Weight gain in marine animals when exposed to hypoxic or cold-water environments

by

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Abstract

Hypoxia is a common factor found in many human degenerative and inflammatory conditions. It is most often associated with an expanded extracellular matrix and high concentrations of proteoglycans. These proteoglycans are hydrophilic and can therefore affect hydration which in turn may lead to increased tissue pressure and decreased blood flow. This common pathophysiology may be a cellular response to stress and exist in mammals as a vestige. Marine animals when subjected to a hypoxic environment have been shown to exhibit a secretion of enzymes and/or an increased resistance to peripheral circulation. If this resistance to circulation is due to proteoglycan secretion and increased tissue pressure the fish will gain in mass. In this experiment three species of marine animals, *Gasterosteus aculeatus, Nereis vexillosa*, and *Hermissenda crassicornis* were subjected to the stressors of a cold-water environment (45˚F/7.22˚C) and *N. vexillosa* and *H. crassicornis* were also subjected to a hypoxic environment (~2 mg/L dissolved oxygen concentration). They were weighed before being introduced to either stressor, during exposure to the stressor and finally after being returned to their normal water environment (53˚F/11. 67˚C temperature for the cold-water experiment, and ~8-9 mg/L for the hypoxic experiment). All three normal water/control treatment replicates of *G. aculeatus* significantly increased in total wet weight and one cold water replicate significantly decreased in total wet weight ($p < 0.05$). For *N. vexillosa*, all three normal water treatment replicates and one cold-water replicate significantly increased in total wet weight ($p < 0.05$). Only one cold-water replicate for *H. crassicornis* significantly decreased in weight ($p < 0.05$). For the hypoxic conditions, there was no significant weight gain in *N. vexillosa* or *H. crassicornis* however, *N. vexillosa* did exhibit an increase in weight overall. Future studies should be conducted to determine if a significant weight gain would be seen in marine animals when exposed to stressors such as hypoxia. These future studies should also incorporate testing tissue samples and/or gene expression of the marine animals exposed to the stressor for presence of proteoglycans.

Keywords

Gasterosteus aculeatus, Hermissenda crassicornis, Hypoxia, *Nereis vexillosa*, Proteoglycans

Introduction

Life is sustained through differences in pressure. Basic physiology such as blood flow and the permeation of gases are due to a pressure gradient. In the circulatory system, the greatest pressure gradient is within the chambers of the heart but at the capillary level the gradient is far less. It is not difficult to see why any increase in interstitial fluid

pressure will curtail blood flow and oxygenation to those tissues as the pressure would not be great enough. Hypoxia, characterized as having dissolved oxygen levels below 2.8 mg/L and thus causing a deficiency of oxygen to the tissues, has been shown to exist in many conditions ranging from cancer to tendinopathy to inflammatory arthritides. The lack of oxygen is the cause rather than an effect of many of these diseases. The same conditions are also associated with edema and elevated concentrations of proteoglycans (Wu, 2002; Negrini, Passi and Moriondo, 2008).

Hypoxia is also a risk to marine animals and its effects are becoming more pronounced in recent years (Fitzgerald et al., 2017). A physiological response to an environmental stress in marine animals such as fish when exposed to hypoxia is an increased resistance to peripheral circulation. Bradycardia and partially shifting from aerobic to anaerobic metabolism is also seen. These reflexes seem to be similar in many fishes (Satchell, 1971). Other marine animals, such as invertebrates, also have physiological responses to hypoxia such as secreting enzymes. The low dissolved oxygen levels increase oxidative stress, which can damage cells due to the production of radicals or reactive oxygen species (ROS) within the animal. These enzymes secreted are found to increase after being exposed to the stress of low dissolved oxygen (Sui et al., 2017). This is shown to be a tissue-specific phenomenon, at least in many fish (Leveelahti et al., 2014). An increase in enzymes may be triggered by an increase in other molecules being secreted into the tissue such as proteoglycans, as ROS are capable of cleaving proteoglycans (Regan et al., 2005). These enzymes may be found in other invertebrates or even vertebrates.

The gap in current knowledge is the relation between proteoglycans and elevated interstitial fluid pressures and therefore weight gain. Proteoglycans (PG's) are made up of a protein bonded to a glycosaminoglycan (GAG) group, which is a long unbranched polysaccharide (repeating unit of carbohydrate molecules) that contains a repeating disaccharide unit (sugar carbohydrate unit) (Kwok et al., 2008; Matsui and Oohira, 2004). These molecules are quite varied in both their size and function and are produced by most cells. Large sulfated PG's such as chondroitin sulfate (CSPG) and

heparin sulfate (HSPG) are hydrophilic and therefore affect hydration. This is partly due to their negative charge creating a net osmotic pressure within the tissue by reducing the outward movement of water particles from the area (Han et al., 2011; Chahine et al., 2005). It is also due to certain PG's having a larger number of GAG chains, making those PG's capable of binding large amounts of water (Bancroft 2008). As hydration takes place the interstitial fluid pressure is going to increase. The magnitude of the increase will be determined by the hydrophilic nature of the PG's, the concentration of the PG's and the compliance of the tissue. The HSPG's and CSPG's this experiment is concerned with are those secreted by the nerves and their related connective tissue when stressed. The response of PG secretion when cells are stressed may be a vestige of a normal adaptive reflex of marine animals.

This study is important because it addresses one reason why hypoxia might occur in chronic degenerative and inflammatory conditions, whether that hypoxia occurs in relation to weight gain, and if that weight gain may in fact be due to proteoglycan secretion. If the null hypothesis is rejected, that is if the test subjects gain weight, then the cause of increased extracellular proteoglycan concentrations in mammals is likely vestigial. Also, if the null hypothesis is rejected, it is significant because future zoological studies can investigate: which animals exhibit the response, what other stressors cause this response, what proteoglycans are secreted (if at all), and what enzymes were involved in reducing the proteoglycan concentration when the stressor was removed. Future medical studies could involve, neurology, psychiatry, urology, respirology, cardiology, oncology, ophthalmology, gynecology, rheumatology, sports medicine, traditional Chinese medicine, orthopedics, internal medicine, infectious diseases, and manual therapies.

The purpose of this study is to determine if three different marine animals will exhibit a weight gain when introduced to stressors of a cold-water environment or hypoxic environment. The marine animals being tested include: the stickleback *Gasterosteus aculeatus,* the annelid *Nereis vexillosa*, and the nudibranch *Hermissenda crassicornis*. I hypothesize that these three species will gain weight when exposed to cold-water or

hypoxic environments. Predictive outcomes include: if the marine animals being tested gain weight, the cause may in fact be due to proteoglycan secretion and if a tissue sample is taken and preserved, when tested it will contain concentrations of proteoglycans.

Background information on test subjects

The three-spine stickleback, *Gasterosteus aculeatus,* is found in both marine and freshwater environments. It is in the phylum Chordata, class Actinopterygii, and infraclass Teleostei. Their circulatory system is a simple circuit that moves from the heart to the gills to the rest of the body and then back again to the heart, similar to fishes as a whole. Pressure is maintained by valves present in sections of the heart. There are also branchial arteries that move blood between the heart to the gills and the gills to the body, and play a role in peripheral circulation (Helfman et al., 2009). As stated above, increase resistance to peripheral circulation has been demonstrated when fish are exposed to hypoxia. When it comes to hypoxia, stickleback have a low tolerance and have exhibited behavioural and biochemical effects such as fewer aggressive acts, and physiological responses such as increased gill movement in response to low dissolved oxygen concentrations. The adaptation of these ecological strategies decreases the negative effects imposed on *G. aculeatus* when exposed to hypoxia (Fitzgerald et al., 2017).

Nereis vexillosa, like stickleback fish, have a closed circulatory system. Their common name is the banner sea-nymph and they are part of the class Polychaeta and phylum Annelida. *Nereis vexillosa* can be found buried in the sediment under rocks in the mid-intertidal zone. Circulation occurs by blood vessels and hearts, and can also occur by the movement of fluids in the coelom. The dorsal blood vessel carries blood anteriorly and the ventral blood vessel carries blood posteriorly. These two major vessels are contractile and are connected to allow blood flow between them by a capillary network/plexus. This is a closed circulatory system using blood vessels and hearts to

move blood throughout the *N. vexillosa*, and throughout annelid specimens in general (Ruppert et al., 2004).

Hermissenda crassicornis, commonly known as the opalescent sea slug, are a species of nudibranch and are part of the class Gastropoda and phylum Mollusca. They can be found moving along different substrates in tidepools, mudflats, and from the low-tide water line to depths of approximately 110 feet along the ocean floor (McKinley, 2000). They differ from *N. vexillosa* and *G. aculeatus* as their circulatory system lacks a cellular lining and is characterized as being open, rather than closed. There are vessels including a heart, dorsal aorta, and blood vessels which distribute blood to the foot for example, but otherwise the hemolymph (a fluid analogous to blood in vertebrates) is moved to sinuses or open cavities in the tissue forming the open system. Many nudibranch species, like *H. crassicornis* have several pairs of external gills projecting off their dorsal surface called cerata, which function for both respiration and circulation. Other nudibranch species have feathery gills that are also external and project from the dorsal surface. The cerata transport oxygen to the tissues by the hemolymph. From the ctenidia, the oxygenated blood is moved through blood vessels to the aorta which splits into smaller arteries and distributes the hemolymph into sinuses within the hemocoel oxygenating the tissues. This is done in conjunction with the respiratory water current moving in the opposite direction, creating a countercurrent gas-exchange mechanism (Ruppert et al., 2004; Pechenik, 2015).

Materials and Methods

There were 32 marine *Gasterosteus aculeatus* collected using minnow traps from the Straight of Georgia at Courtney Lagoon on June 29th. They were placed in 30-gallon tanks with a fake plant, filter and air stone, with automatic water changes daily to maintain the water chemistry. Animal care staff fed them blood worms every morning and night to satiation. I collected 42 *G. aculeatus*, with a couple other peers, from Burt's Island in the Bamfield inlet amongst the sea grass and in shallow waters, using a seine and waders. They were transported in a cooler back to the lab and placed in 30 gallons

tanks with the additions mentioned above. I used 24 *G. aculeatus* from Courtney Lagoon and 12 from Burt's Island for my treatments.

Thirty-six *Hermissenda crassicornis* were collected at three different locations: Bamfield Marine Sciences Centre south dock on June 18th, Ohiatt and Seppings island BC on June 19th, and Ross Islets on June 21st. They were placed in round mesh and plastic containers to keep them all separated, in a shallow sea table. They were fed an assortment of food every other day including small pieces of mussels, hydroids, tunicates and sea anemones until the beginning of the experiment July 10th, 2017.

I collected 50 *Nereis vexillosa* from Dixon Island the morning of June 27th (48'51.122' N, 125'7.313' W). Only 36 were used in the experiment, the remaining 14 were used as fish bait for scientific angling. They were collected in the mid-intertidal zone in sulfurous muddy sandy sediment underneath overturned rocks. I placed them in a sea table at its normal temperature of 53˚F (11. 67˚C) in buckets with some of the sediment I collected them from. They were given various types of algae and some mussels over the course of the 2 weeks they were held until the beginning of the experiment, but I did not observe them feeding.

I set up chillers beside each sea table with a tube connecting the in-flow to an air pump in one corner and a tube connected to the out-flow extending into the sea table in the opposite corner. The chiller in one sea table was set to 53˚F (11. 67˚C) for the normal treatment to match the temperature of the seawater coming out of the line, and the other two connecting chillers were set to 45˚F (7.22˚C) for the cold-water treatment. For the cold treatment, I used two chillers connecting to one another to lower the water temperature enough. I collected 18 plastic tanks and their lids (approx. 5 gallons) that were yellowish brown tinted. Nine containers were placed in the control/normal water deep sea table and nine were placed in the cold-water environment. Two lock-lines were connected to spouts releasing sea water into each of the sea tables at a low flow and aimed towards the air-pump. This was done so there was some water flow to keep oxygen levels high enough but not too high so the chillers could keep the water

temperature fixed. I placed one fake plant and two rocks in each of the 18 tanks. A line of malleable plastic tubing connected to one air line and branched off to 18 different air stones that were in each of the individual tanks. Then I placed lids on the tanks with a few openings for water flow and the air stone line.

Six individuals of one species were placed in a tank, and there were three trials per species, making a total of 18 specimens of each species per treatment group. Once all the specimens were in their respective tanks, I set the cold-water chillers to 45˚F (7.22˚C) to bring the water temperature down. For the stickleback, the Courtney Lagoon and Burt's Island populations were kept in separate tanks, with four containers containing Courtney Lagoon specimens (2 in cold-water and 2 in normal water) and two containers with the Burt's Island specimens, one in the cold-water deep-sea table and one in normal water. Each *H. crassicornis* was kept in a small individual container as they exhibited aggressive behaviour towards one another when they came into contact. The stickleback tanks were noted as N1, N2, N3 for the normal water table and C1, C2, C3 for the cold-water table. The annelid tanks were noted as N4, N5, N6 for the normal water table and C4, C5, C6 for the cold-water table. The nudibranch tanks were noted as N7, N8, N9 for the normal water table and C7, C8, C9 for the cold-water table (Fig. 1).

I kept all three species of marine animals in the cold-water treatment for 5 days (120 hours) and in the normal water for approximately 4 days and 5 hours (77 hours). This was done so all the specimens were housed in their respective conditions for the same number of degree days being approximately 225°F. I weighed all individuals in a single treatment tank together for the total biomass wet weight in grams every three hours for the first 24 hours of the experiment, every six hours from hour 24 to 48 and at hour 56, 72, 80, and 96. For day 4, that is after hour 96, I weighed the individuals in the normal tank at hour 101 which was the last weighing for the normal water treatment. The cold-water treatment was also weighed at hour 101, and then again at hour 114 and 120. The nudibranchs were weighed on a different scale than the stickleback and annelids because the sticklebacks and annelids needed a scale that could hold more weight.

Shortly after weighing at hour 18, there was a power outage for two and a half hours which ceased the flow from the air stones and chillers for the normal water deep sea table only. At hour 42, I measured the oxygen levels and both the normal and cold-water treatments had levels above 8 mg/L.

All stickleback normal and cold-water tanks began with n = 6. There were four stickleback deaths: at hour 18 in tank N1, at hour 24 in tank N2, shortly after hour 96 in tank N2, and at hour 114 in tank C1. The sticklebacks were fed blood worms after the weighing at hour 18, between weighing at hour 42 and 48, and after the weighing at hour 80. All annelid tanks began with n = 6 and there were no deaths. A few individuals had epitokes which broke off the posterior end of their body, and those pieces were still included with the individual in each weighing. They were fed blood worms at hour 80 and after the last weighing. The nudibranch cold-water treatment tank, C9 started with n = 5. There were three nudibranch deaths: 2 at hour 48 in tank C7, and at hour 72 in tank N9. All deaths were of nudibranchs that were less than half a centimeter in length, that is, the smallest ones of the 36. They were fed at hour 30, and after the last weighing. At hour 96, I added 15 mL of Tank Buster, which is a solution added to tanks with there are nitrite spikes, to each of the stickleback individual tanks in the normal and cold-water treatments. This was due to the cold-water tanks having a NH_3/NH_4^+ concentration of 3-4 mg/L. The normal water had a concentration of approximately 0 mg/L but I still added the Tank Buster solution to keep the two treatments as similar as possible.

After taking the last weighing of the cold-water treatment group, I set the temperature of the chiller to 53˚F (11. 67˚C) to bring the water temperature back up to normal for the animals and increased the flow. Three days later I placed 12 tanks in one deep sea table each with two rocks and a fake plant as a closed system with no water flow into the tanks. There was water in the deep sea table continuously running to keep the water temperature constant. Only half the tanks had an air stone for the normal water treatment to keep a dissolved oxygen concentration of about 8-9 mg/L, and the other six had no air stone for the hypoxic water treatment at a dissolved oxygen concentration of about 2 mg/L. Six individuals of *Nereis vexillosa* were placed in a tank, and there were

three trials per species, making a total of 18 specimens of each species per treatment group. *Hermissenda crassicornis* were placed in their respective tanks but had n = 5 for a hypoxic and normal water tank, and n = 4 for the other four hypoxic and normal tanks.

I weighed all individuals in a single treatment tank together for the total biomass wet weight in grams at hour zero, 24 and 48. When weighing them, they were placed in normal sea water at a temperature of 53˚F (11.67˚C). I also recorded the oxygen level in each tank a few hours after beginning the experiment, at hour 24 and at hour 48. The temperature of the water ranged from 11-12°C during the 48-hour period. Wet weight changes in each of the 18 containers for both the cold-water and hypoxic experiments were analyzed using a linear regression. All statistical analysis tests were run using RStudio Version 0.99.903 (R Core Team, 2016).

Results

Cold-water experiment

All three stickleback tanks in the normal water treatment did exhibit a significant change in wet weight (N1 linear regression, b = - 0.07026±0.01368, t = - 5.137, df = 16, p = $9.94x10^{-5}$); (N2 linear regression, b = - 0.06288±0.01408, t = - 4.466, df = 16, p = 0.00039); (N3 linear regression, b = - 0.029414±0.008261, t = - 3.561, df = 16, p = 0.00261). All three replicates followed a pattern of decreasing in weight over time with N1 and N2 decreasing about three times faster than N3. There were three deaths: one in N1 at hour 18 resulting in a 4 g decrease in total biomass and two in N2 at 24 hrs and at 96 hrs resulting in an 8 g and 2 g decrease, respectively (Fig. 3). For the cold-water treatment sticklebacks, the stickleback tanks C1 significantly changed in wet weight (C1 linear regression, b = - 0.027731±0.006358, t = - 4.361, df = 18, p = 0.000376). The sticklebacks in tank C2 and C3 both did not exhibit a significant change in wet weight (C2 linear regression, b = - 0.003998±0.006616, t = - 0.604, df = 18, p = 0.553); (C3 linear regression, b = - 0.005644±0.004227, t = - 1.335, df = 18, p = 0.198). *G. aculeatus* demonstrated a decrease in weight over time for tank C1, whereas in tanks

C2 and C3 there was almost no change in weight over time. One death occurred in tank C1 at hour 114 resulting in a 2 g decrease in total biomass (Fig. 4).

Nereis vexillosa in all three normal water treatment groups significantly changed in wet weight (N4 linear regression, b = 0.034691±0.008093, t = 4.287, df = 16, p = 0.000566); (N5 linear regression, b = 0.030988±0.008289, t = 3.739, df = 16, p = 0.00179); (N6 linear regression, b = 0.019053±0.003645, t = 5.227, df = 16, $p = 8.31x10^{-5}$). All three replicates slightly increased in weight over time (Fig. 5). For the cold-water treatments, the annelids in tank C4 significantly changed in wet weight (C4 linear regression, b = 0.021530±0.005857, t = 3.676, df = 18, p = 0.00173). The annelids in tanks C5 and C6 did not significantly change in wet weight (C5 linear regression, b = 0.007439±0.005919, t = 1.257, df = 18, p = 0.225); (C6 linear regression, b = 0.008984±0.007002, t = 1.283, df = 18, p = 0.216). There was a small increase in weight over time for tank C4, and the other two replicates exhibited a very small and insignificant increase in weight over time (Fig. 6).

The nudibranchs in all the normal water treatment tanks did not exhibit a significant change in wet weight (N7 linear regression, b = 0.005124±0.005586, t = 0.917, df = 16, p = 0.373); (N8 linear regression, $b = - 3.166x10^{-5}\pm2.86x10^{-3}$, t = - 0.012, df = 16, p = 0.991); (N9 linear regression, b = - 0.004443±0.002281, t = - 1.948, df = 16, p = 0.0692). Two of the replicates, tanks N8 and N9 slightly decreased in weight over time, whereas N7 slightly increased. One death occurred in tank N9 at hour 72 which did not affect the total biomass as the specimen that died was approximately 0.1 g and less than half a centimeter in length (Fig. 7). For the cold-water treatment, the nudibranchs in tank C7 significantly changed in wet weight (C7 linear regression, b = - 0.009385±0.001880, t = - 4.993, df = 18, $p = 9.42x10^{-5}$). The nudibranchs in tanks C8 and C9 did not significantly change in wet weight (C8 linear regression, b = - 0.003120±0.002853, t = - 1.094, df = 18, p = 0.289); (C9 linear regression, b = - 0.002141±0.001929, t = - 1.11, df = 18, p = 0.282). All three tanks demonstrated a decrease in weight, with C7 decreasing about three times more than both C8 and C9.

There were two deaths, both from tank C7 at hour 48 resulting in a total biomass 0.4 g less than the weighing before (Fig. 8).

Hypoxic experiment

For all three normal water treatment tanks, there was not a significant change in wet weight for *Nereis vexillosa* (N1 linear regression, b = - 0.025000±0.009623, t = - 2.598, df = 1, p = 0.23391); (N2 linear regression, b = - 0.006250±0.006014, t = - 1.039, df = 1, p = 0.48775); (N3 linear regression, b = - 0.020833±0.002406, t = -8.66, df = 1, p = 0.07319). All three replicates follow a trend of a slight decrease in weight (Fig. 9). In the hypoxic water conditions, *N. vexillosa* in all replicates did not significantly change in wet weight (H1 linear regression, b = 0.079167±0.007217, t = 10.97, df = 1, p = 0.05787); (H2 linear regression, b = 0.02917±0.02646, t = 1.102, df = 1, p = 0.4691); (H3 linear regression, b = 0.029167±0.009623, t = 3.031, df = 1, p = 0.20287). However, all three replicates did exhibit an overall increase in wet weight over the 48-hour period. Tank H1 increased in weight by 2.2 g from hour 0 to 24, and by 1.6 g from hour 24 to 48. Tank H2 increased in weight by 1.8 g from hour 0 to 24, and slightly decreased by 0.4 g from hour 24 to 48. For tank H3, the total wet weight increased by 1.1 g in the first 24 hours, and by 0.3 g from hour 24 to 48 (Fig. 10).

Hermissenda crassicornis did not exhibit a significant change in wet weight for all of the normal water treatment replicates (N4 linear regression, b = 0.00625±0.01323, t = 0.472, df = 1, p = 0.719); (N5 linear regression, b = - 0.025000±0.004811, t = - 5.196, df = 1, p = 0.1210); (N6 linear regression, b = 0.002083±0.001203, t = 1.732, df = 1, p = 0.3333). Tanks N4 and N6 increased a small amount in wet weight whereas tank N5 slightly decreased in weight (Fig. 11). For the hypoxic treatment, *H. crassicornis* also did not exhibit a significant change in total wet weight in any of the three replicates (H4 linear regression, b = 0.002083±0.001203, t = 1.732, df = 1, p = 0.3333); (H5 linear regression, b = - 0.008333±0.009623, t = - 0.866, df = 1, p = 0.5456); (H6 linear regression, b = - 0.010417±0.003608, t = - 2.887, df = 1, p = 0.2123). Both replicates,

H5 and H6 slightly decreased in weight overall, whereas H4 increased in the first 24 hours by 3.39 g, but decreased from hour 24 to 48 by 1.66 g (Fig. 12).

Discussion

The purpose of this experiment was to determine if three species of animals, *Gasterosteus aculeatus*, *Nereis vexillosa*, and *Hermissenda crassicornis* would gain weight when introduced to the stressor of a cold seawater environment at 45˚F (7.22˚C) versus the normal water temperature at 53˚F (11. 67˚C), and to determine if *Nereis vexillosa* and *Hermissenda crassicornis* would gain weight when exposed to hypoxic water (~2 mg/L dissolved oxygen) versus normal oxygen levels (~8-9 mg/L dissolved oxygen). My results demonstrated that there was a statistically significant effect of water temperature on the change in wet weight in each of the three species for at least one replicate. My results also showed there was no significant change in wet weight for *Nereis vexillosa* or *Hermissenda crassicornis* in hypoxic water however, *N. vexillosa* for all three hypoxic replicates did increase in weight overall.

Cold-water experiment: *G. aculeatus*

All three normal water treatment tanks of *G. aculeatus* significantly decreased in wet weight over the five-day experimental period. The sticklebacks in one replicate of the cold-water treatment also significantly decreased in wet weight. This is may not be consistent with my current hypothesis. One reason *G. aculeatus* may have lost weight, rather than gained weight from this environmental stress is because they were not feeding. Also, out of the four tanks that did show a significant decrease in weight, three tanks experienced fish mortality which was likely a major driver behind the decline in total wet weight. One of those three tanks was a cold-water replicate, C1, and there was elevated levels of nitrites and nitrates (approximately 3-4 mg/L) detected in the water which may have contributed to the death of the stickleback in combination with being in the cold-water environment. The other two tanks with fish mortality were N1 and N2, however the nitrite and nitrate levels in the normal water treatment were at

approximately 0-1 mg/L so it may have been the amount of handling they were exposed to as well as lack of eating that contributed to these mortalities.

Another reason for the decrease in wet weight may be in relation to how fishes are able to partially shift to anaerobic metabolism when exposed to hypoxia as stated by Satchell, 1971. This shift is in response to an environmental stress, and a cold-water environment is another environmental stress to stickleback. In a study done by Barrett et al., 2011, *G. aculeatus* were seen to tolerate a decrease of 2.5˚C in water temperature. I, however, decreased the water temperature by approximately 4.45˚C and so perhaps the response of shifting to anaerobic metabolism was elicited by *G. aculeatus* in the cold-water tanks because they could not tolerate a larger decrease in water temperature than a 2.5˚C shift. Hypoxia also reduces the feeding and growth of individuals and when marine animals are exposed to hypoxia they attempt to conserve energy through actions such as metabolic depression (Wu, 2002). It may also be true for these stickleback in the cold-water experiment that they depressed their metabolisms which would lead to decreased eating and thus weight loss.

Cold-water experiment: *N. vexillosa*

Overall, only one tank, C4, in the cold-water treatment containing *Nereis vexillosa* showed a significant increase in weight over the 120-hour period. This weight may in fact be due to secretion of proteoglycans, however, future studies would have to be conducted to test if they are present. It may have also been experimental error when taking the wet weights of the worms causing a significant increase in only one of the three tanks. Though all *N. vexillosa* were collected from the same area, the other cold-water tanks may not have gained weight because in general, *N. vexillosa* live intertidally and thus are exposed to great fluctuations in the ambient temperature. As the water temperature was only decreased to 45˚F (7.22˚C), it may not have been low enough to cause stress to these worms because they can burrow deep into the sediment under rocks where the temperature is cooler due to the combination of lack of sunlight and being immersed in cold seawater when the tide is high enough.

Cold-water experiment: *H. crassicornis*

One tank in the cold-water treatment, C7 decreased in weight significantly which is not consistent with my alternative hypothesis that the marine animals will increase in weight. This may have been due to the nudibranchs only being fed every other day, whereas in their natural habitat they have been observed to eat quite regularly, even to the point of being the cause of large declines in populations of some of their prey (Hoover et al., 2012). There were also two mortalities in tank C7, thus contributing to the decrease in overall wet weight. Almost all of the nudibranchs also were laying egg masses in their individual containers, on the leaves of the fake plant in the individual tanks and also on the tank itself which would contribute to the significant decrease in mass of the *H. crassicornis* in tank C7, and also to the insignificant decrease in weight for tanks C8 and C9.

Similar to *N. vexillosa*, *H. crassicornis* also live intertidally and therefore are exposed to the ambient temperature fluctuating. It may be possible that cold-water at 45˚F (7.22˚C) is not a stress and they are able to withstand cooler temperatures, or instead warmer water temperatures are a stress for these invertebrates. *H. crassicornis* are also found in a wide variety of habitats in differing climates from Alaska to Mexico, which may indicate their ability to adapt to varying water temperatures (Hoover et al., 2012).

Hypoxic experiment: *N. vexillosa*

Though all three hypoxic treatment tanks increased in weight overall it was not significant. Perhaps if they were left in hypoxic conditions for a longer period of time then they would have gained significant weight. Also, the dissolved oxygen concentration did not stay at or below 2.8 mg/L for the entire experiment for all three replicates, it fluctuated from 2.09 to 7.75 mg/L between the three tanks (Table 1). The fluctuation in the amount of dissolved oxygen in the water may have be due to the lids of the tanks having holes in them, as they were the same tanks that were used in the

cold-water experiment. Additional oxygen may have dissolved into the water when I took the lids off to measure the dissolved oxygen concentrations and weigh the worms. If they were housed in air tight containers instead with no holes, the levels might have stayed in hypoxic conditions, that is at or below 2.8 mg/L and the worms potentially could have gained more weight as other polychaete species have been observed to increase in growth rate when the dissolved oxygen levels were at 2.23 mg/L (Wu, 2002). Furthermore, *N. vexillosa*, as well as other annelid worms, burrow into the sediment, as mentioned above, which exposes them to hypoxic conditions if they burrow deep enough. One behaviour seen in another species of Nereid worm to avoid these hypoxic conditions is oxidizing the sediment by ventilating it either actively or passively (Diaz and Cutter, 2001). Though there was no sediment for *N. vexillosa* to burrow in or possibly ventilate, the holes in the tank lids may have been sufficient ventilation for the worms to withstand the levels of dissolved oxygen in their tanks.

Hypoxic experiment: *H. crassicornis*

Two of the replicates in the hypoxic tanks decreased in weight overall, though it was not significant. This slight decrease in weight may have been due to not eating, as I did not feed the nudibranchs for the 48 hours during the experiment. As seen in the cold-water experiment, the nudibranchs were also laying egg masses, another factor that likely contributed to their decrease in weight. The majority of the nudibranchs in all three hypoxic replicates were observed upside down on the surface of the water using the surface tension to crawl. I have observed this behaviour of *H. crassicornis* in the field and this is also seen in other nudibranch species (Kjerschow-Agersborg, 1921). Coming from Latin and Greek roots, nudibranch translates to “naked gill” and this is with reference to how they have their gills located externally on their dorsal surface and are called cerata (Ruppert et al., 2004; Pechenik, 2015). It may have been possible that they were using surface tension crawling to attain oxygen from the water at the very surface where the dissolved oxygen levels may not be at or near hypoxic conditions.

Future studies

Overall, follow-up experiments are needed to test the tissue for the presence of proteoglycans of the specimen being tested for weight gain. Differences in gene expression may also be another variable to look at when comparing marine animals in normal versus hypoxic water. If another experiment was conducted with *Nereis vexillosa* in hypoxic waters for a longer period of time and with weighing each annelid individually in order to compare the mean change in weight over time in hypoxic conditions, there may be potential for results showing a significant increase in weight. Experimental error that may have prevented a significant increase in weight include weighing the marine animals in water that was not hypoxic, not having the dissolved oxygen levels stay below 2.8 mg/L for the entire duration of the experiment, and potentially not having a large enough sample size.

Furthermore, if future experiments found proteoglycans present in the marine animal being tested, in can aid in future medical studies because it may be the secretion of these molecules causing pockets of hypoxia within the animal's tissue and potentially compromising blood flow. By determining if it is in fact proteoglycans involved in this process, this also allows for future experiments to test which enzymes are responsible for and capable of breaking down these proteoglycans, thus aiding in research towards the medical implications of these physiological responses.

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References

Bancroft, J.D., 2008. Carbohydrates. In: Theory and Practice of Histological Techniques. Churchill Livingstone Elsevier, London, pp. 163-166.

Barrett, R.D.H., Paccard, A., Healy, T.M., Bergek, S., Schulte, P.M., Schluter, D., Rogers, S.M., 2011. Rapid evolution of cold tolerance in stickleback. Proc. R. Soc. B. 278: 233-238.

Chahine, N.O., Chen, F.H., Hung, C.T., Ateshian, G.A., 2005. Direct measurement of osmotic pressure of glycosaminoglycan solutions by membrane osmometry at room temperature. Biophys. J. 9(3): 1543-1550.

Diaz, R.J., Cutter, G.R., 2001. In situ measurement of organism-sediment interaction: Rates of burrow formation, abandonment and sediment oxidation, reduction. In: Aller, J.Y., Woodin, S.A., Aller, R.C., eds., Organism– Sediment Interactions, University of South Carolina Press, SC, USA, pp. 19–31.

Fitzgerald, J.A., Katsiadaki, I., Santos, E.M., 2017. Contrasting effects of hypoxia on copper toxicity during development in the three-spined stickleback (*Gasterosteus aculeatus*). Environ. Pollut. 222, 433-443.

Han, E.H., Chen, S.S., Klisch, S.M., Sah, R.L., 2011. Contribution of proteoglycan osmotic swelling pressure to the compressive properties of articular cartilage. Biophys. J. 101(4): 916-924.

Helfman, G.S., Collette, B.B., Facey, D.E., Bowen, B.W., 2009. The Diversity of Fishes: Biology, Evolution and Ecology, 2nd ed. Wiley-Blackwell, UK.

Hoover, R.A., Armour, R., Dow, I., Purcell, J.E., 2012. Nudibranch predation and dietary preference for the polyps of *Aurelia labiata* (Cnidaria: Scyphozoa). Hydrobiologia. 690: 199-213.

Kjerschow-Agersborg, H.P.V.W., 1921. Contribution to the knowledge of the nudibranchiate mollusk, *Melibe leonina* (Gould). Am. Nat. 55(638): 222-253.

Kwok, J.C.F., Afshari, F., Garcia-Alias, G., Fawcett, J.W., 2008. Proteoglycans in the central nervous system: Plasticity, regeneration and their stimulation with chondroitinase ABC. Restor. Neurol. Neurosci. 26, 131-145.

Leveelahti, L., Rytkonen, K.T., Renshaw, G.M.C., Nikinmaa, M., 2014. Revisiting redox-active antioxidant defenses in response to hypoxic challenge in both hypoxia-tolerant and hypoxia-sensitive fish species. Fish Physiol. Biochem. 40, 183-191.

Matsui, F., Oohira, A., August 12, 2004. Proteoglycans and injury of the central nervous system. Congenit. Anom. 44, 181-188.

McKinley, G., 2000. "Hermissenda crassicornis" (On-line), Animal Diversity Web. Electronic document: http://animaldiversity.org/accounts/Hermissenda_crassicornis/ accessed 2017-06-14

Negrini, D., Passi, A., Moriondo, A., 2008. The role of proteoglycans in pulmonary edema development. Intensive Care Med. 34, 610-618.

Pechenik, J.A., 2015. Biology of the Invertebrates, 7th edition. McGraw Hill Education, New York.

R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Regan, E., Flannelly, J., Bowler, R., Tran, K., Nicks, M., Carbone, B.D., Glueck, D., Heijnen, H., Mason, R., Crapo, J., 2005. Extracellular superoxide dismutase and oxidant damage in osteoarthritis. Arithritis Rheum. 52(11): 3479-3491.

Ruppert, E., Fox, R., Barnes, R., 2004. Invertebrate Zoology: A Functional Evolutionary Approach, 7th ed. Cengage Learning, India.

Satchell, G. H., February 2, 1971. The Control of the Heart. In: Circulation in Fishes. Cambridge University Press, London, pp. 91-92.

Sui, Y., Hu, M., Shang, Y., Wu, F., Huang, X., Dupont, S., Storch, D., Portner, H.O., Li, J., Lu, W., Wang, Y., 2017. Antioxidant response of the hard shelled mussel *Mytilus coruscus* exposed to reduced pH and oxygen concentration. Ecotoxicology and Environmental Safety 1(37): 94-102.

Wu, R.S.S., 2002. Hypoxia: from molecular responses to ecosystem responses. Marine Poll. Bull. 45: 35-45.

Tables and Figures

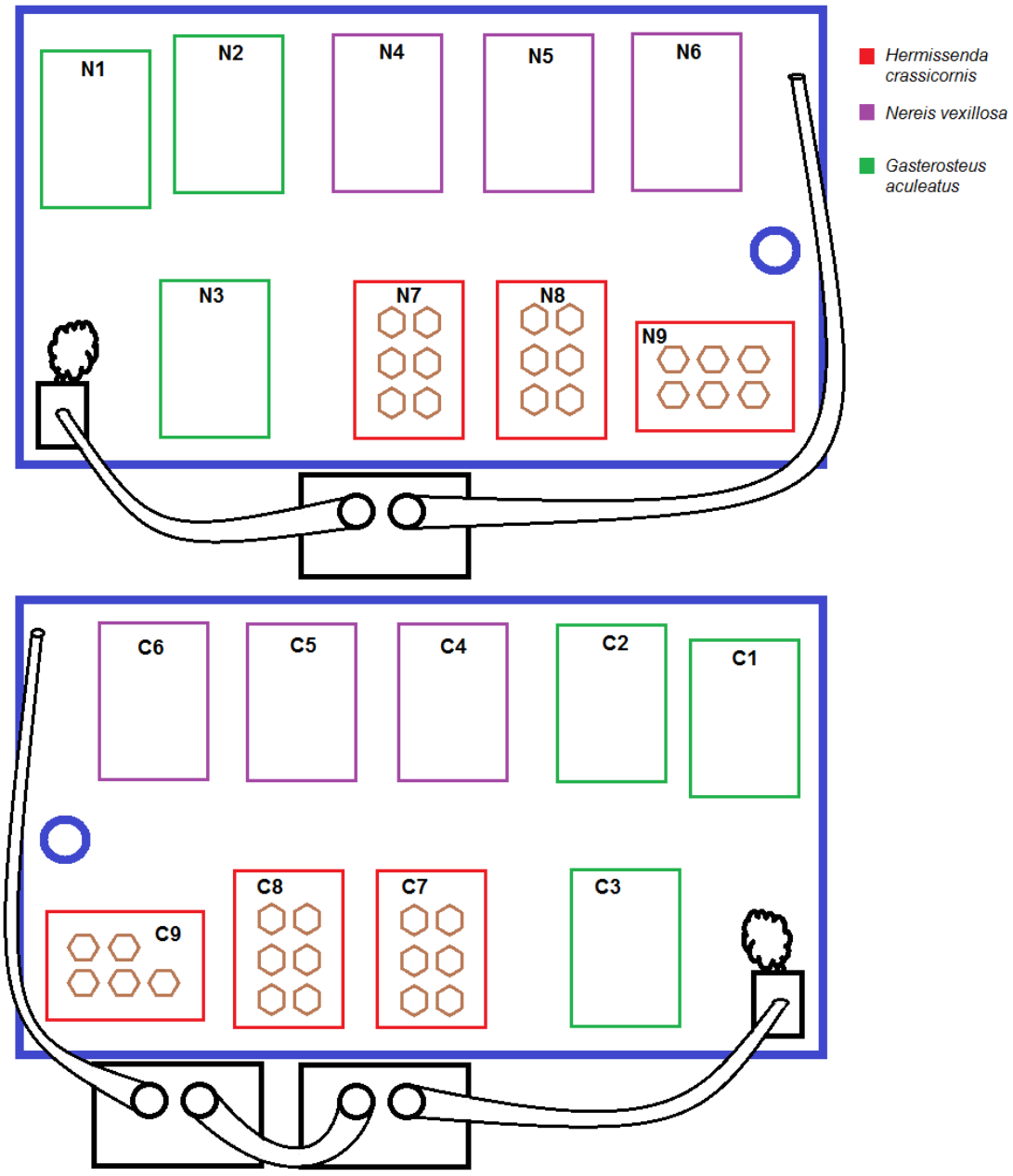

Fig. 1. Experimental set-up of the normal water treatment (top) and cold-water treatment for the three marine animals being tested for weight gain: *Gasterosteus aculeatus* (green), *Nereis vexillosa* (purple) and *Hermissenda crassicornis* (red). The blue square represents the deep sea table, each coloured box is the individual tanks for n=6 individuals except for C9 being n = 5, the black boxes with two circles represent the chillers attached to an air-pump and the other end flowing into the sea table. Each tank also has two rocks, one fake plant and an air stone.

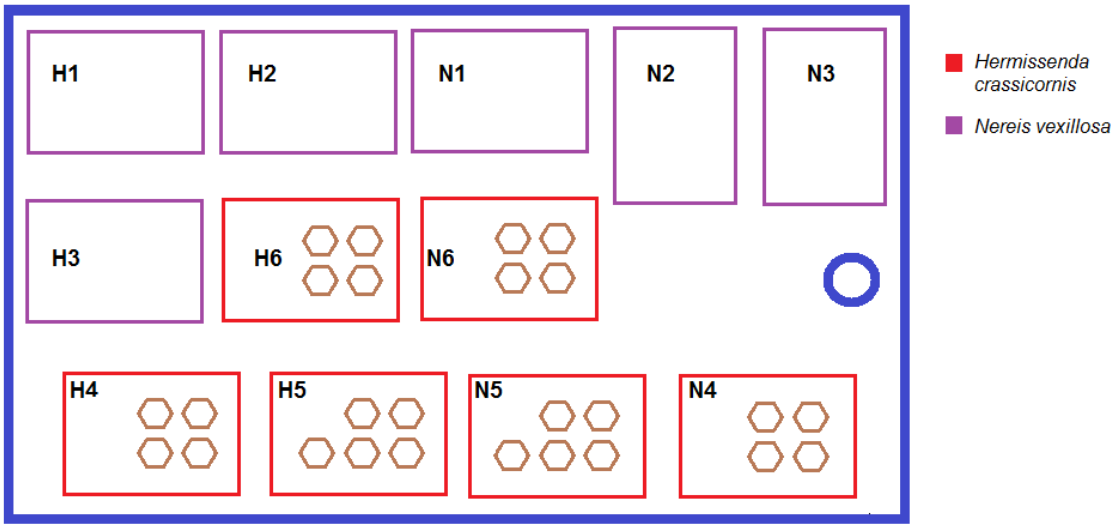

Fig. 2. Experimental set-up of the hypoxic water treatment for the two marine animals being tested for weight gain: *Nereis vexillosa* and *Hermissenda crassicornis*. The blue square represents the deep sea table, each coloured box are the individual tanks. There were three hypoxic tanks and three normal tanks for each of the two species and each tank also has two rocks, one fake plant and an air stone. For all six *N. vexillosa* tanks, n = 6. For tanks H4, H6, C4 and C6, n = 4 and tanks H5 and C5, n = 5.

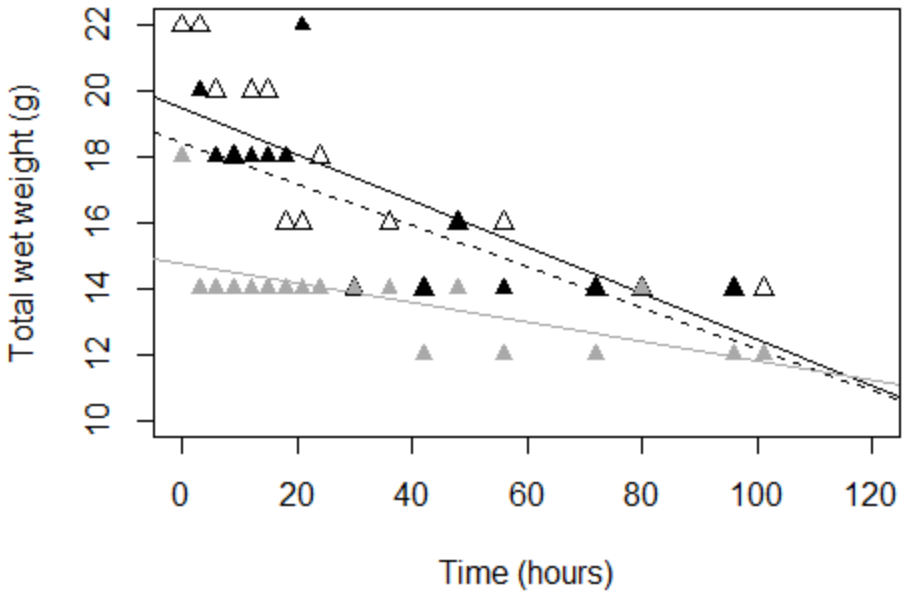

Fig. 3. Change in weight (g) of the total biomass as a function of time (hours) for *Gasterosteus aculeatus* in seawater at 53˚F/11. 67˚C for the normal water treatment tanks. The white triangles represent N1, the black represent N2 and the gray represents N3. At 0hrs, n = 6 for all three tanks. At 18 hrs n = 5 for N1, at 24 hrs n = 5 for N2, and at 96 hrs n = 4 for N2. A linear regression line is shown fitting the data.

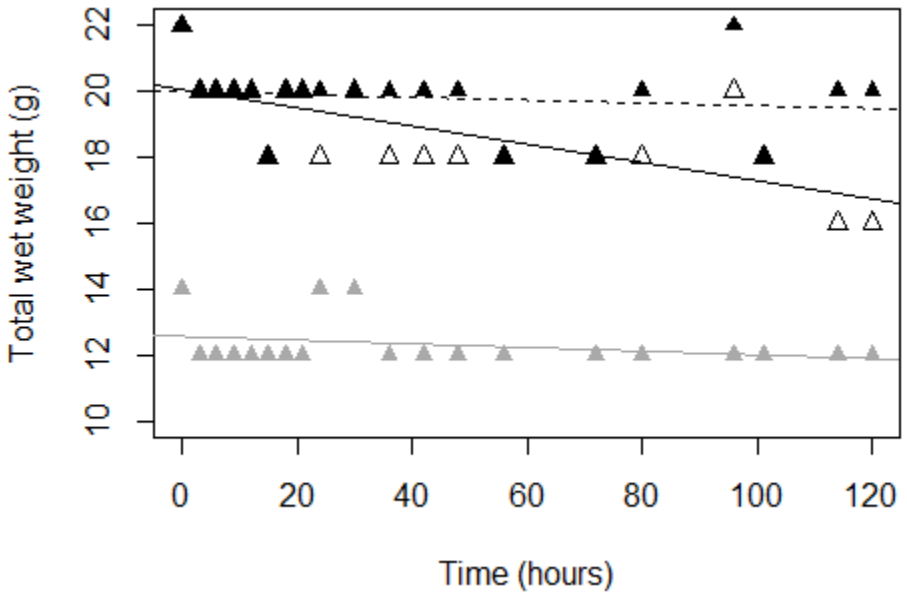

Fig. 4. Change in weight (g) of the total biomass as a function of time (hours) for *Gasterosteus aculeatus* in seawater at 45˚F/7.22˚C for the cold-water treatment tanks. The white triangles represent C1, the black represent C2 and the gray represents C3. At 0hrs, n = 6 for all three tanks. At 114 hrs n = 5 for C1. A linear regression line is shown fitting the data.

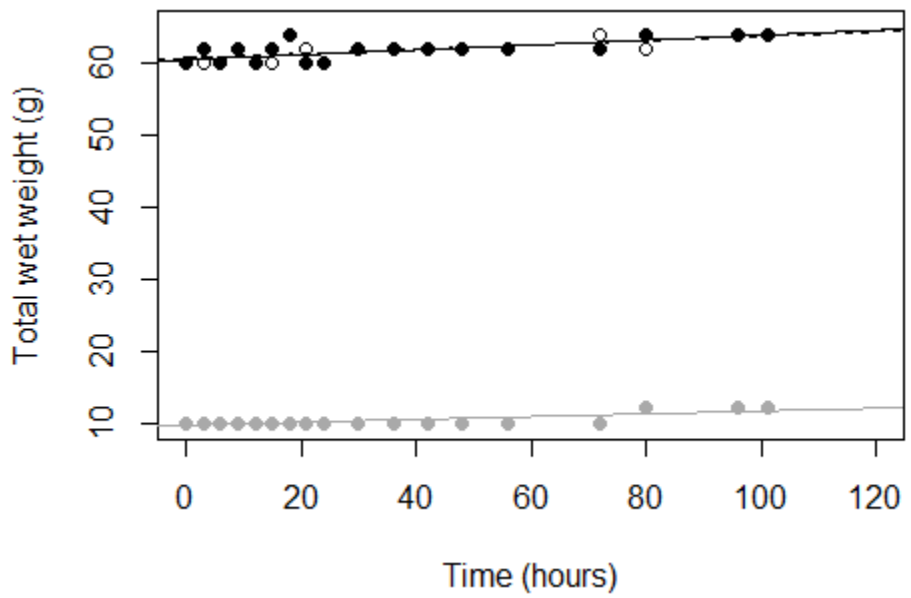

Fig. 5. Change in weight (g) of the total biomass as a function of time (hours) for *Nereis vexillosa* in seawater at 53˚F/11. 67˚C for the normal water treatment tanks. The white circles represent N4, the black represent N5 and the gray represents N6. For the duration of the experiment n = 6 for all three tanks. A linear regression line is shown fitting the data.

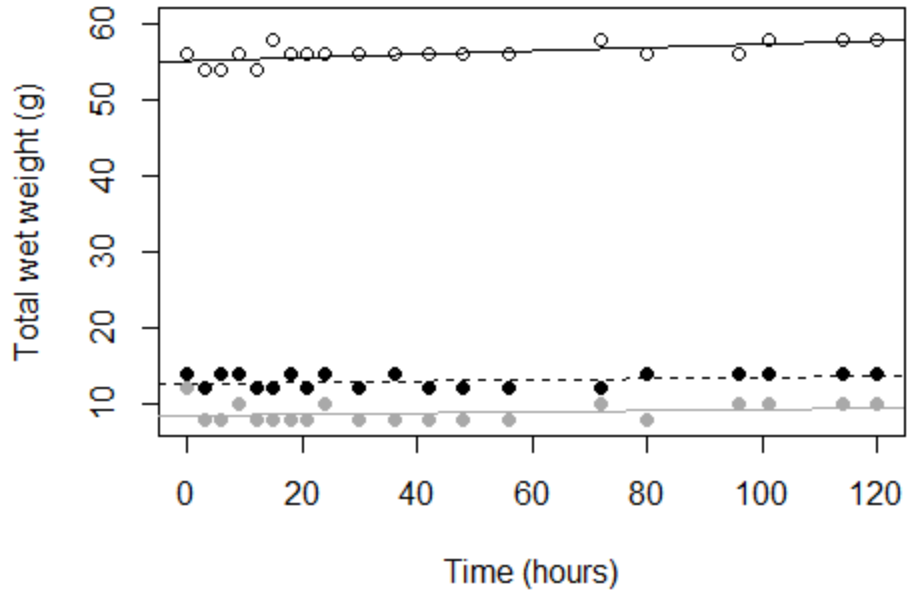

Fig. 6. Change in weight (g) of the total biomass as a function of time (hours) for *Nereis vexillosa* in seawater at 45˚F/7.22˚C for the cold-water treatment tanks. The white circles represent C4, the black represent C5 and the gray represents C6. For the duration of the experiment n = 6 for all three tanks. A linear regression line is shown fitting the data.

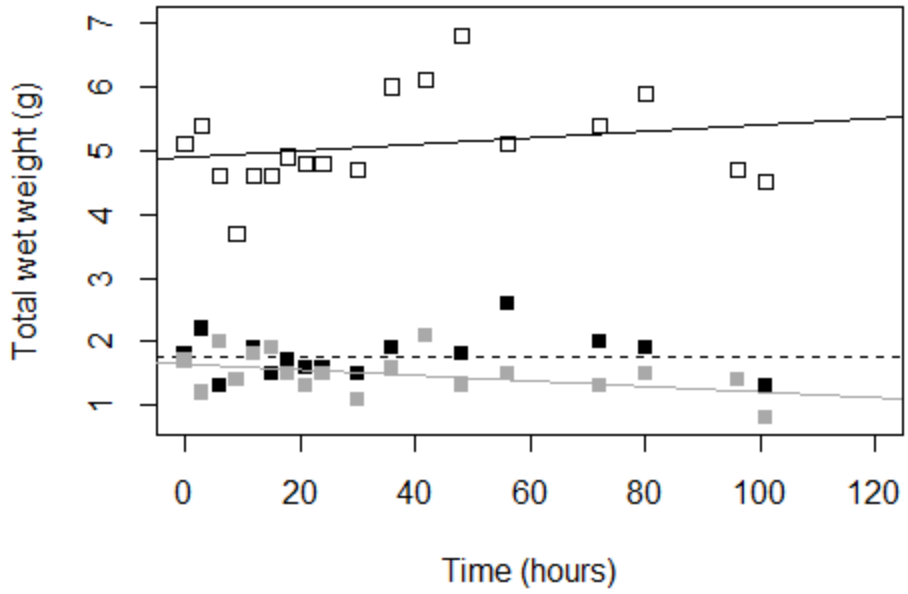

Fig. 7. Change in weight (g) of the total biomass as a function of time (hours) for *Hermissenda crassicornis* in seawater at 53˚F/11. 67˚C for the normal water treatment tanks. The white squares represent N7, the black represent N8 and the gray represents N9. At 0hrs, n = 6 for all three tanks. At 72 hrs n = 5 for N9. A linear regression line is shown fitting the data.

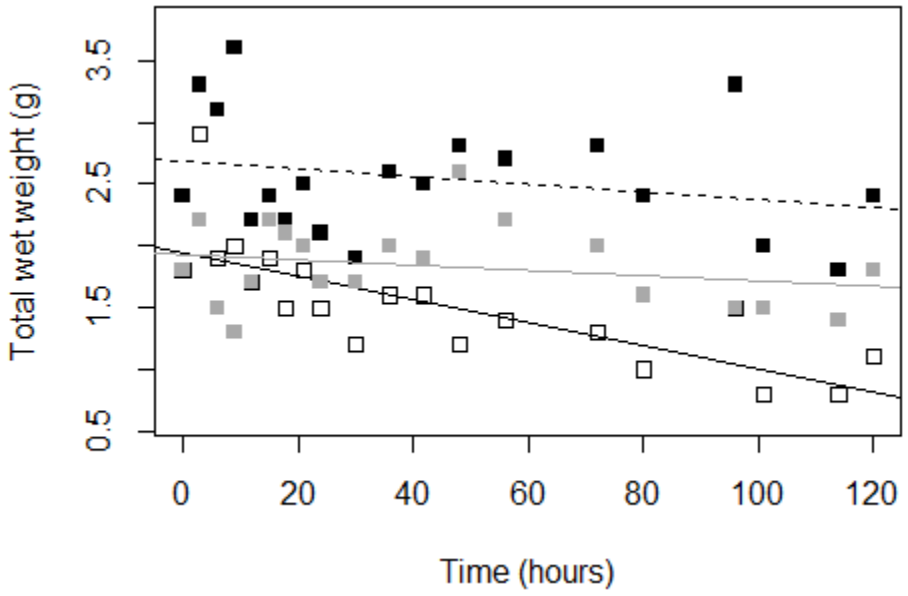

Fig. 8. Change in weight (g) of the total biomass as a function of time (hours) for *Hermissenda crassicornis* in seawater at 45˚F/7.22˚C for the cold-water treatment tanks. The white squares represent C7, the black represent C8 and the gray represents C9. At 0hrs, n = 6 for C7 and C8, and n = 5 for C9. At 48 hrs n = 4 for C7. A linear regression line is shown fitting the data.

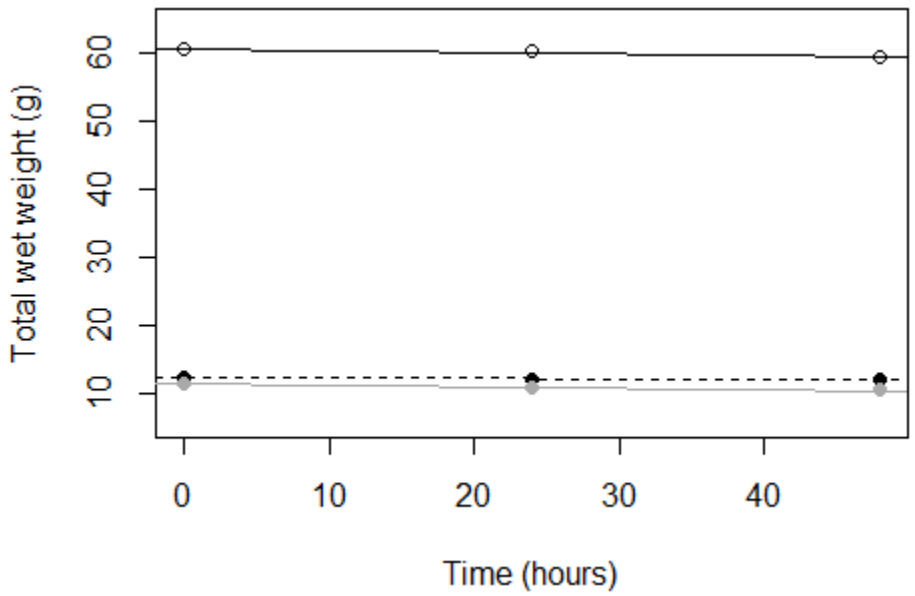

Fig. 9. Change in weight (g) of the total biomass as a function of time (hours) for *Nereis vexillosa* in seawater at 53˚F/11. 67˚C for the normal water treatment tanks. Each tank had an air stone and were closed systems. The white circles represent N1, the black represent N2 and the gray represents N3. For each of the three tanks, n = 6. A linear regression line is shown fitting the data.

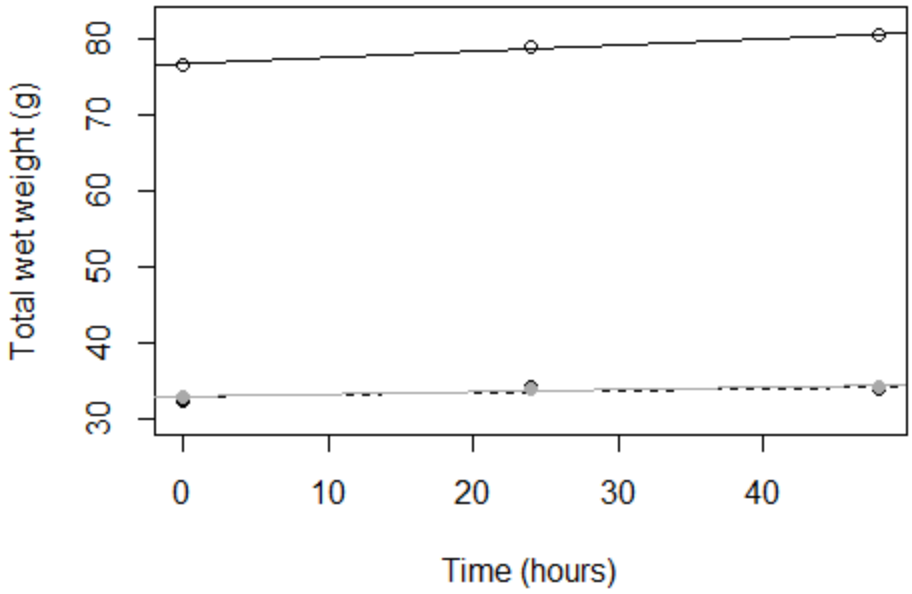

Fig. 10. Change in weight (g) of the total biomass as a function of time (hours) for *Nereis vexillosa* in seawater at 53˚F/11. 67˚C for the hypoxic water treatment tanks. Each tank did not have an air stone and were closed systems. The white circles represent H1, the black represent H2 and the gray represents H3. For each of the three tanks, n = 6. A linear regression line is shown fitting the data.

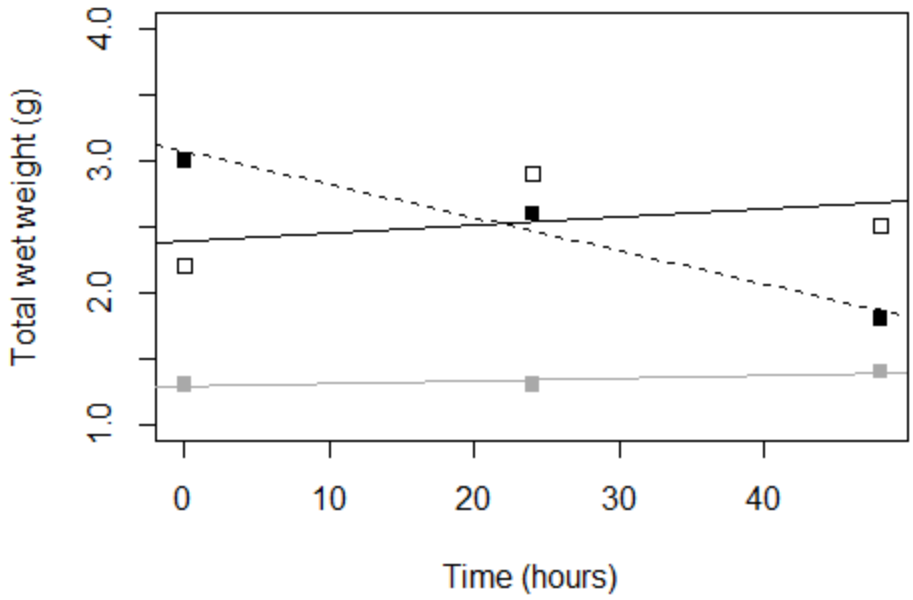

Fig. 11. Change in weight (g) of the total biomass as a function of time (hours) for *Hermissenda crassicornis* in seawater at 53˚F/11. 67˚C for the normal water treatment tanks. Each tank had an air stone and were closed systems. The white circles represent N4, the black represent N5 and the gray represents N6. At 0hrs, n = 4 for N4 and N6 and n = 5 for N5. At 48hrs, n = 3 for N4, n = 4 for N5 and n = 4 for N6. A linear regression line is shown fitting the data.

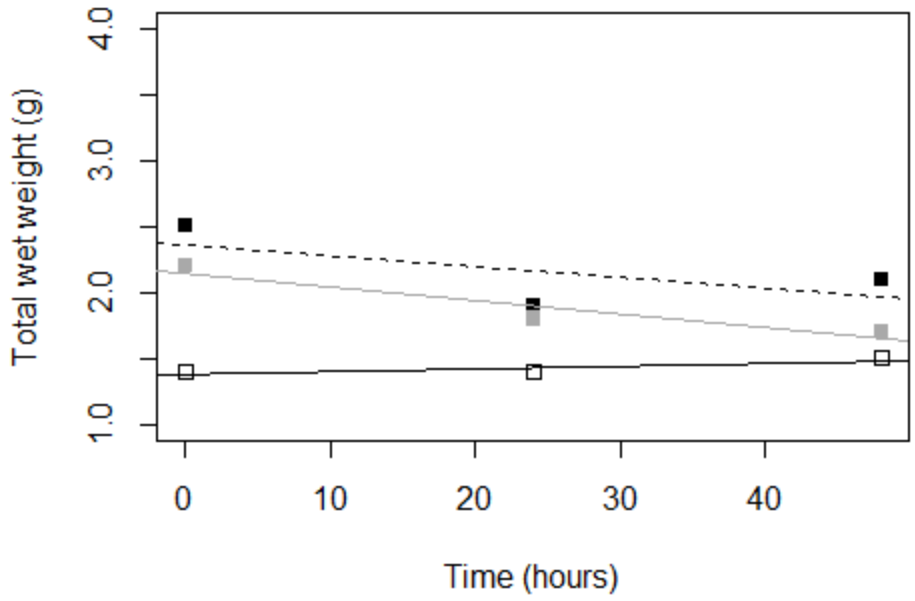

Fig. 12. Change in weight (g) of the total biomass as a function of time (hours) for *Hermissenda crassicornis* in seawater at 53˚F/11. 67˚C for the hypoxic water treatment tanks. Each tank did not have an air stone and were closed systems. The white circles represent H4, the black represent H5 and the gray represents H6. For each of the three treatments, n = 4 for H4 and H6 and n = 5 for H5. A linear regression line is shown fitting the data.

Table 1. Dissolved oxygen levels (mg/L) measured at 0, 24 and 48 hours with an oxygen probe for each individual closed system tank (~5 gallons) covered with a lid with 1-3 holes and containing either *Nereis vexillosa* or *Hermissenda crassicornis*. H1-H3 represents *N. vexillosa* and for each replicate n = 6. H4-H6 represents *H. crassicornis* and for H4 and H6, n = 4 and for H5, n = 5.

Time (hours)	Replicates' dissolved oxygen level (mg/L)					
	H1	H2	H3	H4	H5	H6
0	2.10	2.07	2.25	2.29	2.09	2.13
24	2.93	5.35	5.12	5.52	4.74	7.75
48	3.19	3.67	3.65	3.86	3.03	6.69