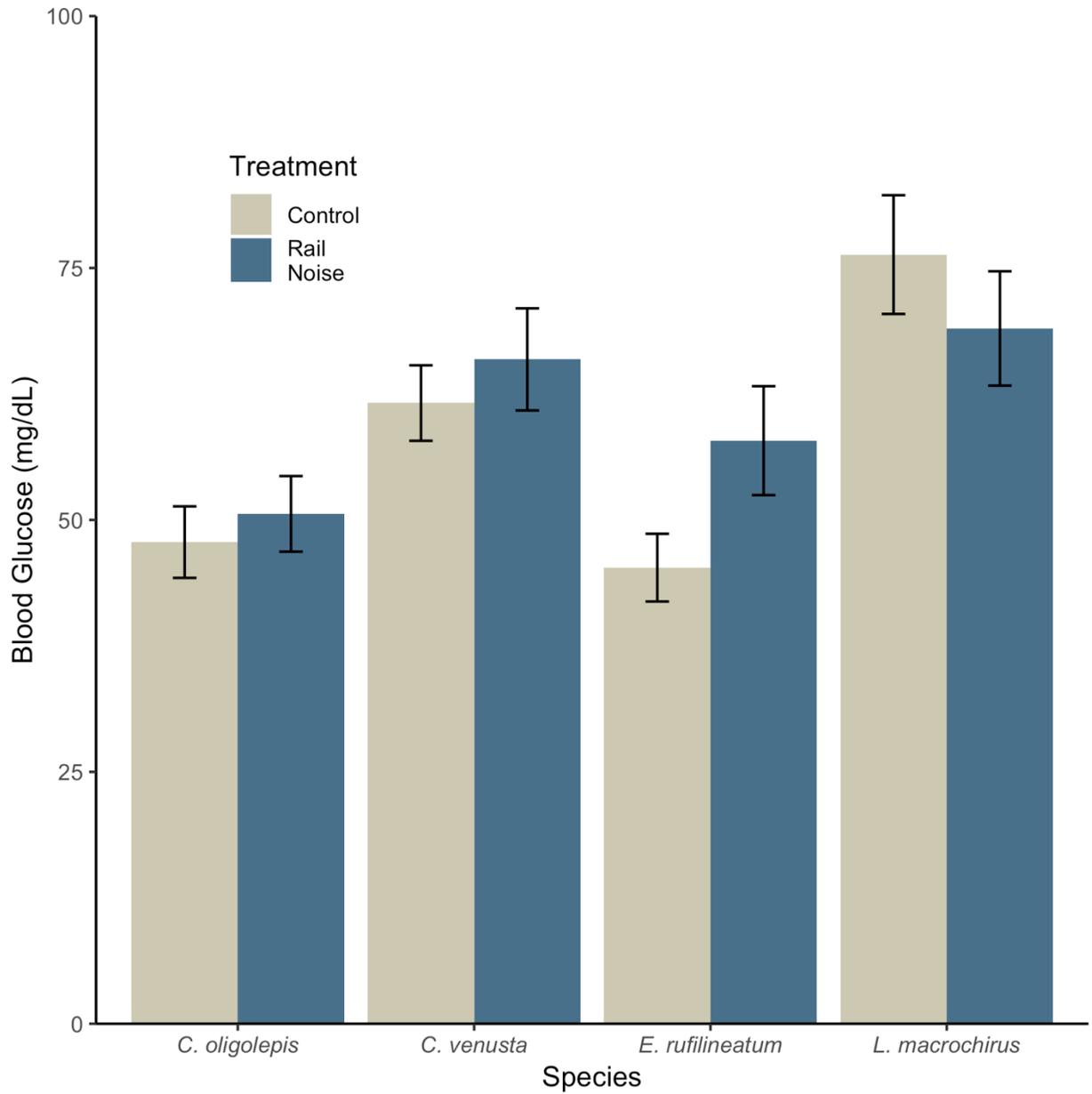
Figure 8. Box plot comparing water-borne cortisol concentrations (ng/mL/30 min) across control and noise exposed individuals from each species. Thick lines and boxes denote medians and interquartile ranges respectively. (n=15)
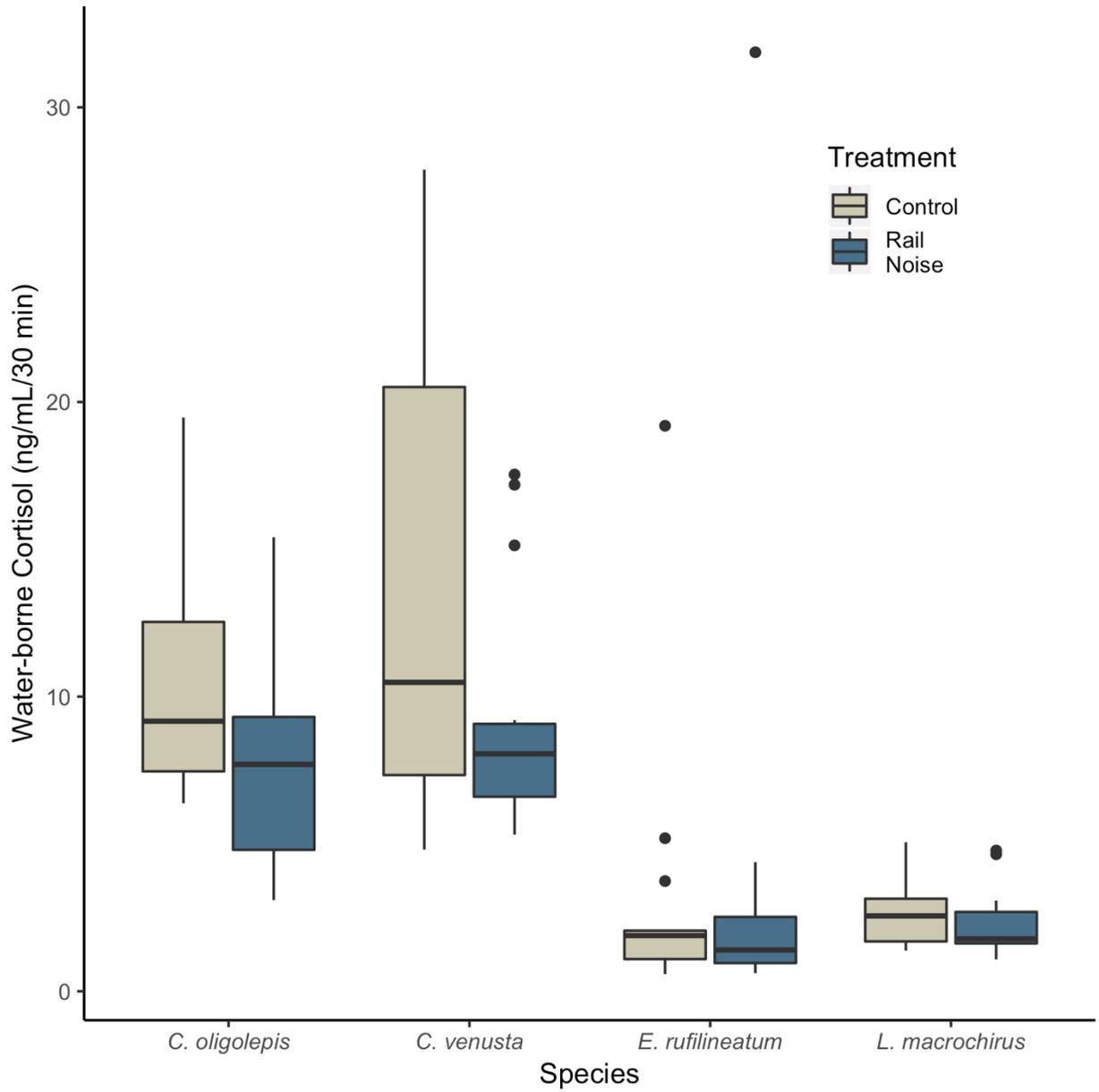
Figure 9. Box plot comparing water-borne cortisol concentrations (ng/mL/30 min) across different temporal arrangements of rail-noise from *C. venusta*. Thick lines and boxes denote medians and interquartile ranges respectively. (n=15)
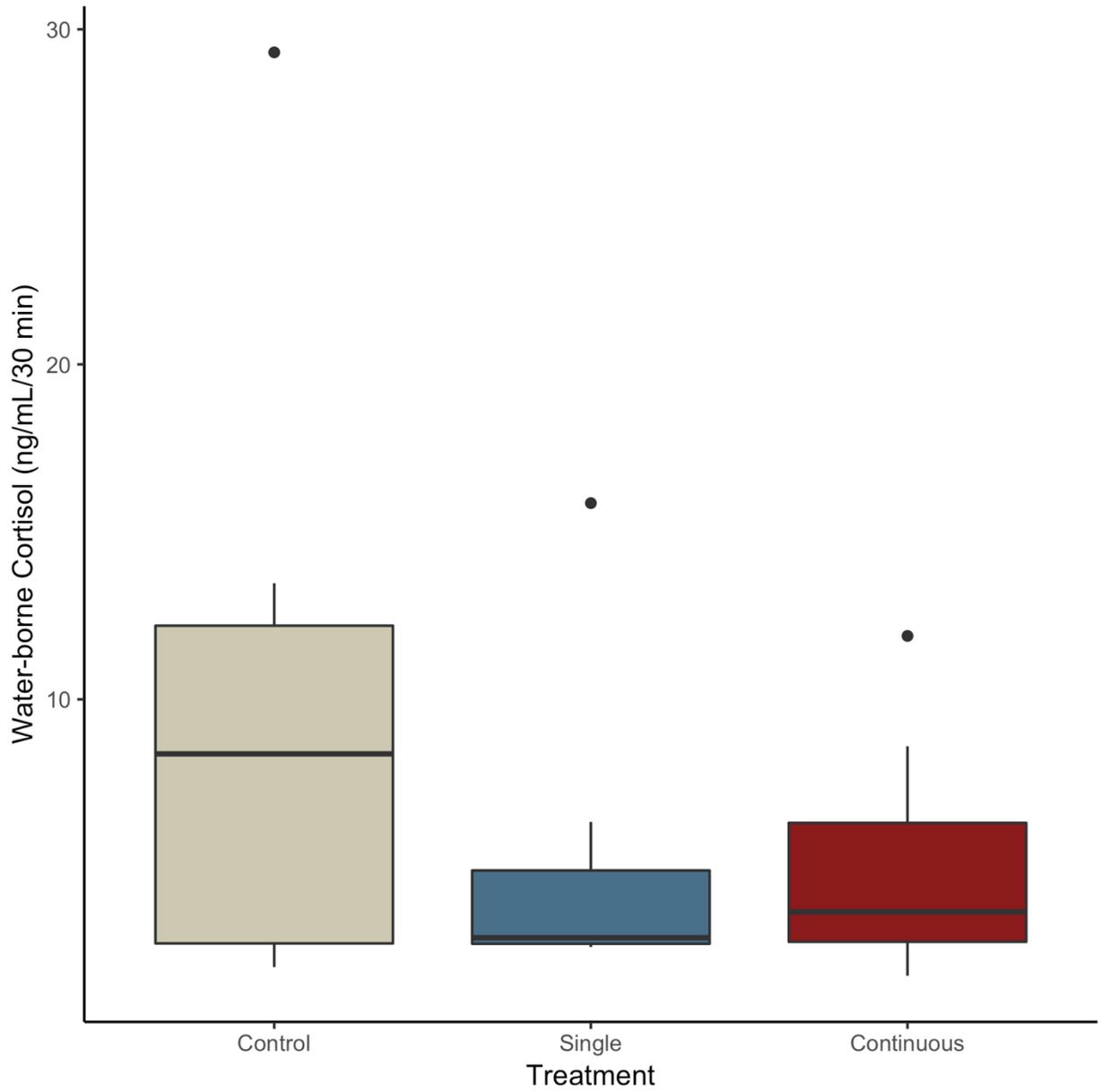
Figure 10. Bar graph comparing ventilation rate (bpm) across control and rail-noise exposed individuals from each species. Presented as means ± SE. "*, **, ***, and ****" denote increasing levels of statistical significance. (n=15)
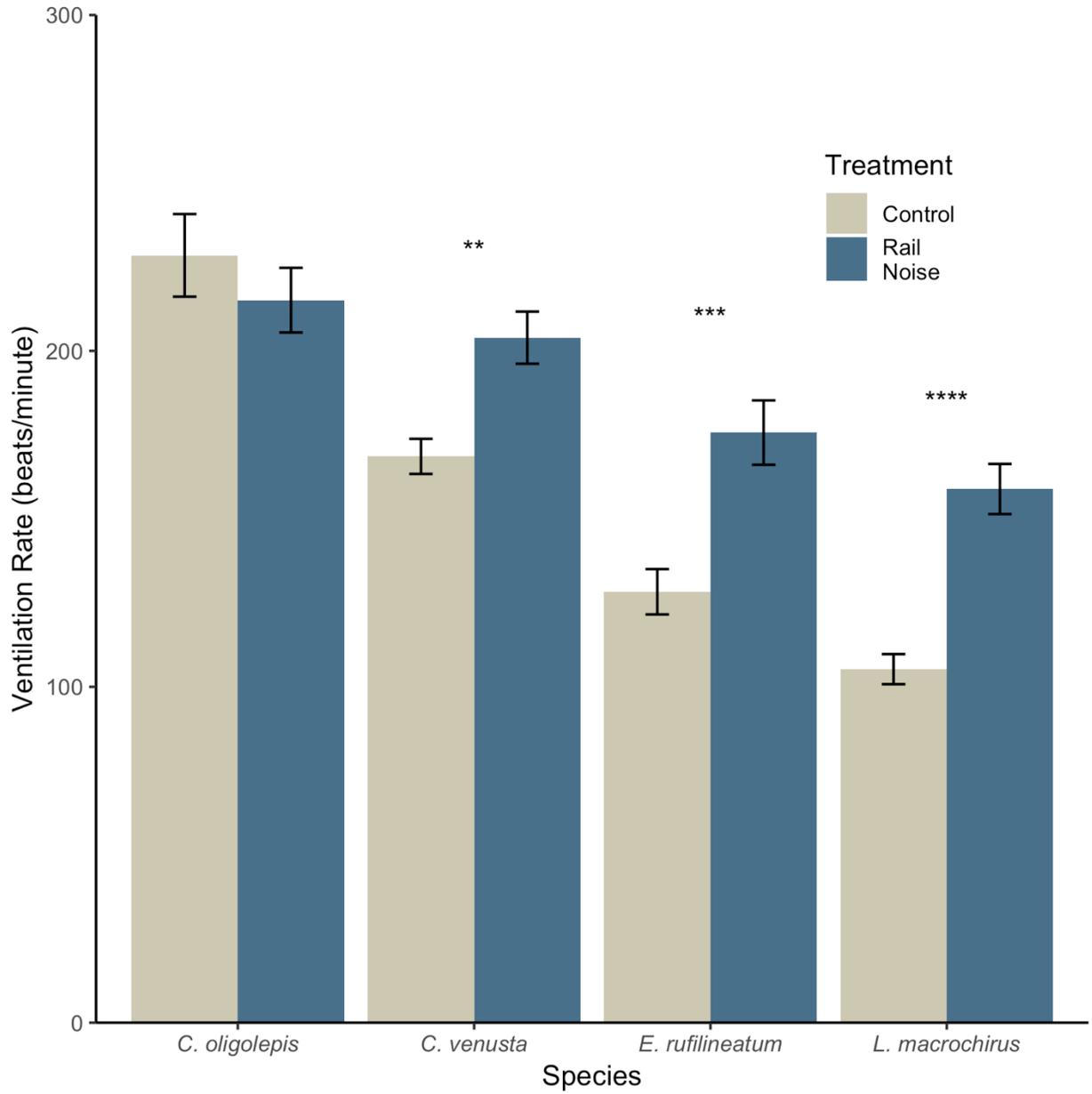
Figure 11. Bar graph comparing total lineal distance swam per trial (cm/trial) across control and rail-noise exposed individuals from each species. Presented as means ± SE. "**" denotes statistical significance. (n=15)
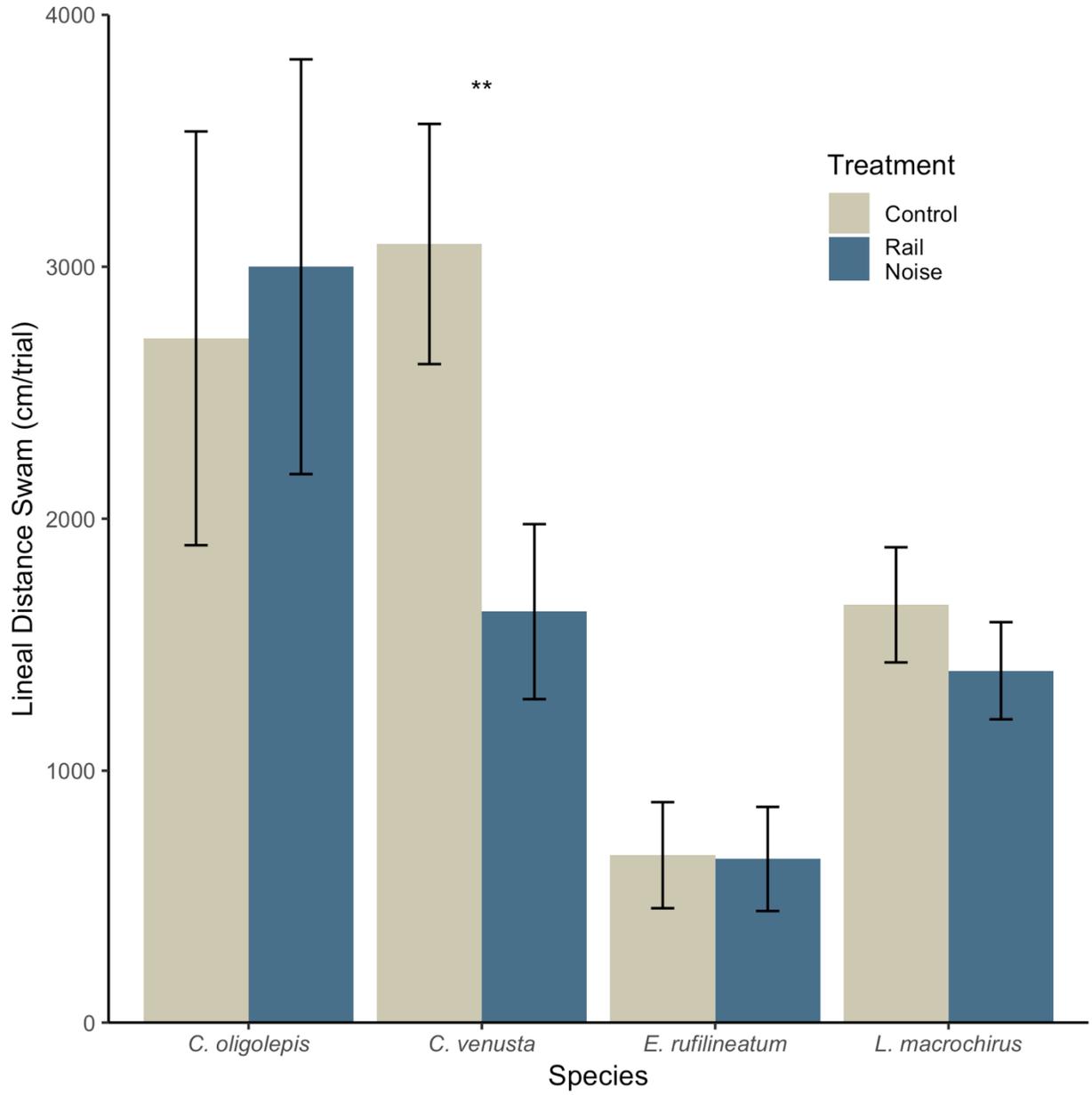
Figure 12. Bar graph comparing average swimming velocity per trial (cm/s/trial) across control and rail-noise exposed individuals from each species. Presented as means ± SE. "**" denotes statistical significance. (n=15)
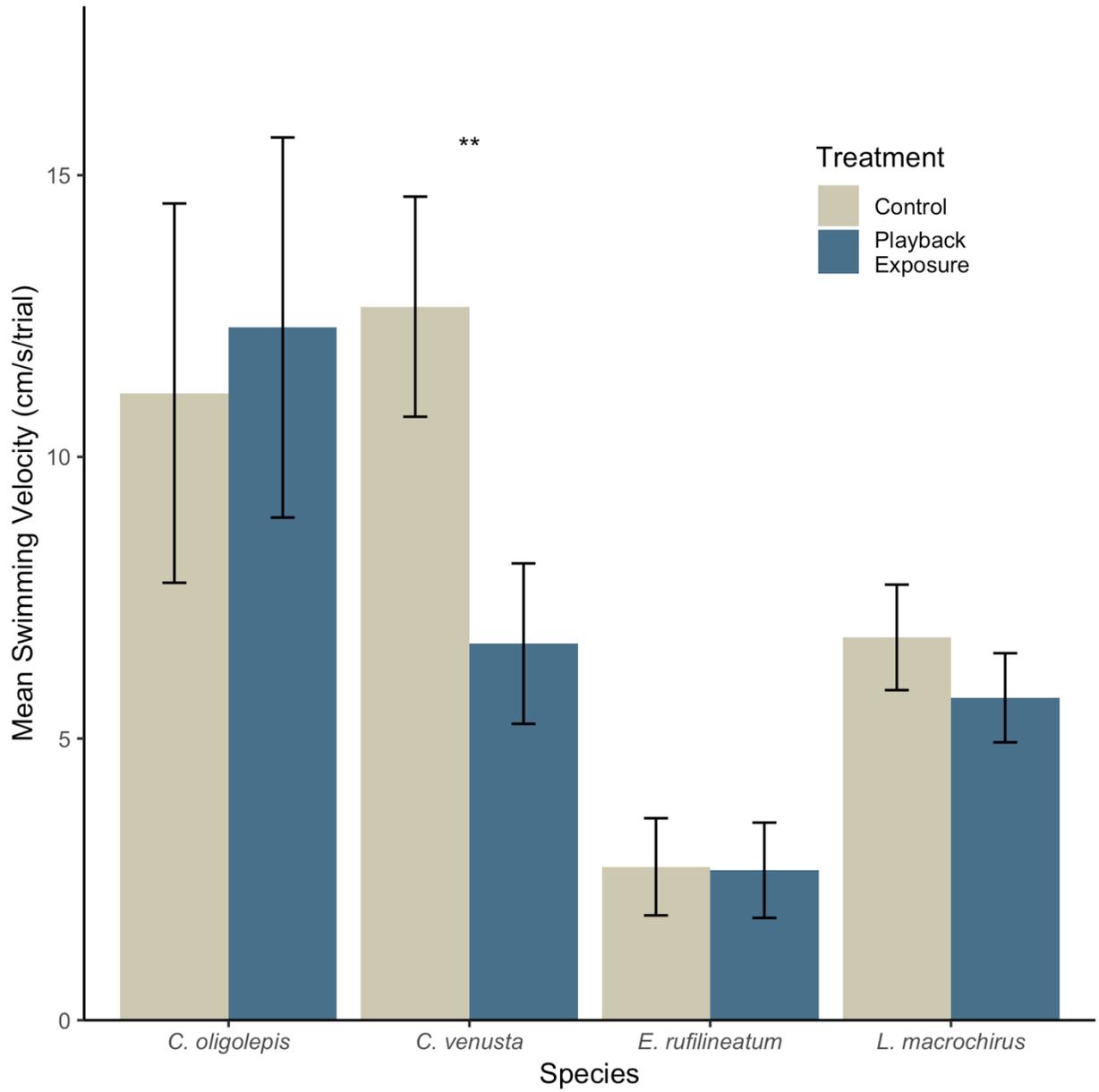
Figure 13. Bar graph comparing average swimming velocities (cm/s) across 10-second time periods in order to quantify the elicitation of a startle response due to rail-noise playback. "*, **, ***, and ****" denote increasing levels of statistical significance. (n=15)