The effects of sound on the survivorship and embryonic development of a marine gastropod *Stylocheilus striatus*, (aplysiidae).

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How anthropogenic noise pollution affects marine organisms is drawing increasing international concern. There is evidence for anthropogenic noise having negative and harmful effects on the health, development and behavior of many terrestrial species; however, there are few examples of how specific frequencies of sound affect the survivorship and embryonic development of marine invertebrates. This experiment examines the effects of specific frequencies of sound on the survivorship and embryonic development of a marine gastropod, *Stylocheilus striatus* on the island of Mo'orea, French Polynesia. It was found that high frequency sound treatments caused a delay in the embryonic development of *S. striatus* embryos by 3 days while decreasing veliger survivorship by 37%. Additionally, high frequency treatments were shown to cause an observed morphological difference in shell morphology as compared to control and low frequency treatment groups. This study can be used to aid in the management and planning of future conservation polices regarding sound pollution and marine invertebrate gastropods as their presence is crucial for reef health and community structure.

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Abstract

How anthropogenic noise pollution affects marine organisms is drawing increasing international concern. There is evidence for anthropogenic noise having negative and harmful effects on the health, development and behavior of many terrestrial species; however, there are few examples of how specific frequencies of sound affect the survivorship and embryonic development of marine invertebrates. This experiment examines the effects of specific frequencies of sound on the survivorship and embryonic development of a marine gastropod, *Stylocheilus striatus* on the island of Mo'orea, French Polynesia. It was found that high frequency sound treatments caused a delay in the embryonic development of *S. striatus* embryos by 3 days while decreasing veliger survivorship by 37%. Additionally, high frequency treatments were shown to cause an observed morphological difference in shell morphology as compared to control and low frequency treatment groups. This study can be used to aid in the management and planning of future conservation polices regarding sound pollution and marine invertebrate gastropods as their presence is crucial for reef health and community structure.

Key words: Anthropogenic, embryonic, *Stylocheilus striatus,* Mo'orea, veliger, survivorship gastropods, community.

Introduction

Anthropogenic (human-produced) sound disturbance is drawing increasing concern from the scientific community (McCauley & Fewtrell, 2008; Kight and Swaddle, 2011; Morley et al., 2014; Nedelec et al., 2014). Since the beginning of the industrial revolution, anthropogenic sound disturbance has continually increased as technology, transportation, resource acquisition and human civilization expands (Morley et al., 2014). This lead to anthropogenic noise becoming classified as a worldwide pollutant within the US National Environmental Policy Act (1972) and the European Commission Marine Strategy Framework Directive (2008). Similarly, the effects of noise pollution on terrestrial ecosystems have undoubtedly increased as compared to the past (Barber et al., 2010).

Anthropogenic noise pollution research has been suggested to help aid in conservation management and policy (Kingsford et al., 2002; Barber et al., 2010; Nedelec et al., 2014). For instance, increases in acoustic pollution reduced the distance and area in which some terrestrial birds infer their own acoustic signals (Brumm & Slabbekoorn, 2005). Evidence for noise altering terrestrial vertebrates' foraging behavior, anti-predator responses, reproductive success, and community structures has also been found (Brumm & Slabbekoorn, 2005; Arch & Narins 2008), all of which can be assessed for conservation management.

In marine environments anthropogenic sound disturbance is centered between economic transport routes, commercial harvesting fisheries, or underwater drilling sites (Slabbekoorn et al., 2010). Research associated with marine anthropogenic noise pollution has primarily investigated mammals and fish (Wells et al., 1999; McCauley & Fewtrell, 2008; Morley et al., 2014). Larval stage reef-dominate fish have been found to locate and settle on coral reefs due to sound cues made by fish and shrimp currently living on the reef (Montogomer et al., 2006). This demonstrates how sound is guiding behavior and how anthropogenic noise pollution is interfering with recruitment patterns (Kingsford et al., 2002; Simpson et al., 2004, 2005; Montgomery et al., 2006).

There have been considerably fewer investigations into invertebrate behavior and response to increases in anthropogenic sound. One study found coral larvae respond to acoustic cues which aid in their detection of habitat from large distances and from up-current of their preferred settlement locations (Vermeij et al., 2010). This was the first observed auditory response in the invertebrate phylum Cnidaria (Vermeij et al., 2010), demonstrating the urgency needed for additional research on invertebrate response to anthropogenic noise pollution.

How anthropogenic noise pollution affects an organism's fitness in early life stages is also an understudied area (Morley et al., 2014; Nedelec et al., 2014), despite the impacts it might have on population dynamics (Lindström, 1999; Nedelec et al., 2014). Fortunately, the life cycles of some marine invertebrates are relatively short (Morley et al., 2014; Nedelec et al., 2014), allowing fitness and population dynamics to be tested with methods logistically different than compared to many vertebrates (Morley et al., 2014; Nedelec et al., 2014). The embryonic development of the sea hare *Stylocheilus striatus* (a gastropod in the family Aplysiidae) found on Moorea, French Polynesia, was discovered to become impaired by anthropogenic noise playback (Nedelec et al., 2014). Increased mortality rates from noise in the veliger (the final larval stage of this mollusk, often denoted by small ciliated flaps used for swimming and feeding) stage was also observed (Nedelec et al., 2014). It is not known which frequency of sound decreases survivorship of the juvenile veligers of *S. striatus*.

The biological importance of *Stylocheilus striatus* measures large in terms of herbivorous maintenance of algal reefs (Nedelec et al., 2014), and as invertebrates are vital parts of trophic webs (McCauley & Fewtrell 2008; Morley et al., 2014). It is, therefore, important to understand the effects of specific frequencies of noise on early life stages in invertebrates and their possible implications in marine ecosystems. This experiment elaborates on Nedelec et al. (2014), while serving as a quantitative approach to identifying how varying frequencies of sound affect the survivorship and embryonic development of the early life stages of *S. striatus*. This experiment will also contribute to understanding invertebrate fitness at crucial, early life stages, potentially aiding in future conservation management and policy.

Materials & Methods

Study site and species

Forty-six adults of the sea hare *Stylocheilus striatus* were acquired courtesy of the CRIOBE (Centre de Recherches Insulaires et Observatoire de l'Environnement) research facility located on the Island of Mo'orea in French Polynesia. The specimens acquired from the CRIOBE facility were located from the lagoon around Moorea. *S. striatus* specimens were kept in an aerated flowing seawater aquarium at the University of California Gump Field Research Station. The specimens grazed on cyanobacteria (*Lyngyba majuscule*) collected from white sandy substrates within the lagoon around Mo'orea at two locations (Figure 1; Table 1).

Sea hare reproduction

Individual *S. striatus* specimens were paired with similar sized individuals in 0.5 L cylindrical breeding containers that allowed seawater flow while preventing specimens from mixing with the main population (Nedelec et al. 2014). Specimens were monitored hourly throughout the night until copulation was observed (Nedelec et al. 2014). Following mating, sea hare mothers were separated into 50 mL falcon tubes thus allowing maternity to be known (Nedelec et al. 2014). This opisthobranch gastropod lays a gelatinous string of eggs (an egg ribbon) with each egg containing 1-6 embryos (Nedelec et al. 2014). The following morning, mother specimens were removed from the falcon tubes and the egg ribbons were left to develop (Nedelec et al. 2014).

Egg preparation

Egg ribbons were cut into three similarly sized segments using a scalpel and randomly separated and prepped for treatment four days after deposition; denoted by the embryotic formation of villi and defined shell morphology (Figure 2). This stage of the embryonic development was chosen to minimize chances of disease or an early developmental issue causing mortality while allowing accurate counts of hatched and unhatched veligers. Three cross sections of each egg ribbon (the middle and both ends) were taken and the numbers of eggs and veligers were counted using a compound light microscope. The mean of the cross section's counts was used as an extrapolation multiplier against the number of eggs stacked lengthwise within the ribbon to provide an egg number estimation for each segment of ribbon.

Sound experiments

To examine the effect of sound on development of *S. striatus* eggs, 6 hour tones of pure sine wave function MP3 clips of 100 hertz (low frequency treatment) and 1000 hertz (high frequency treatment) were generated using a multi-track audio editor and recorder, Audacity (v2.1.2, Carnegie Mellon University, 2016). Tones were played through a waterproof Bluetooth speaker (Toshiba DT-B660, 4W, frequency response 0.1-20kHz, Japan) via a Bluetooth (v4.0, A2DP, EDR, LE, aptX) connection to a Samsung Galaxy S5-Active phone (Operating system: Android 2014, Samsung Galaxy, Suwon, South Korea).

Decibel levels of underwater sound recordings were measured using a digital voice recorder converted into a hydrophone (Olympus DM- 620, 3 Microphone, Linear Pulse Code Modulator, input level -70dB, Tokyo, Japan). Decibel levels ranged from 76-101 decibels with a reference level of 20 μPa per second squared per hertz. These measurements were then converted to an underwater decibel level by adding 61.5 decibels to the readings (DOSITS, 2016; NOAA, 2016).

Petri dishes containing a sea hare egg ribbon segment in 20mL of seawater were placed directly above the speaker and secured to the speaker to prevent the petri dish from vibrating on the speaker. The 100 Hz clip was played to egg ribbon segments between the hours of 12:00 and 18:00 while the 1000 Hz clip was played between 22:00 and 06:00. The control egg ribbon segment did not receive sound treatment. Control and previous treated egg ribbons were kept in a separate laboratory on the Gump facility to control for sound. After treatments were finished, eggs hatched 1-3 days later.

The following categories were counted from October 9th to November 10th, 2016 using the methods adopted and modified from Nedelec et al. (2014): (1) dead eggs or eggs failed to undergo organogenesis (i.e. failed to develop); (2) unhatched eggs with dead developed embryos; (3) total number of dead embryos in unhatched eggs; (4) hatched eggs; and (5) eggs containing veligers with shell abnormalities. Post treatment egg segments were shuffled before counting to reduce counting biases. These egg segment lengths were measured again to identify shuffled segments. Survivorship was measured as the percentage of hatched eggs.

Statistical analyses

To avoid pseudoreplication, tests were conducted with each individual sea hare that produced an egg mass (i.e., mother) as the unit of analyses (Nedelec et al. 2014). All tests used the statistical program RStudio (R Core Team, 2013). Kruskal-Wallis analyses were performed to examine differences between means ($n = 12$ mothers) of control groups and treatment groups; as the survivorship data was not normally distributed. A post-hoc Tukey analysis was performed to identify where survivorship differences occurred between groups.

Results

Embryonic developmental time

Stylocheilus striatus eggs hatched 1 to 3 days after sound treatments, totaling 5 to 7 days in embryonic development (Figure 3). Egg hatching times were significantly affected by sound treatments (Kruskal-Wallis Ranked Sum, $\chi^2 = 9.0474$, df = 2, $p < 0.05$). High frequency treated eggs hatched, on average, 3 days after treatment while the control and low frequency treated eggs hatched after 2 and 1.5 days respectively (Figure 3). Post-hoc analysis of means ($n = 12$) indicated eggs treated with high frequency tones hatched significantly later than control and low frequency treated eggs (Kruskal-Nemenyi post-hoc analysis, $p < 0.005$). However, developmental time between controls and low frequency treated eggs were not significantly affected by sound treatments (Kruskal-Nemenyi, $p > 0.05$).

Egg hatching and percent survivorship

Of the 12,267 eggs counted, 615 failed to develop to 4 day old embryos (Figure 4). Of the 11,652 surviving eggs 2,583 failed to hatch (Figure 4). High frequency treated eggs had an average survivorship of 52.0% while the control and low frequency treated eggs had a survivorship of 89.1% and 89.8% respectively (Figure 5). The percentage of hatched eggs (survivorship) was significantly affected by high frequency sound treatment (Kruskal-Wallis Ranked Sum test, $\chi^2 = 23.447$, $df = 2$, $p < 0.005$). Additional exploration of the differences between survivorship means via a post-hoc pairwise analysis revealed high frequency treatments had the greatest impact on survivorship (Kruskal-Nemenyi post hoc analysis, $p < 0.005$)

Interestingly, the pair-wise comparison between low and high frequency groups and the control and high frequency groups indicated the low and high frequency treatments had the greatest significance difference between mean survivorship (32.8%) (Kruskal-Nemenyi, $p < 0.005$). The number of hatched eggs varied greater between the low frequency and high frequency treatments (Figure 5). The pair-wise comparison between the low frequency group and the high frequency group demonstrated high frequency treatments had the greatest impact on the number of hatched eggs (Kruskal-Nemenyi, $p < 0.005$). There was no significant difference between the survivorship of the control and low frequency treatments (Kruskal-Nemenyi, $p > 0.05$).

Morphological differences

Of the 2,583 eggs that failed to hatch, 159 eggs contained veligers with an observed difference in their shell morphology as compared to a healthy, surviving veliger (Figure 4). Veligers considered morphology different contained abrupt edges or protrusions within their shell (Figure 4). High frequency treated eggs had a mean of 10 eggs containing veligers with morphological differences, while the control and low frequency treated eggs had means of 1 and 2 eggs with deformed shells, respectively (Figure 6). The number of eggs with deformed veligers was significantly affected by high frequency sound treatment (Kruskal-Wallis Ranked Sum test, $\chi^2 = 12.253$, df = 2, $p < 0.05$). Similarly, the pair-wise comparison between deformed shell means revealed high frequency treatments had the greatest impact on the number of eggs containing deformed veligers (Kruskal-Nemenyi, $P < 0.05$), with no significant difference between control and low frequency treatment groups (Kruskal-Nemenyi, $p > 0.05$).

Discussion

High frequency noise treatment significantly slowed hatching times of *S. striatus* eggs (Figure 3). Past research has found anothropogenic noise can delay development in New Zealand scallop larvae (Aguilar de Soto et al., 2013). This is not the first evidence for sound exposure to decrease developmental life-history rates (see Lagardère, 1982). Lagardère (1982) discovered high level sound exposure also slowed developmental rates of *Crangon crangon,* a species of brown marine shrimp found in the northeastern Atlantic Ocean. However, *in situ* research also reported anthropogenic sound from boat noise playback may have no apparent effect on embryonic developmental rates (Nedelec et al., 2014). Although the results of the current study contrast with those of Nedelec et al., (2014), a number of methodologically different measures allow the current experiment to provide evidence for anthropogenic noise slowing hatching times of marine invertebrates in a controlled setting. The sea hare embryos were subject to 6 hours of constant sound treatment at 137.5-162.5 decibels with a reference level of 1 μPa per second squared per hertz at either 100 or 1000 hertz frequencies. It is possible that the level of sound and egg ribbon treatment time may be the greatest factors causing the observed delay in hatching time of these ecologically and economically important organisms (McCauley & Fewtrell 2008; Wale et al., 2013; Morley et al., 2014).

This study was performed in a laboratory with the goal of discerning which frequencies of noise have the greatest impact on the survivorship *of S. striatus* embryos (Figure 3); however, because it was executed *in vitro*; it may be difficult to pinpoint how specific frequencies of noise pollution affects organisms in their natural environments. Nedelec et al., (2014) suggested it is possible that vibrations of substrates from sound pollution may affect sound transmission differently. Regarding the current experiment, egg ribbons were placed in a petri dish above the speaker, potentially altering the vibrational patterns of sound through the plastic dish into the sea water containing the egg ribbon. Although, an increase in developmental time and a decreased survivorship was observed, the laboratory apparatus may have affected sound vibrations, pressure and subsequently the hatching time and survivorship of sea hare eggs.

High frequency noise treatment also significantly decreased survivorship of *S. striatus* veligers. A potential mechanism for this observed response to anthropogenic noise may be due to pressure induced injuries caused by cavitation or barotrauma. Cavitation is when gas bubbles collect and implode causing high levels of heat and pressure (Brennen, 1995); while barotrauma is the rupturing of gas filled cavities from changes in pressure (Nedelec et al, 2014). There are few studies of noise-induced cavitation stress and how they affect organisms, yet cavitation can be the result of long exposure times of high frequency sound (Leighton, 1995). *S. striatus* embryos may have gas filled chambers within their tissues where cavitation and barotrauma may occur when they are subject to relatively long term and constant high frequency noise.

Another possible mechanism for the observed survivorship and morphological difference is molecular vibrations within the developing organisms (Silva et al., 2002; Aguilar de Soto et al., 2013; Nedelec et al., 2014). Low frequency noise and whole-body vibrations caused increased levels of sister chromatid exchange in mice (Silva et al., 2002), and high frequency and sound level caused a deregulation of calcium transport systems in rats (Siegel & Mooney, 1987; Aguilar de Soto, 2013). Because of the small size of scallop larvae and the absence of strong tissue density gradients in early development phases, molecular vibrations have been suggested to decreases survivorship and cause body malformations as well (Aguilar de Soto et al., 2013). According to Aguilar et al., (2013) these observed processes may be related to particle motion rather than to the pressure component of noise exposure. As an herbivore, invertebrates like *S. striatus* often provide a balance between algal and coral health, specifically regarding their specialized grazing of toxic cyanobacteria (Paul & Pennings, 1991; Nedelec et al., 2014). With increasing noise pollution in marine environments, invertebrate response and behavior, like that of *S. striatus*, require further investigation to determine appropriate mitigation policies.

Anthropogenic noise pollution continues to increase across the world. Conservation policy and management should consider the effects of anthropogenic noise on invertebrates like *S. striatus,* as their herbivory is important to marine ecosystem health as well as their role within trophic webs. Research from studies directly accessing how organisms respond to specific frequencies of noise, as this experiment has done, are crucial to understanding and implementing future domestic and international conservation and management policy, ultimately addressing the worldwide concern of sound pollution.

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Figures and Tables

Figure 1: Map of the island of Mo’orea (Scale bar = 5km). The stars and numbers represented field collection sites corresponding with Table 1. The grey line around the island represents coral reefs.

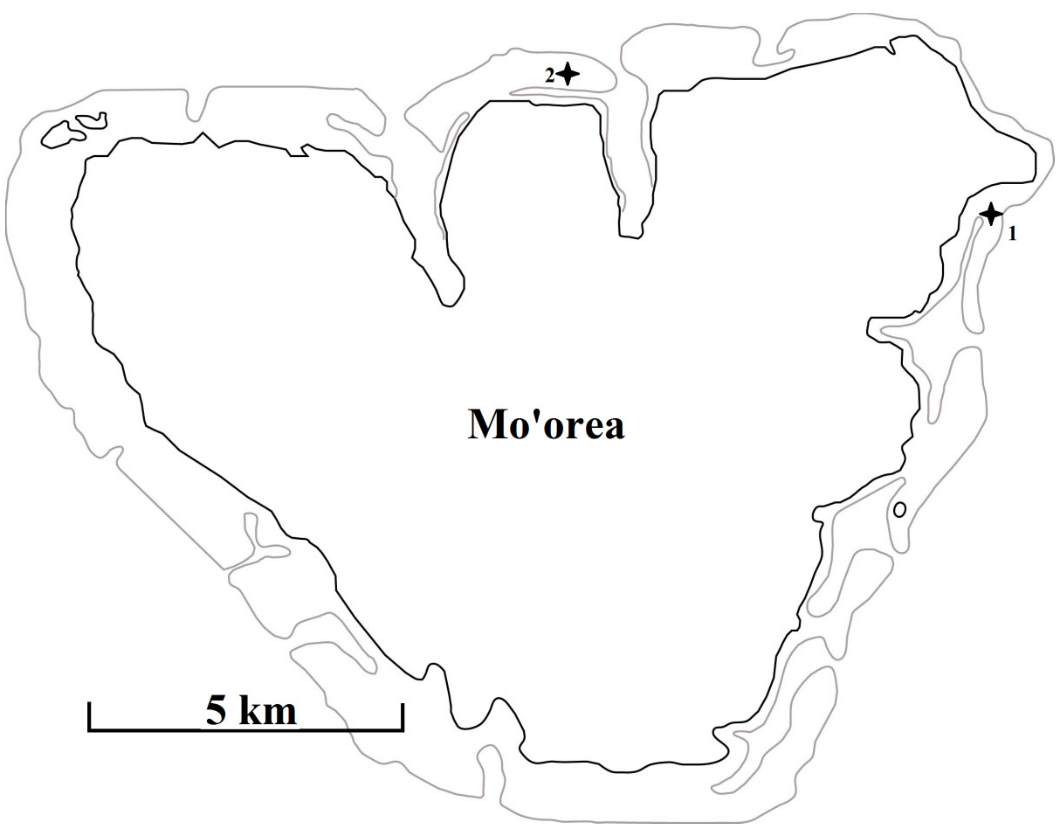

Table 1: Cyanobacteria collection sites and GPS Coordinates.

Collection Site		GPS Coordinates
Temae Public Beach	1	S 17° 29' 56.0544" W 149° 45' 32.778"
Piha'ena Public Beach	2	S 17° 29' 0.6125" W 149° 50' 0.9318"

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Figure 2: Pretreatment *Stylocheilus striatus* embryos four days after copulation. (Scale bar = 0.5mm).

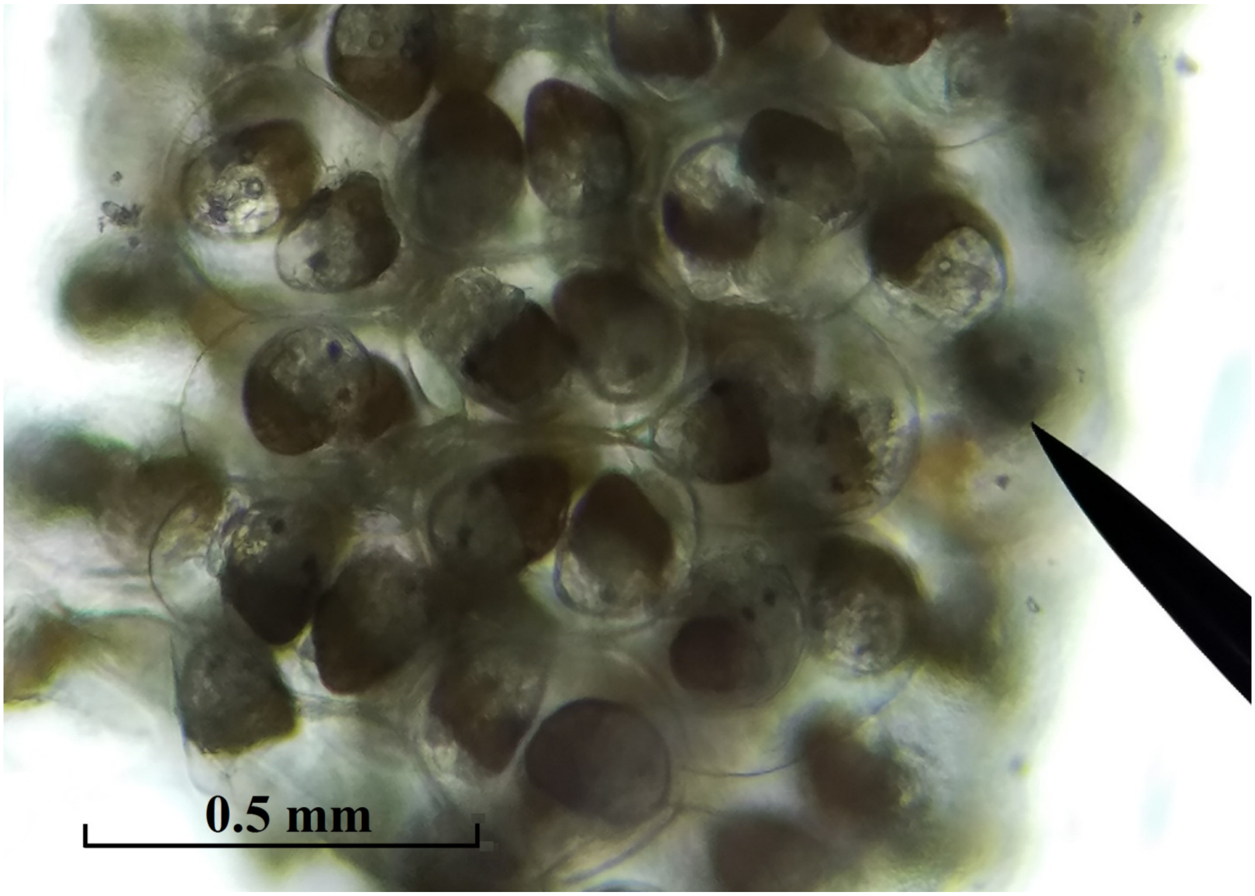

Figure 3. A boxplot of *Stylocheilus striatus* egg development time in days, grouped by frequency treatment (n =12). A frequency of 0 Hz represents the control, while 100 Hz and 1000 Hz represent the low and high frequency treatment groups respectively.

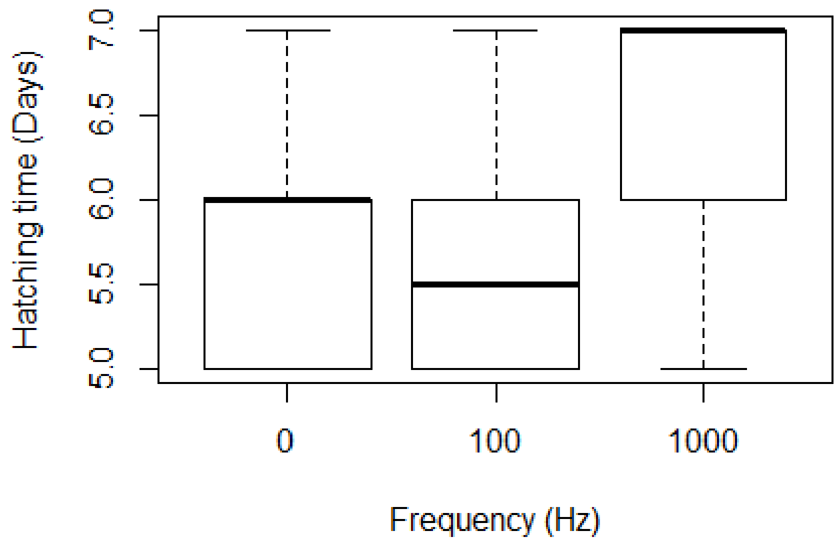

Figure 4. *Stylocheilus striatus* egg ribbon segment post high frequency (1000 Hz) treatment. Red arrows indicate eggs that failed to reach day 4 of development. Light blue arrows specify unhatched eggs with dead embryos. Yellow arrows indicate eggs with abnormal shell morphology denoted by abrupt edges or protrusions within the shell (Scale bar = 0.5 mm).

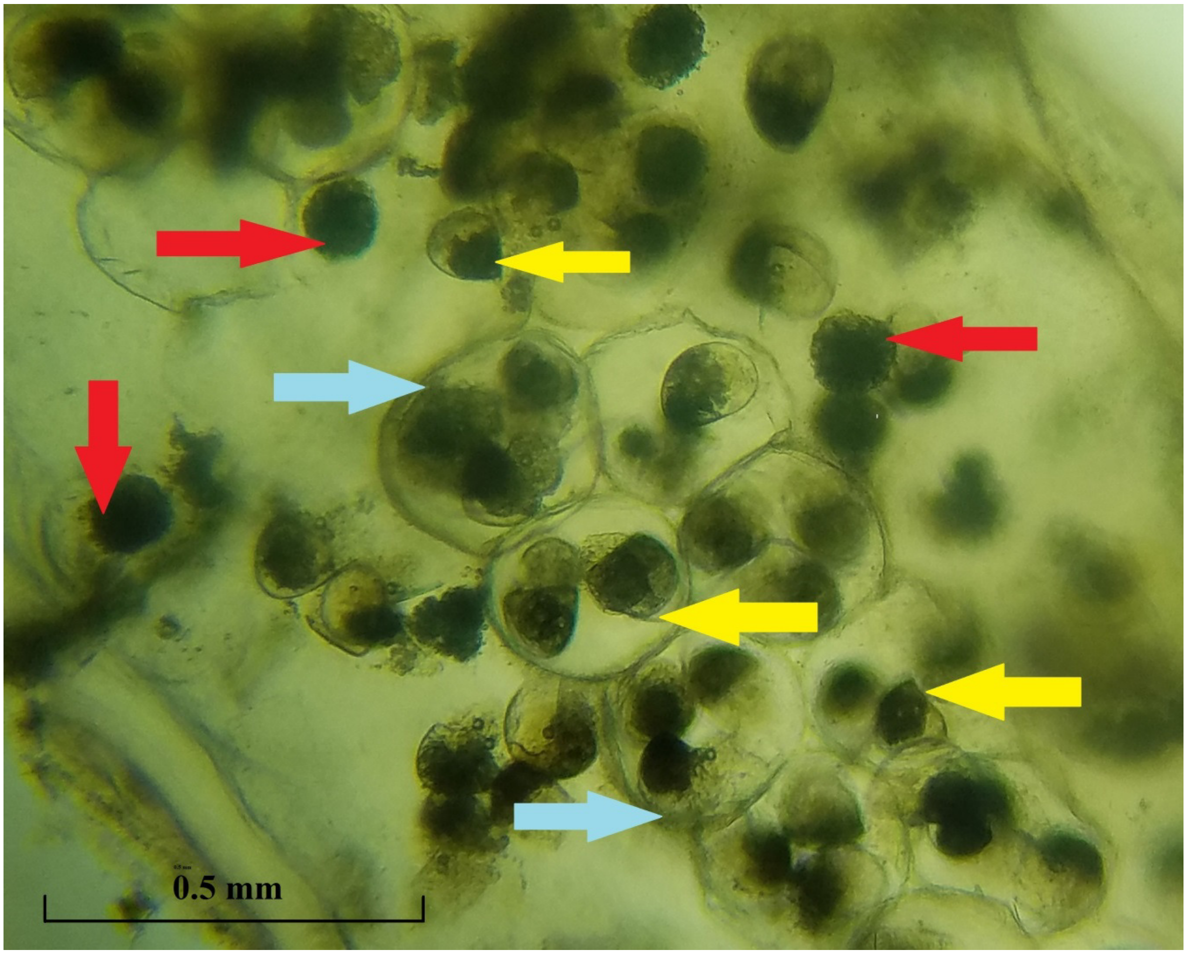

Figure 5. A boxplot of hatched eggs as percent survivorship (n=12 mothers), grouped by frequency treatment. A frequency of 0 Hz represents the control, while 100 Hz and 1000 Hz represent the low and high frequency treatment groups respectively.

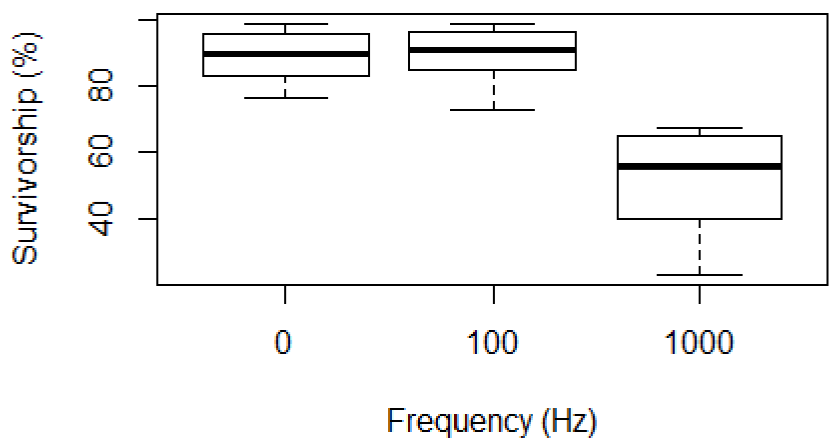

Figure 6. A boxplot of the number of eggs containing deformed veligers (n=12 mothers), grouped by frequency treatment. A frequency of 0 Hz represents the control, while 100 Hz and 1000 Hz represent the low and high frequency treatment groups respectively. The hollow circle represents an outlier.