THERMAL TOLERANCE OF *SIPHONARIA NORMALIS* EMBRYOS ON O‘AHU, HAWAI‘I

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By
Michaela Johnson

Thesis Advisor

Amy Moran

I certify that I have read this thesis and that, in my opinion, it is satisfactory in scope and quality as a thesis for the degree of Bachelor of Science in Global Environmental Science.

THESIS ADVISOR

Amy L. Moran
School of Life Sciences

For my family and friends who have supported me throughout my entire journey.

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ABSTRACT

As average global temperatures continue to rise leading to hotter conditions, there is a need to understand the impact of heat stress on the vulnerable life stages of marine organisms. Thermal tolerance studies assessing the impact of elevated temperatures on ecologically important intertidal organisms are necessary to predict future species distributions. This study focuses on an abundant but rarely studied intertidal species on the island of O‘ahu, the false limpet *Siphonaria normalis*, by examining the thermal tolerance of the embryos. *S. normalis* embryos develop intertidally in benthic egg masses, in the same environment as the adults. Embryos are often more vulnerable to thermal stress than adults. Egg masses that were 1-2 days old were exposed to a range of elevated temperatures between 35 °C and 50 °C in an incubator for 2 hours. These ranges of temperature and time of exposure have been observed in field settings. Treated egg masses were subsequently maintained at a constant temperature of 24 °C until embryos matured to an encapsulated crawling stage, in which they metamorphose into a juvenile. Video data, taken every 2-3 days to follow the development of the mass, were evaluated for percent survival and deformities in growth. Results showed that embryonic survival was significantly lower following the treatment of 45 °C and 50 °C than of lower temperatures. We observed that after thermal treatments, most embryos continued to develop for some time before dying. We therefore explored the relationship between temperature and the stage that embryos developed into before they died. These data suggest that the survivorship of *Siphonaria normalis* embryos on the island of O‘ahu experience higher instances of mortality at elevated temperatures. This study has broader implications for the survivorship of *S. normalis* in a changing climate.

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1.0 INTRODUCTION

1.1 Thermal Stress on Marine Organisms

As global climate change progresses, the frequency and intensity of high temperature events is predicted to increase as well (IPCC, 2014; Weisheimer & Palmer, 2005; Diffenbaugh et al., 2017). Thermal stress has many consequences for performance of intertidal species, including increased mortality, and altered energetic demand, all of which may impact the distribution and abundance of species (Stillman, 2002; Tsuchiya, 1983; Peck & Buckley, 2007; Tomanek & Helmuth, 2002; Harley, 2008). Some species and populations have differing capacities for temperature acclimation compared to others (Miller et al., 2013; Kuo & Sanford, 2009; Sorte et al., 2011). The effects of temperature change across the life cycle in many organisms; early life stages can be particularly vulnerable to both high and low temperatures (Crisp & Ritz, 1967; Miller et al., 2013; Wang et al., 2017). Environmental stressors that harm embryos potentially impact the distribution of the adult population (Andronikov, 1975; Wang et al., 2017; Gosselin & Chia, 1995). In order to accurately predict how marine species distributions may be altered by global warming, it is necessary to examine the thermal tolerances of a variety of species in their early life stages.

1.2 Stress Within the Intertidal Zone

The rocky intertidal zone is an historically important model system for measuring the ecological consequences of environmental stressors. The intertidal ecosystem is

readily accessible, and a majority of the species are slow-moving and abundant (Connell, 1972). In addition, the intertidal zone is an environment of intense stress and rapid thermal change (Denny & Wethey, 2001; Harley, 2008). A multitude of environmental conditions impact the vertical distribution of a species in the rocky intertidal, with lower limits being defined by biotic factors such as interspecific interactions, and upper limits by physical stressors such as thermal stress, wave force, and desiccation (Connell, 1961a; Connell, 1961b; Paine, 1974). Climate-change-induced shifts within the intertidal zone can occur rapidly and include changes to species abundance, distribution, and latitudinal range (Helmuth et al., 2006). High intertidal species are often already living in conditions fairly close to their thermal limits, thus making them potentially the most vulnerable to global warming (Stillman & Somero, 2000).

According to a zonation model by Bird et al. (2013), the Hawaiian intertidal habitat can be defined as a shoreline with three distinct, tidally driven zones: (1) the emergent tidal zone, in which wave patterns dictate periodic aerial emersion, (2) the wave zone, which undergoes constant wave action regardless of tide height, and (3) the submergent tidal zone, in which tides drive submersion. The emergent tidal zone has the most aerial exposure, and potentially has the greatest amplitude of temperature ranges on the substrata due to tide-driven exposure to multiple thermal sources such as the water, atmosphere, and solar irradiation (Bird et al., 2013). Global climate change impacts on the highly variable thermal conditions that exist on Hawai‘i’s rocky shores may have a future negative impact on the ecological balance of the Hawaiian intertidal zone. A change in the thermal extremes has the potential to directly affect intertidal organisms, many of which are ecologically important marine gastropods.

1.3 Gastropods: Ecological Role and Adaptations to Stress

Gastropods, the most vast and diverse class within Mollusca, inhabit diverse ecosystems around the world, including the intertidal zones (Brown & Lydeard, 2010). These include limpets, snails with a conical shell that suction onto the rocky shore. Limpet is a common name containing several very distantly related taxa that have convergently evolved similar morphology from different ancestral origins (Vermeij, 2017). While many species of snail have the conical shell and large foot that characterizes the term "limpet", diversity is seen in the respiratory organs across groups (Branch et al., 1985; Lindberg & Ponder, 2001). A majority of limpets possess gill structures to allow underwater respiration ("true limpets"). Another group, the pulmonate limpets, a group of snails that is only capable of breathing air but may still inhabit the intertidal, are referred to as "false limpets" (Branch et al, 1985).

Many species of gastropods reside in the intertidal and occupy ecologically important roles as grazers. This mode of feeding is their most important role in the ecosystem, contributing to the overall algal distribution on the rocky shore and thus altering the assortment of organisms within the marine community (Branch et al, 1985). Herbivorous gastropods have been found to feed mostly on very small algae, such as sporelings and diatoms, as well as larger macrophytes and crustose algae (Steneck & Watling, 1982). Experimental removal of grazers causes ecological changes, in which algae overgrowth occurs due to the lack of herbivore control (Lodge, 1948; Underwood, 1980).

Due to their ecological importance, grazer responses to environmental stressors in

the intertidal have been widely examined. Studies comparing several marine gastropods that occupy different regions of the intertidal zone (low versus high) show that upper intertidal gastropods have higher thermal tolerances than lower-level gastropods, even within their own genus (Tomanek, 2002). Organisms that inhabit the upper intertidal are particularly subject to harsh environmental conditions during their aerial emersion, as they are exposed to longer periods of ultraviolet radiation, heat, and desiccation stress (Bjelde & Todgham, 2013; Stillman & Somero, 2000; Przeslawski, 2004). Intertidal gastropods protect themselves, to an extent, from thermal stress with a range of mechanisms. Behavioral responses to heat stress by true limpets within the intertidal zone include seasonal vertical migration, lifting of the shell to expel heat, scar formation around the shell to reduce water loss, and aggregating to reduce collective evaporation (Lewis, 1954; Branch et al., 1985; Lowell, 1984; Garrity, 1984). In addition, adult gastropods have some adaptations to maximize heat loss and reduce overheating, such as ribbed shells to increase the surface area interaction with air (Branch et al., 1985), taller shell shapes, and an increased gap within the extravisceral cavity to retain more water (Vermeij, 1973). Many gastropods lay egg masses that develop intertidally, rather than free-spawning or producing pelagic egg capsules (Przeslawski, 2004). These intertidal gastropod egg masses are often more vulnerable to environmental stressors, including temperature, than adults; temperature impacts embryonic development and mortality rates of multiple species (Przeslawski, 2004).

1.4 *Siphonaria*: The False Limpet

The focus of this study is the thermal tolerance of encapsulated embryos of one

species of false limpet, a pulmonate snail within the genus *Siphonaria* (Sowerby I, 1823). Species in the genus *Siphonaria* are widespread across the globe but are particularly common throughout the Indo-Pacific. The diets of *Siphonaria* limpets include several varieties of algae, such as foliose macroalgae, lichens, cyanobacteria, diatoms, and microalgae (Allanson, 1958; Jara & Moreno, 1984; Simpson, 1976; Underwood & Jernakoff, 1981; Santelices & Correa, 1985; Quinn, 1988; Davenport, 1997). As grazers of diverse phyla, *Siphonaria* may have an impact on the algae assemblages in the intertidal zone.

The species *Siphonaria normalis* (Gould, 1846) is an abundant grazer in the Hawaiian intertidal zone that deposits gelatinous egg ribbons of direct-developing embryos to the substratum in adult habitat (Butler et al., in prep.). Some species of adult *Siphonaria* lift their shells to promote evaporative cooling or moving to vertical rocks in order to escape thermal stress (Garrity, 1984). According to Chambers & McQuaid (1984), among *Siphonaria* species with direct development, egg masses typically develop in the upper regions of the intertidal zone, leaving them vulnerable to desiccation stress. While embryos in benthic egg masses develop in the intertidal and experience similar environmental conditions as adults, embryos do not have the same ability to choose or alter their position in the intertidal zone to mitigate heat stress.

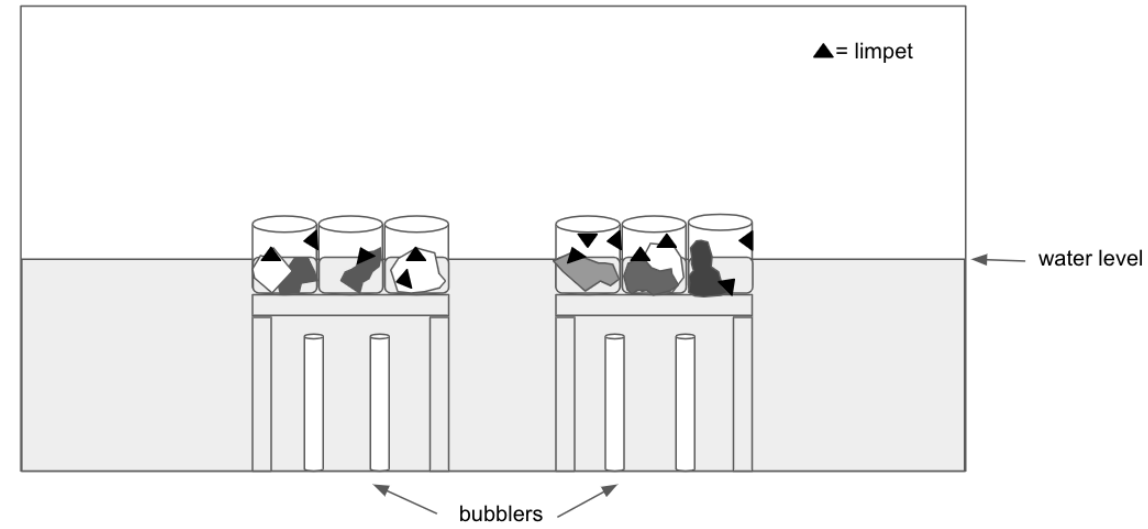
Siphonaria have two main modes of egg mass production: gelatinous egg capsules which are cemented to a rocky substratum, or pelagic egg ribbons released into the water column (Hodgson, 2002). A majority of *Siphonaria* species lay benthic egg masses that emerge as planktotrophic, free-swimming larvae, while others deposit eggs that carry out direct development before exiting the egg mass as juveniles (Hodgson,

2002; Chambers & McQuaid, 1994). Most studies examining the early life stages of *Siphonaria* have thus far focused on the pelagic veliger form; however, few thermal tolerance studies exist for either reproductive strategy, but especially for the direct-developing embryos (Wang et al., 2017; Kessel & Phillips, 2018). Wang et al. (2017) found that encapsulated *S. japonica* embryos experienced lower hatching success following high temperature exposures, while post-hatching planktotrophic larvae exhibited a much higher thermal tolerance than embryos. *S. australis* showed abnormal embryonic development within the egg mass and reduced hatching size of pelagic larvae in response to thermal stress (Kessel & Phillips, 2018). The tropical species of *Siphonaria* located on O‘ahu, *Siphonaria normalis*, lays benthic egg masses that hatch as direct-developing juveniles (Moran, personal communication, 2020). Direct-developing *Siphonaria* are likely to undergo extended periods of thermal stress in their early life compared to a free-swimming larval form (Chambers & McQuaid, 1994). The purpose of these experiments is to assess how thermal stress within early development of *Siphonaria* embryos impacts survivorship and developmental success into the hatching stage. During experimentation, even after exposure to a lethal stress, I observed that many embryos continued developing for some time before they eventually died. This raised the question of whether the magnitude of thermal stress that an embryo is exposed to during early development has an impact on its ability to continue some developmental processes. I decided to explore the stage that was reached before death of these dead *Siphonaria* embryos following various temperature exposures. This is an area within thermal stress studies that may contribute to further understanding of the mechanisms in early development that respond to higher temperature exposures.

2.0 METHODS

2.1 Limpet Collection and Maintenance

Siphonaria normalis were collected from the rock retaining wall at Koko Marina Center, O‘ahu, Hawai‘i (21°16’40.4”N, 157°42’21.2”W). Adult limpets were collected from the site periodically between July 2020 to March 2021 and kept in a closed recirculating aquarium, in batches of approximately 30-50 at a time. Adult rearing and egg mass culture methods were based on lab protocols and equipment from the “limpet farm” of the Moran and Marko labs at the University of Hawai‘i at Mānoa. In brief, limpets were divided among five cages which were covered with a mesh lid to prevent escape but allow oxygenation. Animals were elevated above the water line on rocks collected from the site. A bubbler was used to gently foam water underneath the cages and keep the animals moist. Cages were inspected daily for egg masses by examining and overturning every rock within each cage.



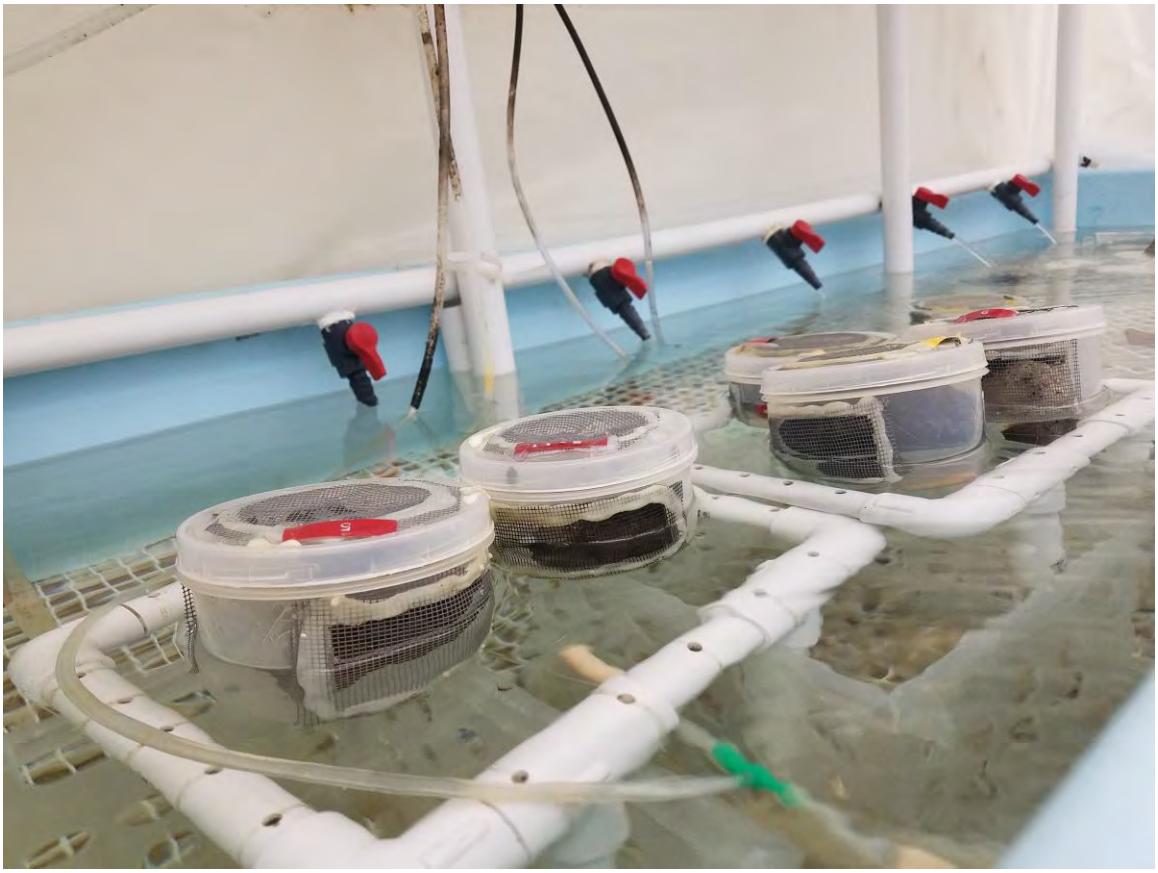


Figure 1. A diagram and photo of the limpet farm set up, showing aerial emersion of limpets within their cages.

2.2 Thermal Treatments of Egg Masses

Egg masses were used for temperature experiments within 1-2 days of deposition while embryos were in the uncleaved zygote stage. Egg masses were gently scraped off of the rock using plastic tweezers and placed inside 12-well non-treated cell culture trays. A thin layer of filtered sea water was pipetted into each well to avoid desiccation, providing enough moisture to coat the eggs but not fully submerge the mass. Promptly following egg mass placement into culture trays, masses were labelled and video recorded using a Jenoptik Gryphax® Subra camera attachment on a dissecting microscope. When imaging egg masses, multiple angles were imaged in order to properly observe any individual embryos that were optically distorted by the curvature of the mass. Video recordings were used in place of individual photographs so that the fine focus could be adjusted throughout and record as many embryos in focus as possible.

After initial video recording, 12-well trays containing egg masses were immediately placed in a VWR® Forced Air General Incubator 414005-120 for a duration of 2 hours. Eggs were kept in the incubator at 24 °C (n=5), 35 °C (n=2), 40 °C (n=2), 45 °C (n=6), or 50 °C (n=6). Pilot experiments had shown close to 100% survival at 24 °C. Temperatures were based off of data loggers at two intertidal sites on O‘ahu, outplanted in *Siphonaria* habitat. Data loggers showed fluctuations between lower temperatures (low 20s) and a maximum temperature of approximately 50 °C (Shishido et al., in prep.). According to Shishido et al. (in prep.), daily maximum temperatures were often maintained in the field for approximately 2 hours. During temperature treatments, trays were taped shut tightly in order to prevent evaporation within each well. After 2 hours, eggs were removed from the dry oven and video recorded a second time from multiple

angles to capture any immediate changes. Masses from all treatments were then kept at 24 °C for the duration of the experiment and monitored by capturing video recordings every 2-4 days. To prevent salt buildup within the culture tray wells, egg masses were rinsed with deionized water and replaced with a new coating of filtered sea water at least every 2 days. Measurements were ended either when embryos reached the encapsulated crawling stage or when all embryos in a given mass were dead.

2.3 Embryo Mortality

For each egg mass, an initial count of embryos was made from the video recording taken on Day 1. This was to ensure that any late-stage embryos that were not visible in the final imaging because of being blocked by other embryos were counted as well. Day 1 imaging was used as a reliable measurement of the total number of individuals in each egg mass because Day 1 embryos were smaller than late-stage embryos. The initial stage, the uncleaved zygote, had a smooth, round shape prior to cleaving. The next stage was cleavage with individual cells still visible. Development continued into a veliger, which grew cilia and a velum. A shell and eyes also began to form during the veliger stage. Lastly, the veliger developed into an encapsulated crawling stage, with a shell, eyes, and foot (Figure 2). Individual embryos that I could not confidently stage were classified as undetermined. Survivorship of embryos within each egg mass was determined by the ratio of total number of embryos that were able to successfully reach the encapsulated crawling stage, to the initial number of embryos counted on Day 1.

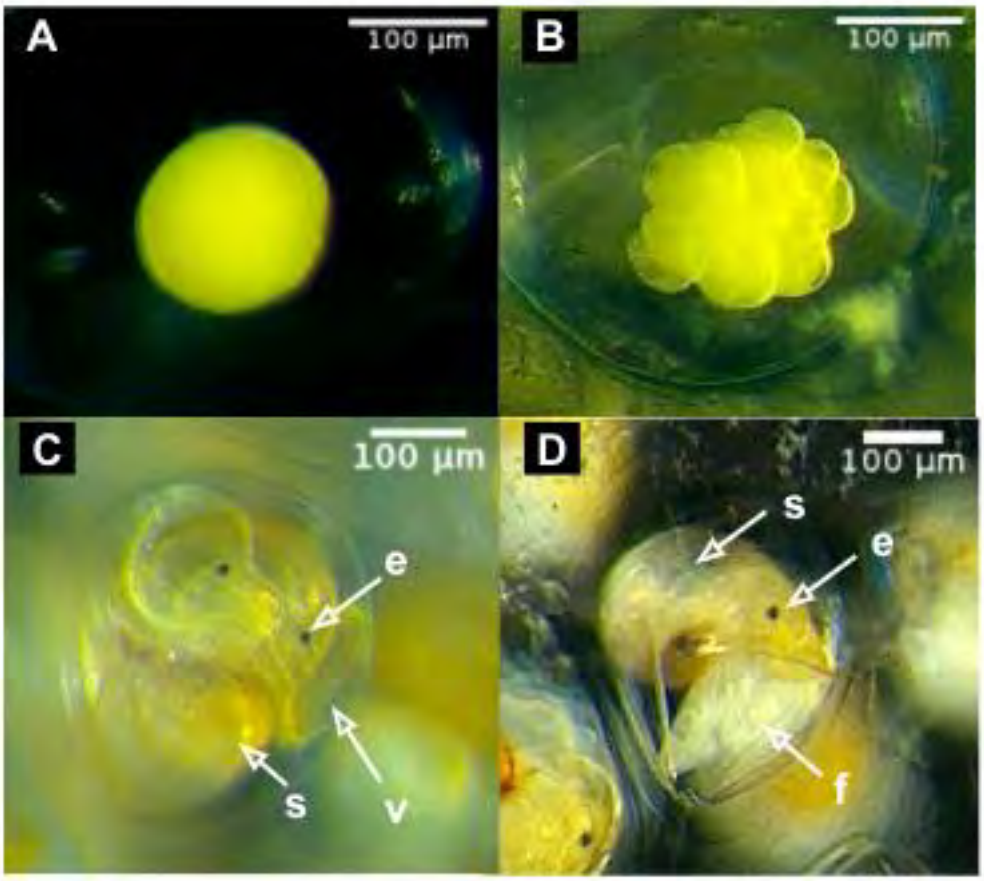


Figure 2. A guide depicting how *Siphonaria* early life stages are classified for the purpose of this study: A) day 1 egg, B) egg undergoing cleavage, C) early-stage veliger, showing a velum (v) and occasionally the early signs of a shell (s) and eyes (e), D) encapsulated crawling stage, showing a shell (s), eye (e), and foot (f) formation.

Unsuccessful development was characterized by any of the following criteria: failure to develop eyes, failure to develop a shell, deformed cleavage, death before complete development, and discoloration. Deformed growth was visible in cleaving cells when the individual blastomeres began to physically separate from each other, or when cells show blebbing. Blebbing was defined as when the membrane of a cell begins to bulge and separate from the cell, creating “blebs”. Normal colors throughout

development were yellows, greens, and browns. Discoloration was apparent when embryos turned off-white (Figure 3).

During temperature experiments, I observed that some embryos were continuing development for some time before dying. To determine whether embryos exposed to higher temperatures died at an earlier stage, the stage reached before death (SRBD) of each dead embryo within an egg mass was recorded.

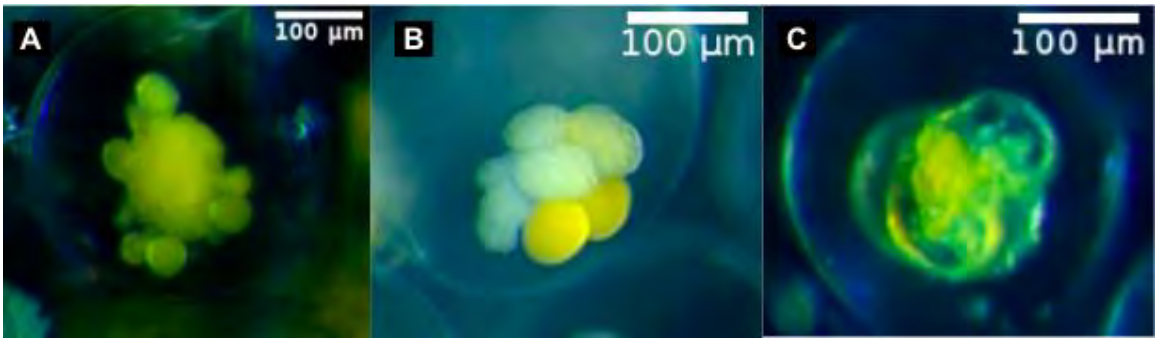


Figure 3. Criteria for unsuccessful development: A) separating cells in the cleavage stage, blebbing B) discoloration in the cleavage stage; color changes from green-yellow to an off-white, C) abnormal development in the early veliger stage; blebbing.

2.4 Data Analysis

As data did not follow normal distributions, nonparametric Kruskal-Wallis tests were used to assess the effects of different temperature treatments on embryo survival. Dunn's tests were used following Kruskal-Wallis tests to determine which temperature groups were significantly different. Both tests were carried out in Microsoft Excel 365 Real Statistics Resource Pack ((Release 7.6), Copyright (2013 – 2021) Charles Zaiontz). To determine whether survival was different among experimental temperatures, the proportion of survivors to the total number of embryos within each egg mass was

analyzed with a Kruskal-Wallis and Dunn's test. Undetermined embryos, which are embryos that could not confidently be categorized in final imaging, were counted and their distribution in different treatments was assessed to ensure they were not biasing the results. In order to decide whether undetermined embryos could be left out of analyses without misinterpreting the results, the proportion of undetermined embryos to total embryos by each egg mass was compared among temperatures using a Kruskal-Wallis and Dunn's test. Egg masses that did not contain any dead embryos were not included in SRBD analyses. The proportion of SRBD to the total dead embryos within each egg mass was calculated and analyzed with a Kruskal-Wallis and Dunn's test.

3.0 RESULTS

3.1 Mortality After Temperature Treatments

The number of individual eggs within each mass ranged from 25 to 132 ($\bar{x}$=74, SD=28, n=21), and development of the encapsulated crawling stage took between 14-20 days ($\bar{x}$=18, SD=2.6, n=10) (Table 1). The proportion of undetermined embryos to the total number of embryos (Table 2, $\bar{x}$=0.0347, SD=0.0347, n=21) was not significantly different between treatments (Figure 4, H=1.0640693, p=0.8960427, df=4). We therefore removed undetermined embryos from all data prior to any analyses.

Table 1. Initial count of embryos and final counts of surviving, undetermined, and dead embryos within each mass at each temperature treatment. For masses that had embryos die before the encapsulated crawling form, the stage reached before death (SRBD) is also shown.

Temperature (°C)	Collection Date	Final Image Date	Initial Survived	Final Survived	Final Undetermined	Final Dead	SRBD - Egg	SRBD - Cleavage	SRBD - Early Veliger
24	9/3/20	9/22/20	77	76	1	0	-	-	-
24	9/24/20	10/8/20	70	65	4	1	0	0	1
24	9/24/20	10/8/20	54	50	3	1	0	1	0
24	11/18/20	12/3/20	90	83	4	3	0	0	3
24	1/27/21	2/16/21	125	106	2	17	0	0	17
35	9/4/20	9/24/20	25	22	0	3	0	0	3
35	9/4/20	9/24/20	64	59	5	0	-	-	-
40	9/4/20	9/24/20	69	66	3	0	-	-	-
40	9/4/20	9/20/20	53	48	2	3	0	0	3
45	9/17/20	10/6/20	76	57	6	13	0	8	5
45	10/17/20	11/6/20	66	0	2	64	0	64	0
45	10/18/20	11/3/20	104	0	13	91	3	0	88
45	10/20/20	11/6/20	51	0	0	51	0	0	51
45	10/20/20	11/6/20	75	0	0	75	0	0	75
45	11/15/20	12/3/20	92	0	0	92	0	92	0
50	9/17/20	9/29/20	41	0	0	41	0	41	0
50	9/22/20	10/6/20	132	0	11	121	0	121	0
50	9/22/20	10/1/20	50	0	1	49	0	49	0
50	9/24/20	10/6/20	115	0	2	113	0	113	0
50	9/24/20	10/6/20	45	0	0	45	0	45	0
50	9/24/20	10/6/20	71	0	2	69	0	69	0

Table 2. The proportion of undetermined embryos to total number of initial embryos within each egg mass ($\bar{x}$=0.0347, SD=0.0347, n of egg masses=21).

Temperature (°C)	Proportion (Undetermined/Total)
24	0.0130
24	0.0571
24	0.0556
24	0.0444
24	0.0160
35	0.0000
35	0.0781
40	0.0435
40	0.0377
45	0.0789
45	0.0303
45	0.1250
45	0.0000
45	0.0000
50	0.0000
50	0.0833
50	0.0200
50	0.0174
50	0.0000
50	0.0282

Kruskal-Wallis Test

	24°C	35°C	40°C	45°C	50°C	
median	0.0444	0.0391	0.0406	0.0152	0.0187	
rank sum	63	21.5	27	62.5	57	
count	5	2	2	6	6	21
r^2/n	*793.8*	*231.125*	*364.5*	*651.0417*	*541.5*	2581.9667
H-stat						1.0640693
H-ties						1.0888151
df						4
p-value						0.8960427
alpha						0.05
sig						no

DUNN's TEST			alpha	0.05	0.005
group	*R-sum*	*size*	*R-mean*	*z-crit*	
24°C	63	5	12.6		
35°C	21.5	2	10.75		
40°C	27	2	13.5		
45°C	62.5	6	10.416667		
50°C	57	6	9.5		
		21		1.959964	

D TEST

group 1	*group 2*	*R-mean*	*std err*	*z-stat*	*R-crit*	*p-value*
24°C	35°C	1.9	5.1	0.37	10	0.71
24°C	40°C	0.9	5.1	0.18	10	0.86
24°C	45°C	2.2	3.7	0.59	7.3	0.56
24°C	50°C	3.1	3.7	0.84	7.3	0.4
35°C	40°C	2.8	6.1	0.46	12	0.65
35°C	45°C	0.33	5	0.07	9.8	0.94
35°C	50°C	1.3	5	0.26	9.8	0.79
40°C	45°C	3.1	5	0.62	9.8	0.54
40°C	50°C	4	5	0.8	9.8	0.42
45°C	50°C	0.92	3.5	0.26	6.9	0.79

Figure 4. A Kruskal-Wallis (H=1.0640693, p=0.8960427, df=4) and Dunn's test of the proportion of undetermined embryos to total dead embryos within each egg mass is shown by temperature treatment. Both the Kruskal-Wallis and Dunn's test show that there were no significant differences in undetermined embryos between any temperature groups.

A steep decline in survivorship occurred above the 40 °C treatment to low level viability at 45 °C and ultimately no survivors at the highest treatment (Figure 5). The effect of temperature on mortality is significantly different between treatments (Kruskal-Wallis, Figure 6, H=15.0727, p=0.001487, df=4).

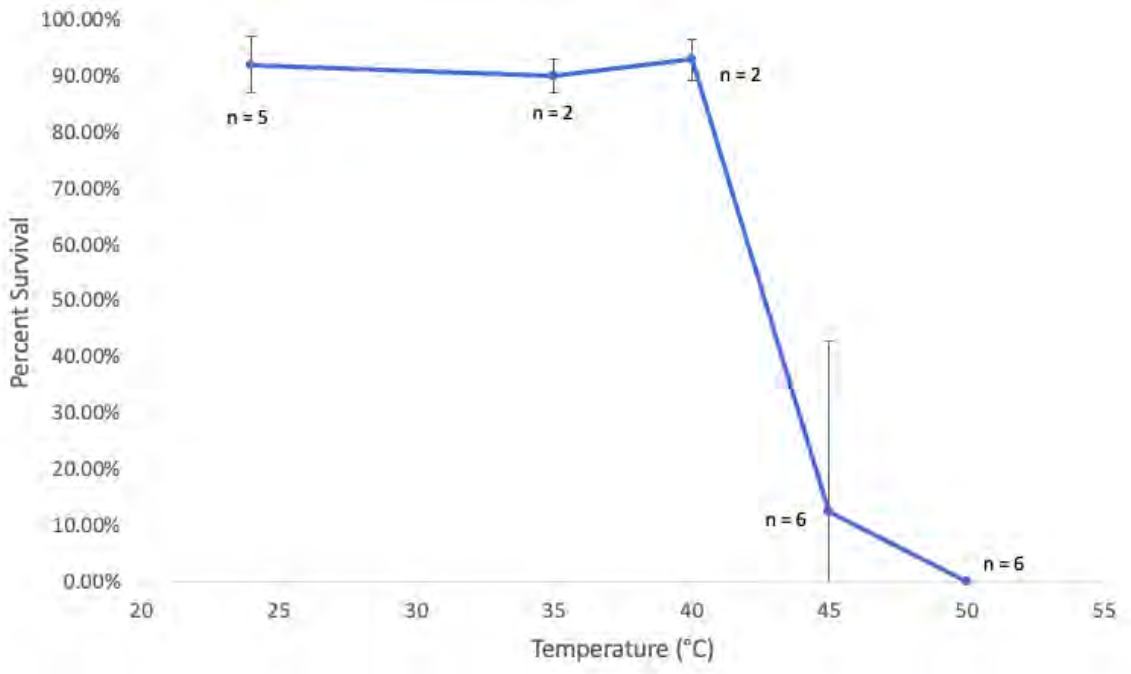


Figure 5. The average percent survival of *Siphonaria* embryos by temperature treatment (24 °C, 35 °C, 40 °C, 45 °C, and 50 °C).

Kruskal-Wallis Test

	24°C	35°C	40°C	45°C	50°C	
median	92.59	90.10	93.11	0.00	0.00	
rank sum	88	30	35	42	36	
count	5	2	2	6	6	21
r^2/n	1548.8	450	612.5	294	216	3121.3
H-stat						15.07272727
H-ties						17.58484848
df						4
p-value						0.001487267
alpha						0.05
sig						yes

DUNN's TEST			alpha	0.05	0.005
group	*R-sum*	*size*	*R-mean*	*z-crit*	
24°C	88	5	17.6		
35°C	30	2	15		
40°C	35	2	17.5		
45°C	42	6	7		
50°C	36	6	6		
		21		1.959964	

D TEST

group 1	*group 2*	*R-mean*	*std err*	*z-stat*	*R-crit*	*p-value*	
24°C	35°C	2.6	4.8	0.54	9.4	0.59	
24°C	40°C	0.1	4.8	0.02	9.4	0.98	
24°C	**45°C**	10.6	3.5	3.0	6.9	**0.002**	*
24°C	**50°C**	11.6	3.5	3.3	6.9	**0.0009**	*
35°C	40°C	2.5	5.7	0.44	11	0.66	
35°C	45°C	8	4.7	1.7	9.2	0.089	
35°C	50°C	9	4.7	1.9	9.2	0.057	
40°C	**45°C**	10.5	4.7	2.2	9.2	**0.03**	*
40°C	**50°C**	11.5	4.7	2.4	9.2	**0.02**	*
45°C	50°C	1	3.3	0.3	6.5	0.76	

Figure 6. A Kruskal-Wallis (H=15.0727, p=0.001487, df=4) and Dunn's test of the percent survivorship within each egg mass is shown by temperature treatment. Asterisks indicate significant differences between temperatures.

3.2 Latest Stage Reached Before Death

Following temperature exposures, almost all embryos showed apparently normal early cleavage patterns. Of 1,545 embryos total, only 3 were unable to cleave, all of which were from a single egg mass in the 45 °C treatment (Table 1). All other embryos died at either the cleaving or early veliger stage. Dead early veligers had incomplete velum formation, lack of eyes, and lack of shells. At all temperatures up to 45 °C, most embryos that failed to develop normally reached the early veliger stage prior to dying; at 45 °C, embryos died at either the early veliger, cleaving, or for three embryos, the egg stage (Figure 7, Table 3). At 50°C, all embryos died and they reached only the cleaving

stage (Table 3). Temperature had a marginally nonsignificant effect on the SRBD (Kruskal-Wallis, Figure 8, H=7.2317, p=0.05722, df=4).

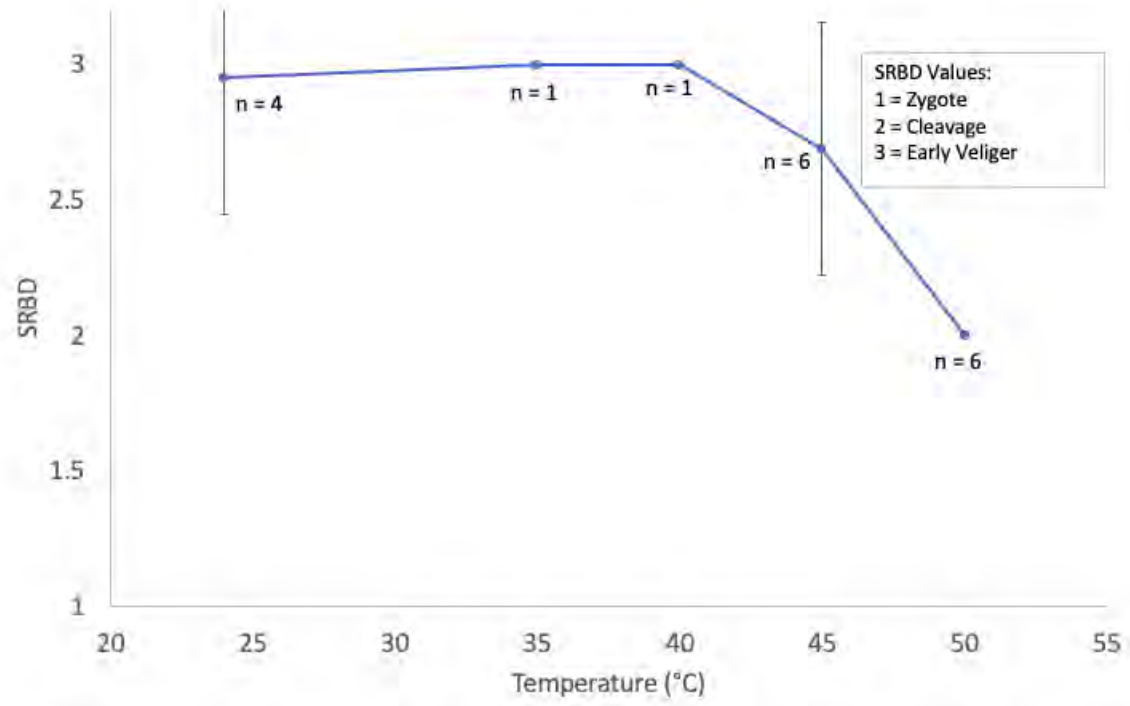


Figure 7. The mean SRBD of *Siphonaria* embryos by temperature treatment (24 °C, 35 °C, 40 °C, 45 °C, and 50 °C). Only egg masses that had dead embryos were included in the means. Stages shown in this figure are the egg, cleavage, and early veliger stages as defined by Figure 2. Criteria for unsuccessful development is defined by failure to develop eyes or shells, halted development, deformed growth, and discoloration (Figure 3).

Kruskal-Wallis Test

	24°C	35°C	40°C	45°C	50°C	
median	3	3	3	2.69	2	
rank sum	48.5	14.5	14.5	63.5	30	
count	4	1	1	6	6	18
r^2/n	588.0625	210.25	210.25	672.041667	150	1830.60417
H-stat						7.23172515
H-ties						9.16018519
df						4
p-value						0.05721797
alpha						0.05
sig						no

DUNN's TEST alpha 0.05 0.005

group	*R-sum*	*size*	*R-mean*	*z-crit*
24°C	48.5	4	12.125	
35°C	14.5	1	14.5	
40°C	14.5	1	14.5	
45°C	63.5	6	10.5833333	
50°C	30	6	5	
		18		1.95996398

D TEST

group 1	*group 2*	*R-mean*	*std err*	*z-stat*	*R-crit*	*p-value*	
24°C	35°C	2.4	5.3	0.45	10	0.65	
24°C	40°C	2.4	5.3	0.45	10	0.65	
24°C	45°C	1.5	3.1	0.48	6.1	0.63	
24°C	**50°C**	7.1	3.1	2.3	6.1	**0.02**	*
35°C	40°C	0	6.7	0	13	1	
35°C	45°C	3.9	5.1	0.76	10	0.45	
35°C	50°C	9.5	5.1	1.9	10	0.06	
40°C	45°C	3.9	5.1	0.76	10	0.45	
40°C	50°C	9.5	5.1	1.9	10	0.06	
45°C	**50°C**	5.6	2.7	2.1	5.3	**0.04**	*

Figure 8. A Kruskal-Wallis (H=7.2317, p=0.05722, df=4) and Dunn's test of mean SRBD within each egg mass is shown by temperature treatment. Within the Dunn's test, asterisks indicate areas where groups were significantly different from each other (24 °C and 50 °C, p=0.02; 45 °C and 50 °C, p=0.04).

The 45 °C egg masses were the only treatments to have the SRBD of unsuccessful embryos show a combination of the cleaving eggs and early veliger stage *Siphonaria* within any single egg mass (Table 3). However, a majority of dead embryos in the 45 °C treatment was able to reach the early veliger stage. By 50 °C, 100% of dead embryos arrested development during the cleavage stage. Within the 50 °C egg masses, a majority of dead embryos developed to the 16-cell cleavage stage. *Siphonaria* that died at the cleaving stage often changed color from a healthy green-yellow to an off-white or pale blue color. The individual blastomeres of several embryos came apart from one another and free-floated in the fluid within the egg capsule.

Table 3. The proportions of SRBD to the total number of died embryos within each egg mass, where 0 represents none of the embryos dying at the given stage, and 1 represents all dead embryos within the egg mass (N=18).

Temperature (°C)	Proportion: Egg	Proportion: Cleavage	Proportion: Early Veliger
24	0	0	1
24	0	1	0
24	0	0	1
24	0	0	1
35	0	0	1
40	0	0	1
45	0	0.615	0.385
45	0	1	0
45	0.034	0	0.967
45	0	0	1
45	0	0	1
45	0	1	0
50	0	1	0
50	0	1	0
50	0	1	0
50	0	1	0
50	0	1	0
50	0	1	0

4.0 DISCUSSION

My experiments showed that *Siphonaria normalis* embryos could tolerate temperatures up to 40 °C for 2 hours with near-100% survivorship. These results are consistent with previous studies; Wang et al. (2017) reported that *S. japonica* embryos

experienced increased rates of mortality at higher temperature exposures, and Kessel & Phillips (2018) showed abnormal development in *S. australis* embryos following thermal stress. In our study, the maximum tested temperature that the embryos tolerated with partial survivorship to the encapsulated crawling stage was 45 °C, while a single 2-hour exposure of 50 °C within the first 1-2 days of deposition led to 0% survivorship of embryos within an egg mass. These conditions are similar to the duration and magnitude of temperature events during low tides within the Hawaiian intertidal, measured in the habitat of *S. normalis* (Shishido et al., in prep.). Shishido et al. (in prep.) report that temperatures in the rocky intertidal in Makapuʻu, Oʻahu, commonly reach at least 45 °C for two hours. In the same report, temperatures in the intertidal zone of Kaiwi, Oʻahu, also occasionally reach 50 °C for two hours. Thus, current Hawaiian intertidal temperatures are already high and long-lasting enough to impact survivorship of *Siphonaria normalis* embryos.

At lower temperatures, our results suggest *S. normalis* embryos may survive temperature stress initially, but experience increased mortality from latent effects during early development. This is supported by data from nine of the twelve egg masses between 24 °C and 45 °C had embryos that developed through to the veliger stage before dying. However, by 50 °C, no embryos in any mass continued developing beyond the 16-cell stage. In all masses, all but three embryos appeared to have normal early cleavage across the tested temperature groups. While there was a visible pattern suggesting that the mean SRBD of an egg mass had a negative relationship with temperature, this relationship was marginally nonsignificant. This pattern may still provide insight on the specific development mechanisms that are disrupted by early temperature exposures. At lower

temperatures, where embryos were able to develop into the veliger stage, data suggest that the mechanisms that are obstructed differ from those at very high temperatures. Our data show that the highest temperature exposure prevents embryonic development beyond cleavage, which may suggest that mechanisms for cell division are disrupted. The field of embryo stress response may benefit from future studies that explore the SRBD by pinpointing the temperatures where mechanisms such as damage to DNA or disrupted cell division impact embryonic development.

For future studies, varied duration and frequency of temperature exposures should be incorporated to provide more complex, realistic conditions. The possibility of multiple intermediate and high temperature exposures during development may provide additional insight on egg mass thermal tolerance. Consecutive exposures of high temperatures lasting 1.5 hours and 3 hours have shown to increase mortality rates in a species of mussel, when a single occurrence of these same temperatures resulted in no mortality (Seuront et al., 2019). The opposite effect is also possible; corals have been shown to experience lessened mortality to multiple 2-3 hour high temperature events when inhabiting a thermally variable pool, indicating that some organisms may acclimatize (Oliver & Palumbi, 2011).

Another limitation of this study is that the time frame excludes any heat stress impacts following hatching. *S. normalis* embryos within this study were only observed from deposition until the encapsulated stage directly prior to hatching. While temperature treatments were not fatal for all embryos, there may be sublethal impacts on fitness after hatching. Temperature stress experiments conducted on multiple life stages of *Siphonaria australis* created carry-over effects into the adult stage and across generations, including

reduced hatching size and decreased embryonic viability of future generations (Kessel & Phillips, 2018). Organisms that inhabit the intertidal zone also experience many additional environmental factors that can contribute to the fitness and survival of a species (Strain et al., 2014; Li & Brawley, 2004; Negri & Hoogenboom, 2011). These include physiological and environmental factors, such as temperature, and tend to be control the upper limits of an intertidal species; there are also biotic factors, such as predation or interspecific competition for resources, which often control the lower limits (Connell, 1961a; Connell, 1961b; Paine, 1974). Experiments show a combination of abiotic conditions, such as temperature, salinity, pH, and pollutants, can have a synergistic impact on the survival of early marine life stages in multiple invertebrate phyla, including mollusks (Przeslawski et al., 2015). *In situ* observational experiments or controlled lab environments that consider interactive effects can benefit our understanding of embryonic survivorship under more realistic conditions. This is necessary to test whether survival in the field is lower than expected from the results of this study, due to the combination of multiple stressors.

Under global warming scenarios exceeding 2 °C above preindustrial levels, marine heat waves are projected to increase in intensity, frequency, and duration, particularly in the tropical Pacific (Frölicher et al., 2018). Under a 3.5 °C global warming simulation, the increase in maximum temperature intensity of marine heat waves rises by approximately 2.5 °C (Frölicher et al., 2018). According to Shishido et al. (in prep.), some areas of the Hawaiian intertidal are currently reaching daily maximum temperatures at or above 50 °C. With these projections in mind, our study indicates that *S. normalis* will be at a higher risk of mortality and possibly see a reduction in the abundance of

future populations. Grazers, including siphonariids, play an important intertidal role in algal control as well as interspecific interactions (Lodge, 1948; Branch et al, 1985). Comprehensive thermal tolerance studies that incorporate synergistic factors in the intertidal zone can better predict the future conditions of Hawai‘i’s rocky shore species distributions.

5.0 CONCLUSION

Global climate change is a complex problem that is expected to push the boundaries of many species’ thermal tolerances. Rapid changes in global temperatures highlight the need for thermal stress assessments of many ecologically necessary species. As an abundant grazer in the Hawaiian intertidal, it is important to examine the thermal tolerance of *S. normalis*. The next step for predicting *S. normalis’* thermal tolerance should be a multifactor study incorporating additional abiotic conditions, such as pH, salinity, and desiccation, to assess if and how tolerance is impacted. A deeper understanding of ecologically important species, such as *S. normalis*, is necessary to anticipate risk with warming conditions and gauge the potential changes in the Hawaiian intertidal ecosystem.

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