Title

Short-term effects of levonorgestrel exposure on the reproductive system of the California warty sea cucumber (*Apostichopus parvimensis*) and potential repercussions on its fishery management

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Abstract

Levonorgestrel (LNG), an endocrine disrupting compound that falls under the pharmaceutical and personal care products category, has become a contaminant of emerging concern globally. Like other steroidal hormones, LNG can enter the aquatic environment through wastewater treatment plant effluent and agricultural run-off and is expected to adsorb to the sediment. The California warty sea cucumber (*Apostichopus parvimensis*), an ecologically important epibenthic detritovore and an economically desirable fishery species of interest, may be affected by exposure to pollutants such as LNG. How a species reacts to threats in the environment—whether it be from the fishery itself, mortality events, or pollution—is data that can be incorporated into management decisions. There is little data on *A. parvimensis* available despite the economic demand for it, and there are limited regulations in place to manage its stocks in California. Conservation and fishery management use best available science to create policies and plans to keep fishery populations sustainable. This study aims to capture a snapshot of how short-term exposure of LNG can potentially affect gonad development in *A. parvimensis* and discuss how this could affect fishery management.

Keywords: warty sea cucumber, echinoderm, levonorgestrel, synthetic hormone, progestin

Background

The California warty sea cucumber *Apostichopus parvimensis* (Clark, 1913) is a member of the phylum Echinodermata. It is an epibenthic Holothuroidea, occupying depths between the subtidal zone to as deep as 50m (180ft), with a range from Fort Bragg, California, USA to Puerto San Bartolome, Baja California, Mexico (CDFW 2019). *Apostichopus parvimensis* spawns by releasing gametes into the water column through the months of May and June. Over the course of July through February, the gonads of *A. parvimensis* will undergo a cycle of resorption of the emptied gonadal tubules and regrowth of the tubules (Muscat 1983). The annual reproductive development in *A. parvimensis* can be divided into five main stages: resting (stage I), developing (stage II), maturing (stage III), spawning (stage IV), and spent (stage V) (Muscat 1983). Observations of differences in spawning season between different populations of *A. parvimensis* have been recorded and are associated with latitude, with higher latitudes having earlier spring-summer spawning periods and lower latitudes having winter-spring spawning periods (Chavez et al. 2011). *Apostichopus parvimensis* ingests sediment off the top of the epibenthos (CDFW 2019; Yingst 1982). This mode of eating evidences the importance of *A. parvimensis* in the natural ecosystem as a bioturbator for the sandy bottom and a nutrient and mineral recycler (Yingst 1982).

The fishery

Apostichopus parvimensis is a fisheries species of interest (CDFW 2019; Chavez et al. 2011; Toral-Granda et al. 2008). In California, the dive fishery for *A. parvimensis* opened in 1989 (CDFW 2019). Until the early 1990s, the number of commercial fishery participants was small with a maximum of six fishermen and ten vessels during the first ten years of the fishery according to information collected by the California Department of Fish and Wildlife (CDFW) (CDFW 2019). After the first decade of the dive fishery being open, participation in the fishery increased drastically. Participation in the form of number of vessels increased by 100% from 1989 to 1990, and by 950% from 1990 to 1991 (CDFW 2019). Over the same course of time, landings in thousands of pounds multiplied by nearly 7 and 3.5, respectively (CDFW 2019). One reason for the increase in landings can be attributed to increased demands for sea cucumber in Asian markets (Chavez et al. 2011; Toral-Granda et al. 2008). High demand for sea cucumber in international markets has made all fishery-targeted sea cucumber species vulnerable to illegal, unreported, and unregulated (IUU) fishing (Toral-Granda et al. 2008). The vulnerability marks all edible sea cucumber species as a species of concern with regards to conservation. Economic interest in *A. parvimensis* has necessitated regulation of the commercial fishery in number of participants, gear, and temporal closures (CDFW 2019).

Conservation efforts utilize best available science and population structure in order to form policy (Toral-Granda et al. 2008). Although *A. parvimensis* is a species of economic interest, particularly in Asian markets where it is used in cultural cuisine and pharmaceuticals, the species is considered data-poor (CDFW 2019). There has been no formal stock assessment on the species by the CDFW to date (CDFW 2019). Population estimations have been calculated based on scuba-collected data and landing receipts by commercial fisheries (CDFW 2019). According to information provided by the latter, the commercial fishery has exhibited a continuous decline in landings from 2011 to 2016 while the value for the warty sea cucumber has remained relatively high (CDFW 2019). The CDFW recognizes that more data is needed for *A. parvimensis* on age and growth (CDFW 2019). More data on how potential threats, such as pollutants, can affect age, growth, reproduction, and mortality is necessary to gather a more-encompassing picture of trends on population dynamics. This information is especially important for a species like *A. parvimensis* for which demand has rapidly increased while landings have decreased. Figure 1 provides a visualization of fishery landings, value, and participation for the *A. parvimensis* fishery over time.

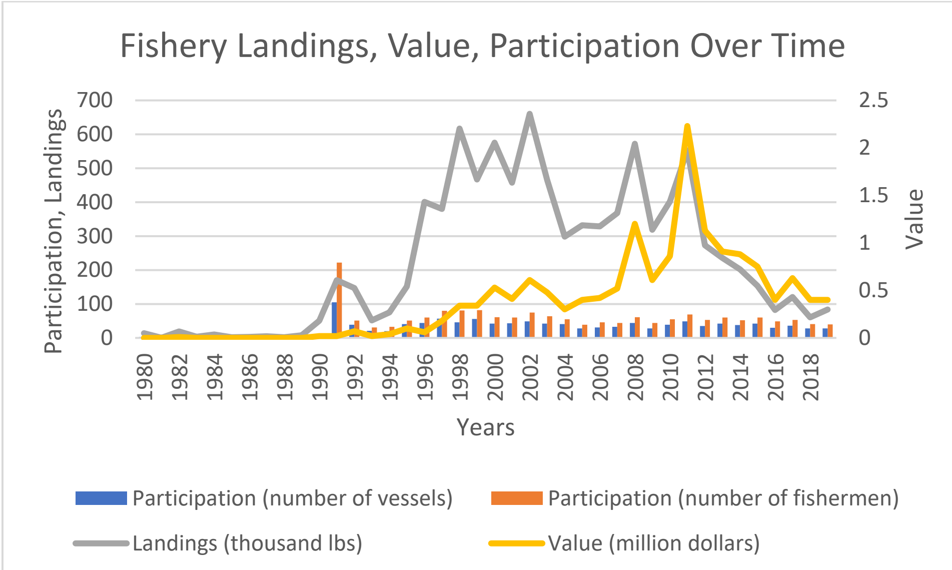

Figure 1. Fishery landings, vessels, and participation over time (1980-2019). Information from CDFW Marine Species Portal Website. On the left axis: landings in thousands of pounds, participation in number of vessels, and participation in number of fishermen. On the right axis: value of the commercial dive fishery for Apostichopus parvimensis *in millions of USD. Note shift of trend between thousands of pounds of landings in comparison to value in millions of USD in 2011.*

Levonorgestrel

Pollution is a factor that can affect population dynamics. Understanding potential correlations between pollution in the marine environment on *A. parvimensis* gonadal development becomes necessary when evaluating conservation efforts and establishing fishery management policy.

Pharmaceuticals and personal care products (PPCPs) and endocrine disruptor compounds (EDCs), such as synthetic hormones, have been found in wastewater effluents, even after the water has been treated (Anderson et al. 2012; Dai et al. 2021; King et al. 2016; Orlando & Ellestad 2014; Oropesa et al. 2020). Of particular interest is an EDC, the synthetic hormone levonorgestrel, which has been found in wastewater effluent in California (Kolodziej et al. 2003) and documented as affecting freshwater and estuarine vertebrate species (King et al. 2016; Orlando & Ellestad 2014; Oropesa & Guimaraes 2020) and marine species (Johnston et al. 2018; Loganimoce 2016). Levonorgestrel (LNG) is a synthetic progestin that is used in contraception and to treat other disorders related to menstruation, such as endometriosis (The Human Metabolome Database 2020; Medicine Plus 2016). It is a steroid lipid molecule, which indicates it is essentially insoluble in water (Medicine Plus 2016) and is predicted to adsorb into sediments based on its Koc coefficient (Oropesa & Guimaraes 2020). Concentrations of LNG have been found in aquatic sediment samples in Spain, Belgium, Canada, and China (Oropesa & Guimaraes 2020). LNG is a contaminant of emerging concern (CEC) in the PPCP category (King et al. 2016; Orlando & Ellestad 2014).

The effect of PPCP synthetic hormones has been observed in other echinoderms to interfere with developmental physiology, such as in crinoids (Sugni et al. 2008) as well as sea urchins (Para-Luna et al. 2020) and a different species of sea cucumber (Parra-Luna et al. 2020; Martin et al. 2020). Benthic and epibenthic organisms, such as the sea cucumber, are used as bioindicators for contaminants (Martin et al. 2020; Parra-Luna et al. 2020). Studies have found hormones similar to those in mammals in the gonad of echinoderms (Donahue 1940; Wang et al. 2020; Wasson et al. 2000). LNG is not as well studied as other synthetic hormones involved in contraception (King et al. 2016; Oropesa & Guimaraes 2020) and may become an issue in California coastal waters as use of LNG continues to increase (Anderson et al. 2012). *Apostichopus parvimensis*, a species of fishery interest and an epibenthic organism with an annual reproductive development cycle, could prove to be a bioindicator of detrimental LNG concentrations in the marine environment.

Purpose

The goal of this study was to explore whether gonad maturation in *A. parvimensis* is affected by exposure to the synthetic progestin levonorgestrel, specifically testing the hypothesis that exposure to LNG will cause malformations in gamete appearance and thickening of the gonad tubule membrane. Information uncovered through the course of this experiment could add to the data available for this species of growing fishery interest.

Methodology

The aquarium experiment was conducted over 37 days from 3 April 2021 to 10 May 2021. Over the course of this time, salinity was measured and recorded in the three tanks used for the experiment and in the ambient seawater used to regulate the tanks' temperature and used for water changes on a weekly basis. LNG was introduced into the dosed tanks through water changes, which is explained further down.

Specimen collection and preparation

Eighteen live specimens were collected by hand through SCUBA at depths between 15m to 17m (48ft to 56ft) west of La Jolla Cove in southern California on 26 February 2021. Large specimens were collected to increase likelihood that the individual was sexually mature. Upon collection, specimens were stored in a floating holding basket (no sediment) for 17 days then transferred into a holding tank with a sandy bed and fed the same fish flake diet. After 19 days, specimens were gendered then transferred into one of three tanks for the experiment. Tank placement was based on maintaining a sex ratio of 3 males : 2 females in each tank based on the initial biopsy. Except for this ratio, specimens were assigned to tanks randomly. Each tank held five individuals. Three of the collected specimens were not used because one individual had eviscerated before the first round of biopsies, one individual had eviscerated during the first round of biopsies, and the third individual was not used to keep numbers consistent. These three individuals were kept in the sandy bed holding tank.

Specimens' measurements were taken using a tape measure and calipers to obtain total contracted length and total contracted width, respectively. Photographs of each specimen were taken at time of measurement (Figure 2) and used to serve as a reference for identification at each round of biopsy.

Tank set-up

Three 37.85L (10gal) glass tanks with measurements of 50.8cm x 25.4cm x 30.48cm (20in x 10in x 12in) were used to house specimens during the trial in a closed system. The tanks were designated as follows: control (Tank C), high dose concentration (Tank H), and low dose concentration (Tank L). Each tank was filled with 5.08cm (2in) of sand and filled to 1.27cm beneath the tank lip with ambient seawater and placed into a black cement-mixing tray with a 2.54cm (1in) clearance off the bottom of the tray. Sand was collected from the end of Scripps Pier, baked in the sun, then rinsed with tap water to remove detritus from baking. Ambient seawater was pumped from the end of Scripps Pier and filtered before use. Cement-mixing trays were filled with running ambient water to regulate tank temperature. Each tank was given an airline and an airstone weighed down by a 2.54cm (1in) PVC elbow. Airflow into the tank was set so air would break the surface at the top of the tank. Tanks were set-up on 15 March 2021. An Onset HOBO Pendant MX Water Temperature data logger was placed in the control tank to monitor and record temperatures.

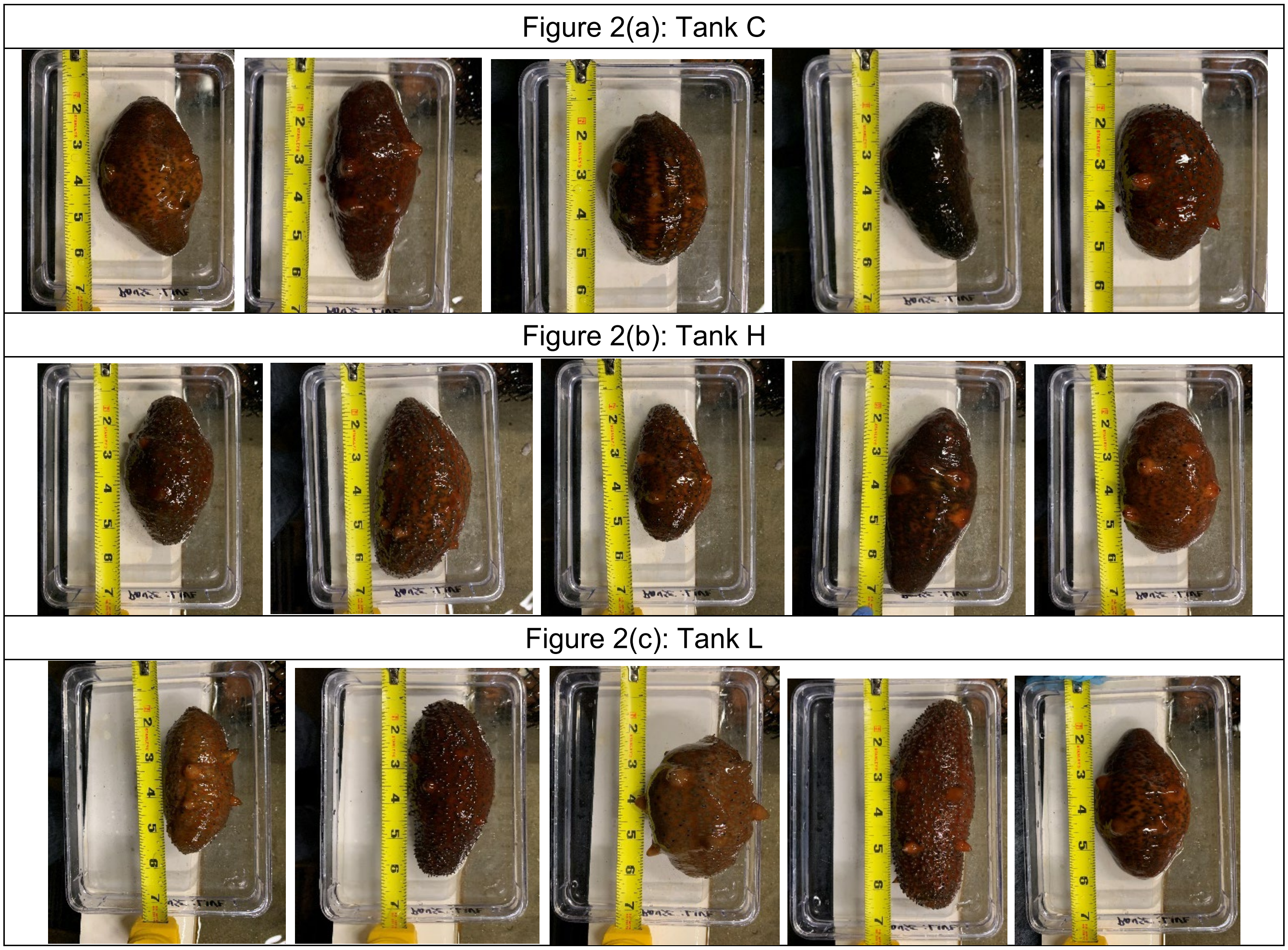

*Figure 2. Specimens on measurement day (5 days after first biopsy, 8 April 2021), contracted. (**a**) Tank C specimens, left to right: C1, C2, C3, C17, C15. (**b**) Tank H specimens, left to right: H14, H6, H7, H12, H14. (**c**) Tank L specimens, left to right: L10, L11, L9, L8, L13.*

Feeding

Specimens were fed Tetra® TetraColor Tropical Fish Flakes through the duration of the trial. The initial amount of food fed out was 1.5g of flakes per tank, which was added after serial dilution was complete for the initial set-up (8 April 2021) and after the dosing water changes that occurred over the course of the trial until 26 April 2021. This initial amount was chosen so there would be approximately 1g of flake for 1000cm^2 of sediment. The amount of feed administered to each tank was readjusted to 0.5g of flakes per tank starting on 26 April 2021 and fed once every three days because bacterial mats began to develop on the sediment. Food was dropped at the surface of the tank over the airstones.

Dosing protocol

LNG was purchased as an over-the-counter medication from the pharmacy in tablets containing 1.5mg of LNG. The initial target concentration of LNG was set to 0.132µg LNG/L of seawater in Tank L and 0.264µg LNG/L of seawater in Tank H. The concentration in Tank L was chosen because it is above the instrument detection limit of 0.1ng/mL (0.1µg/L) for this compound when using solid-phase extraction and liquid chromatography-tandem mass spectrometry (Al-Odaini et al. 2010). This concentration was achieved through serial dilution by water change. Water was removed from each tank. LNG was mixed into ambient seawater in a bucket and poured into the tank while still suspended. After new water was added to the tank, water was stirred, then siphoned out, and refilled with clean seawater. This process was repeated until the desired concentration could be achieved. Tank C was subjected to the same water change protocol without LNG added to the water. Specimens were temporarily removed from the tank for this sequence to avoid stress. Table 1 outlines the serial dilution process.

Table 1. Serial dilution for setting initial concentration of Tank C, Tank L, and Tank H.

	LNG concentration (µg/L) after H_2O Δ		
Percent H_2O Δ	Tank C	Tank H	Tank L
Pre-H_2O Δ	0.000	0.000	0.000
50%	0.000	132.0	66.00
90%	0.000	13.20	6.600
90%	0.000	1.320	0.660
20%	0.000	0.264	0.132

For all subsequent dosing, water was prepared in large batches through serial dilution. A stock solution was created by dissolving one tablet containing 1.5mg tablet of LNG into 18L of ambient seawater and diluted until a concentration of 0.264µg/L was obtained. Water was prepared every 3 days using the same stock solution.

Treatment tanks were dosed by replacing water taken out during water changes with LNG water at 0.294µg/L for Tank H and 0.167µg/L for Tank L. Water changes occurred regularly beginning 4 days after the *A. parvimensis* specimens were placed into the tanks with two days of consecutive water changes followed by a day of no water changes. Tank C experienced the same water change regiment with ambient seawater. Water in the amount of ten percent of the tank's volume were taken out on each day water changes occurred. Water changes did not occur on the day following the second biopsy to allow organisms a chance to recuperate.

Gonadal tissue collection

Gonad tissue was collected in two ways. For the first and second rounds of tissue collection, gendering, and photographing, samples were biopsied using needle aspiration with an 18 gauge needle and 5mL syringe. The needle was inserted at a 25-35 degree angle in the dorsal anterior and left of center, between 4cm-5cm from the oral end on each specimen. If gonad was not pulled into the syringe but was pulled out of the body from the needle insertion site, a scalpel was used to cut the gonad. Collected tissue was placed on a glass slide for observation and photographing under a dissection microscope.

In the last round of gonad tissue collection, collection was first attempted using needle aspiration. In the circumstance that gonad could not be collected from needle aspiration, a small incision, 1cm to 1.5cm in length, was made into the body cavity close the needle insertion site and gonad was either pulled from this incision or from content that was eviscerated after incision. Gonads in the final round of biopsy were collected from 9 individuals: 4 from Tank C, 1 from Tank L, and 4 from Tank H. This is further elaborated in the discussion section.

Table 2. Dates when biopsies were taken.

Biopsy round number	Date(s) of biopsy	Number of days passed since first biopsy
1	3 April 2021	0
2	24 April 2021, 25 April 2021	21-22
3	10 May 2021	37

Photography of slides under the microscope

Two main microscopes were used for gonad tissue photography. The compound microscope used for many of this data collection is the Leica DMR at various magnifications. A dissecting microscope, Leica MZ9.5, was used to take external photographs of female and male gonad tubules at 1.25x magnification.

Histology slide preparation

A total of 27 histology slides were mounted and stained. The gonads collected during the final round of biopsy were fixed using Bouin's fluid, then dehydrated through an ethanol series of 50%, 70%, 80%, 95%, and 100%. Xylene was used as the transitional fluid between the ethanol dehydration and embedding in Fisher Tissue Path Paraplast® Tissue Embedding Medium, a paraffin wax. Slides were sectioned at 10µm. Slides were prepared for staining with two xylene washes and a decreasing serial ethanol dilution into de-ionized (DI) water. Gill's Hematoxylin (GHS3) (Sigma-Aldrich) was used for staining and slides were exposed to the stain for a minimum of 5 minutes. Slides were cleaned with an increasing serial dilution before mounting in Fisher Scientific Permount. Three slides were also stained with 0.05% azure A in DI water for 1 minute.

Gonad analysis

Analysis began with physical descriptions of biopsy tissue color and cells present in the tubules based on photographs taken of the biopsy slides. Some cells that had been photographed were also measured. Measurements were conducted on the diameter of randomly selected gametes in each respective specimen and of collected gonad tubule width. Oocytes were compared based on measurements made to the diameter. Spermatozoa was compared based on percentage of dense area over the cross-section of the gonad tubule. Measurements were made by either measuring divisions in the eye piece of the microscope and calculating measurements or by using the scales taken at its respective magnification and comparing it to photographs for the histology slides. Individuals were also compared based on gamete counts of ten random areas per individual. Areas measured 50µm by 50µm for males and 1000µm by 1000µm for females. Means for each tank were calculated from these counts, and comparisons were made based on sex. The sampling site area for males was determined to provide a sample that would encompass roughly 10%, at minimum, of the entire surface area of the tubule measured. The sampling area for females was determined to obtain comparable count by maintaining the size ratio between sperm and egg.

Notes on observations made in the laboratory (on site) were written in real time. Further analysis took place off site based on photographs of biopsy slides and histology.

Post-treatment sediment sample collection

Sediment samples were collected for future LNG analysis. Analysis for these samples extended beyond the timeframe for this study. Samples were collected from all tanks into small plastic bags from three random spots in each tank. In order to collect sediment samples, water was drained from each tank and collected with the spoon end of a stainless steel lab spatula. Samples were stored at -20^{o}C.

Results

Mean initial measurements on contracted width and contracted length were 10.80cm and 6.74cm in Tank C, respectively; 11.78cm and 7.08cm in Tank H, respectively; and 11.73cm and 6.61cm in Tank L, respectively. Final measurements were not taken because many individuals eviscerated before measurements could be made. After evisceration, individuals did not contract, and comparable data to initial measurements could not be taken.

Some observations and results of note (Table 3) include: early evisceration, especially in Tank L; mortalities that occurred before the contaminant exposure trial was complete; the occurrence of white bacterial mats that appeared on the sediment; the tendency of specimens to live at the top of the tanks; and the proliferation of copepods in only the contaminant-dosed tanks, Tank H and Tank L. Mortalities during the aquarium experiment occurred following evisceration and occurred within 36 hours for the first observed mortality and within 12 days for the second.

Over the course of the experiment, gonads continued to develop to successive stages in all tanks. Based on descriptions of holothurian gonad development by Christophersen et al. 2020, Fajardo-Leon et al. 2008, Tolon & Engin 2019, and Whitefield & Hardy 2019, collected specimens were either in stage II or stage III of gonad development at the beginning of the aquarium experiment and in stage IV or stage V at the conclusion of the aquarium experiment (Figure 3).

Table 3. Key event and condition dates for the experiment starting from collection date (26 February 2021) until last round of biopsy (10 May 2021). B = biopsy, Ev = eviscerated, X = mortality. Last round eviscerations on 10 May 2021 not included on chart as the majority of specimen eviscerated that day.

Date	Event	Condition Notes
26 Feb	Collected	
15 Mar	Transferred to pre-treatment holding	
3 Apr	Placed in Experiment Tanks, B	
8 Apr	Length/width measurements taken	
24 Apr	B (Tank C, Tank L)	Ev (Tank L x3)
25 Apr	B (Tank H)	X (Tank L)
26 Apr		Ev (Tank L)
8 May		X (Tank L)
9 May		Ev (Tank H)
10 May	B (Tank C, Tank H, Tank L)	

At the end of the aquarium experiment, there was no observable delayed or deformed physical effect on gonad or gamete development after exposure to LNG. One fully mature female specimen in Tank C produced 12.6g of gonad at the end of the experiment. One fully mature female specimen in Tank H produced 16.5g of gonad at the end of the experiment. These gonads were pulled from individuals were the largest in their tanks, with initial contracted measurements of 7.35cm by 10.80cm and 7.50cm by 13.65cm in Tank C and Tank H, respectively. More comparisons of gamete count and gonad development can be found under the histology subsection of Results.

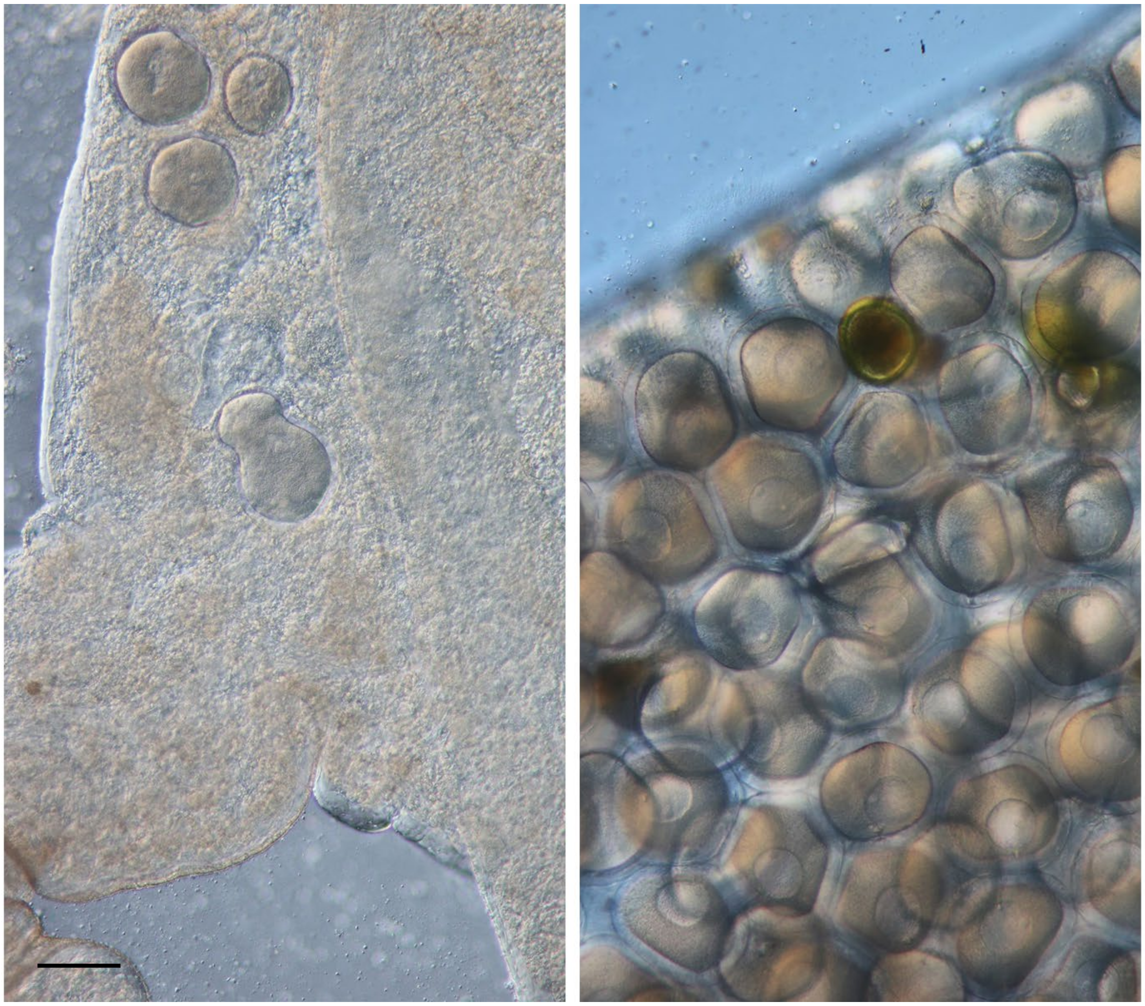

Figure 3. Gonad biopsies from the same female in Tank H at different dates. Both images at 20M. Scale bar = 100µm. Left gonad pulled on 24 April 2021, right gonad pulled on 20 May 2021.

Biopsy

Specimens in Tank C and Tank H did not eviscerate within 72 hours of undergoing biopsy by needle aspiration in round 1 and round 2. Four specimens in Tank L did eviscerate within 72 hours of undergoing biopsy by needle aspiration in round 2. Three specimens in Tank C, three specimens in Tank H, and one specimen in Tank L eviscerated after undergoing biopsy by incision in round 3. One of the three specimens in both Tank C and Tank H did not have gonad present in the eviscera produced during round 3 of biopsies. Individuals were sexed during each of the three rounds of biopsy completed. Initial results yielded a total of 6 females and 9 males. This ratio, however, appeared to change between rounds (Table 4) due to misidentification of parasites (Figure 4) as eggs in male gonads. Sex was finitely determined at the third round of biopsies when histology slides were prepared using the tissue collected as almost all the specimens eviscerated during the final round of biopsies.

Table 4. Sex ratio based on biopsy photos taken during each round. M = Male, F = Female, U = Unidentifiable.

	Round 1		Round 2			Round 3		
Tank	M	F	M	F	U	M	F	U
C	3	2	2	3	0	3	1	1
H	3	2	2	2	1	1	3	1
L	3	2	1	2	2	1	0	4

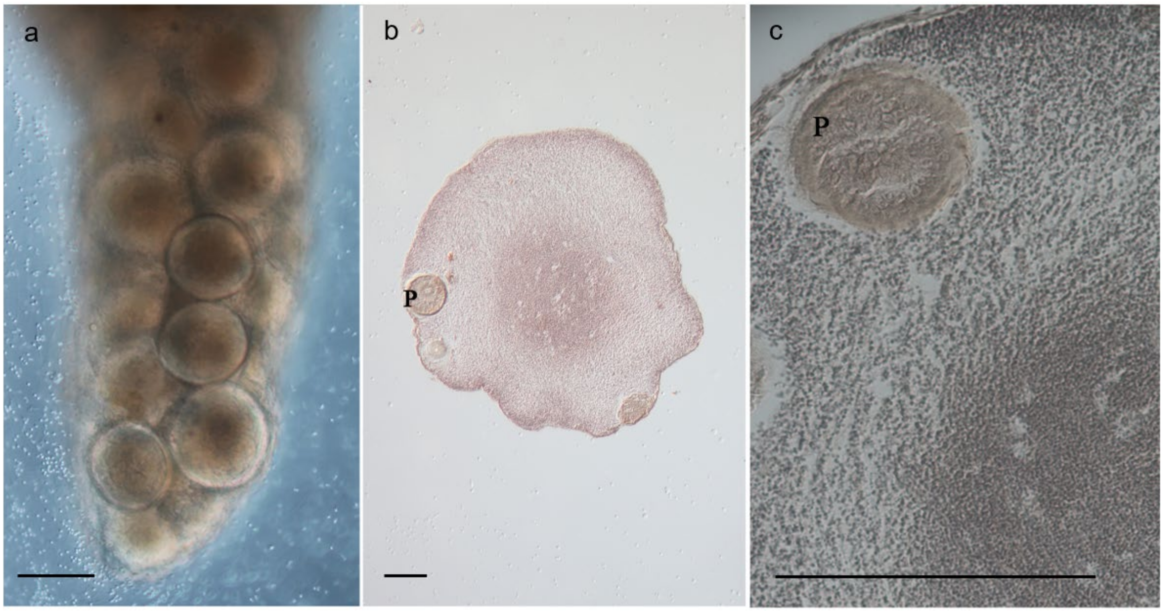

*Figure 4. Photographs taken using the Leica DMR of the same specimen in Tank C from round 3 (10 May 2021) of biopsies. P = parasites. Scale bar = 100µm. (**a**) Biopsy of a male gonad tubule with parasites, 20M. (**b**) Histology cross-section of a different gonad tubule from the same specimen, 10M. (**c**) Histology of the same tubule in **b** but at a different cross-section location, 63M.*

Histology

A total of 4 histology slides for each of the 9 surviving, viable (had gonad material to use) specimens were generated from gonad collected from the last biopsy. As previously stated, there was no discernable visual difference between specimen samples across the three tanks when comparing gonad development in both males (Figure 5) and females (Figure 6) in similar stages of reproductive development.

Male *A. parvimensis* had a spermatozoon count of 241.8 ± 91.5 in Tank C, 299.1 ± 101.0 in Tank L, and 294.0 ± 112.4 in Tank H. Membrane width in testes was measured at 9.9µm ± 0.82µm for all males, 9.8µm ± 5.8µm for males in Tank C, 11.0µm ± 5.7µm for the male in Tank L, and 9.0µm ± 5.9µm for males in Tank H. In males, the testes had a greater density of mature gametes towards the center. The percent of the tubule cross section composed of dense area was 48.5% ± 30.8% in Tank C, 44.5% ± 5.1% in Tank L, and 43.18% ± 4.6% in Tank H. Gonads were uniform throughout with mature spermatozoa; no spermatogonia were observed in the collected histology stains.

Female *A. parvimensis* had an oocyte count of 21.1 ± 3.7 in Tank C and 18.7 ± 2.8 in Tank H. Membrane width in ovaries measured at 15.2µm ± 1.2µm for all females, 14.0µm ± 4.3µm for females in Tank C, and 16.4µm ± 3.8µm for females in Tank H. Gonads had irregular boundaries and were filled with oocytes that assumed irregular shapes inside the gonad. Upon exiting the gonad and entering seawater, oocytes rounded out and assumed an elliptical shape. Oocytes measured 180.8µm ± 11.4µm in Tank C specimens and 184.8µm ± 6.3µm in Tank H specimens. Oocytes were uniform in level of development with a clearly defined nuclei and nucleolus in each cell.

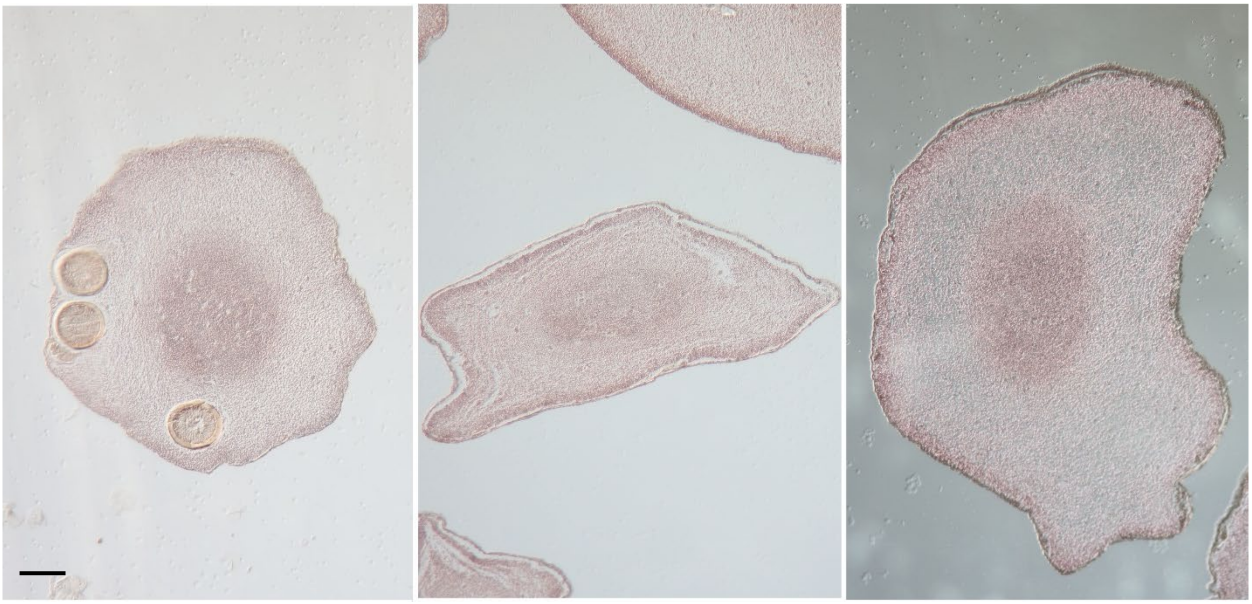

Figure 5. Cross-section of male gonad tubules at 10M under the Leica DMR. Scale bar = 100µm Left to right: specimen from Tank C, specimen from Tank L, specimen from Tank H.

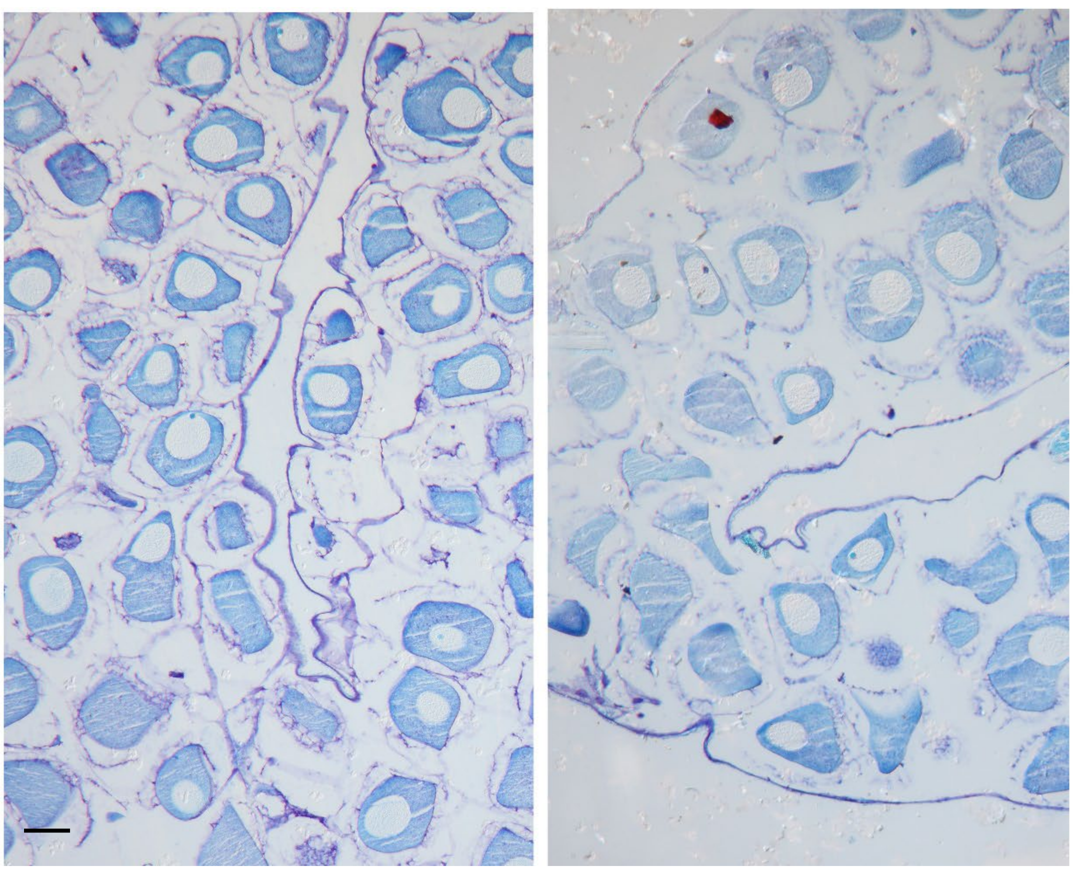

Figure 6. Cross-section of female gonad tubules at 10M under the Leica DMR. Scale bar at 100µm. Left: specimen from Tank C, right: specimen from Tank H.

Tank conditions

All tanks were subject to the same water temperature condition. Temperature ranged between 14.63°C and 18.49°C (Figure 7). During the aquarium experiment, ambient seawater temperature and salinity were affected by weather variability and conditions of the source water. Salinity had noticeable differences between the ambient seawater, Tank C, Tank H, and Tank L. Mean salinity was 36.5ppt in the ambient source, 38.4ppt in Tank C, 37.8ppt in Tank H, and 37.8ppt in Tank L (Figure 8). Tank C experienced different, higher salinities on average in comparison to Tank H and Tank L; Tank H and Tank L had the same salinity as one another.

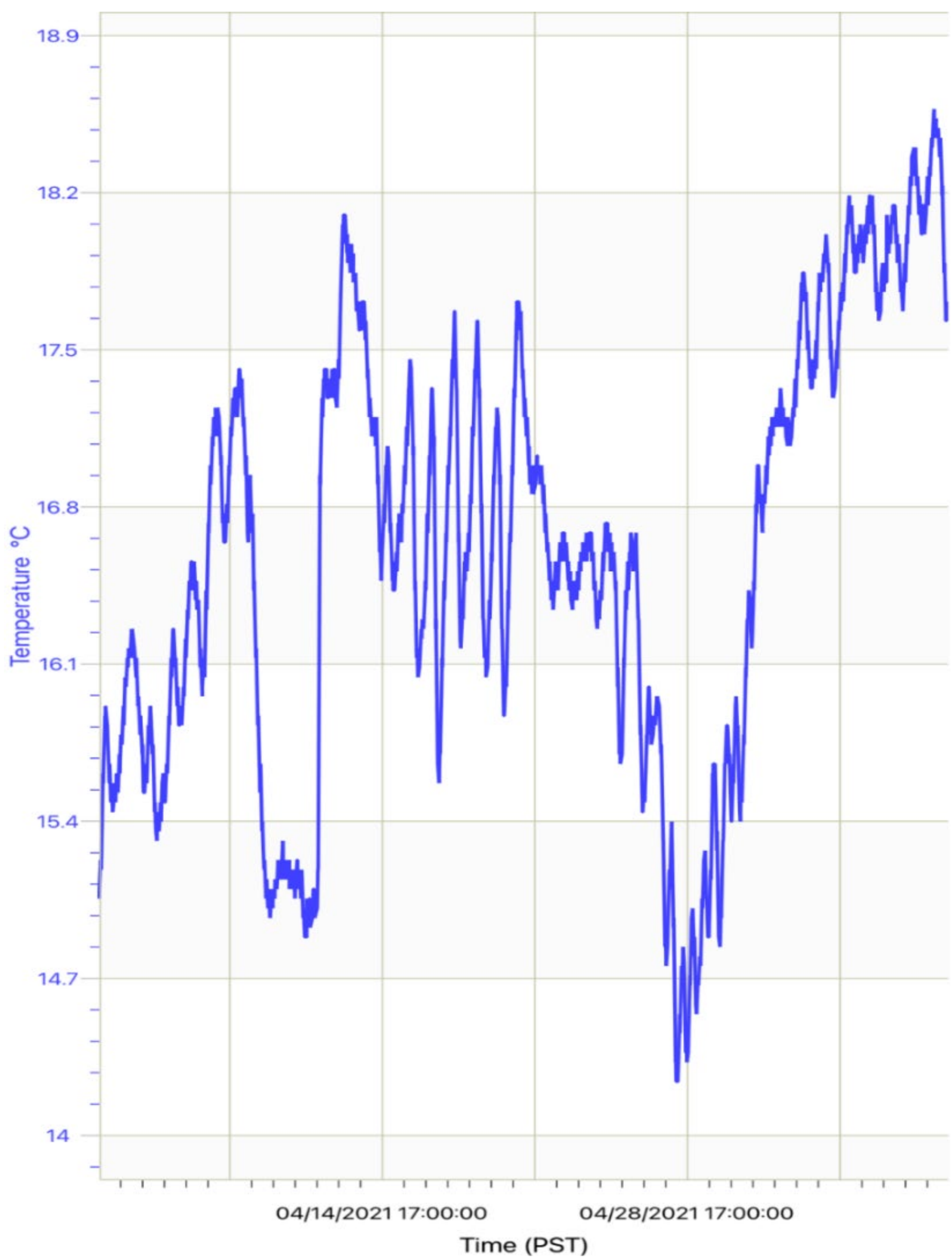

Figure 7. Ambient water temperature from tank set-up (17 March 2021) until third round of biopsy (10 May 2021).

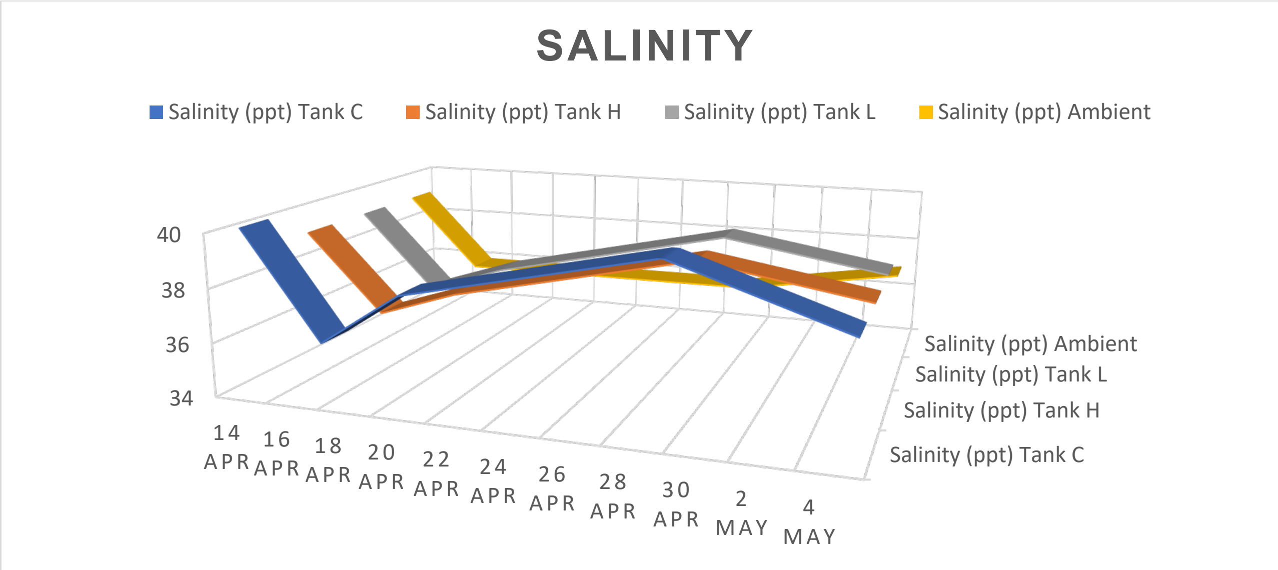

Figure 8. Salinity (ppt) of tanks and source ambient water. Measurements taken on 14 April 2021, 17 April 2021, 20 April 2021, 29 April 2021, 5 May 2021.

Discussion

All specimens, regardless of sex, were in late stage III (developing) or in stage IV (spawning) of gonad development. According to Muscat 1983, *A. parvimensis* from Santa Catalina is in stage III from March through April and is in stage IV from May through June. The observations from this experiment fit this timeline, which indicate that the stock the collected specimen originate from have a similar development cycle. Results from this experiment on gonad development are congruent with the spawning season cited by the CDFW for seasonal fishery closures.

The null hypothesis cannot be rejected for this experiment because there is no evidence of a visual physical effect on the gonad development in *A. parvimensis* after exposure to LNG. Temperature variability may have played a role in metabolic rate for LNG, however, all individuals were subject to the same temperature changes, which implies results should still be comparable between each tank. The temperature spike to 18.49°C at the end of the experiment and anecdotal evidence correlating *A. parvminensis* spawning with the arrival of warm water (CDFW 2019) could explain the absence of gonad tissue in the unidentifiable individuals in Tank C and Tank H. Salinity in Tank L and Tank H may have been affected by the presence of LNG, which could potentially account for the differences seen between the experimental tanks and the control. Using more accurate methods to measure salinity and frequent environmental analysis for LNG could provide the data needed to ascertain this postulation.

Levonorgestrel on A. parvimensis *reproduction development*

Based on physical, visual observations, exposure to LNG does not cause thickening or the gonad membrane or malformations in gamete development. A lack in the number of individuals for measurements to be taken from and comparisons calculated could be affecting the results observed. Membrane thickness and gamete count in both testes and ovaries were not significantly different between specimens in Tank C, Tank H, and Tank L. In male specimen, Tank C and Tank H had thinner membranes than that from the male in Tank L. This could be due to increased nutrient intake due to reduced feeding competition in Tank L in comparison to Tank C and Tank H. The dense areas composed of mature spermatozoa were composed nearly half of the width of area inside a testis tubule. In female specimens, the gonad membrane was thicker in

Tank H than in Tank C, however the difference is not significant enough to prove the hypothesis of this study partially true.

Limitations and considerations

Limitations that may have resulted in not rejecting the null hypothesis include the duration of the experiment, the season it occurred, and size of the experiment. The aquarium experiment occurred over the course of 37 days, which may not have been enough time to show any potential effects on the reproductive system of *A. parvimensis* after exposure to LNG. Additionally, with the experiment occurring during mid to late gonad maturity and within close proximity of spawning season, effects on gametogenesis and gonadal development may not be exhibited with the short time frame and low concentrations of contaminant used. The size of the experiment may have been a variable that affected the results. This, in conjunction with the mass premature evisceration exhibited in Tank L, caused an insufficient number of specimens on which comparisons could be made at the end of the experiment.

Another confounding factor was the presence of an unknown Apicomplexa parasite, which led to misidentification of specimens and culminated in further skewing the number of individuals that could be used in comparison of results. This parasite bore a similar appearance to those found in *Apostichopus japonicus* that were photographed and described by Unuma et al. 2020, with a conspicuous difference in where it was found. The parasite described by Unuma et al. 2020 was only found in ovaries, and the Apicomplexan found in this experiment was present in the gonads of all individuals regardless of which tank it was housed in. Parasites had a diameter that measured between 80µm-100µm, which is similar in diameter with developing oocytes. Its presence in all gonads was the major contributor to misidentification of *A. parvimensis* sexing in this experiment and further research can be done to analyze how or if this parasite has a significant influence on the reproductive capability of *A. parvimensis*.

For future experiments, some improvements include starting the aquarium experiment earlier in gonadal development, increasing the number of specimens used in the trail, separating specimens into individual containers with lower heights to avoid surface feeding, placing containers on a closed recirculating system with consistent contaminant-dosing occurring at regular intervals throughout the day, screening out specimens with parasites, and using a sediment that better matches those that *A. parvimensis* prefers.

Conclusion

This experiment did not have results that reflected LNG exposure affected *A. parvimensis* gonad development. However, other studies do show that LNG exposure causes biological repercussions in the aquatic environment (Johnston et al. 2018; King et al. 2015; Loganimoce 2016; Orlando & Ellestad 2014; Oropesa & Guimaraes 2020). Endocrine disrupting pollutants alter the reproductive success of aquatic life, and thus, pose a threat to population structures and conservation efforts.

Further information is needed to prove a statistically significant case on the adverse impacts of LNG on *A. parvimensis* and other marine invertebrates of fishery interest that may also serve as a biological indicator of this or other remnant hormonal PPCPs still present in treated wastewater.

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