**No evidence for heat-triggered wasting in giant California sea cucumbers (*Parastichopus californicus*)**

Introduction, Methods and Results (Nov 2021)

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**Introduction:**

Anthropogenic climate change is increasing the intensity, duration, size and frequency of marine heat waves across the globe with potentially catastrophic effects on organism fitness, marine ecosystems and human economies (Allan et al., 2021; Frölicher et al., 2018). These extreme climatic events often cause immediate and mass mortalities at all trophic levels from thermal stress, starvation, toxicity and hypoxia (Cavole et al., 2016; Di Lorenzo & Mantua, 2016; Suryan et al., 2021; von Biela et al., 2019). For example, the 2014-2015 warm water anomaly in the Northeast Pacific Ocean triggered a harmful algal bloom, reduced the abundance of key prey species, and caused the starvation of species from salmon to sea birds (Cavole et al., 2016; von Biela et al., 2019). As such, this marine heat wave harmed recreational, commercial and subsistence fisheries of salmon, tuna and shellfish (Cavole et al., 2016; Di Lorenzo & Mantua, 2016; Frölicher et al., 2018). Marine heat waves can also impact marine ecosystems more subtly by impacting biological processes including foraging behaviour (CITE), movement (CITE), reproductive behaviour (CITE), and disease dynamics (CITE).

The adverse effects of marine heat waves on marine invertebrates can profoundly impact disease dynamics (Aalto et al., 2020). This occurs in part because thermal stress can compromise organism’s immune response (Branco et al., 2012; Matozzo et al., 2012). However, pathogen transmission and growth may also be facilitated by heat depending on the microbial response to high temperatures (Marcogliese, 2008; Rubio-Portillo et al., 2016). For example, temperature has been shown to regulate the virulence of marine diseases in corals and algae (Case et al., 2011). Abnormally warm temperatures have also been accredited to triggering and exacerbating sea star wasting disease (Aquino et al., 2021; Bates et al., 2009; Eisenlord et al., 2016; Harvell et al., 2019; Hewson et al., 2018). Over the past decade, sea star wasting disease has decimated the populations of over 20 sea star species in the Northeast Pacific Ocean (Hewson et al., 2018). Wasting in sea stars is described as a broad set of symptoms including twisted arms, lesions, deflation/loss of turgor, lost arms, lack of grip strength in tube feet, and disintegration (Bates et al., 2009; Hewson et al., 2018; Menge et al., 2016). Although wasting-like symptoms are ambiguous, recent evidence has emerged that wasting may occur in echinoderms other than sea stars.

Giant California sea cucumbers (*Parastichopus californicus)* displaying wasting symptoms have been reported in small numbers throughout the Salish Sea and the Northwest Coast of British Columbia and Alaska since 2014 (Hewson et al., 2020; Schroeder, 2017). Symptoms of wasting in sea cucumbers are anecdotally reported as similar to those observed in sea stars: non-focal lesions and fissures across the body wall, epidermal tissue sloughing and rapid liquefaction (Hewson et al., 2020b). A recent giant California sea cucumber wasting event occurred in Nanoose Bay, B.C., Canada, from August – October 2021 (Em Lim, *personal communication*). Reports from citizen scientist divers indicated that up to 75 sea cucumbers exhibited wasting symptoms and no healthy individuals were observed in the top 20 meters of the water column. Sea star wasting disease was reported at the same time as wasting sea cucumbers were observed. This event followed several regional heat waves that decimated populations of intertidal invertebrates and warmed subtidal temperatures XX amount. As such, there is a potential link between thermal stress and wasting in giant California sea cucumbers. However, understanding the cause of wasting in sea cucumbers also requires knowledge of how their unique behavioural and physiological characteristics are affected by thermal stress.

Giant California sea cucumbers have several unusual and poorly understood traits that distinguish them from other echinoderms in which wasting symptoms have been reported. To begin, sea cucumbers have collagenous catch connective tissue that allows them to change the mechanical properties of their dermis in response to environmental stimuli (Motokawa & Tsuchi, 2003). Under normal seawater conditions, sea cucumber dermal stiffness can rapidly increase, which is thought to be adaptive for posture maintenance and antipredator defense (Motokawa & Tsuchi, 2003; Yamada et al., 2010). Wasting symptoms first present as small lesions in the dermis (Hewson et al., 2020a), and therefore may affect or interact with changes in stiffening behaviour under varying thermal regimes. Another unique physiological behaviour of sea cucumbers is evisceration, the ability to expulse and subsequently regenerate their digestive tract. Evisceration can occur in response to disease stress (Deng et al., 2008), intense stimulus, parasite infestation or predator presence (Ding et al., 2019), or even seasonally during low-productivity months (Swan, 1961). Further emphasizing the importance of understanding this unique behaviour and others, Hewson et al. (2020a) proposed that seasonal evisceration may be linked to wasting disease because wasting has been reported during the same season when evisceration occurs (Swan, 1961). Improving the baseline knowledge of sea cucumber response to thermal stress, which may relate to wasting, is therefore essential for predicting if, when, and where wasting may occur.

In light of recent marine heat waves and reports of sea cucumber wasting, this study uses a lab simulated marine heatwave to assess whether thermal stress as an isolated factor causes wasting symptoms in giant California sea cucumbers. We also aim to evaluate how behaviours that are both unique to sea cucumbers (e.g., stiffening and evisceration) and more widespread (e.g., movement, spawning) are affected by thermal stress to advance scientific knowledge of how increasing global sea temperatures may impact sea cucumbers. We hypothesized that prolonged elevated temperature exposure will cause wasting symptoms, direct mortality and changes in behaviour due to physiological stress. Based on our hypothesis, we make three predictions: 1) we will observe wasting symptoms only in sea cucumbers in high temperatures; 2) we will observe greater mortality with higher temperatures; and 3) we will observe changes in stiffening, movement, spawning and evisceration in temperature treatments.

**Methods:**

*Study organisms*

63*Parastichopus californicus* were collected by divers Em Lim and Siobhan Gray in Scott’s Bay and the entrance to Bamfield Inlet in Barkley Sound, British Columbia (48°50'02"N, 125°08'45"W) in July 2021. All were gathered from the shallow subtidal, between {NUMBER} and {NUMBER} depth. We placed the cucumbers in 19 cm deep flow-through sea tables at the Bamfield Marine Sciences Centre, which had a constant flow of seawater from Barkley Sound. The sea cucumbers were fed with kelp, plankton cultures and bloodworms. The cucumbers remained in the lab for {NUMBER} days prior to the start of the experiment. As part of a separate study, individuals were tagged with several types of tags and monitored; the results of this study indicated that the tags did not affect the sea cucumbers’ behaviour or survival (Lim et al., unpublished data).

We measured sea cucumber size as some studies have shown that body size can affect the thermal tolerance of marine organisms (Di Santo & Lobel, 2017; Kelley et al., 2011). We weighed sea cucumbers twice, 24 hours apart, to get an average wet weight. We conducted a pilot study before the start of our experiment to determine how many cucumbers had their internal organs. Prior to the start of the experiment, we isolated cucumbers into individual containers for 24 hours to determine if they were defecating.

*Experimental Design*

Our experiment exposed sea cucumbers to 12ºC, 17ºC, and 22ºC temperature treatments. The 12ºC control treatment represents the average seawater temperature 50 meters below surface in Barkley Sound, while 17ºC mimics a high subtidal temperature and 22ºC represents an extreme heat event that is unlikely to occur under natural circumstances (Chen et al., 2021b; Pawlowicz, 2017; Xuereb et al., 2018).

We separated the sea cucumbers into three temperature treatments (N22ºC treatment = 18, Ncontrol & 17ºC treatments = 19, N = 56). Sea cucumbers were separated into 30 bins with 2 cucumbers in each (except 4 bins in which only housed one cucumber). Each treatment consisted of two sea tables with a total of 10 bins. Cucumbers were randomly assigned to bins, and bins were randomly assigned to sea tables. The bins sat in 5cm deep water baths that were either at the temperature of the sea tables’ flow through system (12ºC), standing at ambient (room) temperature (17ºC), or heated with two 800W aquarium heaters to 24ºC, to keep the bins at 22ºC. The temperature treatments lasted from 09:00 on November 09 (Day 1) to 01:00 on November 13 (Day 5) for a total of X hours of heat exposure, and bucket temperature was ramped up over 24h on Day 1 and decreased over 8h on Day 5 to limit temperature shock. In the 22ºC treatment, the water bath was circulated around the sea table with powerhead aquarium pumps to ensure even temperature distribution. We monitored temperature in every bin at least twice daily. We used a water-permeable divider to separate cucumbers within bins to allow for individual identification throughout the experiment. We did not provide sea cucumbers with food during the experiment.  Water changes were done as need to keep nitrate and ammonium levels below 0.5. Fresh sea water was heated to the appropriate treatment temperature prior to water changes. We exposed sea cucumbers to treatments for 82 hours and monitored them for 7 days afterwards for mortality and wasting symptoms.

*Measuring Sea Cucumber Stress & Wasting*

Sea cucumbers stiffen as a defense mechanism and for structural maintenance (Motokawa & Tsuchi, 2003). We determined these two metrics of stiffness using two different ordinal scales. First, we gently palpated the cucumber for 10 seconds to mimic an attempted predation event. We assigned the cucumber a score of 0 if it failed to stiffen and felt like the texture of fat, a score of 1 if it stiffened to the level of un-flexed muscle and a score of 2 if the cucumber got comparably stiff to a flexed bicep muscle. Secondly, we removed each cucumber from their tank and placed them on a 5 cm x 5 cm elevated platform to measure their ability to maintain their structure over 5 seconds. We assigned the cucumber a score of 0 if it failed to stiffen at all, a score of 1 if it failed to remain stiff when placed on the platform, a and a score of 2 if it maintained its structure for the entire 5 seconds (Figure 1). Each stiffness test was performed daily.

To measure cucumber activity, we recorded movements each minute over a 15-minute period for every morning of the experiment. We used a 5 by 5 cm grid on the bottom of the containers as a reference to standardize movement classifications (Figure 2). We considered cucumbers to have moved if their head travelled from one junction of the grid to another.

We checked sea cucumber tanks every 12 hours for evisceration, an act where they partially or fully expulse their inner organs. We also determined if spawning had occurred, since heat-stress induced spawning has been reported in other sea cucumber species (Battaglene et al., 2002). Spawning was only able to be measured per-bucket instead of per-cucumber because the gametes, especially sperm, would flow from one side of the bucket to the other and we were unable to determine which of the cucumbers had spawned.

We assessed cucumbers for ulcers on the 4th, 5th, 6th, and 12th days of the experiment. We classified ulcers as either minor or major ulcers based on their size and visual appearance (Figure 3). We considered minor ulcers to be small lesions on the ends of spines, which did not have severe discoloration, where the dermis was not fully removed. Scars and closed ulcers were not counted. We considered major ulcers to be open wounds that were fully through the dermis, where white tissue could be seen beneath. We then determined the maximum number of minor and major ulcers per cucumber across the experiment’s duration.

*Statistical Methods*

To assess which variables affected the maximum number of minor ulcers, we used a generalized additive model. We determined that the number of minor ulcers best fit a geometric distribution. We modelled the maximum number of minor ulcers as a function of treatment, weight, evisceration, and defecation status. We included sea table and bucket as random effects.

To examine how sea cucumber mortality varied across treatments, we tested the data for parametric assumptions and then used a Kruskal-Wallis (K-W) test followed by a Dunn’s Kruskal- Wallis (Dunn’s K-W) test for multiple comparisons. We then built a logistic regression model with cucumber mortality as the response variable and evisceration, defecation status, initial droop and squeeze scores, and initial weight as explanatory variables. The objective of this analysis was to understand what factors, other than temperature treatment, affected mortality. We used forward GAIC model selection to investigate which combination of these variables best explained the variation in sea cucumber mortality.

We determined if the two sea cucumber stiffness metrics (antipredator response and structure maintenance) were correlated using spearman’s rank correlation coefficient. We assumed that these metrics represent two different types of stiffening behaviour, regardless of correlation strength, and modelled them separately. For both response variables, we constructed full ordinal regression models with the following predictor variables: temperature treatment, date as a categorical variable for each individual date, and the interaction term between treatment and date. We restricted our measurement period to the days preceding (Day 1), during (Day 2-4) and immediately after the heat treatment (Day 5). We included individual cucumber identity as a random effect to account for repeated measures on the same individuals over time. We also included bucket ID and sea table ID as random effects to account for our paired (two cucumbers per bucket) and blocked (five buckets per sea table) experimental design. We used AIC model selection to determine the most parsimonious models (ΔAIC > 2). From top selected models we calculated 95% confidence intervals and considered effects to be significant if confidence intervals did not overlap zero.

We compared activity scores between treatments on specific days of interest with K-W and Dunn’s K-W tests. We looked at Day 1 (pre-experiment), Day 2 initial heat shock and Day 12 (long-term recovery). We then fit a generalized additive model to activity score, which we identified as having a zero-adjusted negative binomial distribution. We included temperature treatment, date (measured as categorical) and the interaction between treatment and date as fixed effects in the full model. We included individual, sea table and bucket ID as random effects. We used backward AIC step-selection to find the variables that best explained the variation in activity scores.

We used a Kruskal-Wallis rank sum test to compare the number of buckets with spawning cucumbers across temperature treatments. To examine the effect of temperature on the likelihood of a cucumber eviscerating, we created a logistic regression model with treatment, weight, and defecation status as explanatory variables in the full model, and sea table as a random effect. We conducted forward AIC model selection to determine appropriate explanatory variables.

All statistical analyses were conducted in R version 4.0.3. Dunn’s K-W tests were done using the *dunnTest* function in package FSA (Derek et al., 2021). Models for mortality effects, activity score, evisceration, and minor ulcers were conducted using the *gamlss* function, and model selection was done using the *stepGAIC* function, both from package GAMLSS (Rigby et al., 2021). We determined the most appropriate distribution for activity scores and the number of minor ulcers using the *fitDist* function in package GAMLSS (Rigby et al., 2021). Ordinal regression models were conducted using the *clmm* function in package ordinal (Christensen, 2019); model selection for these models was conducted using the *dredge* function in package MuMIN (Barton & Barton, 2020). We considered results to be significant at P < 0.05 or if 95 % confidence intervals did not overlap zero.

**Results**

The temperature treatments for the experimental heatwaves varied slightly from the target temperatures (Figure 4). The mean temperature of the 12°C treatment was 12.5°C, but varied from 11.2-14.0°C; the mean of the 17°C treatment was 16.8°C but ranged from 14.9°C to 17.9°C; the mean of the 22°C treatment was 21.9°C but varied from 20.0°C to 23.3°C.

Five mortalities occurred in the 22°C treatment on day 3 (*N*=2), day 4 (*N*=1), day 6 (*N*=1) and day 7 (*N*=1) of the experiment (Figure 4). These mortalities all occurred in different buckets and in no cases did both sea cucumbers in a shared bucket die. Mortality was significantly different across temperature treatments (K-W χ2 = 11.383, df = 2, p = 0.00337). There was no significant difference between the 12°C and 17°C treatments (Dunn’s K-W Z = 0, p = 1), but the 22°C treatment had significantly higher mortality than the 12°C (Dunn’s K-W Z = 2.935, adj. p = 0.00334) and 17°C (Dunn’s K-W Z = -2.935, adj. p = 0.00334) treatments. Based on forward AIC selection, our null model was the best fit, indicating that evisceration, defecation status, initial droop and squeeze score, and weight did not significantly predict mortality in the 22°C treatment.

Over the course of the experiment, skin ulcers occurred in all three treatments. Minor ulcers were observed on 17 cucumbers in the 12ºC treatment, 15 cucumbers in the 17ºC treatment, and 10 individuals in the 22ºC treatment (Figure S1). Major ulcers were observed in only the heat treatment: on the final day of the treatment, two cucumbers had 1 and 4 ulcers respectively. Neither of these individuals died, and after the heat treatment stopped their major ulcers appeared to heal and were re-classified as minor ulcers on day 12 of the experiment. The maximum number of minor ulcers per individual was not significantly explained by treatment, weight, evisceration, or defecation status, because all explanatory variables were excluded from the final backward-selected model (Table S1).

Sea cucumber stiffness, as measured by antipredator response and structure maintenance, was lower in the 22ºC and 17ºC treatments compared to the 12ºC treatment (Figures 5, 6). Antipredator response and structure maintenance were significantly positively correlated with each other (rs = 0.53, p < 0.001; Figure S2). Backward-selected models indicated that temperature treatment and day affected both stiffness metrics (Table S2, S3). There was a significantly lower likelihood of high antipredator stiffness values for the 17°C (p = 4.80 x 10-5) and 22°C (p = 5.88 x 10­-9) treatments relative to the control. Sea cucumber antipredator stiffness scores were significantly likely to be lower on day 2 (p = 1.16 x 10­-6), day 3 (p = 1.33 x 10­-7), day 4 (p = 2.06 x 10­-6) and day 5 (p = 5.05 x 10­-8) than on day 1 (Table S2). For structural stiffness, cucumbers were significantly less likely to have high stiffness scores relative to the control treatment in the 17°C (p = 1.99 x 10-7) and 22°C (p = 2.44 x 10­-11) treatments. Structural stiffness values were significantly likely to be lower than day 1 on day 3 (p = 1.37 x 10­-5), day 4 (p = 2.50 x 10­-5) and day 5 (p = 8.66 x 10­-5), but not on day 2 (p = 0.0627; Table S3). In both antipredator and structural stiffness metrics, there were no significant differences in the likelihood of low stiffness values between 17°C and 22°C treatments, as indicated by the high degree of overlap in 95% confidence intervals (Figure S3).

Minor differences in activity scores were observed between treatments over the duration of the experiment, and overall, they decreased compared to day 1 (Figure 7). Seven days after the heat treatment ended (day 12), activity scores appeared comparable to those observed on day 1. Notably, on day 2 (the first day of the heat treatment), activity scores in the 22ºC treatment were much lower (median = 0) than the 12°C (median = 2.5) or 17°C (median = 3) treatments. Differences between treatments were not statistically significant on day 1 (K-W χ2 = 2.28, df = 2, p = 0.319) or day 12 (K-W χ2 = 1.81, df = 2, p = 16). On day 2 the difference was significant (K-W χ2 = 6.21, df = 2, p = 0.0448), but not between treatment pairwise comparisons of 12°C and 17°C (Dunn’s K-W, Z = 0.540, p = 0.589), 12°C and 22°C (Dunn’s K-W Z = 2.39, p = 0.0506) or 17°C and 22°C (Dunn’s K-W Z = -1.87, p = 0.124). Model results indicated that treatment did not have a significant overall effect on activity scores, but elapsed treatment days did (Table S4). Activity scores on day 3 (p = 0.0105), 4 (p = 0.00274) and 5 (p = 0.00991) were all significantly lower than on day 1. Day 2 was not significantly lower (p = 0.797).

Over the course of the experiment, evidence of stress spawning (eggs or sperm) occurred in 11 buckets. Nine of these buckets were in the 17ºC and 22ºC treatments. There was no significant difference in per-bucket spawning between temperature treatments (K-W χ2 = 1.94, df = 2, p = 0.379). We also observed *N* = 13 cucumbers eviscerate their digestive organs in the 12 ºC (n = 3), 17ºC (n = 5) and 22ºC (n=5) treatments. Logistic regression results indicated that treatment temperature does not explain a significant amount of the variance in evisceration data because it was excluded from our forward selected model (Table S5). However, weight (p = 0.0383) and defecation status (p = 0.0163) were included in the top model as significant predictors of evisceration (Table S5). Two cucumbers in the heat treatment eviscerated their respiratory trees in addition to their internal organs (Figure 8). One cucumber died 12 hours after respiratory evisceration, the other was observed to have died 68 hours after. The latter cucumber was severely impacted by eviscerating its respiratory tree; stiffness values and activity scores all dropped to zero for the days it remained alive. Despite the traumatic evisceration, it continued to move extremely slowly and wave its mouth parts until it died.

**Discussion**

*Broad overview*

The objective of our study was to determine if heat stress can induce wasting symptoms in giant California sea cucumbers (*Parastichopus californicus*). Our hypothesis that heat stressing caused by exposure to elevated temperatures *P. californicus* would trigger wasting symptoms, mortality, and changes in behaviour was partially supported. Unexpectedly, we observed minor skin ulcers at all treatments and major ulcers in the heat treatment(cite a figure), but neither were characteristic wasting symptoms(cite source maybe of wasting symptoms or basically something saying this isn’t a symptom).We observed mortality in the high temperature treatment, indicating that sea cucumbers were indeed experiencing extreme physiological stress. We also observed decreased stiffness of cucumbers in the elevated temperature treatments. In contrast to our predictions, activity scores were not significantly different across temperature treatments and rather indicated decreased movement in cucumbers over the duration of the experiment (figure). Spawning and evisceration were also not significantly explained by temperature treatments, which was unexpected.

*Minor and major ulcers*

Neither the minor or major ulcers that we observed matched the wasting symptoms observed in *P. californicus* in Nanoose Bay, B.C., or the isolated wasting events throughout the Pacific coast (Hewson et al., 2020b). The minor ulcers we observed were small lesions on the epidermis of the spines, rather than white open lesions and fissures which grew to cover the whole aboral body surface, as seen on the wasting cucumbers in Nanoose. We also did not see any sloughing of body tissues or liquefaction, as has been anecdotally reported in previous literature (Hewson et al., 2020a; Schroeder, 2017). From the major ulcers (N = {NUMBER}) that we did observe on two *P. californicus,* they did not match the full suite of wasting symptoms, though they bore a closer resemblance in colour, texture, and location. These major ulcers had fully worn through the spine or body wall epidermis, were white and oozing, and some were located on the aboral body wall as opposed to on the ends of spines. Unlike reports of widespread mortality resulting from wasting in wild *P. californicus*,the major ulcers in both of our specimens healed within the 7-day recovery period (is there a figure that shows this). In summary, there is no evidence that the sea cucumbers in our experiment were afflicted by the fatal wasting condition that has been previously reported (Hewson et al., 2020a; Schroeder, 2017).

The ulcers that we observed may have been the product of intensive daily handling. During the experiment, specimens were handled daily and measured for their posture maintenance. This would have represented a large increase in the potential for abrasion of the epidermal tissue to occur across treatments, and we saw an even distribution of sea cucumbers presenting lesions across all treatments (Figure S1). Major ulcers may have begun as minor ulcers that had become infected in sea cucumbers that were under high levels of stress in the 22°C treatment. However, our experiment did not specifically test the effect of handling on sea cucumbers and we cannot confirm the origin of the observed ulcers.

*Mortality*

Five sea cucumbers died in our extreme heat treatment and mortality was not explained by any measured variables outside of temperature treatment. None of these mortalities occurred on the first day of the heat treatment, indicating that acute heat shock was not responsible for deaths. We did not observed mortality of both cucumbers in a bucket, meaning that bucket water quality or contagious disease presence was unlikely to have affected mortality. Our findings therefore suggest that 22°C exceeds the thermal tolerance of *P. californicus*, but 17ºC does not. These findings align with those of Ren et al. (2018) who found that 22°C but not 16°C or 18°C temperatures limited the survival , specific growth rate, and metamorphosis of larval giant California sea cucumbers. Together, these findings provide evidence that temperatures above 22°C are physiologically determinantal to both life stages of giant California sea cucumbers.

*Stiffness*

Sea cucumbers have a band of longitudinal muscle along each ambulacral zone, and rings of circular muscle throughout the length body which allow them to contract and lengthen. The dermis layer of sea cucumbers is rich in mutable collagenous tissue which allows the dermis to rapidly stiffen as a predator defense mechanism, and to maintain posture (Gao & Yang, 2015). Protein-mediated changes in the attractive forces between macromolecules in the extracellular matrix (the bulk of the cell material in mutable collagenous tissue) drives this stiffening (Takehana et al., 2014). Observed stiffening measured both the muscle contractions and dermal stiffening. Based on the lack of stiffening observed at higher temperatures, heat stress may increase the production of the de-stiffening protein softenin (Takehana et al., 2014), or denature/decreases the production of the stiffening protein tensilin (Tamori et al., 2016; Yamada et al., 2010). It may also {}. Although the 17°C treatment did not cause any mortality, the sublethal impact of this treatment on stiffening behaviours was almost to the same extent as the 22°C treatment, suggesting that the physiological mechanisms behind stiffening are disrupted by extreme and moderate temperature effects. Even less extreme temperature fluctuations could have adverse effects on sea cucumber stiffening in a broader ecological context.

*Activity*

We did not find strong evidence that sea cucumber movement behaviour (based on activity scores) was affected by temperature. Instead, variation in activity scores was most explained by experimental day for all three treatments. This indicates that other factors, such as the daily handling that all cucumbers experienced or the lack of seawater input during temperature treatment days had the strongest negative impacts on sea cucumber movement. Without having measured or controlled for these factors, we cannot infer what may have caused this strong daily reduction in sea cucumber activity throughout the experiment. We observed a non-significant decrease in activity in heat treated cucumbers on the first day of the temperature treatment that may have been indicative of thermal shock. In some aquatic invertebrates, high temperatures only have short-term effects on movement behaviour (Lagerspetz, 2003), and this may explain the pattern that we observed in the sea cucumbers. However, more work is needed to better quantify and describe sea cucumber movement in response to temperature stress.

*Spawning*

While we did not observe any significant trends in per-bucket spawning; 9 of 11 spawning events occurred in temperature treatments. This trend suggests that giant California sea cucumbers are among the sea cucumbers that release gametes in response to thermal stress (Battaglene et al., 2002; Rakaj et al., 2018; Schagerström et al., 2021). Our ability to connect spawning to individual sea cucumber traits (e.g., size, stiffness, evisceration status) was limited due to the paired bucket experimental design. To better understand spawning, we would recommend keeping sea cucumbers in isolated containers to better track and identify spawning individuals.

*Evisceration*

We did not observe a treatment-related trend in digestive tract evisceration. The lack of a trend could have occurred because sea cucumbers were all overstimulated and stressed from extensive handling during the experiment (Ding et al., 2019). However, model results indicated that both weight and defecation status had significant effects on evisceration. In particular, defecation status had a strong magnitude and directionality (defecating cucumbers were less likely to eviscerate), implying a strong biological importance (Nakagawa & Cuthill, 2007). This may provide further evidence of seasonal evisceration because evisceration was significantly more likely when sea cucumbers were not observed to be using their digestive tract (Swan, 1961). Additionally, sea cucumbers that were entering a seasonal senescence may have been more likely to eviscerate their digestive tract in response to handling-induced stress (Ding et al., 2019), as the energetic cost of eviscerating could be lower than sea cucumbers that continued to gather energy from feeding. Our findings in support of seasonal evisceration do not align with the claims of Fankboner and Cameron (1985), who provided evidence of seasonal atrophy, rather than evisceration of sea cucumber internal organs. We propose that these two explanations may not be mutually exclusive. Instead, intraspecific phenotypic or genetic variation in seasonal organ evisceration or atrophy could maximize fitness in a highly variable environment (Ellis et al., 2006; Smith & Blumstein, 2008).

Respiratory tract evisceration was an unexpected event that only occurred in two individuals in the high temperature treatment. As it was followed by mortality on both occasions, we do not believe there is an adaptive advantage to respiratory evisceration in giant California sea cucumbers. Instead, we postulate that this traumatic event was a physiological response to extreme temperature stress.

*Limitations*

There are several limitations to our study that are worth noting. First, we did not find any wasting symptoms and thus, are unable to refute or support evidence that a link may exist between seasonal evisceration and wasting disease in sea cucumbers (Hewson et al., 2020a). Second, we obtained sea cucumbers from Bamfield, B.C., a population where wasting symptoms have not been reported (Hewson et al., 2020b). While no population genetic studies have specifically examined *P. californicus* in Bamfield, we assume that they are closely related to the Tofino, B.C. subpopulation (Xuereb et al., 2018). The Tofino subpopulation receives significant genetic influx from the *P. californicus* population in the Strait of Georgia where wasting has been reported (Xuereb et al., 2018). Therefore, we do not expect that genetic differentiation could confer differential vulnerability to wasting in *P. californicus* from Bamfield compared to those in Nanoose. Third, our experiment was conducted during November 2021 while the wasting event in Nanoose Bay occurred between August and October 2021. Seasonality could explain the lack of wasting symptoms in our specimens, but sea cucumber wasting has been previously reported between September and January in other populations (Hewson et al., 2020b). Hewson et al. (2020) proposed a link between season and wasting due to *P. californicus*’ seasonal evisceration of their viscera; this is unlikely given the August 2021 event. Minor and major lesions were also not correlated with the defecation status of the cucumbers, or evisceration activity, suggesting there is no significant link. The final limitation of our study was its short duration. We only exposed sea cucumbers to heat stress for a total of 82 hours and the majority of minor and major ulcers formed in the later days of our experiment. We cannot confirm that they would not have developed into more severe, wasting-like lesions provided with a more extended duration of extreme temperature. However, during this period we observed mortality and a significant stiffness-based behavioural response, indicating that sea cucumbers were stressed to the limits of their thermal tolerance and if wasting was induced by heat stress alone we expect it would have occurred under these conditions.

*Synthesis*

White skin ulcerations like those that we observed are a well-documented disease phenomenon in sea cucumbers, described as a Skin Ulceration Disease (SKUD) or Skin Ulceration Syndrome (SUS), especially in *Apostichopus japonicus*, and *Holothuria scabra* (Delroisse et al., 2020). SUS has been of particular concern in China, where *A. japonicus* is farmed extensively and SUS is threatening the sustainability of aquaculture operations (Choo, 2008). SUS has been characterized by very high transmissibility and mortality, and by white ulcers on the oral and aboral sides of the body wall (Deng et al., 2009; Li et al., 2012; Wang et al., 2007; Zhang et al., 2018). SUS, like Sea Star Wasting Disease, has a largely unknown cause. Several experiments have determined bacterial pathogenetic origins including through injection experiments, however SUS has not been linked to a specific strain or family of bacteria (Delroisse et al., 2020; Liu et al., 2010a; Zhang et al., 2018). Viruses have also been considered as a causal agent, though there have been no injection experiments to support the tissue-sampled viruses (Deng et al., 2008; Hewson et al., 2020b; Liu et al., 2010a; Wang et al., 2007).Much of what we know about SUS comes from research on *A. japonicus*, as it is a large fishery in the North West Pacific Ocean that has experienced significant mass mortality events, especially in aquaculture environments (Choo, 2008). Both *P. californicus* and *A. japonicus* are temperate sea cucumbers that play similar ecosystem roles, but as they do not share a genus, it is difficult to fully apply knowledge about SUS to the *P. californicus* context. However, based on the photographic evidence provided by Zhang et al. (2018) and Deng et al. (2009), there are some remarkable similarities between SUS-associated ulcers and the ‘major ulcers’ seen in our *P. californicus* cucumbers. Unlike in SUS reports, we did not see any indication of swelling or discolouration of the peristomes, and we did not see an initial abundance of ulcers around the mouth or cloaca (Becker et al., 2004; Delroisse et al., 2020; Wang et al., 2007).

We observed major ulcers only in 2 of 18 sea cucumbers in the 22ºC treatment, and the lack of similarity between these ulcers and wasting symptoms further suggests that the wasting event in Nanoose was not triggered solely by thermal stress. Future research into the etiology of sea cucumber wasting should investigate the potential for a pathogenic causal agent, as this has been previously linked to similar symptoms both in sea cucumbers (Deng et al., 2008, 2009; Liu et al., 2010b) and sea stars (Hewson et al., 2014, 2018; Work et al., 2021). Infection experiments examining agents of varying size fraction (parasitic, bacterial, viral) will be an essential part of this future research. Future research on sea cucumber wasting in Nanoose should further investigate the potential connections to SUS, given the symptomatic similarities between *P. californicus* wasting and SUS in *A. japonicus* Future research should also focus on identifying abiotic factors that may act alone or in combination with pathogenic agents to trigger wasting events in *P. californicus*. In the face of widespread environmental degradation and climate change (Allan et al., 2021; Marcogliese, 2008), multifactorial stressors will likely increase the severity of wasting across the Pacific Northeast Ocean.

Determining the factors that cause and increase the severity of wasting in *P. californicus* is essential for preventing the mass mortality of these ecologically important echinoderms, as seen in sea star wasting (Hewson et al., 2014, 2018; Work et al., 2021) and SUS (Choo, 2008). Sea cucumbers, often underappreciated, provide several important ecological and economic services. As benthic detritivores, sea cucumbers break down organic matter, recycle nutrients and maintain sediment health (Purcell et al., 2016; Wheeling et al., 2007). In North America, sea cucumber harvest is a growing industry (Hannah et al., 2013; van Dam-Bates et al., 2016), that is worth 10.2 million dollars in British Columbia alone (Fisheries and Oceans Canada, 2021).

In this study we have shown that sea cucumber wasting is not

Efforts to protect, manage and harvest giant California sea cucumbers in the face of global climate change and environmental degradation will require a comprehensive understanding of sea cucumber wasting.

Overall I thought you both did a great job in stating the data and using previous literature. The flow of it is great as well. I think overall theres some language that needs to be rephrased and your results summary would be much stronger if you cited the relevant figures and tables to statements (if you have matching ones of course). Lastly towards the end I would work on a wrap up summary of your findings/hypothesis! Great job Kucumber Kids! Few more days til symposium ☺

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**Figures**

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**Figure 1**. Structural maintenance stiffness tests on *Parastichopus californicus* indicating a score of 0 (left), 1 (center) or 2 (right).

A picture containing indoor, appliance, kitchen appliance

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**Figure 2**. Plastic containers with 5 x 5cm grids used to house *P. californicus* throughout the experiment. Bins were divided by a permeable divider. Sea cucumbers were provided with oxygen using a bubbler and water changes were done as needed to keep ammonia and nitrate levels low.

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**Figure 3**. Minor (left) and major (right) ulcers on *Parastichopus californicus*. Scale bars represent ~1 cm on each image.

**Diagram

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**Figure 4**. Water temperatures over the duration of our experiment. Day 1, the start of the warming, was November 09. Each black line indicates a single bin housing 1 or 2 cucumbers. The 3 coloured lines indicate target temperatures for the heat treatments.

Chart

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**Figure 5**. *Parastichopus californicus* stiffness as measured by antipredator defense over the duration of the heat treatment (days 1-5) and on recovery days after the treatment ended (day 7 and 12). Dashed lines indicate the days during which heat was applied. Antipredator response was categorized by manually palpating the cucumber to mimic the occurrence of an attempted predation event.

Chart

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**Figure 6.** *Parastichopus californicus* stiffness as measured by structural maintenance over the duration of the heat treatment (days 1-5) and on recovery days after the treatment ended (day 7 and 12). Structural maintenance stiffness was categorized by placing a stiff cucumber on an elevated platform for 5 seconds and determining if they could maintain their posture. Dashed lines indicate the days during which heat was applied.

Chart, box and whisker chart

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**Figure 7.** Boxplot visualizing *Parastichopus californicus* activity scores across heat treatments throughout the duration of the experiment (days 1-5) and the days after the heat treatment ended (day 7 and 12). Activity scores were determined by counting the number of movements made by each individual sea cucumber over a 15-minute period between 09:00 and 11:00. Dashed lines indicate the days during which heat was applied.

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**Figure 8.** The eviscerated digestive tract (left) and respiratory tree (right) of *Parastichopus californicus* in the 22ºC treatment. Respiratory tree (A) and digestive tract (B) were both eviscerated in right panel. Sea cucumbers frequently expel their digestive tract as a stress response, but respiratory tree evisceration only occurred twice in the 22ºC treatment and was followed by mortality.

**Supplementary Information**

**Table S1**. Results from geometric regression model examining the effect of treatment and weight on sea cucumber maximum lesion counts.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Variable | Coefficient | Standard error | T | P |
| (intercept) | 0.965 | 0.157 | 6.15 | 9.35e-08 \* |

**Table S2**. Results of ordinal regression model examining the effect of treatment and date on sea cucumber antipredator defense stiffness. Asterisks indicate significant effects.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Variable | Coefficient | Standard error | T | P |
| Treatment: 17C | -3.03 | 0.748 | -4.07 | 4.80e-05 \* |
| Treatment: 22C | -4.76 | 0.818 | -5.82 | 5.88e-09 \* |
| Date: 2 | -3.09 | 0.634 | -4.86 | 1.16e-06 \* |
| Date: 3 | -3.39 | 0.643 | -5.28 | 1.33e-07 \* |
| Date: 4 | -3.00 | 0.632 | -4.75 | 2.06e-06 \* |
| Date: 5 | -3.62 | 0.664 | -5.45 | 5.05e-08 \* |

**Table S3**. Results of ordinal regression model examining the effect of treatment and date on sea cucumber structural maintenance stiffness. Asterisks indicate significance.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Variable | Coefficient | Standard error | T | P |
| Treatment: 17C | -2.99 | 0.575 | -5.20 | 1.99e-07 \* |
| Treatment: 22C | -4.05 | 0.606 | -6.68 | 2.44e-11 \* |
| Date: 2 | -0.976 | 0.524 | -1.86 | 0.0627 |
| Date: 3 | -2.30 | 0.528 | -4.35 | 1.37e-05 \* |
| Date: 4 | -2.22 | 0.527 | -4.21 | 2.50e-05 \* |
| Date: 5 | -2.12 | 0.541 | -3.93 | 8.66e-05 \* |

**Table S4**. Results from the backward-selected regression model with a zero-adjusted negative binomial distribution examining the effect of treatment and date on sea cucumber activity scores. Asterisks indicate significance.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Variable | Coefficient | Standard error | T | P |
| (intercept) | 1.78 | 0.0740 | 24.05 | < 2e-16 \* |
| Date: 2 | 0.0279 | 0.109 | 0.257 | 0.797 |
| Date: 3 | -0.289 | 0.112 | -2.58 | 0.0105 \* |
| Date: 4 | -0.331 | 0.109 | -3.03 | 0.00274 \* |
| Date: 5 | -0.328 | 0.126 | -2.60 | 0.00991 \* |

**Table S4**. Results from forward-selected logistic regression model examining the effect of evisceration as a function of defecation status and weight. Asterisks indicate significant effects.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Estimate | Std. Error | t-value | P |
| (Intercept) | 1.7126 | 1.1281 | 1.518 | 0.135 |
| Poop (yes) | -2.780 | 1.1202 | -2.482 | 0.0163 \* |
| Weight | -0.004309 | 0.002029 | -2.124 | 0.0383 \* |

Chart, box and whisker chart

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**Figure S1**. Maximum number of minor ulcers found on each individual *Parastichopus californicus* during the heat treatment, compared between the three temperature treatments.  We examined sea cucumbers for ulcers and classified them as minor or major (Figure 3) based on their apparent severity.

Chart, scatter chart

Description automatically generated

**Figure S2:** Scatter plot displaying the relationship between *Parastichopus californicus* stiffness metrics. Spearman’s rank correlation coefficient indicated a high degree of correlation between metrics (rs = 0.53, p < 0.001). Stiffness metrics were measured daily for each sea cucumber throughout the experiment.

Chart, box and whisker chart

Description automatically generated

**Figure S3**. Ordinal regression coefficient and 95% confidence intervals from two AIC selected models with stiffness as antipredator defense or structural maintenance as the response variables. Variable reference categories were Temp: 12°C and Day 1. If confidence intervals overlap 0 (dashed line) the effect is non-significant.