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

Introduction, Methods and Results (Nov 2021)

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BMSC Directed Studies

**Introduction:**

Anthropogenic climate change is increasing the intensity, duration, size, and frequency of marine heat waves and warm water anomalies with consequences across the globe (Allan et al., 2021; Frölicher et al., 2018; Oliver et al., 2021).  Marine heatwaves have damaging and potentially catastrophic effects on marine ecosystems and human economies (Di Lorenzo & Mantua, 2016). They have bottom-up trophic impacts on ecosystems, driving sudden and large-scale die-offs 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 (nicknamed “the Blob” drastically reduced the abundance of subarctic copepods, krill, and sand lace, which caused starvation-induced die-offs of species from salmon to sea birds (Cavole et al., 2016; von Biela et al., 2019). This marine heat wave significantly harmed commercial, recreational, and subsistence fisheries, and triggered the closures of open water fisheries including salmon and tuna (Cavole et al., 2016; Di Lorenzo & Mantua, 2016). It also triggered a large harmful algal bloom which resulted in closures of economically important shellfish fisheries (Cavole et al., 2016; Di Lorenzo & Mantua, 2016; Frölicher et al., 2018). The multi-year impacts of the 2014-2015 warm water anomaly and others of comparable severity have driven sudden and significant change as they can push ecosystem resilience past ecological tipping points (Harley & Paine, 2009; von Biela et al., 2019; Wernberg et al., 2015).

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At an individual level, marine heatwaves directly impact marine organisms in a number of ways. Certain species already close to their thermal tolerances are killed directly (Dong et al., 2011; Oliver et al., 2017). Thermal stress can negatively impact an organism’s fitness by modifying their behaviour and food/nutrient requirements (Kühnhold et al., 2017). Marine heat waves also exert indirect effects on marine organisms, modifying natural processes such as disease dynamics. For example, temperature has been shown to regulate the virulence of marine diseases in corals and algae (Case et al., 2011). In the NE Pacific, sea star wasting disease epidemics have decimated populations of several sea star species over the last decade. Wasting is assumed to be infectious (Work et al., 2021), with epidemics being exacerbated or triggered by environmental factors including warm temperatures (Aquino et al., 2021; Bates et al., 2009; Eisenlord et al., 2016; Harvell et al., 2019; Hewson et al., 2018, 2020). Sea star wasting disease is an ambiguous set of usually-lethal 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). Recent reports have indicated that wasting may affect more than just 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).

A recent sea cucumber wasting event occurred in Nanoose Bay, B.C., Canada, from August – October 2021 (Em Lim, *personal communication*). This event followed several severe regional heat waves (Kotyk, 2021). There is insufficient evidence to confirm that wasting-like symptoms were *not* caused by direct heat stress, as reported in farmed *Holothuria scabra* (Delroisse et al., 2020), or a heat-unrelated viral or bacterial disease as observed in farmed *Apostichopus japonicus* (Deng et al., 2008; Liu et al., 2010). However, based on our knowledge of SSWD, warm water anomalies could plausibly play an etiological role though heat stress interactions with disease dynamics, as reported in corals, algae, oysters, and sea stars (Case et al., 2011; Hewson et al., 2018; Oliver et al., 2017). Understanding whether giant California sea cucumber wasting is linked to MHWs is important for informing management efforts seeking to protect this important species.

Sea cucumbers, often underappreciated, provide several important ecological and economic services. As benthic detritivores, sea cucumbers break down organic matter and are thus important for nutrient recycling (Wheeling et al., 2007). Sea cucumbers maintain sediment health for bacteria and other detritivores through bioturbation and sediment cleaning (Purcell et al., 2016). Giant California sea cucumbers seasonally lose their internal organs in a poorly understood process that may be caused by absorption of the internal organs or expulsion by evisceration (Fankboner & Cameron, 1985; Swan, 1961). This loss of internal organs is hypothesized to be part of a seasonal senescence that could affect their behaviour and therefore confound our experiment (Brothers et al., 2015). North America is also seeing a growing sea cucumber harvest industry (Hannah et al., 2013; van Dam-Bates et al., 2016); in British Columbia alone the total industry is worth 10.2 million dollars (Fisheries and Oceans Canada, 2021). Given their importance, evaluating how marine heat waves may impact sea cucumbers is a pressing concern, especially considering recent heat stress events the NE Pacific Ocean (Harvell et al., 2019).

This study seeks to assess the direct lethal and sublethal effects of marine heat waves ongiant California sea cucumbers (*Parastichopus californicus*), and to enhance our understanding of the cause of sea cucumber wasting. Evaluating how marine heat waves affect giant California sea cucumbers is important to understand the risks facing the sea cucumber harvesting industry and to inform future research into the impacts of marine heat waves on sea cucumbers. We simulated a MHW in a controlled laboratory setting to test three hypotheses. We hypothesized that prolonged elevated temperature exposure will cause direct mortality, changes in behaviour, and increased wasting symptoms due to physiological stress. Based on our hypothesis, we make three predictions: 1) we will observe greater mortality with higher temperatures; 2) we will observe changes in movement rates and stress responses as temperature increases; and 3) we will observe wasting symptoms only in warmer 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 to account for this potential confound, 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 also 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 (Pawlowicz, 2017). Seventeen degrees Celsius mimics a high, but realistic, subtidal temperature (Xuereb et al., 2018). Twenty-two degrees 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. 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 posture maintenance (Motokawa & Tsuchi, 2003). We 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 posture 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 score of 2 if it maintained its posture for less than 2 seconds, and a score of 3 if it maintained its posture for more than 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.

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

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 lesions on the 4th, 5th, 6th, and 12th days of the experiment. We classified lesions as either minor or major lesions based on their size and visual appearance (Figure 3). We then determined the maximum number of minor and major lesions per cucumber across the experiment’s duration.

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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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Description automatically generated**Figure 3**. Minor (left) and major (right) lesions on *P. californicus*. Scale bars represent ~1 cm on each image.

*Statistical Methods*

To examine how sea cucumber mortality varied across treatments, we used a Kruskal-Wallis rank sum test followed by a Dunn 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 AIC model selection to investigate which combination of these variables best explained the variation in sea cucumber mortality.

We first examined how cucumber stiffness metrics, indicated by antipredator response and structure maintenance, were correlated using spearman’s correlation coefficient. However, we modelled these two metrics as separate responses because we assume they represent different types of stiffening behaviour. 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 used Kruskal-Wallis non-parametric analysis of variance tests followed by Dunn tests for multiple comparisons to compare the activity scores between treatments on specific days to assess pre-experiment differences across treatments (Day 1), initial heat shock (Day 2), initial recovery (Day 5) and long-term recovery (Day 12). We then fit a generalized additive model with a zero-adjusted negative binomial distribution. Again, we only used data from the days preceding, during and immediately after the heat treatment. We included temperature treatment, date (measured as categorical) and the interaction between treatment and date as fixed effects. 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 pooping 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.

To assess whether weight and treatment affected the maximum number of minor lesions, we used a generalized additive model with a geometric distribution. We included sea table and bucket as random effects.

All statistical analyses were conducted in R version 4.0.3. Kruskal-Wallis tests were conducted using the *Kruskal.test* function in base R, and Dunn tests were conducted using the *dunnTest* function in package FSA (CITE). Models for mortality effects, activity score, evisceration, and minor lesions were conducted using the *gamlss* function, and model selection was done using the *stepGAIC* function, both in package GAMLSS (CITE). Ordinal regression models were conducted using the *clmm* function in package Ordinal (CITE); model selection for these models was conducted using the *dredge* function in package MuMIN (CITE). We considered results to be significant at P < 0.05 or if 95 % confidence intervals did not overlap zero.

**Results**

The temperature in the buckets increased over 24h on day 1 and decreased over 8h on day 4. The temperature treatments lasted from 09:00 on November 09 to 01:00 on November 13 for a total of X hours of heat exposure. Temperature treatments varied slightly from the target temperatures (Figure 4). The mean temperature of the 12°C 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 16°C to 17.9°C, and the 22°C treatment had a mean temperature of 21.9°C but varied from 20.0°C to 23.3°C.

**Diagram

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**Figure 4**. Water temperatures over the duration of our experiment. Each line indicates a single bin housing 1 to 2 cucumbers. Red dots indicate a single cucumber mortality.

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Five mortalities occurred in the 22°C treatment. The mortalities occurred on day 3 (*N*=2), day 4 (*N*=1), day 6 (*N*=1) and day 7 (*N*=1) of the experiment (Figure 4). The results of a Kruskal-Wallis rank sum test indicated that the treatment-based difference was significant (Kruskal-Wallis χ2 (2) = 11.383, p = 0.00337). A Dunn’s Test indicated that cucumbers experienced significantly higher mortality in the 22ºC treatment than in the cooler treatments (Z = 2.94, p = 0.00334; Supplementary Table 1). Forward AIC selection on the logistic regression mortality model selected our null model, indicating that the variables evisceration, defecation status, initial droop and squeeze scores, and initial weight did not significantly predict mortality.

Chart

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Chart

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Sea cucumber stiffness, as measured by antipredator response and structure maintenance, varied across the duration of the experiment (Figure 5, 6). Stiffness metrics were significantly positively correlated (r = 0.53, p < 0.001; Figure S1). For both stiffness metrics, the backward-selected models with the lowest AIC values indicated that treatment and day both had significant effects on stiffness metrics (Table S2, S3). There was a significantly lower likelihood of being fully stiff for all days compared to the day preceding the experiment and for both 17°C and 22°C treatments relative to the control. However, there were no significant differences in the likelihood of stiffness between 17°C and 22°C treatments as indicated by the high degree of overlap in 95% confidence intervals.

Activity scores varied across treatments over the duration of the experiment (Figure 7). Kruskal-Wallis tests investigating differences between treatments on days 1, 2, 5 and 12 only found a significant difference on day 2 (the first day of the heat treatment; Table S4). The Dunn test conducted on day 2 did not discern any significant differences between temperature treatments (Table S5), although the difference in activity scores between the 12°C and 22°C treatment was almost significant (*Z* = 2.39, P-adjusted = 0.0506). The absence of a treatment-related effect on activity scores was further compounded by the exclusion of treatment as a predictor variable in our forward selected generalized additive model output as treatment day was the only variable retained in the top model (Table S6).

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**Figure 7.** Boxplot visualizing activity scores across heat treatments for each day of the heat treatment (denoted by the space between dashed lines), 2 days after the heat treatment, and 7 days after the heat treatment.

Evidence of stress spawning (eggs or sperm) occurred in 11 buckets over the course of the experiment. Nine of these buckets were in the 17ºC and 22ºC treatments. However, a Kruskal-Wallis rank sum test indicated that there was no significant difference between any of the temperatures (χ2 (2) = 1.94, p = 0.487).  Our logistic regression model suggested temperature treatment does not explain a significant amount of the variance in evisceration data because it was excluded from our forward selected model. However, weight (p = 0.0383) and defecation status (p = 0.0163) were included in the top model as significant predictors of evisceration (Table S7). Two cucumbers in the heat treatment eviscerated their respiratory trees in addition to their internal organs (Figure 8). One cucumber died within 12h, the other survived for more than 60 hours. The latter cucumber was severely impacted by eviscerating its respiratory tree; its stiffness rates and activity scores all dropped to zero, however it continued to move extremely slowly and wave its mouth parts.

ADD SENTENCE HERE DESCRIBING LESIONS Major lesions were observed in two individuals in the heat treatment that had 4 and 1 lesions, respectively, on the final day of heating (day 4). Neither of these individuals died and their lesions appeared to heal and were re-classified as minor lesions on day 12 of the experiment. Varying numbers of minor lesions were observed in 17, 15, and 10 individuals in the 12°C, 17°C and 22°C treatments and the maximum number of lesions per individual was not statistically significant across treatments (Figure S8). Based on generalized additive model results, the maximum number of lesions per individual was not significantly affected by weight or treatment (Table S8).

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**Figure 8.** The eviscerated digestive tract and respiratory tree of a *P. californicus* in the 22ºC treatment.

**Table** **1**. The response variables measured to quantify the effect of a marine heat wave on giant California sea cucumbers (*Apostichopus californicus*).

|  |  |  |  |
| --- | --- | --- | --- |
| **Category** | **Response Variables** | **Measurement Method** | **Variable Type** |
| **Direct lethal** | Mortality | Time to Death | Continuous |
| **Direct sublethal** | Stiffening (antipredator defense) | Repeated poke test | Ordinal (3 level) |
| Stiffening (posture maintenance) | Platform test | Ordinal (4 level) |
| Evisceration | Inner organs observed | Binary |
| Spawning | Sperm or eggs observed | Binary |
| Movement | Dispersal distance | Continuous |
| **Disease (indirect or direct)** | Wasting | Presence of wasting lesions | Binary |

**Supplementary Information**

**Table S1**. Results from Dunn test of multiple comparisons examining differences in mortality across temperature treatments. Asterisks indicate signifance based on α = 0.05

|  |  |  |
| --- | --- | --- |
| Comparison | Z-score | Adjusted p-value |
| Control – heat | -2.935 | 0.00334 \* |
| Control – room | 0.0000 | 1.000 |
| Heat – room | 2.935 | 0.00334 \* |

**Table S2**. Results from AIC model selected top ordinal regression model examining the effect of treatment and date on sea cucumber antipredator defense stiffness.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| 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 from an AIC model selected top ordinal regression model examining the effect of treatment and date on sea cucumber structural maintenance stiffness.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| 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 multiple Kruskal Wallis tests used to compare activity scores across treatments for the days 1, 2, 5, and 12 of the experiment, where the heat treatment ran on days 1-4.

|  |  |  |  |
| --- | --- | --- | --- |
| Day | Kruskal-Wallis chi-square | df | P (not adjusted) |
| 1 (pre-treatment) | 2.28 | 2 | 0.319 |
| 2 | 6.21 | 2 | 0.0448 |
| 5 (initial recovery) | 3.55 | 2 | 0.169 |
| 12 (delayed recovery) | 1.81 | 2 | 0.16 |

**Table S5**. Results from Dunn test of multiple comparisons between temperature treatments on day 2 of the experiment. P values were adjusted for multiple comparisons using the Holm method.

|  |  |  |
| --- | --- | --- |
| Comparison | Z -score | P. adjusted |
| 12C - 17C | 0.540 | 0.589 |
| 12C - 22C | 2.39 | 0.0506 |
| 17C - 22C | -1.87 | 0.124 |

**Table S6**. 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. The final model only included the date variable.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| 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 S7**. Results from forward-selected logistic regression model examining the effect of evisceration as a function of defecation status and weight. Asterisks indicate significance based on α of 0.05.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Estimate | Std. Error | t-value | P-value |
| (Intercept) | 1.7126 | 1.1281 | 1.518 | 0.135 |
| poop1 | -2.780 | 1.1202 | -2.482 | 0.0163 \* |
| weight\_g | -0.004309 | 0.002029 | -2.124 | 0.0383 \* |

**Table S8**. 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) | 0.649 |  | 1.12 | 0.269 |
| Weight | 0.0004 | 0.0009 | 0.458 | 0.649 |
| Treatment : 17°C | 0.0171 | 0.387 | 0.044 | 0.965 |
| Treatment: 22°C | -0.331 | 0.411 | 0.319 | 0.751 |

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**Figure S1:** Scatter plot displaying the relationship between sea cucumber stiffness metrics. Spearman’s rank correlation coefficient indicated a high degree of correlation between metrics (r = 0.53, p < 0.001)

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**Figure S2**. 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 Date: Nov 09 and Temp: 12°C.

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**Figure S3**. Maximum number of minor lesions found on each individual during the heat treatment, compared between the three temperature treatments.

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