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Behavioural and morphological changes in fish exposed to ecologically-relevant boat noises

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Abstract

There is increasing concern about the effect of underwater noise on fish due to rising levels of anthropogenic noise. We performed experiments on the black bullhead (*Ameiurus melas*), a species with known hearing specializations and located within the Laurentian Great Lakes where there is significant commercial and recreational boat traffic. We tested and compared physiology (baseline cortisol), behaviour (activity, sheltering) and morphology (ciliary bundles of hair cells) of bullhead to boat noise. At 140 dB re 1 μ Pa (-54.84 dB re 1 m s⁻²) we saw clear behavioural effects in terms of both activity and sheltering levels despite no obvious morphological or physiological stress. Following both short and long period acute exposure to higher — but environmentally relevant — noise levels, bullhead were less active and sheltered more and also exhibited a decrease in ciliary bundles. These results suggest that there are sublethal effects of anthropogenic noise on fish behaviour and ciliary bundles which may have direct implications on population health. Moreover, commonly used metrics such as stress hormones may not always offer the most relevant biomarker of the response to anthropogenic boat noise.

48 **Introduction:**

49 Anthropogenic noise is now common in aquatic ecosystems, although the effects this has on
50 aquatic animals, particularly freshwater fishes, remains unclear (Slabbekoorn et al. 2010; Popper
51 & Hawkins 2012). There has been a notable increase in anthropogenic noise due to
52 industrialization primarily caused by the expansion of transport networks and various resource
53 extraction methods (Wale et al. 2013; Solan et al. 2016), all of which have the potential to
54 disrupt acoustic communications (Wysocki et al. 2006; Popper & Hastings 2009; Wale et al.
55 2013). Although there has been a greater focus on effects of noise sources such as sonar,
56 airguns and pile driving (Shannon et al. 2015), recreational and commercial boats are the
57 predominant source of anthropogenic noise at low frequencies underwater (Ross 1976; Dyndo
58 et al. 2015; Nichols et al. 2015; Shannon et al. 2015; Solan et al. 2016), having considerable
59 overlap with the sound production and hearing range of most fish species examined to date
60 (20-1000 Hz) (Kasumyan 2005; Ladich & Fay 2013; Nichols et al. 2015).

61 Noise pollution research is well studied in marine environments with a particular focus
62 on marine mammals and fish (Popper 2003; Slabbekoorn et al. 2010), indicating that noise
63 impacts on animals can range from non-lethal behavioural and physiological stressors to death
64 (Weilgart 2007; Mickle & Higgs 2017). Research that has been performed regarding noise
65 impacts on freshwater fish is generally focused on aquaria fish such as: goldfish (*Carassius*
66 *auratus*; Wysocki & Ladich 2005; Smith 2003), zebrafish (*Danio rerio*; Neo et al. 2015; Sabet et
67 al. 2015) and cichlids (Cichlidae; Hastings et al. 1996; Brintjes & Radford 2013), resulting in a
68 gap in our knowledge of noise effects on wild, economically important freshwater fish.
69 Freshwater ecosystems have a disproportionately high fish diversity (Combes 2003) but
70 comparatively less attention on effects of anthropogenic noise. Given the importance of both

freshwater lakes and fish to shipping and the economy, more research is also needed to determine the full impact of shipping noise in these high traffic environments.

Hearing is an important sensory modality in fish for communication and orientation with a great diversity in hearing abilities between species (Hawkins 1981; Aalbers & Drawbridge 2008; Fay 2009). To date the majority of research regarding noise pollution incorporates either behavioural or physiological measures alone, but seldom have single studies integrated techniques (Cooke et al. 2014; Mickle & Higgs 2017). Previous work on fishes leads to the suggestion that some individuals exposed to anthropogenic noise show physiological stress responses such as increased levels of stress hormones (Barcellos et al. 2007), change in cardiac output (Graham & Cooke 2008), and further hypothesize changes in gene expression and immune function (Mommsen et al. 1999; Barton et al. 2002; Shannon et al. 2015). Fish exposed to loud sounds may also suffer from physical impairments such as hair cell damage (Hastings et al. 1996; Wysocki et al. 2007) or a shift in their hearing threshold (Enger 1981; Smith et al. 2006). Finally, behavioural changes have also been seen in response to loud sounds with changes in overall behaviour level (Ona & Godø 1990), feeding behaviour (Payne et al. 2014) and predator/prey interactions (Sabet et al. 2015; Simpson 2016). While these individual effects are informative, more integrative work may more accurately identify possible noise effects on fish. Thus, increased efforts on integrative studies in freshwater fish will help to better understand possible effects on anthropogenic noise in aquatic environments.

Here we take an integrative approach to examine potential impacts of noise on the phenotypic responses of black bullhead (*Ameiurus melas*), a species hypothesized to have specialized hearing capabilities based on previous research on Siluriformes (Poggendorf 1952; Kleerkoper & Roggenkamp 1959; Lechenr & Ladich 2008) and located within the Laurentian

Great Lakes where there is significant commercial and recreational boat traffic. To obtain a holistic measure of the phenotypic response to noise in this species, we measured behavioural, physiological and morphological changes across biologically relevant noise levels. First, we examined the impacts of noise levels at 140 dB re 1 μ Pa RMS (ranging in frequency from 100-10,000 Hz) on bullhead behaviour and physiology, and then to further explore these results we exposed fish to differing intensities of noise (160 and 170 dB re 1 μ Pa RMS; 100-10,000 Hz) across two time points, 1 hour and 24 hours. We hypothesized that bullhead exposed to 140 dB re 1 μ Pa would exhibit behavioural changes, while bullhead exposed to 160 and 170 dB re 1 μ Pa would exhibit changes in behaviour, physiology and morphology in response to noise. More specifically, we hypothesized that bullhead under acute noise exposure would exhibit behavioural and physiological responses to noise, while those with chronic exposure will exhibit physical damage to hearing-related tissues.

Methods

Experimental Design:

All work was conducted under approved Canadian Council for Animal Care (CCAC) protocols (University of Windsor AUPP 14-11). Non-reproductive black bullhead, ranging from 22.02-117.60g were obtained from a fish farm in Harrow, Essex County, Ontario (42°01'14.5"N 83°00'04.1"W). Due to the constraints of catching wild/farmed fish these species were a range in size. Fish were housed at a temperature of 22.2°C and a pH of 6.5-7 in animal quarters at the University of Windsor. The fish were fed daily and kept at a 12:12 light-dark cycle to mimic natural conditions, however as these species prefer dark environments all housing tanks were covered with black garbage bags and opaque tank covers.

Noise levels were collected from housing and experimental tanks using a hydrophone system (Inter Ocean system inc. – Acoustic Calibration and System Model 902) and, where possible, using a waterproofed accelerometer (model 4524 cubic triaxial deltatron, Brüel & Kjær) to estimate noise levels as pressure and particle motion. Both the hydrophone and the accelerometer were suspended in the middle of the tank and both were used because the fish ear is fundamentally responsive to acceleration but pressure measurements are more easily understood for those concerned about noise exposure in a field setting (see Hawkins & Popper 2018). We also recognize the inherent problems with complex acoustics in small tanks (Parvulescu 1964; Rogers et al. 2015) but the current set of experiments would not be possible in a field setting and can still provide useful information about possible noise effects. The background noise in the experimental tanks was below 120 dB re 1 μ Pa RMS and below the noise floor of the accelerometer system. Bullhead likely detect both pressure and particle components of sound, as can other Ostariophysan fishes (Higgs et al. 2006), but because we ultimately want to refer these results to levels experienced in the natural habitats where anthropogenic sound is most relevant, and where particle motion is still difficult to accurately measure, more emphasis is placed on the pressure units.

Two noise experiments were conducted, the first consisted of 140 dB re 1 μ Pa treatment and the second consisted of a 160/170 dB re 1 μ Pa noise treatment, however both treatments played the same boat noise file (Fig. 1). Boat noise was recorded from a recreational vessel using a hydrophone (Loggerhead Instruments, Model # HTI-96-Min/3V/Exp/LED) placed at a depth of approximately 2m at a distance of 4m from a boat launch in a local Great Lakes habitat (Chewitt bay, Ontario, Canada). This sound file was then played through an underwater speaker (UW-30, Lubell Labs) at 140 (equivalent to -54.84 dB re 1 m s^{-2}), 160 (equivalent to -50.61 dB re 1 m s^{-2})

and 170 dB re 1 μ Pa (equivalent to -46.55 dB re 1 m s^{-2}) (all sound levels are in RMS; Fig. 1). Only one recording was chosen to standardize the exposure across treatments so we do not expect these results to necessarily reflect all boat recordings possible (see Slabbekoorn & Bouton 2008).

The experimental setup included an underwater speaker (Electro-Voice UW-30), connected to an amplifier (Scosche SA300), a 12 Volt PBS car battery for power and an mp3 player to play the noise (Sony Walkman NWZ-E464). The speaker was placed in the middle of the tank and background noise was quantified using a hydrophone which measured decibel levels at 8 locations and two depths in the tank, and reliably ranged from 116- 122 dB re 1 μ Pa.

During the 140 dB re 1 μ Pa level experiment, six individual bullhead (total $n=60$), similar in size, were randomly collected from housing tanks and added into separate plastic experimental tanks (55 liters) in a dark room equipped with red light, a PVC shelter, an underwater speaker and single air stone in each tank. During the high intensity noise experiment (160/170 dB re 1 μ Pa), three fish (total $n=24$) similar in size were added into one plastic experimental tank, with the same set-up as the lower intensity experiment, however equipped with three PVC tubes so each fish could have an individual shelter (Fig. 2).

Behavioural Assays:

During the first experiment (140 dB re 1 μ Pa level noise exposure) ($n=60$), six bullhead were given an acclimation time of one hour after which a baseline control treatment took place for four hours followed by a four-hour noise treatment (Fig. 3a). There were two separate controls for this experiment, a “baseline control” and a “non-treatment control”. The baseline control took place after the acclimation period but before the noise was played (Fig. 3A), while the non-

treatment control replicated entire experimental conditions without the presence of noise (Fig. 3). To quantify a change in behaviour, experiments were recorded using a GoPro Hero3+ (Go Pro). Sheltering and general swimming behaviours were analyzed and compared during the last hour of both the baseline control and noise treatments. We quantified a sheltering response when the fish were residing in Polyvinyl chloride (PVC) tubing (one tube in each tank) and activity levels were quantified as a measure of time spent swimming throughout the videos. Activity and sheltering accounted for the total behaviours observed during the experiment.

Based on the results from 140 dB re 1 μ Pa we decided to perform a second experiment to observe the impacts of higher noise levels on bullhead (160 dB re 1 μ Pa and 170 dB re 1 μ Pa). During the second experiment black bullhead ($n=24$) were exposed to either 160 or 170 dB re 1 μ Pa of boat noise for either one hour (short period acute noise exposure) or twenty-four hours (long-term acute noise exposure). Three bullhead were placed in the experiment tank (as opposed to six bullhead in individual tanks as performed in the lower intensity experiment) and allowed to acclimate for one hour before noise treatment (at either 160 or 170 dB re 1 μ Pa) began. Two separate controls (baseline and non-treatment) were also implemented for this experiment. During short period noise exposure (1 hr), fish acclimated for one hour, after which a one-hour baseline control treatment began followed by one hour of boat noise (at either 160 or 170 dB re 1 μ Pa) (Fig. 3b). During the long-term noise exposure (24 hr), fish were also allowed to acclimate for one hour, followed by a one-hour baseline control treatment and then 24 hours of boat noise (at either 160 or 170 dB re 1 μ Pa) (Fig. 3c). To keep consistency in behavioural videos, we recorded the fishes' behaviour during the last hour of the long-term noise experiment. There were two experimental replicates ($n=6$) for both short and long-term acute exposure experiments, at both 160 and 170 dB re 1 μ Pa, totalling 24 fish (Fig. 3c). We quantified

sheltering response and activity levels using the same methods presented in experiment 1. Noise experiments started at approximately the same time each day to avoid diurnal differences in behaviour

Physiological Assays:

At the end of each experiment, bullhead were anaesthetized using 2-phenoxy ethanol (SIGMA-ALDRICH Product #: 1 mL of 2-phenoxy ethanol per 2 L of water) and each tail was removed within two minutes so that blood from the caudal artery could be collected using a heparanized capillary vial. Once blood was collected, the fish were decapitated and heads fully submerged in paraformaldehyde (4%) for preservation before further dissection of ears. Plasma was isolated via centrifugation and cortisol was subsequently extracted from the plasma using a standard ELISA protocol. Cortisol levels were determined using a commercially-available enzyme immunoassay (Cayman Chemical Company, Ann Arbor, MI) with assays performed according to kit instructions. Before beginning assays, a pool of black bullhead plasma was assayed both raw and after dichloromethane extraction. Serial dilutions of both were found to be parallel to the standard curve. As extracted samples showed reduced values due to recovery losses and raw plasma showed no indication of interference, samples were run on raw plasma without extraction. To ensure sample cortisol values fell within the kit detection range, bullhead samples were assayed at 1:20 dilution (10uL of plasma and 190uL of assay buffer). Sample concentrations were determined using an 8 point standard curve run in duplicate on each assay plate. Standards ranged in concentration from 4000pg/mL to 6.6pg/mL while the minimum detection limit of the assay is 35pg/mL. At the end of incubation, the absorbance values for each well were measured at 412nm using a BioTek Synergy H1 plate reader. All samples were

assayed across 7 plates yielding an inter-assay variation of 19.96% and intra-assay variation of 5.3% for bullhead.

Ciliary Bundle Counts:

The catfish saccule is an irregular structure, twice the length of the lagena with rounded anterior/posterior ends (Jenkins 1977), the saccule location in bullhead was determined based on the schematic depicted in Jenkins (1977) (Fig. 4c). Saccules were dissected (using a Leica L2 10445930 dissecting scope) from 1 of every 6 bullhead ears randomly selected from the low intensity noise experiment (total n=6), and 1 of every 3 bullhead from the high intensity noise experiment (total n=8). After saccules were collected, they were preserved in paraformaldehyde (4%) until stained with 12.5 µl of fluorescent green phalloidin mixed with 200 µl of phosphate buffer (Higgs et al. 2002). Once saccules were properly stained, ciliary bundles of hair cells were visualized through images collected from a Leica microscope, (Leica DM IRB inverted fluorescence microscope, Las A.F. 4.5). As there are thousands of ciliary bundles of hair cells present along the saccular epithelium (Higgs et al. 2003), ciliary bundles were counted in three regions along the anterior, middle and posterior saccule using a magnified view of the epithelium. Images were imported into Adobe Photoshop (V3.0; Adobe Systems) to create 3 identical boxes of 225 µm² cm in size (in magnified view) representing 19% of the total saccular area (Higgs et al. 2003) (Fig. 4). Ciliary bundles within each box were then counted using Image J software (NIH) (Fig. 4). Hair cell damage was characterized as a difference in absolute number of ciliary bundles between fish exposed to noise and control fish. Comparisons in ciliary bundles of hair cell number were made between bullhead in the no-treatment control and sound exposure experiments.

228 *Statistical Analyses*

229 Both controls (baseline and no- noise treatment) in the two experiments yielded similar
230 behavioural results, therefore for the purposes of this study, statistics are only reported for
231 comparisons between the baseline control and noise exposure treatments. There was no
232 difference in activity levels between the acclimation period and the no-noise control period, thus
233 time of residence in the tank was accounted for. Once data were collected, a one-way analysis of
234 variance (ANOVA), designating fish ID as a random factor, was performed using SPSS (IBM,
235 2014) to analyze behavioural differences of black bullhead when exposed to noise. Ciliary
236 bundle data were analyzed using an independent sample t-test between no-noise controls and
237 noise exposures, designating hair cell position as a random factor. To examine differences in
238 cortisol levels, data were log-transformed (as cortisol data were not normally distributed) and an
239 ANOVA was performed on differences between no-noise control animals and noise exposed
240 animals. The dependent variables in this experiment were: behavioural markers
241 (activity/sheltering response), cortisol levels and ciliary bundle count. The fixed factor in the low
242 intensity treatment consisted of sound exposure (no-noise control or 140 dB re 1 μ Pa),

243 During the second high-intensity noise experiment (160 and 170 dB re 1 μ Pa), a one-way
244 ANOVA was also used to examine behavioural differences of bullhead during baseline control
245 and noise treatments. We used a Tukey-post hoc test to further investigate where differences
246 were present. Cortisol data were log transformed and analyzed using an ANOVA and ciliary
247 bundle data were compared using an independent sample t-test. The dependent variables in the
248 high intensity treatment were: activity/sheltering, cortisol levels and ciliary bundle counts,
249 however the fixed factor consisted of sound exposure (control or 160 or 170 dB re 1 μ Pa) and
250 time of exposure (short and long period acute exposure).

Results:

When exposed to 140 dB re 1 μ Pa boat noise bullhead exhibited significant changes in behavioural characteristics. Activity levels decreased from 2.63 to 0.97 ($\pm$ 0.43 SEM) minutes per hour when fish were exposed to boat noise played at 140 dB re 1 μ Pa ($F_{1,5}=8.4$, $p=0.034$; Fig. 5a). Sheltering behaviour increased from 21.37 to 24.27 ($\pm$ 1.21913 SEM) minutes per hour when fish were exposed to noise ($F_{1,5}=8.6$, $p=0.033$; Fig. 5b). There was no significant difference in cortisol levels relative to resting levels ($F_{8,50}=4.2$, $p=0.184$; Fig. 5c) exposed to 140 dB re 1 μ Pa for four hours. When comparing ciliary bundle data in the control and noise treatment during the 140 dB re 1 μ Pa noise exposure there was no significant difference in counts ($t_6=0.78$, $p=0.902$; Fig. 5d).

During the 160 and 170 dB re 1 μ Pa noise exposure treatments, differences were present within the short period acute exposure experiment (1 hour) for both activity and sheltering. Activity levels significantly decreased from 21.69 to 1.97 ($\pm$ 1.0 SEM) minutes per hour during 160 dB re 1 μ Pa exposure and from 21.69 to 8.90 ($\pm$ 1.0 SEM) minutes at 170 dB re 1 μ Pa ($F_{2,12}=32.987$, $p<0.001$; Fig. 6a). Sheltering behaviour significantly increased from 27.13 to 57.70 ($\pm$ 0.80509 SEM) minutes during 160 dB re 1 μ Pa and from 27.13 to 50.70 ($\pm$ 1.85 SEM) minutes per hour during 170 dB re 1 μ Pa when bullhead were exposed to noise ($F_{2,12}=11.236$, $p<0.001$; Fig. 6b). During the long period acute exposure (24 hour) there were differences in both activity and sheltering behaviours. Overall, activity levels significantly decreased from 2.51 to 0 ($\pm$ 0.034 SEM) minutes per hour at 160 dB re 1 μ Pa treatment and increased from 2.51 to 8.36 ($\pm$ 1.0 SEM) minutes at 170 dB re 1 μ Pa, ($F_{2,12}=9.989$, $p=0.002$; Fig. 6a). Post-hoc tests demonstrated significant differences in activity levels between the control treatment and 170 dB re 1 μ Pa of noise ($p=0.020$) and between 160 and 170 dB re 1 μ Pa ($p=0.002$), indicating that fish

274 were more active during longer (24 hour) exposure of 170 dB re 1 μ Pa compared to the control
275 treatment. However, there was no significant difference in activity levels between the control and
276 160 dB re 1 μ Pa treatment ($p=0.442$). Sheltering behaviour differed overall when bullhead were
277 exposed to noise ($F_{2,12} = 10.799$, $p= 0.001$; Fig. 6b). Post-hoc tests indicated a non-significant
278 difference in sheltering between the control treatment and 170 dB re 1 μ Pa ($p=0.072$) of noise
279 and also between control treatment and 160 dB re 1 μ Pa ($p=0.095$). However, bullhead sheltered
280 more at 160 dB re 1 μ Pa (60 minutes per hour) compared to 170 dB re 1 μ Pa (50 minutes per
281 hour) ($p=0.001$). During acute exposure of both noise levels of 160 and 170 dB re 1 μ Pa, bullhead
282 did not exhibit a change in cortisol levels compared to the control ($F_{2,14} = 1.305$, $p=0.302$; Fig.
283 6c). Cortisol data collected during the chronic noise treatment uncovered no significant
284 differences ($F_{2,15} = 3.268$, $p=0.066$; Fig. 6c). Post-hoc analyses revealed no significant difference
285 between cortisol levels in the no-noise control experiment compared to 160 dB re 1 μ Pa
286 ($p=0.147$) and 170 dB re 1 μ Pa ($p=0.992$); and between the no-noise control and 170 dB re 1 μ Pa
287 ($p=0.075$).

288 There was a significant effect of 160 and 170 dB re 1 μ Pa noise exposure on ciliary bundle
289 number ($F_{2,30} = 18.458$, $p < 0.001$; Fig. 6d), resulting in fewer ciliary bundles present in noise
290 treatments than no-noise controls. Post-hoc tests further uncovered a significant difference in
291 ciliary bundles of hair cells when comparing the no-noise control and 170 dB re 1 μ Pa treatment
292 ($p < 0.001$) during short term acute exposure. During long term exposure of noise, post-hoc
293 analyses determined a significant difference in ciliary bundle number between the control and
294 170 dB re 1 μ Pa ($p < 0.001$), 160 vs 170 dB re 1 μ Pa ($p=0.001$), however there was not a significant
295 difference between the no-noise control and 160 dB re 1 μ Pa ($p=0.062$) (Fig. 6d). There was no
296 significant difference in the number of ciliary bundles present in each box placed along the

saccular epithelium in both control and exposed ears ($p=0.0727$), showing no regional effects of sound exposure on hair cell damage (Fig. 4).

Discussion

Anthropogenic noise caused a change in behavioural characteristics and ciliary bundles in black bullhead. Bullhead exhibited an increase in sheltering behaviour and a decrease in activity levels even when exposed to 140 dB re 1 μ Pa and had fewer ciliary bundles when exposed to 170 dB re 1 μ Pa during both short and long period acute exposure. Noise pollution research is not commonly studied in freshwater environments (Mickle & Higgs 2017), even though these environments are species rich and important economically and recreationally as we rely on fish as a major source of protein for the world's population (16%) (Tidwell & Allan 2001). As such, we would suggest that more resources be dedicated to better understanding possible fitness effects of anthropogenic noise in these critical habitats.

Behavioural Responses

The decreased activity patterns demonstrated by bullhead when exposed to 140 dB re 1 μ Pa suggest this level of anthropogenic noise impacts behavioural responses which can be a precursor to a physiological stress response (Eriksson & Van veen 1980; Valdimarrson & Metcalfe 1998). Contrary to some literature (Smyly 1957; Lelek 1987), bullhead are not normally sedentary in nature, but instead are mobile (usually under dark conditions) to detect prey species and find suitable spawning habitats (Eriksson & Van veen 1980; Knaepkens et al. 2004). Research has indicated that fish can often exhibit avoidance behaviours (Ona & Godø 1990; Fewtrell & McCauley 2012) in response to noise; however, due to constraints of tank size, we used activity levels to indicate a change in behavioural characteristics. McLaughlin and Kunc

(2015) examined the behavioural impacts of boat noise on the convict cichlid (*Amatitlania nigrofasciata*) and found that although the presence of a boat noise increased time spent sheltering and decreased spent time foraging, it did not alter their overall activity level. Activity effects in bullhead may be due to the enhanced hearing capability of bullhead and suggests caution in extrapolating effects between species with different hearing abilities.

Physiological Responses

Cortisol levels were used as a measure of physiological stress when fish were exposed to noise (Donaldson 1981; Wysocki et al. 2006; Barcellos et al. 2007). While our behavioural results suggested that fish were showing a stress response, this was not indicated by the physiological marker of cortisol level. This apparent discrepancy can be explained by the principle that animals first respond to stress through a behavioural mechanism (Dawkins 2003; Moberg & Mench 2005). It is also possible that there was no clear pattern associated with cortisol data due to sampling at the end of the experiment. If bullhead exhibited a spike in cortisol at the beginning of the experiment when fish were first introduced to the noise, it is possible cortisol levels returned to baseline levels after a certain amount of time had passed. Thus, our findings do not suggest that bullhead do not exhibit signs of physiological stress, to confirm this, more stress markers such as: glucose, lactate, cardiac output and changes in oxidative stress or immune response could be measured (Graham & Cooke 2008; Dantzer et al. 2014). Finally, physiological responses are highly context specific and can be modified by a number of intrinsic and extrinsic factors (Madliger & Love 2014). As a result, physiological stress and changes in growth and condition may only be apparent after longer time periods or repeated exposures to noise stressors, and the extent of these responses may be different during different life history stages (Dantzer et al. 2014; Shannon et al. 2016).

342 *Ciliary Bundle Data*

343 Based on results obtained from the first noise treatment of 140 dB re 1 μ Pa, we decided to
344 expose bullhead to both short and long term acute periods of 160 and 170 dB re 1 μ Pa to
345 determine what the impacts were at these higher noise levels. As fish were being housed in
346 communal tanks, we changed the experimental design during the higher intensity noise treatment
347 to more accurately represent normal housing conditions and reduce stress in the chronic
348 treatments that necessitated holding fish for longer time. Therefore, we had 3 fish in an
349 experimental tank as opposed to 1 fish in 6 separate tanks. The boat noise played to the bullhead
350 at these higher noise levels is still ecologically relevant — small boats generally produce noise
351 ranging from 140-167 dB re 1 μ Pa and merchant ships produce noise ranging from 178-192 dB re
352 1 μ Pa up to an approximate distance of 2 m away (Arveson & Vendittis 2000; Amoser et al.
353 2004). Cargo ships have been shown to reach up to noise levels of 212 dB re 1 μ Pa at 1 m away,
354 this level can create shock waves emitted from the propeller (Arveson & Vendittis 2000). During
355 long term acute exposure, fish were less active at 160 dB re 1 μ Pa but were more active during
356 170 dB re 1 μ Pa when compared to the control. These behavioural effects may be attributed to the
357 ciliary bundle data; if there is a decrease in ciliary bundles of hair cells after both short and long
358 term acute stressors of noise played at 170 dB re 1 μ Pa, bullhead hearing sensitivity will likely
359 decrease (Smith et al. 2003) so they may no longer perceive the noise to be as loud and therefore
360 stressful. If fish are no longer sensitive to the noise it is likely they will no longer exhibit signs of
361 stress, explaining why bullhead exposed to 170 dB re 1 μ Pa for 24 hours did not exhibit a change
362 in cortisol levels and were more active during this treatment, even relative to the controls which
363 still had background noise present in the holding conditions.

Higgs and colleagues (2002) looked at regional differences in hair cell density along 14 regions of the saccular epithelium of zebrafish and only found density differences at the caudal end of the epithelium. Smith and colleagues (2003) counted hair cells along 4 locations (2500 μm^2 size boxes) along the saccular macula in goldfish, therefore, we focused on hair cell number along three locations of the bullhead sacculus. We found no significant regional differences in both the controls and exposed ears. Previous research suggest topographic frequency dependent loss of hair cells in fish (Furukawa & Ishii 1967), however our boat noise file featured a broad spectral range (100-10,000 Hz), limiting the topographic effect of frequency specific hair cell damage.

Future Considerations

There are a few considerations when analyzing the current data. First, some fish have higher baseline cortisol levels than others, which can cause variability in results. Second, cortisol levels fluctuate seasonally and diurnally (Laidley & Leatherland 1988); to avoid this confounding variable, all experiments were started at approximately the same time each day, over the period of three months. Due to the capture of live fish, we had a large size range in bullhead, however the fish were non-reproductive (fish were dissected to visualize presence of gonads) to avoid the impacts reproduction could have on behaviour. As we did not test other sources of noise, we cannot definitively say fish are responding to the boat noise specifically, however, we can conclude that bullhead display behavioural changes and fewer ciliary bundles when exposed to noise. As our research includes intensities of noise at 160 and 170 dB re 1 μPa , we need to determine the frequency of these noise levels in fishes environment. Most research involving soundscape data is carried out in marine environments (McWilliam & Hawkins 2013; Staaterman et al. 2014; Erbe et al. 2015) but the data that do exist for freshwater (e.g. Amoser et

al. 2004; Graham & Cooke 2008) do indicate that anthropogenic noise levels in freshwater ecosystems often exceed those used here..

Possible next steps for future study would be to measure sound levels in local areas across areas such as the Great Lakes impacted by boat noise to determine the source, timing and duration of noise levels. Further research is also needed to determine boat noise impacts on freshwater fish with general hearing capabilities. Another component to consider is that the fish were kept in captivity and could not escape, therefore in the wild fish may simply leave the area to avoid the noise. However, depending on the noise source, health status of the animal and how loud it is, this may not always be possible. Recommendations to decrease noise impacts on freshwater habitats include: the addition of protected areas, restricting human access to specific sites (particularly spawning grounds for endangered fish), the use of physical barriers to noise and widespread quiet technology (Shannon et al. 2015). Noise pollution research is not commonly studied in freshwater environments (Mickle & Higgs 2017), even though these environments are species rich and important for human survival (Tidwell & Allan 2001). More focus should be given to noise impacts on freshwater environments to be able to truly assess the impact of anthropogenic stressors on survival and fitness of these key species.

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Figure1: A spectrogram comparing the output of the boat noise file from the field and a hydrophone recording of the noise file collected from the experimental tank when played at 140, 160 and 170 dB re 1 μ Pa.

Figure 2: The experimental tank set-up used in both the 140 dB re 1 μ Pa and 160/170 dB re 1 μ Pa trials (although only one fish was placed in this tank during the 140 dB re 1 μ Pa trial). The test tanks were fitted into a Styrofoam holding structure and placed on top of two acetal plastic sheets, 2 cm thick, to minimize acoustic disturbance and vibrations from the floor.

Figure 3:

- a) Six bullhead were placed in separate experimental tanks during the 140 dB re 1 μ Pa exposure experiment. Bullhead acclimated for one hour, after which time a four hour baseline control trial took place followed by a four hour treatment (either noise or silence). Bullhead were anesthetized and blood sampling took place immediately following the experiment.
- b) Three bullhead were added into one experimental tank during the higher intensity (160 or 170 dB re 1 μ Pa) short-term acute noise experiment. Bullhead acclimated for one hour, followed by a one hour baseline control trial and one hour treatment (either noise or silence). Bullhead were anesthetized and blood sampling took place immediately following the experiment.
- c) Three bullhead were added into one experimental tank during the high intensity (160 or 170 dB re 1 μ Pa) long-term acute noise experiment. Bullhead acclimated for one hour, followed by a one hour baseline control trial and a twenty-four hour treatment (either noise or silence). Bullhead were anesthetized and blood sampling took place immediately following the experiment.

A)

B)

C)

Figure 4:

- a) A dissected bullhead ear is shown in this image, exposing the sacculus, which is further sectioned into three identical boxes ($15 \mu\text{m}^2$ in size in magnified counting view) to allow for hair cell counts.
- b) A stained lagena in the bullhead ear is shown here to allow for comparison to the sacculus. The hair cells in the lagena were not quantified.
- c) A schematic of a catfish ear from Jenkins (1977) is referenced here as we used this diagram to help us identify the ear organs (u= utricle; l=lagena; s=sacculus; sag= sagitta (saccular otolith)).

Figure 5:

a) Mean activity levels of black bullhead during exposure to 140 dB re 1 μ Pa of boat noise and during control trials. Significant differences are indicated by different letters at the $p=0.05$ level, error bars are representative of mean ($\pm$ S.E).

b) Mean levels of sheltering behaviour in black bullhead when exposed to 140 dB re 1 μ Pa of boat noise and during control trials. Significant differences are indicated by different letters at the $p=0.05$ level, error bars are representative of mean ($\pm$ S.E).

c) Mean log cortisol levels of black bullhead after exposure to 140 dB re 1 μ Pa of boat noise compared to controls. Significant differences are indicated by different letters at the $p=0.05$ level, error bars are representative of mean ($\pm$ S.E).

d) Mean hair cell counts of black bullhead when exposed to 140 dB re 1 μ Pa of boat noise and in control trials. Significant differences are indicated by different letters at the $p=0.05$ level. Error bars are representative of mean ($\pm$ S.E).

Figure 6:

a) Mean activity levels of bullhead when exposed to 160 and 170 dB re 1 μ Pa of boat noise at two time points. Significant differences are indicated by different letters at the $p=0.05$ level. Error bars are representative of mean ($\pm$ S.E).

b) Mean sheltering levels of bullhead when exposed to 160 and 170 dB re 1 μ Pa of boat noise at two time points. Significant differences are indicated by different letters at the $p=0.05$ level. Error bars are representative of mean ($\pm$ S.E).

c) Mean cortisol levels of bullhead when exposed to 160 and 170 dB re 1 μ Pa of boat noise at two time points. Significant differences are indicated by different letters at the $p=0.05$ level. Error bars are representative of mean ($\pm$ S.E).

d) Mean hair cell counts of bullhead when exposed to 160 and 170 dB re 1 μ Pa of boat noise at two time points. Significant differences are indicated by different letters at the $p=0.05$ level. Error bars are representative of mean ($\pm$ S.E).