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THE EFFECTS OF CLIMATE CHANGE ON THE HEART RATES & GROWTH OF SEA SLUGS IN THE GULF OF MAINE

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ABSTRACT

THE EFFECTS OF CLIMATE CHANGE ON THE HEART RATES & GROWTH OF SEA SLUGS IN THE GULF OF MAINE

By

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In the next 80 years, sea surface temperatures are expected to increase by 1.5^{o} to 2^{o}C and ocean pH is expected to drop by 0.06 to 0.32 units, with exacerbated effects seen in coastal waters. Temperature increase has already forced organisms to shift their range polewards and ocean acidification has negatively affected calcifying organisms. Interactive effects, only more recently studied, vary depending on phylum and life cycle stage. This study examined both the upper thermal tolerance and interactive effect of temperature and acidification on the heart rate of five cold-water species of nudibranchs (*Aeolidia papillosa, Cuthona gymnota, Dendronotus frondosus, Flabellina verrucosa,* and *Onchidoris bilamellata*) and one species of sacoglossan (*Placida dendritica*) from the Gulf of Maine. Thermal tolerance was determined by recording heart rate for each organism starting at 4^{o}C and increasing the temperature by increments of 4^{o}C until the organism's heartbeat slowed or ceased. For interactive effects, pH levels used were pH 8 (control) and pH 7 at temperatures: 4^{o}, 8^{o} (control), 12^{o}, and 16^{o}C. Upper thermal tolerance limits ranged from 16^{o} to 20^{o}C for the nudibranchs and 24^{o}C for the sacoglossan. The combined effects of increasing temperature and lower pH were neutral, negatively additive, and antagonistic. Only *F. verrucosa* exhibited an interactive effect, with higher temperature and lower pH leading to decreased heart rate. Although no interactive effect was demonstrated in *C.*

gymnota, *D. frondosus*, and *O. bilamellata*, lower pH slowed heart rates across all temperatures. Subsequently, the relationship between temperature and growth rates was examined in *D. frondosus* and *F. verrucosa*. The nudibranchs were reared for eight weeks at 4º, 10º, or 16ºC and growth was measured weekly. The ideal temperature for growth appeared to be 10ºC, whereas 16ºC was lethal. Additionally, an unsuccessful attempt was made to culture *A. papillosa*, but the number of embryos per egg capsule and larval growth rates were examined. Size of adult sea slug positively impacted the number of embryos per egg capsule, with embryos increasing in length by 50% over the first week and 10% over subsequent weeks. With an interactive effect only seen in one species and upper temperatures being lethal if held constant for a month, temperature appears to be the greatest threat to survival. What is happening to these sea slugs in the GOM is likely happening to other snails and marine invertebrates throughout the ocean. Knowing how organisms will react to the projected changes can help inform future policies and practices.

GENERAL INTRODUCTION

The last 30 years have been the warmest on record: ocean surface waters warmed by 0.33^{o}C, and are predicted to continue to rise as global sea surface temperatures are expected to increase by 1.5^{o} to 2^{o}C in the next 80 years (IPCC, 2014). In addition to increased temperatures, the pH of ocean surface waters fell approximately 0.1 unit over the last 200 years and is expected to drop by another 0.06 to 0.32 units by 2100 depending on the Earth Systems Model Representative Concentration Pathway (RCP) scenario (IPCC, 2014) with coastal waters seeing the most significant changes (Lesser, 2016; IPCC 2014). By 1982, the surface water temperature of the Gulf of Maine (GOM) was increasing three times faster per year than global sea surface temperature and by 2013, it was increasing by 0.23^{o}C year^{-1}, approximately seven times faster (Pershing et al. 2015). The shallow depth and proximity to land surface runoff likely exacerbate climate change effects in coastal areas. Temperature increases and acidification, two of the chief concerns of marine climate change, are already showing intensified effects in coastal zones (Pershing et al. 2015).

Maintaining body temperature and physiological pH are vital to the survival of an organism (Schmidt-Nielsen, 1997). Temperature and pH affect fundamental biological and chemical processes, from protein synthesis to metabolism (Hochachka & Somero, 1994). Ionic regulation within the body is dependent on a specific internal pH range, and if the acid-base balance of bodily fluids is shifted too far to one side or the other, cells do not function properly (Malan et al. 1976). Because poikilotherms rely upon ambient water temperatures and pH to regulate their internal temperature and pH, they are the most susceptible to environmental

changes. Consequently, habitats with temperatures beyond an organism's thermal tolerance will exclude those species (see review by Harley et al. 2006).

Along with temperature increases, marine ecosystems are further stressed by ocean acidification. According to Gledhill et al. (2015) and references therein, acidification is already negatively affecting calcifying organisms, including clams, scallops, and lobsters. Lower pH slows the process of calcification in *Mytilus edulis* (Lesser, 2016) and leads to decreased growth for the sea urchin, *Strongylocentrotus droebachiensis* (Stumpp et al. 2012). Direct effects of lower pH (e.g., thinner shells) has led to indirect effects, such as behavioral changes. The marine snail *Littorina littorea* has responded to a reduced shell thickness by more actively avoiding predators (Bibby et al. 2007).

Early studies have examined the effects of temperature and acidification separately (Newell & Bayne, 1973; Brey, 1999). However, interactive effects may be even more important, because one factor can either lessen or intensify the effects of another. The interactions of temperature and acidification may be positive (e.g., photosynthesis), negative (e.g., calcification, reproduction, survival), or neutral (e.g., growth) depending on life cycle stage or taxonomy of the study organism (see review Harvey et al. 2013).

Most studies examining organismal responses to increased temperatures and lower pH have focused on commercially valuable species of fish, crustaceans, and bivalves (Gledhill et al. 2015; Lesser, 2016), while the majority of non-commercial marine species, such as sea slugs (Opisthobranchia: Mollusca), have been under represented. Although adult sea slugs lack shells,

most of their larvae have fully formed shells (Hyman, 1967). Lower pH may not only affect their larval development by interfering with their ability to form calcified shells (Carr & Podolsky, 2016), but temperature may also affect larval growth and survival. For example, Runnström (1927) has demonstrated that *Dendronotus frondosus* embryos fail to develop at temperatures below 1.4^{o}C or above 12^{o}C. Similarly, embryos of *Aeolidia papillosa* are also temperature sensitive, as the cells breakdown at temperatures above 16^{o}C (pers. obs.). Increasing temperatures have already been shone to increase heart rates in gastropods (Snyder, 1906; Skramlik, 1929), including adult *A. papillosa* (Harris, unpublished data). To date, there are no studies demonstrating interactive effects of temperature and pH on the heart rate of sea slugs. As short-lived species with complex life cycles, sea slugs may prove to be effective bio-indicators of climate change. The goals of this study were to determine the interactive effects of warming temperatures and decreasing pH levels on the heart rate of six species of adult sea slugs native to the North Atlantic and to identify their respective thermal tolerances. Additionally, the effects of temperature on the growth rates of two species of nudibranchs were examined. Lastly, an attempt was made to culture *A. papillosa* and examine the relationship between adult size and embryos per egg capsule, as well as larval growth rate.

Objectives

Chapter 1: Determine the thermal tolerance and interactive effect of temperature and pH on the heart rate of six cold-water species of sea slugs.

Chapter 2: Determine the effect of temperature on growth rates for *Flabellina verrucosa* and *Dendronotus frondosus.*

Chapter 3: Compare number of embryos per egg capsule to adult size of *Aeolidia papillosa.*

CHAPTER 1: THERMAL TOLERANCE & THE INTERACTIVE EFFECTS OF TEMPERATURE & ACIDIFICATION ON THE HEART RATES OF SIX SPECIES OF SEA SLUGS IN THE GULF OF MAINE

INTRODUCTION

Increasing sea water temperature and ocean acidification are among the top threats to the marine environment. Of the two, temperature is likely *the* key component determining habitat suitability as temperature influences all aspects of life including biological processes (Newell & Bayne, 1973; Hochachka & Somero, 1994), larval development, behavior (Harley et al. 2006), and ultimately, survival (Runnström, 1927; O'Conner et al. 2007; pers. obs.). Although assessing the effects of individual factors is important, interactive effects may be more meaningful, because two factors could work cumulatively, synergistically or antagonistically.

According to the metabolic theory of ecology, temperature and body size determine metabolic rate (Gillooly et al. 2001; Brown et al. 2004). As poikilotherms, body temperature and in turn, the metabolic processes of marine invertebrates are determined by ambient water temperatures. Temperature and developmental rate are positively correlated in a wide range of marine invertebrates, whereas temperature and survival are negatively correlated (Byrne & Przeslawski, 2013). Previous studies have found that higher temperatures decrease the survivability of the larvae of the nudibranchs *Dendronotus frondosus* (Watt & Aiken, 2003) and *Armina maculata* (Pires, 2012). Being intertidal organisms, the sea slugs included in this study may already be living near their upper critical temperatures (Hopkin et al. 2006; Somero, 2010).

Although intertidal organisms are accustomed to fluctuations in diurnal temperature, prolonged periods at higher temperatures have been reported to accelerate the life cycle, causing earlier spawning and shorter life spans (Loeb & Northrop, 1916; Miquel et al. 1976; Sohal et al. 1981; McArthur & Sohal, 1982). Additionally, temperature positively affects heart rate and oxygen consumption in marine invertebrates (Ahsanullah & Newell, 1971; Widdows, 1973; DeFur & Mangum, 1979). Hence, knowledge of the thermal tolerance of a species, may allow for inferences about the continued survival of that species in a particular environment.

Along with temperature change, other changes in the water, such as a decrease in pH or less dissolved oxygen, can also impact biological processes in marine organisms (Grieshaber et al. 1993). Furthermore, increased carbon dioxide in seawater (lower pH), resulted in the larvae of *A. papillosa* hatching sooner and having a shorter shell length when compared to larvae raised in ambient seawater pH (Carr & Podolsky, 2016). A study on the combined effect of expected future temperatures and pH levels on the sacoglossan *Elysia clarki* resulted in reduced reproductive output and greater larval mortality, but did not affect feeding in larvae or adults (Dionísio et al. 2017). Interactive effects of increased temperature and acidification on the gastropod whelk *Dicathais orbita* have also resulted in decreased and altered composition of fatty acids in this commercially profitable species (Valles-Regino et al. 2015).

The main goal of this study was to determine the suitability of sea slugs as bioindicators of climate change. To this end, I tested the interactive effects of increased temperature and decreased pH on the heart rates of six cold-water species of sea slugs: *Aeolidia papillosa* (Nudibranchia: Aeolidiidae), *Cuthona gymnota* (Nudibranchia: Cuthonidae), *Dendronotus*

frondosus (Nudibranchia: Dendronotidae), *Flabellina verrucosa* (Nudibranchia: Flabellinidae), *Onchidoris bilamellata* (Nudibranchia: Onchidorididae), and *Placida dendritica* (Sacoglossa: Limapontiidae) (see Appendix A). All species are found in the Gulf of Maine (GOM) which is likely the fastest warming body of water in the northern hemisphere (Pershing et al. 2015). In addition to interactive effects, I also determined the thermal tolerance for each species. I hypothesize that the heart rate of all species will correlate positively with temperature and that the interactive effects of increased temperature and lower pH will result in lower heart rates, as ocean acidification has been shown to cause metabolic depression in mollusks (Parker et al. 2013). Although heart rate cannot be used as a direct measurement of metabolism, both rates are linked in gastropods (Bruning et al. 2013).

METHODOLOGY

Collection & Maintenance of Animals

From November 2017 to July 2018, *Aeolidia papillosa, Cuthona gymnota, Dendronotus frondosus, Flabellina verrucosa, Onchidoris bilamellata,* and *Placida dendritica* were collected from areas in the southern GOM, including intertidally and subtidally from the UNH Marine Research Pier, located at the Judd Gregg Marine Research Complex in New Castle, New Hampshire (43.07, -70.71), subtidally at Cape Neddick, York, Maine (43.16, -70.59), and intertidally at Hilton Park, Dover, New Hampshire (43.12, -70.83). All nudibranchs were kept in a recirculating water system maintained at 7.5^{o} to 9.8^{o}C, with a pH of 7.7 to 7.9 and salinity of 31 to 35 ppt. Within the system, *A. papillosa* were housed separately, in mesh plastic organizers to allow water flow-through and fed *Metridium senile* of appropriate size (smaller than the nudibranch – see Harris, 1986) *ad libitum*. *Cuthona gymnota, D. frondosus,* and *F. verrucosa*

were isolated by species, housed in circular, mesh nets and fed the hydroid *Ectopleura crocea ad libitum*. *Onchidoris bilamellata* were housed in circular, mesh nets and fed the barnacle *Semibalanus balanoides ad libitum*. The sacoglossan, *P. dendritica,* were kept in plastic buckets containing the siphonaceous green alga *Codium fragile.* The water was aerated and kept between 14.6º and 16.3ºC, with a pH of 7.9 to 8.1, and salinity of 32 to 35 ppt. All systems were located on the main Durham campus of the University of New Hampshire in a temperature controlled aquatic room in Spaulding Hall. Tank water temperatures were obtained from Onset® HOBO water temperature Pro v2 data loggers (U22-001), pH was measured using a YSI© Pro 10 pH probe attached to a YSI© Professional Plus multiparameter instrument, and salinity was obtained using a Cole-Parmer® refractometer.

Upper Thermal Tolerance Limit

Between January and July 2018, the sea slugs (*A. papillosa* (n=12), *C. gymnota* (n=20), *D. frondosus* (n=20), *F. verrucosa* (n=20), *O. bilamellata* (n=20), and *P. dendritica* (n=20)) were placed in individual glass bowl specimen dishes containing approximately 220mL of ambient seawater. Specimen dishes were then placed in water/ice baths to drop the temperature to 4ºC over a period of approximately 40 minutes. Once at 4ºC, the sea slug was left to acclimate for approximately 15 minutes. Heart rates (beats/min; BPM), which were visible through the dorsal surfaces of each species, were averaged from three different counts over 30 second intervals using an AmScope© LED stereo zoom microscope with an AmScope© cold light source (HL 150-A) and recorded. The specimen dish was then left at room temperature to warm to 8ºC (control for all nudibranch species), 12ºC, and 16ºC (control for *P. dendritica*). A warm water bath was used to warm the seawater in increments of 4ºC (20º, 24º, and, for *P. dendritica*, 28ºC).

The heart rate procedure described above was used for each temperature. A single individual was used per trial and a trial was considered complete when the heartbeat of the sea slug slowed or ceased. Most trials ended prior to reaching 24^{o}C for all nudibranch species, except *A. papillosa*, and 28^{o}C for the sacoglossan. Seawater was changed after the temperature reached 16^{o}C to avoid oxygen depletion. Prior to testing, the length (cm) of each subject was obtained using a ruler set alongside the relaxed, extended body of the sea slug. Subsequent to testing, damp weight (g) of the animals (except *P. dendritica*) was determined using a Carolina® 150g electronic balance (Model: SLB152-US): animals were blotted on a clean cloth to remove excess water before being placed on the scale.

Interactive Effects of Temperature & pH

To determine potential interactive effects between pH and temperature, carbon dioxide was bubbled into a flask of seawater until the pH dropped to 7.0. Although a pH of 7 is approximately three times the maximum predicted decrease in overall ocean pH (IPCC, 2014), coastal waters experience higher fluctuations in temperature and pH, with the GOM seeing some of the greatest increase on the East Coast (Signorini et al. 2013; Gledhill et al. 2015; Pershing et al. 2015). The flask was placed into the recirculating tank holding the animals and allowed to cool to ambient temperature. Once cooled, the pH of the water was rechecked to ensure it had remained at a pH of 7 and then poured into a specimen dish along with a sea slug (*A. papillosa* (n=10), *C. gymnota* (n=15), *D. frondosus* (n=15), *F. verrucosa* (n=15), *O. bilamellata* (n=15), and *P. dendritica* (n=15)). The same procedure used for temperature trials was used to change temperatures to 4^{o}C, 8^{o}C (control for all nudibranch species), 12^{o}C, and 16^{o}C (control for *P. dendritica*) and to record heart rate. A single individual was used per trial. The length and damp

weight of all individuals (except *P. dendritica*, for which only length was measured) were obtained using the procedure previously stated. The pH level was measured at the end of each trial to ensure pH remained consistent.

Statistical Analysis

The average heart rate (BPM) from three readings for each sea slug at each temperature was determined. Weight was tested as a covariable using regression analysis to find the R^2 and significance was determined using an ANOVA. Due to an inability to obtain a weight for *P. dendritica*, length was tested as the covariable. To meet the assumption of normally distributed data, BPM were log transformed for all species. All statistical analyses were performed in R, a statistical programming package (R Core Team, 2018) and figures were created using ggplot2 (Wickham, 2009).

Upper Thermal Tolerance Limit

A one-way ANOVA with repeated measures using individual subjects as a block was used to test if temperature significantly impacted heart rate. Data were log transformed to meet the assumption of normally distributed data. A Tukey's means separation test was then used to determine significant differences between temperatures (6 treatments for nudibranchs, 7 treatments for *P. dendritica*).

Interactive Effects of Temperature & pH

A two-way ANOVA with repeated measures was used to determine whether an interactive effect of increasing temperature (4 treatments) and lower pH (2 treatments) on the

average heart rates (BPM) of the sea slugs existed. Data were log transformed and interaction plots were created to visualize interactions between temperature and pH.

RESULTS

Observations

Individual sea slugs responded behaviorally to increasing temperature in a similar way. At lower temperatures, the individuals crawled steadily around the side of the glass dish, near the surface edge of the water. Not all, but many individuals from every species spent time crawling upside down on the surface tension of the water. As the temperature increased, however, the sea slugs slowed their crawling and eventually stopped, typically being located at the bottom of the dish. Although there was some variation between individuals, all nudibranch species reacted to their upper thermal tolerance by rolling on their sides, curling into a ball, and swelling their bodies. The sacoglossan, *P. dendritica,* on the other hand, did not roll on their sides, but instead stayed in place, lifting their anterior body portions from the glass and swaying their upper bodies side to side, typically from the waterline on the edge of the dish.

The average damp weight of the nudibranch species used in the study varied as follows: *Aeolidia papillosa* (5.4 $\pm$ 2.4g), *Dendronotus frondosus* (2.9 $\pm$ 1.5g), *Flabellina verrucosa* (0.8 $\pm$ 0.4g), *Onchidoris bilamellata* (2.0 $\pm$ 0.7g), and *Cuthona gymnota* (0.05 $\pm$ 0.02g). Average damp weight was not a significant covariate with heart rate for any nudibranch species: *A. papillosa* (R^2=0.021, $F_{1, 70}$=1.534, p=0.220), *D. frondosus* (R^2=0.007, $F_{1, 111}$=0.744, p=0.390), *F. verrucosa* (R^2=0.024, $F_{1, 113}$=2.761, p=0.099), *O. bilamellata* (R^2=0.0001, $F_{1, 118}$=0.016, p=0.899)

or *C. gymnota* (R^2=0.016, $F_{1, 112}$=1.764, p=0.187). The average length of *P. dendritica* (0.6 ± 0.08cm) was also not a significant covariate with heart rate (R^2=0.006, $F_{1, 138}$=0.826, p=0.365).

Upper Thermal Tolerance Limit

Some individuals from each species survived to a maximum temperature of 24ºC, whereas trials ended at lower temperatures for others when the heartbeat of the nudibranch slowed or stopped. Average heart rates varied depending on species. Once maximum temperatures were reached, heart rates had more than doubled, tripled, or even quadrupled compared to starting heart rates at 4ºC (Table 1.1). A one-way blocked ANOVA revealed that heart rates among individual nudibranchs/sacoglossans within each species differed with increasing temperatures: *A. papillosa* ($F_{11, 55}$=3.003, p=0.003), *D. frondosus* ($F_{19, 88}$=1.772, p=0.039), *F. verrucosa* ($F_{19, 90}$=9.195, p<0.001), *O. bilamellata* ($F_{19, 95}$=4.672, p<0.001), *C. gymnota* ($F_{18, 90}$=13.310, p<0.001), and *P. dendritica* ($F_{19, 114}$=14.353, p<0.001).

Heart rates were positively correlated with temperature in all species: *A. papillosa* ($F_{5, 55}$=30.681, p<0.001, Fig. 1.1a), *D. frondosus* ($F_{5, 88}$=167.850, p<0.001, Fig. 1.1d), *F. verrucosa* ($F_{5, 90}$=132.692, p<0.001, Fig. 1.1b), *O. bilamellata* ($F_{5, 95}$=634.855, p<0.001, Fig. 1.1e), *C. gymnota* ($F_{5, 90}$=191.340, p<0.001, Fig. 1.1c), and *P. dendritica* ($F_{6, 114}$=689.545, p<0.001, Fig. 1.1f). However, the relationship between temperature and heart rate was not entirely linear for all species. Except *C. gymnota*, the greatest temperature coefficient (Q_{10}) for all species was from 4º to 8ºC at which heart rate increased more than two-fold every 10ºC (Table 1.2). After 8ºC, Q_{10} dropped by 0.3 to 0.9 for every 4ºC, dependent on species. At the maximum temperature, Q_{10}

was close to 1 for all species, indicating that heart rate had slowed. For *C. gymnota*, Q_{10} was highest at 2.8 between 16° and 20°C, then fell to 1.3 over the next 4°C increase (Table 1.2).

For *A. papillosa,* except for a significant increase in heart rate between 4°C and 8°C ($p=0.018$), only small increases were noted every 4°C temperature change ($p>0.145$, Fig. 1.1a). However, heart rate did increase significantly over 8°C changes between 8° and 16°C ($p<0.001$) and between 12° and 20°C ($p<0.001$). Once the animal reached 16°C, heart rate began to slow and no change was seen between 16° and 24°C ($p=0.836$). Although all 12 *A. papillosa* survived trials to 24°C, as evidenced by continued heartbeats, they showed signs of stress by curling on their sides at the bottom of the dish at 24°C.

Between 4° and 16°C, a positive linear relationship between heart rate and temperature was found in *D. frondosus* ($p<0.001$, Fig. 1.1d), after which heart rate did not increase between 16° and 20°C ($p=0.308$) or 16° and 24°C ($p=0.999$). Only 13 of 20 *D. frondosus* tolerated 24°C before curling in a ball on their sides. Of the seven *D. frondosus* that did not tolerate 24°C, heart rate decreased between 16° and 20°C.

Flabellina verrucosa also demonstrated a linear relationship between heart rate and temperature until 20°C, with heart rate leveling off between 20° and 24°C ($p=0.194$; Fig. 1.1b). Although only 15 of 20 *F. verrucosa* endured 24°C, all individuals exhibited similar stressed behavior by laying on their sides and enlarging their gonopores.

The only species in which temperature and heart rate had a linear relationship over the entire temperature range (4° to 24°C), was *O. bilamellata* (R^2=0.996; Fig. 1.1e). However, at 24°C, all animals were swollen, curled on their sides, and Q_{10} between 20° and 24°C had dropped to 1.3 (Table 1.2).

The heart rate for *C. gymnota* increased from 4° to 8°C ($p<0.001$), and for every additional 4°C change until 20°C ($p<0.001$), except between 8° and 12°C ($p =0.508$; Fig. 1.1c). After 20°C, heart rate did not increase significantly ($p=0.158$). Nineteen of 20 *C. gymnota* survived to 24°C, albeit with different responses to the maximum temperature: 8 nudibranchs writhed in place, 4 nudibranchs crawled very slowly and occasionally folded over, 5 nudibranchs curled on their side, and 3 nudibranchs continued crawling around the edge of the dish, but their heart rates had slowed.

Between 4° to 24°C, *P. dendritica* exhibited a linear relationship between temperature increases ($p<0.001$), but no increase was seen in heart rate between 24° and 28°C ($p=1.000$; Fig. 1.1f). At 28°C, all 20 *P. dendritica* responded to the high temperature by bending backwards and swaying their upper bodies side to side.

Table 1.1: Back-transformed average heart rate (BPM) with standard deviation for each species at all temperatures, sorted by size.

	4ºC	8ºC	12ºC	16ºC	20ºC	24ºC	28ºC
Large (>4cm)							
A. papillosa	15.9 ± 1.3	22.6 ± 1.2	29.0 ± 1.2	37.6 ± 1.2	45.9 ± 1.2	42.6 ± 1.8	-
D. frondosus	19.9 ± 1.1	28.7 ± 1.1	39.3 ± 1.1	49.7 ± 1.1	54.2 ± 1.2	50.5 ± 1.3	-
Medium (2-4cm)							
F. verrucosa	23.2 ± 1.2	31.2 ± 1.2	38.5 ± 1.2	48.5 ± 1.2	57.7 ± 1.3	64.8 ± 1.5	
O. bilamellata	27.3 ± 1.1	38.2 ± 1.1	50.3 ± 1.1	65.5 ± 1.1	81.0 ± 1.1	90.5 ± 1.2	-
Small (<2cm)							
C. gymnota	23.0 ± 1.2	29.9 ± 1.2	32.5 ± 1.4	46.7 ± 1.4	67.7 ± 1.2	78.5 ± 1.4	-
P. dendritica	29.7 ± 1.2	47.2 ± 1.1	63.9 ± 1.2	82.6 ± 1.1	104.3 ± 1.2	126.3 ± 1.2	125.3 ± 1.2

Table 1.2: Q_{10} temperature coefficients for all species.

	4º to 8ºC	8º to 12ºC	12º to 16ºC	16º to 20ºC	20º to 24ºC	24º to 28ºC
A. papillosa	2.4	1.9	1.9	1.6	0.8	-
D. frondosus	2.5	2.2	1.8	1.2	0.8	-
F. verrucosa	2.1	1.7	1.8	1.5	1.3	-
O. bilamellata	2.3	2.0	1.9	1.7	1.3	-
C. gymnota	1.9	1.2	2.2	2.8	1.3	-
P. dendritica	3.2	2.1	1.9	1.8	1.6	1.0

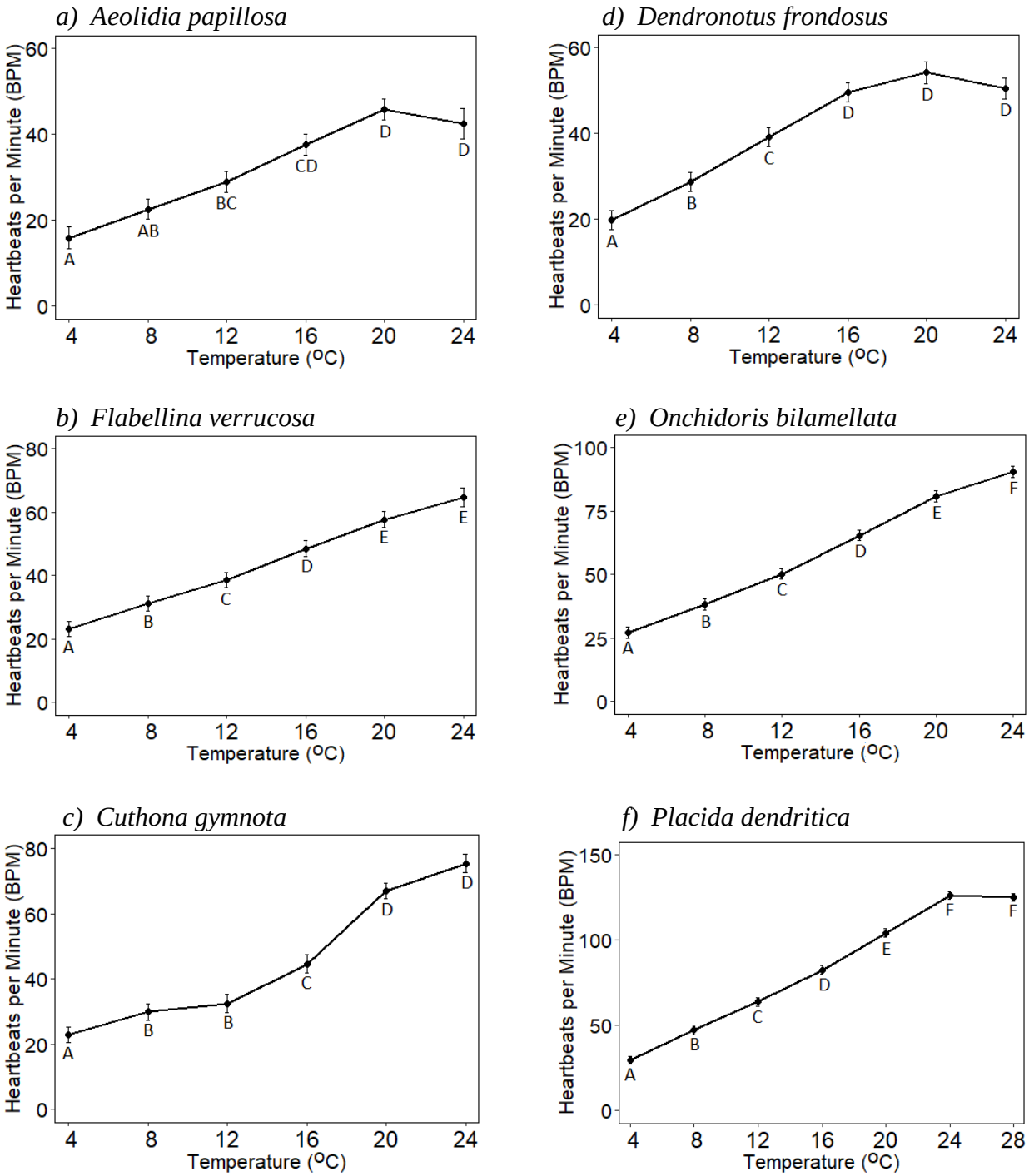

Figure 1.1: Relationship between Heart Rate & Temperature. Back-transformed average heart rate (BPM) of *Aeolidia papillosa* (a), *Flabellina verrucosa* (b), *Cuthona gymnota* (c), *Dendronotus frondosus* (d), *Onchidoris bilamellata* (e), and *Placida dendritica* (f) over increasing temperature (°C). Bars indicate two standard deviations from the mean and letters represent significance between temperatures as assigned by the Tukey test.

Interactive Effects of Temperature & pH

Decreasing pH, as tested at 4º, 8º (control), 12º, and 16ºC, did not influence the heart rates for *A. papillosa* ($F_{1, 9}$=0.08, p=0.8) or *D. frondosus* ($F_{1, 33}$=1.4, p=0.2), thus no interaction between pH and temperature was demonstrated: *A. papillosa* ($F_{3, 54}$=0.4, p=0.8; Fig. 1.2a) and *D. frondosus* ($F_{3, 99}$=1.7, p=0.2; Fig. 1.2d). On the other hand, lower pH slowed the heart rates of *C. gymnota* ($F_{1, 33}$=10.2, p=0.003) and *P. dendritica* ($F_{1, 33}$=22.9, p<0.001). Because heart rate was lowered uniformly throughout the pH range test (8 to 7 pH from 4º to 16ºC), no interaction between pH and temperature was found: *C. gymnota* ($F_{3, 99}$=0.4, p=0.7; Fig. 1.2c) and *P. dendritica* ($F_{3, 99}$=0.6, p=0.6; Fig. 1.2f).

Although the heart rate of *F. verrucosa* did not decrease in the lower pH of 7 ($F_{1, 33}$=2.7, p=0.1), the log transformed data showed a significant interactive effect of pH and temperature at 16ºC ($F_{3, 99}$=2.9, p=0.04; Fig. 1.2b). In contrast, the heart rate of *O. bilamellata* was significantly lowered when pH was decreased ($F_{1, 33}$=10.2, p=0.003), but no interactive effect of pH and temperature was found ($F_{3, 99}$=2.3, p=0.08; Fig. 1.2e).

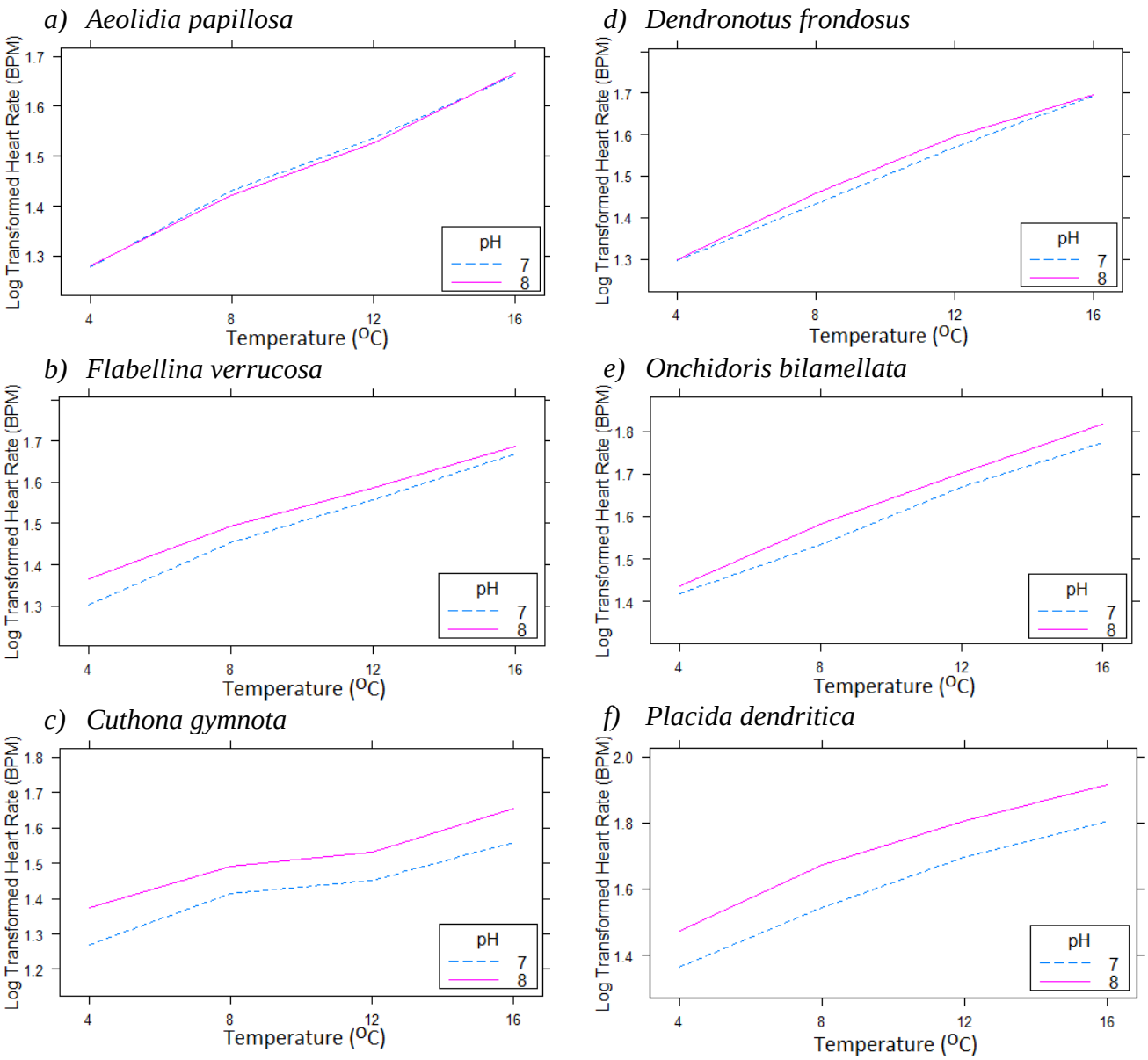

Figure 1.2: Interactive Effect of pH & Temperature: Log transformed interaction effect of pH and temperature for *Aeolidia papillosa* (a), *Flabellina verrucosa* (b), *Cuthona gymnota* (c), *Dendronotus frondosus* (d), *Onchidoris bilamellata* (e), and *Placida dendritica* (f).

DISCUSSION

The major goal of this study was to determine the suitability of cold water sea slugs as a model species for the study of environmental changes, specifically the interactive effects of ocean acidification and temperature. Additionally, thermal tolerance was determined for the six study species. The effects of temperature and ocean acidification on heart rates were observed, because of the non-invasive method of observation and the relationship between heart rate and metabolism (Bruning et al. 2013).

Upper Thermal Tolerance Limit

For all species, heart rate and temperature were found to be positively correlated. Although the differences in heart rates between 8º and 12ºC were not significant for *A. papillosa* and *C. gymnota*, the trend was linear (Fig. 1.1). This is supported by previous studies in snails (Romero & Hoffman, 1996; Stenseng et al. 2005), mussels (Braby & Somero, 2006), and crabs (Wachter & McMahon, 1996). Heart rates varied by species (Table 1.1). However, unlike the mussels studied by Braby & Somero (2006), the heart rates of the aeolid nudibranchs were higher for species that inhabit shallower depths (*F. verrucosa*, up to 450 m deep and *C. gymnota*, up to 30 m deep) and lower for species that can inhabit deeper depths (*A. papillosa*, up to 800 m). The increased heart rate of shallow-depth species corresponds to findings by Stenseng et al. (2005) and may be due to larger, more unpredictable temperature fluctuations. Stenseng et al. (2005) found that *Tegula funebralis*, a shallow water snail, had a higher heart rate than two subtidal *Tegula* spp. Additionally, Stenseng et al. (2005) found *T. funebralis* had a higher critical temperature range, similar to the species in this study. The upper thermal tolerance range for shallow-water nudibranchs (*C. gymnota, F. verrucosa*, and *O. bilamellata*) was between 20º and

24ºC, whereas the upper thermal tolerance range for the deep-water nudibranchs (*A. papillosa* and *D. frondosus*) was between 16ºC and 20ºC. The sacoglossan *P. dendritica* revealed the highest upper thermal tolerance range between 24º and 28ºC. This may be attributable to the distribution of *P. dendritica* from Norway to the Caribbean (Thompson, 1976).

The Q_{10} for all slugs at temperatures above 4ºC and below the start of their respective upper thermal tolerance ranges were approximately 2 (Table 1.2). Similar results have been found in mussels (Braby & Somero, 2006), various marine invertebrates (DeFur & Magnum, 1979), and terrestrial snails (Duval, 1983; Romero & Hoffman, 1996). Except in *C. gymnota*, the largest Q_{10} for the other sea slug species were between 4º and 8ºC. A Q_{10} of 2 is expected when physiological processes are functioning properly, but will slow if biological processes are impeded (Prosser, 1973; Hochachka & Somero. 1994). As the sea slugs neared their respective upper thermal tolerance range, their respective Q_{10} slowed to 1 or less (Table 1.2), indicating that temperature was slowing heart rate. A slowed heart rate at higher temperatures may be an mechanism to withstand the heat. When their respective upper thermal tolerance ranges were reached, heart rates remained constant or decreased as the animals succumbed to the heat (Table 1.1; Fig. 1.1).

As expected, there was no one common heart rate for all species. Although intraspecific body size variation did not impact heart rate, interspecific body size of slugs did. The larger sea slug species studied exhibited lower overall heart rates (Table 1.1). A similar trend was also observed in terrestrial slugs (Duval, 1983). Additionally, body size affected the upper thermal tolerance ranges for each species. Both *A. papillosa* and *D. frondosus*, the largest of the sea slugs

studied, had the lowest upper thermal tolerance range (16ºC to 20ºC), whereas the upper thermal tolerance range for *F. verrucosa* and *O. bilamellata*, the two mid-sized species studied, was between 20º and 24ºC. Although the average heart rate of *O. bilamellata* did not level off or drop significantly, all slugs responded identically to higher temperatures by swelling their bodies and not moving. By staying immobile and lowering heart rate they could be conserving energy which may allow them to withstand the higher temperature of 24ºC, for a longer period of time, but not indefinitely, as their Q_{10} dropped to 1.3 between 20º and 24ºC (Table 1.2). The smallest aeolid nudibranch studied, *C. gymnota*, also had an upper thermal tolerance range of 20º to 24ºC, which may be due to an adaptation to its habitat, rather than a relationship with body size, as *C. gymnota* are often found along with *F. verrucosa* in fouling areas close to the surface of the water, where temperature fluctuations are greatest and often warmest (pers. obs.). The smallest sea slugs studied and the only sacoglossan, *P. dendritica*, had the fastest heart rate and the highest upper thermal tolerance range (24º to 28ºC).

Interactive Effects of Temperature & pH

Ocean acidification, on the other hand, does not greatly influence the heart rates of all adult sea slugs. Two species (*A. papillosa* and *D. frondosus*) maintained the same heart rate over all temperatures regardless of a significant drop in pH. Marchant et al. (2010) found a similar result in the gastropod *Patella vulgata*. Size again may be a factor in their ability to maintain the same heart rate in a lower pH, as the smallest of the species (*C. gymnota* and *P. dendritica*), both responded to the lower pH, by slowing their heart rates across all temperatures. Melatunan et al. (2011) found that the gastropod *Littorina littorea* responded similarly to a drop in pH. If these poikilotherms are unable to maintain their proper physiologic pH in the more acidic water,

biological processes may be negatively impacted, which could be causing their heart rates to slow. However, if the more acidic water slows their heart rates without impacting biological processes, the sea slugs may be able to withstand higher temperatures than their current thermal tolerance allows.

F. verrucosa and *O. bilamellata* responded to the combined effects of temperature and acidification differently. For *F. verrucosa*, the species demonstrated an interactive effect of temperature and acidification. Heart rate depression was exhibited at lower temperatures, but as temperature increased in the lower pH, heart rate rose to equal the heart rate at the higher pH. Howell et al. (1973) found that as temperature increases, so does blood pH in crustaceans. It is possible that similar mechanisms are enabled in *F. verrucosa.* The reaction of *O. bilamellata* to increasing temperature and lower pH did not exhibit an interactive effect, but heart rate was significantly lowered in the lower pH. Although slower in the lower pH, at 4^{o}C, heart rate was only slightly less, whereas the difference in heart rate became more pronounced as seawater temperature increased in the lower pH. Although all species reacted similarly to the increasing temperature, once the additional stressor of lowered pH was added, responses differed among species. The same has been described for other mollusks both in and between classes (Parker et al. 2013).

General Conclusions

As hypothesized the heart rates of all species correlated positively with temperature. Although average heart rates varied between species, the trend between temperature and heart rate was linear for all species. However, neutral, additive, and antagonistic interactive effects of increasing temperature and lower pH were demonstrated. The combined effect of temperature

and pH on the heart rates of *A. papillosa* and *D. frondosus* was neutral. Heart rates responded to increasing temperature the same regardless of pH (Fig. 1.2a & d). Due to the lack of an interactive effect, but having a slower heart rate in the lower pH across all temperatures, the combined effect for *C. gymnota*, *P. dendritica*, and *O. bilamellata* was negatively additive (Fig. 1.2c, e, & f). The only significant interaction demonstrated was in *F. verrucosa* and was antagonistic. In the lower pH at the lower temperatures, heart rate slowed, but as temperatures increased in the lower pH, heart rate increased to match that in the higher pH (Fig 1.2b). Because both heart rate and metabolism are linked in gastropods (Bruning et al. 2013), it is possible that when heart rate slowed, metabolism may also have slowed. Thus, ocean acidification may be causing metabolic depression in *C. gymnota, P. dendritica,* and *O. bilamellata*. A trend seen in other mollusks (Parker et al. 2013).

Due to the varied combined effect of increasing temperature and lower pH, temperature seems to be the most immediate threat to the survival of these species in the GOM. Higher temperatures may possibly hasten reproductive periods (Parker et al. 2013), causing seasonal shifts, phenomena already being seen in the GOM (A. Kuzirian, Marine Biological Laboratory, L. Harris, University of New Hampshire, W. Lambert, Framingham State University, pers. comm., pers. obs.). My study only focused on adults, but knowledge about larval development and mortality under altered environmental conditions may be key in predicting whether sea slugs will continue to inhabit the GOM. Numerous studies on gastropods have already demonstrated the extreme sensitivity of larvae to changes in temperature, salinity, acidification, and ultraviolet radiation (Runnström, 1927; Przeslawski, et al. 2005; Carr & Podolsky, 2016). The species in this study may be especially susceptible to acidification because they have calcium carbonate

shells as larvae (Ross et al. 2011). Additionally, larval studies have shown that development times are shortened by temperature increases for many marine species (O'Conner et al. 2007), including for the nudibranchs *D. fronsosus* (Watt and Aiken, 2003) and *A. proxima* (Thompson, 1958). With the general lifespan of sea slugs being a year or less (Miller, 1962) coupled with the inability of the adults to relocate quickly, made worse by a shortened planktonic life stage, evolutionary changes may be seen in a few years by those that are able to adapt to the changing climate or species may simply disappear from the GOM.

CHAPTER 2: THE EFFECT OF TEMPERATURE ON GROWTH RATES OF *FLABELLINA VERRUCOSA* & *DENDRONOTUS FRONDOSUS*

INTRODUCTION

The effects of temperature on biological processes (Hochachka & Somero, 1994) are among the primary determinants in habitat selection of an organism (Somero, 2002). Although individuals survive and thrive within species-specific temperature ranges, extreme temperatures can be lethal. The temperature increases in ocean waters have already caused marine species to shift polewards 20 times faster than those on land (Sagarin et al. 1999; Sorte et al. 2010). However, not all species can move quickly or long distances as adults and must rely on larval dispersal as a means to shift geographic range. Laboratory larval development studies focused on the effects of temperature have shown repeatedly that warmer water leads to shortened larval stages (asteroids: Hoegh-Guldberg & Pearse, 1995; crabs: Dawirs, 1985; nudibranchs: Runnström, 1927, Thompson, 1958, Dehnel & Kong, 1979, and Watt & Aiken, 2003; snails: Scheltema, 1967), which leads to decreased dispersal (O'Connor et al. 2007). With warmer sea water temperatures reducing time in the water column, shifting a species range takes longer.

In addition to accelerated larval development, warmer waters also increase adult growth rates and result in species reproductively maturing at a smaller size (Daufresne et al. 2009). In nearly 80% of growth-rate studies reviewed by Atkinson (1994), individuals matured at smaller sizes in warmer waters, whereas larger adult individuals were found in colder waters, a finding supported by Partridge & French (1996). Maturing earlier in warmer waters may benefit earlier reproduction (Thompson, 1958), but larger body size often means more energy can be put into

reproduction (Kuzirian et al. 1999; Lambert et al. 2000) and provides better protection from predators (Harris, 1986; Brey, 1999).

Species with higher upper thermal tolerance ranges can be found over wide geographic and temperature ranges, allowing for differing impacts on development. *Flabellina verrucosa* and *Dendronotus frondosus* are two cold-water nudibranchs found on both sides of the Atlantic and Arctic Oceans from intertidal areas to 400m deep (Thompson & Brown, 1984). However, *D. frondosus* has a lower thermal tolerance range (16^{o} to 20^{o}C) than *F. verrucosa* (20^{o} to 24^{o}C) (Chapter 1; Fig. 1.1). The nudibranchs are also taxonomically placed in different families: Dendronotidae for *D. frondosus* and the aeolid family Flabellinidae for *F. verrucosa*, but both are specialist feeders on hydroids. In the Gulf of Maine (GOM), both species are commonly found together feeding on *Ectopleura crocea* (pers. obs.). Although they consume the same prey, *D. frondosus* can grow more than three times larger than *F. verrucosa* (Morris et al. 1980; Thompson & Brown, 1984). Both nudibranchs are used in a variety of studies (see Appendix A), with neurobiological research being a primary focus for *D. frondosus* (Newcomb, 2006; Newcomb et al. 2006; Newcomb & Katz, 2007) and predator-prey interactions for *F. verrucosa* (Tullrot, 1994), including control of jellyfish populations (Hernroth & Gröndahl, 1985; Östman, 1997).

Prior to maturation, growth and metabolism are the primary energy expenditures (Schmidt-Nielsen, 1997). Because temperature directly affects the rate of metabolism (Gillooly et al. 2001; Brown et al. 2004), it may also influence growth rate. Although larval development and temperature have been studied for a variety of nudibranchs (Runnström, 1927; Dehnel &

Kong, 1979; Watt & Aiken, 2003), the focus of growth studies on adult sea slugs has been on the effect of diet (Carefoot, 1967; Hall, 1984; Hall & Todd, 1986; Folino, 1993; Lambert et al. 2016), not temperature. The primary purpose of this study was to determine how temperature impacts the growth rates of the nudibranchs, *F. verrucosa* and *D. frondosus*. The secondary purpose was to determine if the heart rates of *F. verrucosa* and *D. frondosus* over a range of temperatures (4^{o}, 10^{o}, and 16^{o}C) would remain the same over a period of two months. Specifically, the study was aimed at determining if the theory of faster growth rate in warmer waters was supported.

METHODOLOGY

Collection & Maintenance of Animals

Small *Dendronotus frondosus* (1.7 ± 0.1cm; 0.1 ± 0.02g) and *Flabellina verrucosa* (1.8 ± 0.1cm; 0.1 ± 0.02g) were collected from December through June 2019, primarily subtidally, from the UNH Marine Research Pier, located at the Judd Gregg Marine Research Complex (43.07, -70.71) and the floating docks of Wentworth Marina (43.06, -70.73), both in New Castle, New Hampshire. The study was performed in a temperature controlled aquatic room in Spaulding Hall on the main Durham campus, University of New Hampshire. Upon collection, nudibranchs were placed in individual, aerated 1000mL Erlenmeyer flasks, randomly placed in a water bath (10 ^{o} or 16^{o}C) or a temperature-controlled refrigerator set to 4^{o}C and allowed to acclimate for one week prior to the start of a trial. The average water bath and refrigerator temperatures (^{o}C) were obtained from Onset® HOBO water temperature Pro v2 data loggers (U22-001). To determine the average refrigerator temperature, the HOBO logger was immersed in a beaker of salt water to mimic the conditions in the flasks.

Temperature & Growth Rate

Between December and July 2019, growth rates (g/week) of individual nudibranchs, *D. frondosus* (n=7) and *F. verrucosa* (n=7), were measured over an 8 week period at temperatures of 4°, 10°C, and 16°C. Damp weight (g) and length (cm) were recorded weekly. Length of each nudibranch was obtained using a ruler set alongside the relaxed, extended body of the nudibranch and damp weight was determined using a Carolina® 150g electronic balance (Model: SLB152-US). Prior to weighing, animals were blotted on a clean cloth to remove excess water. Two weight readings were taken and averaged to ensure accuracy.

For each trial, flasks were aerated and water was changed weekly. Salinity was recorded at the start and end of each week using a Cole-Parmer® refractometer. Each nudibranch was fed 100-250 polyps of *Ectopleura crocea* two to three times per week depending on species. Prior to feeding, debris and other organisms were removed from the stalks of the hydroids and polyps were counted. To obtain damp weights (g) for the hydroids, they were left on paper towels for approximately 30 minutes before being weighed, using a Carolina® 150g electronic balance (Model: SLB152-US). At the weekly water change, remaining uneaten hydroids from the feedings, were again left on paper towels for 30 minutes and weighed. Differences between the two weights were recorded as amount of food consumed per week (g/week). To asses growth rates, length (cm) and weight (g) of each nudibranch was taken during the weekly water change. The heart rate (beats per minute: BPM) of the nudibranch was also recorded at the initial phase of each trial (weeks 1, 2, & 3) and at the ending phase (weeks 6, 7, & 8). All nudibranchs were released into the local environment at the end of the study.

Statistical Analysis

Growth rates were found from weight differences (g/week) from the average weekly damp weight measurements. Due to a failure to meet the assumption of normally distributed data, needed to run an ANOVA, a Kruskal-Wallis non-parametric test was used to determine if temperature affected growth rates for *F. verrucosa* and pairwise comparisons were made using the Wilcoxon rank sum test. For *D. frondosus*, an independent two-tailed t-test was performed between growth rates in the 4º and 10ºC treatments, because no individuals survived past the third week in the 16ºC treatment. To determine if heart rates remained the same throughout the trial period, paired t-tests were run for each temperature group (4ºC, 10ºC, & 16ºC) and a one-way ANOVA with Tukey's mean separation test was used to determine significant differences between temperatures. To test if food consumption impacted growth rates at the different temperatures, a one-way ANOVA with individual subjects as a blocking variable was used. Statistical analyses were performed in R, a statistical programming package (R Core Team, 2018) and figures were created using ggplot2 (Wickham, 2009).

RESULTS

Observations

Temperature in each of the systems (water bath/refrigerator) fluctuated slightly during the experiment. For the 4ºC treatment, the average temperature was 3.5 ± 1.1ºC. For the 10ºC treatment, the average was 10.5 ± 0.8ºC and for the 16ºC treatment, the average was 15.9 ± 0.6ºC. The initial salinity of 30ppt did not increase more than 4ppt per week, with an average increase of 1ppt.

Flabellina verrucosa

Of the 31 *F. verrucosa* used during the study, only 17 nudibranchs survived the entire 8 weeks. The only treatment in which all 7 *F. verrucosa* completed their trials was 4°C. At 10°C, 6 of 10 nudibranchs survived the 8 weeks and only 4 of 14 nudibranchs survived the 8 weeks at 16°C. For the 4 trials that ended early in the 10°C treatment, one nudibranch crawled out of the flask and was lost in the system (week 3), 2 nudibranchs disintegrated (week 3 & week 6), and one individual was found dead, curled on its side with the gonopore extruded (week 5). For the 10 nudibranchs lost early in the 16°C water bath, 5 nudibranchs did not survive the first week (2 nudibranchs escaped the flask, 2 nudibranchs disintegrated, and one nudibranch was found dead, looking as though it had been attacked by the *E. crocea*), 2 nudibranchs escaped the flask at week 2, one nudibranch was found dead at week 4, and another 2 nudibranchs were dead at week 5. In the 4°C treatment, two individuals appear to have been attacked by the hydroids within the first two weeks and survived but did not regenerate the lost cerata over the remaining 6 weeks.

From the 17 nudibranchs that survived the entire eight weeks, it was determined that heart rates remained the same at each respective temperature (4°C: t_6=0.870, p=0.418; 10°C: t_5=-1.588, p=0.173; 16°C: t_3=0.309, p=0.778) throughout the study, but increased linearly with temperature ($F_{2, 14}$=117.4, p<0.001, R^2=0. 944, Fig. 2.1). Temperature, however, did not affect food consumption ($F_{2, 119}$=0.937, p=0.395) nor did the individual nudibranch ($F_{14, 119}$=1.524, p=0.113). Roughly a gram of *E. crocea* was consumed per week per individual (4°C: 0.9 ± 0.4 g/week; 10°C: 1.0 ± 0.6 g/week; 16°C: 1.0 ± 0.6 g/week).

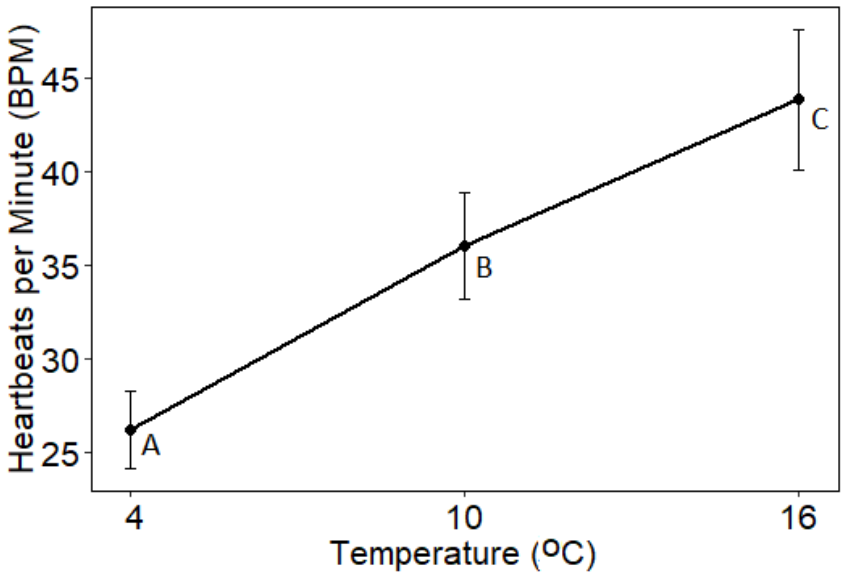

Figure 2.1: Heart Rates & Temperature of *Flabellina verrucosa*. Average heart rates (BPM) of *Flabellina verrucosa* over increasing temperature (°C). Bars indicate two standard errors away from the mean and letters represent significance between temperatures as assigned by the Tukey test.

The Kruskal-Wallis test showed that *F. verrucosa* grew fastest (0.05 ± 0.09 g/week) at 10°C (X^2_2=8.548, p=0.014, Fig. 2.2). Additionally, the average percent increase in body mass in the 10°C treatment (18.2%) was more than 2.5 times greater than at 4°C and more than 4.5 times the increase in body mass at 16°C (Table 2.1). Although growth rates fluctuated over the eight weeks, with occasional negative growth rates, all mean growth rates were positive (at 4°C: 0.02 ± 0.03 g/week and at 16°C: 0.01 ± 0.06 g/week; Table 2.1). The growth rates at 4° and 16°C did not differ significantly (p=0.190).

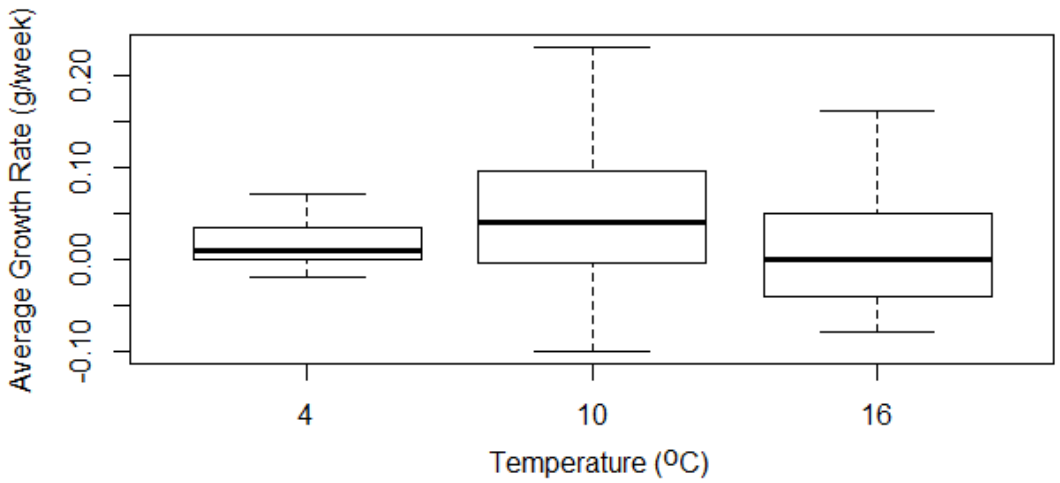

Figure 2.2: Average Weekly Growth Rates at 4°, 10°, & 16°C for *Flabellina verrucosa*. Average growth rates (g/week) for *Flabellina verrucosa* over increasing temperature (°C).

Dendronotus frondosus

Of the 36 *D. frondosus* used, only 9 nudibranchs survived the entire 8 weeks. At 16 °C, none of the 14 nudibranchs used survived: 2 nudibranchs disintegrated within the first week, 5 nudibranchs were found dead at the end of week 2, 6 nudibranchs disintegrated by the end of week 3, and one nudibranch died the first day of week 4. Hence, for statistical analysis, heart rate, food consumption, and growth rate data were used from the 7 individuals that survived for 3 weeks. Only 4 of the 11 nudibranchs used in the 10°C treatment survived to the end of week 8: one nudibranch died within the first week, 2 nudibranchs died by the end of week 2, 2 nudibranchs disintegrated by the end of week 3, one nudibranch disintegrated by the end of week 4, and one nudibranch escaped the flask during week 6. Five of the 11 nudibranchs used in the 4°C treatment survived the entire 8 weeks. Of the 6 nudibranchs that did not survive, 3 nudibranchs died within the first week, 2 nudibranchs died during week 2, and one nudibranch died during week 7. All individuals that died early appeared unhealthy when checked during the water change the week prior to death, typically having stunted or misshapen cerata.

Although heart rates in the 4ºC treatment lowered significantly from 28.8 ± 2.5 BPM to 24.1 ± 2.4 BPM over the 8 weeks (t_4=3.796, p=0.019), average heart rate remained the same throughout the 8 weeks in the 10ºC treatment (t_3=2.205, p=0.115). Heart rates also remained the same for the first two weeks of the study at 16ºC (t_6=-0.159, p=0.879). Starting heart rates from the first two weeks of the trials increased linearly from 4º to 10ºC ($F_{2, 13}$=33.06; p<0.001, R^2=0.836), but the curve leveled off between 10º and 16ºC (Fig. 2.3). Food consumption was not affected by temperature ($F_{2, 71}$=0.782, p=0.461) nor by individual nudibranch ($F_{13, 71}$=0.641, p=0.812). Roughly 0.7g of hydroid prey was consumed per week per nudibranch in each treatment (4ºC: 0.7 ± 0.3 g/week; 10ºC: 0.8 ± 0.3 g/week; 16ºC: 0.7 ± 0.3 g/week).

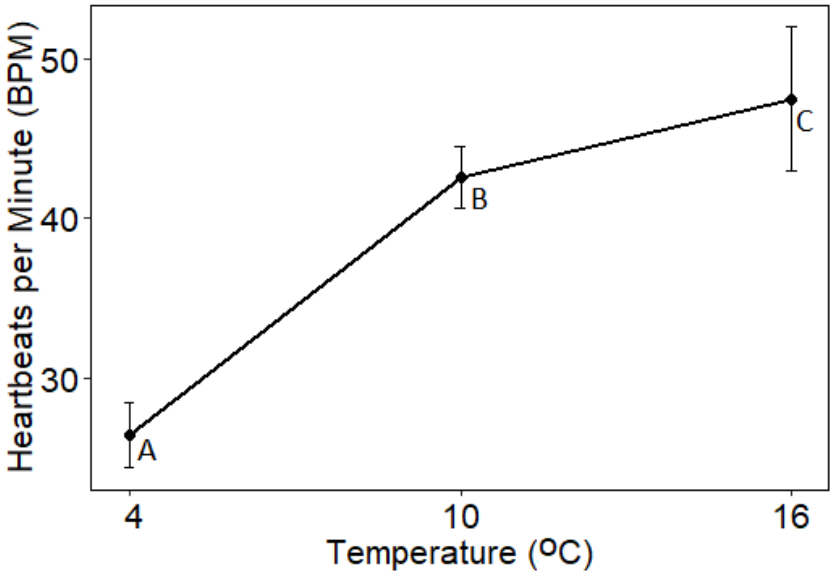

Figure 2.3: Heart Rates & Temperature of *Dendronotus frondosus*. Average heart rates (BPM) of *Dendronotus frondosus* over increasing temperature (ºC). Bars indicate two standard errors away from the mean and letters represent significance between temperatures as assigned by the Tukey test.

Growth rate in the 10°C treatment (0.05 ± 0.15 g/week) was the only positive average weekly growth rate. However, it did not differ significantly from the average weekly growth rate in the 4°C treatment ($t_{32.03}$= -1.946, p=0.060, Fig. 2.4). Although the individual weekly growth rates at 4°C were occasionally positive, the overall growth rate was negative (-0.01 ± 0.02 g/week). For those individuals that survived the first three weeks in the 16°C treatment, either no weight change occurred or body mass was lost, yielding an overall negative growth rate (-0.02 ± 0.03 g/week). Over the first three weeks, nudibranchs lost an average of -27.1% body mass in the 16°C treatment (Table 2.1).

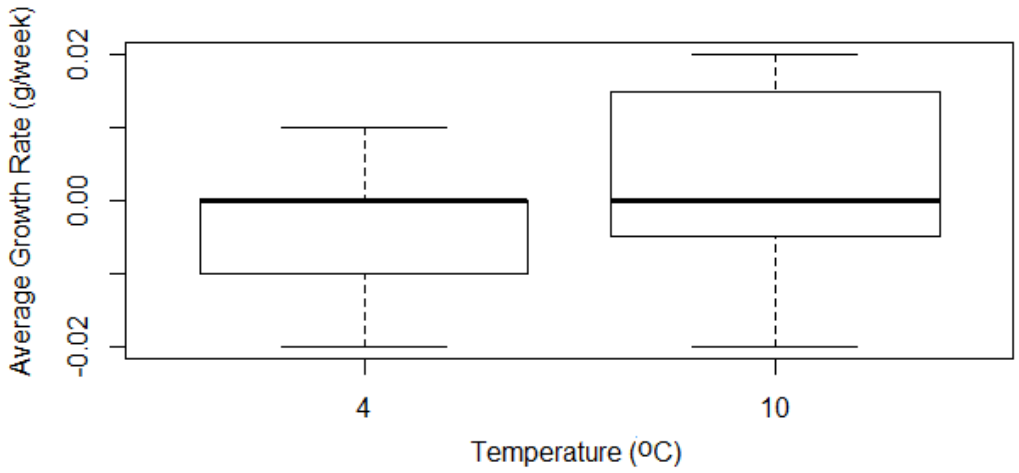

Figure 2.4: Average Weekly Growth Rates at 4° & 10°C for *Dendronotus frondosus*.
Average growth rates (g/week) for *Dendronotus frondosus* over increasing temperature (°C).

Table 2.1: Growth rate data of *Flabellina verrucosa* & *Dendronotus frondosus* in grams per week at different temperatures with percent increase per week over 8 weeks.
*Growth rate data for *D. frondosus* in the 16ºC treatment covered only the first 3 weeks.

		Treatment		
		4ºC	**10ºC**	**16ºC**
Flabellina verruocsa	**N**	7	6	4
	Maximum	0.100	0.330	0.160
	Mean	0.015 ± 0.031	0.048 ± 0.088	0.005 ± 0.058
	Minimum	-0.070	-0.100	-0.080
	% Increase	6.98%	18.2%	4.24%
Dendronotus frondosus	**N**	5	4	7*
	Maximum	0.010	0.850	0.000
	Mean	-0.009 ± 0.022	0.045 ± 0.154	-0.021 ± 0.025
	Minimum	-0.100	-0.040	-0.090
	% Increase	-2.48%	3.19%	-27.1%

DISCUSSION

The principle purpose of this study was to determine if temperature impacted growth rate for *F. verrucosa* and *D. frondosus*. As expected, temperature did influence growth rates for both species (Fig. 2.2 & 2.4; Table 2.1), but the association was non-linear, a finding consistent with other benthic invertebrates (see review Brey, 1999). In addition to growth rates, heart rates were monitored over the two-month trial and found to remain approximately the same over the entire eight weeks at each specific temperature, as was found in the long-term temperature and heart rate studies conducted by Defur & Magnum (1979) on a variety of marine invertebrates.

Consistent with the findings in Chapter 1, the heart rates of *F. verrucosa* increased linearly from 4º to 16ºC and average heart rates at specific temperatures were approximately the same. The heart rates for *D. frondosus*, however, varied slightly. The linear trend that was exhibited in Chapter 1 from 4º to 16ºC for *D. frondosus*, did not continue after 10ºC in the current study. The leveling off of heart rate at the higher temperatures may be due to the average

heart rates in the current study being approximately 6 BPM faster at 10°C (42.6 ± 1.9 BPM) than in the original study. However, the average heart rates between the two studies were roughly comparable at 16°C (47.4 ± 4.5 BPM). Differences in acclimation times might have been a factor. Nudibranchs were left to acclimate for a week in the growth rate study, as opposed to only 15 minutes in the thermal tolerance study (Chapter 1). It is probable the maximum heart rate for *D. frondosus* is approximately 50 BPM and animals are unable to sustain the accelerated heart rate for an extended period of time. This may be why all animals died by the end of the third week in the 16°C treatment. Another factor to be considered was that *D. frondosus* were adults in the earlier study and more than 10 times larger than the juveniles used in the current study. Larger body size typically means slower basic metabolism rates (Schmidt-Nielsen, 1997).

Unlike heart rates, growth rates were not linear and far more variable. Growth rates at 10°C were fastest but also had the greatest variance for both species (Fig. 2.2 & 2.4). The average weekly growth rate at 10°C for both species was approximately 0.05 g/week and animals reared at that temperature grew the largest. Although, weekly growth rates were slower at higher (16°C) and lower (4°C) temperatures for *F. verrucosa* than at the mid-temperature point (10°C), they were still positive, unlike that of *D. frondosus,* which were negative. In an earlier study by Harris (unpublished data), mean growth rate of *Aeolidia papillosa* was also found to be slightly greater at 10°C than at 12°C, but the spread of the data was wide and overlapping, so no significant difference was noted. Additionally, Harris (unpublished data) found that *A. papillosa* at lower temperatures (2° and 5°C) grew very little or not at all, but had a higher survivability than at higher temperatures.

Slower growth rates at the lower temperature were to be expected (Daufresne et al. 2009), but the slower growth rates at the higher temperature and higher mortality seen in this study may be an indication that more energy was used to maintain a higher metabolism, rather than increasing somatic growth (Schmidt-Nielsen, 1997). Once *D. frondosus* were placed into the 16^{o}C water bath, the animals began losing mass (Table 2.1) and only half were able to survive for three full weeks before they died. On the other hand, the weekly growth rates in the 4^{o}C treatment for *D. frondosus* were far more variable: all but two nudibranchs increased in mass over at least one week. All individuals collected in this study were found in warmer ambient water (~ 8^{o} to 12^{o}C) and collected in late spring. They may have been nearing maturity at a much smaller adult size, a trend noted in warmer waters (Daufresne et al. 2009). Thus, they may have been putting less energy into somatic growth and more into reproduction.

Several factors may have played a role in the outcome of this study, the most important being the numerous power-outages that occurred during the trials. Although the average temperatures stayed approximately at the set treatment temperatures (4^{o}, 10^{o}, & 16^{o}C), temperature spikes occurred a few times, especially in the refrigerator which reached a high of 10.6^{o}C one day. When analyzing the data, deaths and heat spikes were examined and no deaths concurred with the spikes, but the heat spikes may have impacted growth. Another possible confounding factor was that individuals used in this study were found later in the year than usual (L. Harris, University of New Hampshire, pers. comm.; pers. obs.). Although one *F. verrucosa* was found in December and two *F. verrucosa* in January, it was not until March that more than two nudibranchs could be found at a time on a collecting trip. Additionally, it was not until April that *D. frondosus* appeared. By spring, the animals should have been adult size and putting effort

into reproduction (Thompson & Brown, 1984), not somatic growth. Additionally, several *F. verrucosa* used in the study were found feeding on tunicates or a different hydroid, possibly *Obelia* spp. or *Sarsia tubulosa*, but were fed *E. crocea* in the study. Changing their diet may have initially shifted their energy away from growth to food acclimation. When *A. papillosa* change anemone prey species, the mucus used to neutralize the nematocysts of their prey changes too, but needs approximately two weeks to do so (Greenwood et al. 2004).

Along with temperature, body size is one of the factors that dictates metabolism (Gillooly et al. 2001) and other physiologic functions (Schmidt-Nielsen, 1997), as well as predator-prey interactions. Harris (1986) found that the body size of prey choice is directly related to the size of the individual *A. papillosa.* Lambert (1991) found that not only does size affect how some nudibranchs feed (*D. frondosus*), but also where they choose to locate themselves within a hydroid colony (*Tergipes tergipes*). If size determines rate of food consumption and nudibranchs can regulate prey populations, such as *F. verrucosa* feeding on *Aurelia aurita* (Hernroth & Gröndahl, 1985; Östman, 1997), then size plays a part in population dynamics. Understanding the role temperature has on growth and metabolism can help determine how these animals will react to changing environments and how that reaction will influence trophic interactions.

CHAPTER 3: LARVAL DEVELOPMENT OF *AEOLIDIA PAPILLOSA*

INTRODUCTION

Aeolidia papillosa is a large, semelparous, hermaphroditic, aeolid nudibranch found mainly in cold waters of the northern hemisphere (Thompson & Brown, 1984; Shine, 2012). Although hermaphroditic, they are not able to self-fertilize and act simultaneously as male and female (Longley and Longley, 1984). Being specialist predators of anemones, *A. papillosa* typically prefer *Metridium senile* (Stehower, 1952) and are often found next to *M. senile* in the Gulf of Maine (GOM) (pers. obs.). Due to having large neurons (Sakharov, 1962), *A. papillosa* have been popular subjects in neurobiology studies (Farber & Grinvald, 1983; Watson & Willows, 1992; Baltzley & Lohmann, 2008), as well as a variety of other studies (see Appendix A). *Aeolidia papillosa* live for approximately one year, with seasonal spawning from winter through spring (Thompson & Brown, 1984) and have only been collected in the wild. Having access to cultured specimens would allow better experimental control and access to a greater number of specimens for studies. Sadly, previous attempts to culture *A. papillosa* have not been successful (L. Harris, University of New Hampshire, W. Lambert, Framingham State University, pers. comm.) and no publications have reported protocols for lab reared specimens.

In general, culturing opisthobranch species has been a sensitive and often unsuccessful venture (Kempf & Willows, 1977; L. Harris, University of New Hampshire, W. Lambert, Framingham State University, pers. comm.). However, culturing laboratory animals not only allows for generational studies, but also compensates for times when local species are difficult to

collect. Since 2015, *A. papillosa* have become scarce in areas of the GOM where previously they were very common (Harris, 1986; L. Harris, University of New Hampshire, pers. comm.). The supposition is that the decline is likely due to the warming of the GOM (Pershing et al. 2015). Successfully culturing *A. papillosa* would promote further research into studying neural network configurations (W. Watson, University of New Hampshire, pers. comm.), genomics, generational studies of adaptability to climate change, and other biomedical research topics. The purpose of this study was to culture *A. papillosa* in the laboratory and to determine if adult size of *A. papillosa* impacted the number of embryos per capsule, as well as the rate of larval growth.

METHODOLOGY

Collection & Maintenance of Animals

From February to March 2017, adult *Aeolidia papillosa* were collected intertidally and subtidally from Cape Neddick, York, Maine (43.16, -70.59) and the UNH Marine Research Pier, located at the Judd Gregg Marine Research Complex in New Castle, New Hampshire (43.07, -70.71). Adult *A. papillosa* were kept in a direct flow, filtered seawater system at the Coastal Marine Laboratory (CML), a part of the Judd Gregg Marine Research Complex. The nudibranchs were housed separately, in mesh plastic organizers to allow water flow-through. All nudibranchs were fed *Metridium senile* of appropriate size (smaller than the nudibranch – see Harris, 1986) *ad libitum*. Egg masses/larvae were kept in a 12^{o}C cold room located in Rudman Hall on the UNH Durham campus. Seawater in the cultures was maintained at a temperature of 11.9 ± 0.3^{o}C, with a salinity of 35.8 ± 1.2ppt and a pH of 7.8. Seawater was obtained from CML and autoclaved prior to use. Deionized water was added to the autoclaved seawater to adjust salinity. Seawater was filtered through 22µm mesh prior to being autoclaved in a loosely capped, 10L Nalgene jug.

Culturing

To facilitate egg laying, two fecund nudibranchs were randomly paired and allowed to mate over 4 to 8 hours, before being separated. Longley and Longley (1984) found that mating usually occurs within 30 minutes and consists of head-to-head apposition followed by the pair moving along their right edge/underside until the gonopores are aligned. At this time, both animals act simultaneously as sperm donors and recipients (Longley and Longley, 1984). Because *A. papillosa* can store sperm (Hyman, 1967), one mating often produced multiple fertile egg masses.

Enclosures were checked every 2-3 days and new egg masses were gently collected using a flat-edged blade. The parent nudibranch and date of collection were recorded. Egg masses were placed into individual baggies and labeled to identify the parent nudibranch prior to returning to the UNH main campus, where masses were transferred to individually labeled, sterilized 1000mL glass Erlenmeyer flasks filled with autoclaved seawater. All seawater used for culturing was treated with a 0.1% solution of Ethylenediaminetetraacetic (EDTA) as a chelating agent to neutralize dissolved metals in the water. The EDTA solution consisted of 50mg of EDTA dissolved in 50mL of deionized water and left to dissolve for 24 hours. For developing eggs masses, 0.25mL of EDTA solution was used per liter of seawater; for larvae, 5mL of EDTA solution was used per liter. EDTA solution was added to the autoclaved seawater at least 24 hours prior to the addition of embryos or larvae (Kuzirian et al. 1999). An air pump connected to a sterilized glass pipette was used to gently aerate the seawater and Parafilm was placed over the top of the flask to reduce evaporation and contamination.

Water was changed weekly for all cultures to minimize handling of fragile embryos/larvae. During water changes, cultures were strained through sterilized 22µm filters. While the embryos were on the filter, a 200µL micropipette was used to remove approximately 100µL of wet embryos/veligers or a 0.5cm piece of the egg mass to check the health of the embryos and take morphometric measurements (embryo diameter/veliger shell length). Any debris on the filter was removed using a plastic pipette and the embryos were washed back into a freshly sterilized 1000mL Erlenmeyer flask. Glass pipettes were re-sterilized between changes using a Bunsen burner.

Health was assessed by the movement of larvae, presence of parasites, and integrity of developing embryos/larvae (i.e. cracked, disintegrating). Three drops of culture containing wet embryos/veligers were placed on glass slides and covered with a cover slip. The slide was examined with an Olympus BH-2 trinocular compound microscope using a creeping line pattern (moving the slide from left to right while scanning up then down alternatively). Twenty embryos/veligers were measured, using every third one. For developing embryos, diameter was measured. For veligers, shell length was measured using the software program cellSens (Olympus Corporation) by drawing a line from the outside tip of the shell opening to the furthest edge of the other side of the shell.

Once larvae hatched, prior to changing the culture, glass pipettes were removed, and dead larvae settled to the bottom of the flask, whereas live larvae remained in the water column. After approximately one hour, larval density was determined by removing 1mL from the center of the flask using a 500µL micropipette into a glass vial. The sample was fixed by adding a couple

drops of formalin. The vial was covered and shaken before removing <3µL by pipette. The subsample was then added to fill the wells of a hemocytometer. Larvae counts were taken from the four large corner squares and the middle square. Using the Android app Cells Calculator (P. Sarapukdee, 2015), cells/mL were calculated. An average of five counts was used to verify that larval density did not exceed 3000 larvae/L.

Following larval density calculations, cultures were filtered through a sterilized 22µm mesh filter, leaving approximately 150mL of the original source culture at the bottom of the flask to minimize transfer of dead larvae. While on the mesh filter, larvae were rinsed with autoclaved seawater, a 200µL micropipette was used to remove 100µL of larvae and a plastic pipette was used to remove any debris. Larvae were then washed back into a sterilized 1000mL roller bottle. Equal parts of *Isochrysis galbana* (clone T-*Iso.*) (7500 cells/mL) and *Dunaliella salina* (7500 cells/mL) were added to the bottles for food. To determine the densities of the algal cultures, the hemocytometer method described above was used with two changes: first, the tube with the algae was swirled prior to the removal of 1mL and second, an average was taken from two counts.

Once the algae were added to the larval culture, the roller bottle was slowly topped off with sterile seawater until a dome of water formed at the top. Three layers of Parafilm were then added to remove any air bubbles and a string was tied around the neck of the bottle. Bottles were placed on a Lab-line Cel-Gro rotator and rotated at approximately 1.5 rpm. A clip-light was placed over the roller bottle and left on a 24-hour light cycle to stimulate algal photosynthesis/growth and oxygen production for larvae until the completion of a trial.

To trigger metamorphosis, after three weeks, small bits of anemone, *M. senile* were rinsed with deionized water and placed in the roller bottle along with the algal culture. A trial was considered completed upon successful larval metamorphosis or when all embryos/larvae were dead.

Measurements & Egg Capsule Capacity

Monthly, the length (cm) of each adult *A. papillosa* (n=9) was obtained using a ruler placed alongside the relaxed, extended body of the sea slug. Damp weights (g) of the animals were determined using a Carolina® 150g electronic balance (Model: SLB152-US).

Upon collection of an egg mass, the number of embryos per egg capsule was averaged from 20 randomly selected egg capsules within a 0.5cm segment of the egg mass. The segment was placed on a glass slide with cover slip and examined under an Olympus BH-2 Trinocular microscope (10x magnification). Moving in a creeping line pattern, embryo counts were taken from every third egg capsule.

In addition to embryo counts, embryo diameter (µm) was measured using cellSens, an Olympus software program linked to the Olympus BH-2 Trinocular microscope that calculates the length of a line (µm) drawn between two points from a live video feed. The average embryo diameter was determined from 20 embryos. The embryo nearest to the center of each counted capsule was measured.

Algal Cultures

Cultures of both *Dunaliella salina* and *Isochrysis galbana* (clone T-*Iso.*) were maintained to feed the larvae. The close T- *Iso. galbana* has repeatedly been found to be a successful microalgal diet for many invertebrates (Gireesh & Gopinathan, 2008; Widowati et al. 2017). *Dunaliella salina* was used due to its use in previous attempts of rearing *A. papillosa* (L. Harris, University of New Hampshire, pers. comm.). The larval cultures were fed equal parts of each microalgal diet.

Algal cultures were kept in a room temperature laboratory in Spaulding Hall on the UNH Durham campus. Cultures were kept in sterilized glass culture tubes fitted with two-holed rubber stoppers: one for a glass pouring spout and another for glass tubing. Cultures were aerated via the glass tubing that ran the length of the culture tube and a 0.2µm sterile venting filter disc (Omicron, Klaus, Austria) was affixed between the glass tubing and air pump to reduce the chance of contamination. Algal cultures were kept in a room temperature water bath and exposed to a halogen light source on a 24-hour light cycle.

Dunaliella salina, already in culture, required deionized water and Guillard's f/2 culture media to be added occasionally to maintain salinity. The starting cultures of the clone T-*Iso. galbana* were obtained from AlgaGen (Vero Beach, FL). The starting algal culture was inoculated with Guillard's f/2 media: 10mL algal culture to 250mL Guillard's f/2 media. Standard sterile inoculation techniques were used.

Statistical Analysis

A regression analysis was performed to determine if adult nudibranch size impacted the number of embryos per capsule. Eleven egg masses from 7 of the 9 reproducing adult nudibranchs in the study were used in this analysis. Embryo counts were included only if the interval between date of egg mass collection and date of adult measurement was within five days.

Only embryos laid and measured within a day of oviposition were included as the initial embryo size (n=26). Additionally, a regression analysis was run to determine whether adult weight correlated with embryo diameter. To determine rate of embryo/veliger growth, only the measured egg masses that survived two weeks were used (n=24). From those egg masses, only the cultures that survived to three weeks (n=7) were included in further analyses. Statistical analyses were performed in Microsoft® Office Excel (2007) and R (R Core Team, 2018). Figures were created using ggplot2 (Wickham, 2009).

RESULTS

Observations

Each of the nine *A. papillosa* (5.4 $\pm$ 2.4cm; 8.9 $\pm$ 7.9g) produced a minimum of two egg masses, with an average of 4.1 $\pm$ 2.2 egg masses laid per individual; one nudibranch produced a total of 8 egg masses while in captivity. It is possible that some of the adult nudibranchs may have spawned prior to collection. Veligers hatched from the capsules at approximately a week after oviposition, but often cultures were infested with ciliates. Unfortunately, none of the larvae metamorphosed and all adults died between mid-May to mid-June. Three of the cultures were

terminated after one week, 23 cultures were terminated after two weeks and 11 cultures were terminated after three weeks. Cultures were typically terminated (in descending order of occurrence) due to an infestation of ciliates and/or nematodes, an overabundance of disintegrating eggs, no live veligers, an infestation of turbellarians, an infestation of copepods (two cultures) and human error (dropped flask – one culture). Of the 11 cultures that were terminated after three weeks, four of the cultures were moved into a 16^{o}C cold room and all eggs disintegrated within a week.

Eggs Capsule Capacity

The number of embryos within an egg capsule ranged from 2 to 14 embryos and body mass of the adult nudibranch was indicative of a strong positive correlation with the number of embryos per capsule (R^2=0.821, $F_{1, 9}$=41.283, p<0.001; Fig. 3.1). Both adult length and mass were also positively correlated (R^2=0.759, $F_{1, 9}$=28.386, p<0.001). The number of embryos per capsule, however, did not depend on the order of egg mass laid ($F_{3, 9}$=0.801, p=0.524).

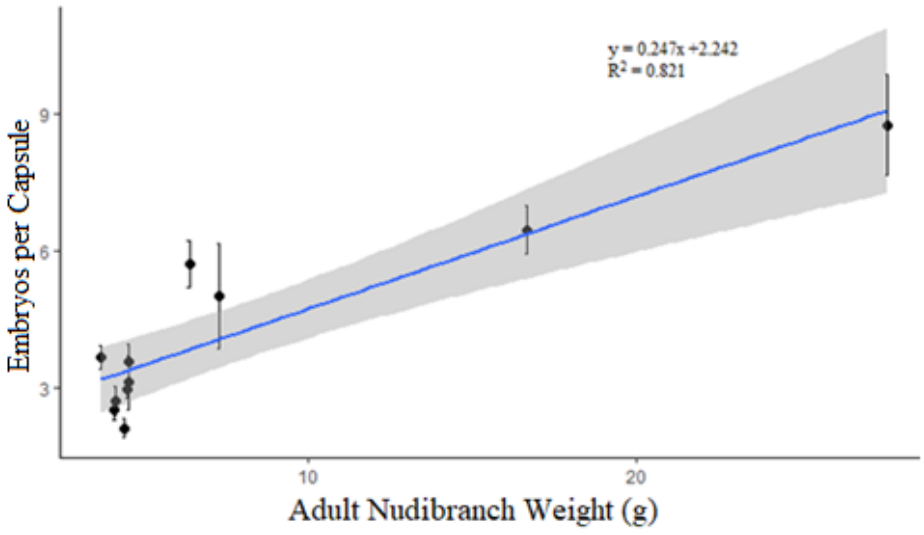

Figure 3.1: Relationship between Adult Nudibranch Weight & Number of Embryos per Capsule. Regression analysis of adult weight of *Aeolidia papillosa* plotted against number of embryos per capsule. The shaded area represents a 95% confidence interval.

Larval Growth

Using embryos at the morula stage from 26 egg masses collected and processed within a day of oviposition, the diameter of embryos was 93.6 ± 5.8µm. Unlike fecundity, adult nudibranch mass did not impact morula size (R^2=0.124, $F_{1, 24}$=3.407, p=0.077). Embryos/Veligers increased in size at an average rate of 6.3 ± 2.1µm/day over the first week, then slowed to 2.1 ± 2.3µm/day during the second week, with hatched, feeding (noted by green coloration in the digestion gland) veligers reaching 140.9 ± 16.1µm long.

DISCUSSION

Like previous culturing attempts of *A. papillosa* (L. Harris, University of New Hampshire, W. Lambert, Framingham State University, pers. comm.), my attempt was also unsuccessful. However, I was able to determine the influence of adult nudibranch mass on fecundity, as well as record embryo size and larval growth rate. The varying number of embryos per capsule (2 to 14 embryos) aligned with other published findings from animals collected off the coast of Washington State (Hurst, 1967: 3 to 19 embryos per capsule; Carr & Podolsky, 2016: 7 to 10 embryos per capsule). Because these studies did not report adult nudibranch mass or length, no comparisons could be made. On the other hand, the initial embryo diameter of *A. papillosa* (93.6 ± 5.8µm) was approximately 20µm larger than that of a Californian population (Williams, 1980), and nearly 10µm larger than embryos from egg masses collected off the Isle of Man (Miller, 1958), but the average embryo diameter in my study was smaller than the 112 to 115µm that Thompson & Brown (1984) found in another population from the British Isles. However, in another UK study, embryo diameter was found to be between 75 and 95µm which aligns closer to the diameters found in this study (Kress, 1971). The variations may be due to

population differences or may represent actual species differences, as *A. papillosa* is a cryptic species complex (Kienberger et al. 2016).

As with embryo diameter, mean veliger length was 30µm longer than veligers reared by Williams (1980). Hatching time, however, was approximately the same at 7 to 8 days (Williams, 1980). Previous studies did not present a growth rate for veligers, but with a growth rate of 6.3µm/day and an initial embryo diameter of 93.6µm, embryos grew approximately 50% after the first week, then slowed to just under a 10% per week once hatched. In all growth rate studies, temperature must be taken into account, as growth-rates are faster in warmer waters and individuals mature at a smaller size (Daufresne et al. 2009; Atkinson, 1994), whereas colder waters slow growth rate and individuals mature at a larger size (Partridge & French, 1996).

Although Williams (1980) and Hall & Todd (1986) found May to be the most productive month, in this study, *A. papillosa* were most prolific in April. Animals produced 21 egg masses in April, twice the number produced in May and five times more than late March. With the death of all adults in June, egg laying stopped. Their deaths were attributed to either contaminants present in the raw seawater used at CML or environmental heat spikes in mid-May to mid-June (Fig. 3.2). Although average monthly temperatures were approximately a degree cooler in 2017 than in 2016 (NEACOOS buoy at UNH CML in New Castle, New Hampshire), adult *A. papillosa* are rarely found during the summer months in the southern GOM (pers. obs.), as summer is usually the end of their life cycle.

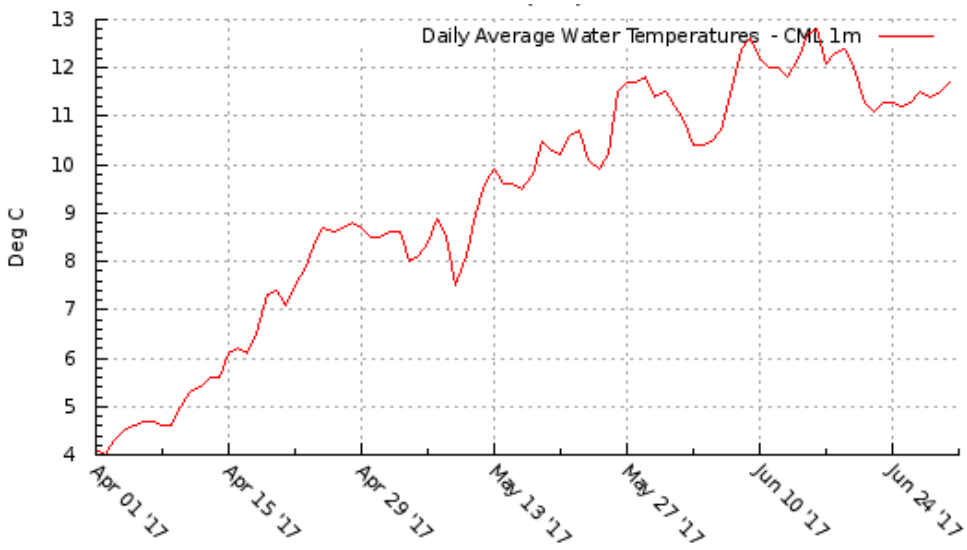

Figure 3.2: NERACOOS UNH Coastal Marine Laboratory Field Station Buoy Temperature Record for 2017. Seawater temperatures 1m below the surface at the UNH CML Field Station at the Judd Gregg Marine ResearchComplex in New Castle, New Hampshire between 1 April and 30 June 2017.

Hall & Todd (1986) suggest that temperature does not only limit life expectancy, but also the ability to reproduce. *Aeolidia papillosa* that did not reproduce survived longer than reproducing individuals (Hall & Todd, 1986). All animals in this study reproduced, and increased productivity as they approached senescence. The first and second egg masses deposited were typically laid 15.9 ± 5.4 days apart, whereas the last two were laid 7.7 ± 2.5 days apart. Increase in oviposition near the end of life was also seen with *Melibe leonina* (T. Duback, University of New Hampshire, pers. comm.) and with *Adalaria proxima* (Lambert et al. 2000).

Adult size of nudibranch had a clear impact on fecundity, but larval survival was not quantified, as no larvae metamorphosed and quantity is not a true measure of quality. To improve this study, both larval and algal density should be revised. Although Harrigan & Alkon (1978) used a larval density of 3000 larvae/L for *Hermissenda crassicornis*, Avila et al. (1997) found that densities of 1000-2000 larvae/L produced larger larvae. Additionally, the algal density

used in this study was the same as that for *H. crassicornis* (Avila et al. 1997) and, although both are aeolid nudibranchs of similar size (Harrigan & Alkon, 1978), algal densities may be species specific as may the algal species being used as food. *Isochrysis galbana* (clone T-*Iso*) was used in both studies rearing *H. crassicornis* as one half of the larval food, but Harrigan & Alkon (1978) used *Monochrysis luther* as the other half whereas Avila et al. (1997) used a variety of different algal diets, including *Rhodomonas salina*, *Dunaliella tertiolecta*, and *Phaeodactylum tricornutum*. However, Avila et al. (1997) found the combination of the clone T-*Iso. galbana* and *R. salina* to be the only diet under which larvae metamorphosed. Thus, different algal diets should be explored if another attempt is made to culture *A. papillosa.*

Other experimental improvements could include leaving egg masses to "harden" over two days prior to removing them to reduce the number of eggs lost during collection. If possible, keeping the nudibranchs in aerated beakers lined with plastic may allow for easier removal of egg masses (M. Litvaitis, University of New Hampshire, pers. comm.). An initial attempt was made to use this method, but a couple nudibranchs got rolled up in the plastic, so the plastic was removed. Other considerations to increase survival of larvae could include adding the antibiotic chloramphenicol to larval cultures to reduce bacterial growth. It has been used successfully and does not have major effects on larval growth (Harrigan & Alkon, 1978). To reduce ciliates, egg masses could be sanitized upon collection in a solution of 70% ethanol solution (A. Kuzirian, Marine Biological Laboratory, pers. comm.). Bubbling air into the larval culture via glass pipettes may have reduced the number of larvae caught in the water tension, but could have increased salinity. Instead, sprinkling cetyl alcohol flakes on the water surface could provide the same benefit without loss of salinity. Although roller bottles eliminated the need for caustic cetyl

alcohol flakes, air pockets were formed in the roller bottles from over oxygenation caused by the added algae. A Kreisel jellyfish aquaculture tank or algal culture tubes may solve the problem of air pockets. Another option could be to reduce the amount of algae added to the larval culture. Another possibility is that a particular dissolved organic compound was missing from the seawater necessary for metamorphosis. Additionally, egg masses and larvae should remain at the same temperature in which they were laid. Thus, adults should be kept in a controlled temperature system, ideally in the same cold room as the larvae being reared. Using some or all of the suggestions made should improve future attempts at culturing *A. papillosa*.

GENERAL CONCLUSION

Temperature was the primary influence on heart rate in all six species of sea slugs studied (*Aeolidia papillosa, Cuthona gymnota, Dendronotus frondosus, Flabellina verrucosa, Onchidoris bilamellata,* and *Placida dendritica*) from the Gulf of Maine (GOM). Temperature and heart rate were positively correlated in a linear fashion (Chapter 1). Both thermal tolerance and reaction to the added effect of lower seawater pH seemed to be associated with sea slug size. Larger sea slugs had lower heart rates and no reaction to the added effect of ocean acidification, whereas smaller sea slugs had faster heart rates, but lowered their heart rates uniformly at lower pH (Chapter 1). Additionally, heart rates for both *D. frondosus* and *F. verrucosa* remained approximately the same over an extended period of time (two months) at a set temperature (Chapter 2). Unlike heart rate, growth rate did not increase linearly with temperature. Both *D. frondosus* and *F. verrucosa* had slower growth rates in the lower and higher temperatures, whereas 10^{o}C appeared to be the optimal temperature for growth (Chapter 2).

The attempt at culturing *A. papillosa* was unsuccessful, but provided insights on how to improve culturing procedures in the future. It was also determined that the number of embryos in an egg capsule was related to adult mass, with larger nudibranchs having more embryos per capsule (Chapter 3).

If the GOM continues to warm at the current rate of 0.23^{o}C year^{-1} (Pershing et al. 2015), and if species are unable to adapt to the increasing temperatures, we could expect to see sea slugs from this study relocate to deeper or more northern waters. A drastic decline in common winter

sea slugs was observed in 2019. Between the end of August 2018 and the end of March (start of spring) 2019, only three *A. papillosa* were found, compared to 18 individuals in 2018, no *D. frondosus* were found, as opposed to over 50 individuals found by mid-February 2018, and only 9 *F. verrucosa* were found, as opposed to well over 70 individuals found during the winter of 2017-2018 (pers. obs.). Additionally, *Dendronotus frondosus* did not appear until late April 2018, and only small (~1cm) individuals were found. With smaller individuals being found later, it is possible that a shift in phenology has happened with *D. frondosus* in the southern GOM. Phenology shifts have already been noted in several species from birds and insects to bivalves and plankton (Visser & Both, 2005).

A factor this study did not account for was increased metabolism possibly hastening the reproductive period of the sea slugs, and the larvae possibly not surviving the extremely hot summer of 2018. Todd (1979a) suggests that an unseasonably warm summer in 1976 caused a population of *O. bilamellata* to mature and reproduce earlier along the coast of northern England, though egg masses have been found year-round along the coast of Britain (Thompson & Brown, 1984). The sea surface temperature in the GOM increased by 1.2^{o}C (NERACOOS buoy at Appledore Island, 43.02, -70.54) from 2017 to 2018 with average monthly sea surface temperatures in June being 1.5^{o}C warmer and 1.9^{o}C warmer in July. For average sea surface temperatures in 2017, only 44 days were above 16^{o}C, eight of those days were above 20^{o}C, whereas 68 days were above 16^{o}C in 2018, with 15 days being over 20^{o}C (NERACOOS buoy at Appledore Island, 43.02, -70.54). Larvae of *D. frondosus* and *A. papillosa* cannot survive seawater temperatures above 16^{o}C (Runnström, 1927; pers. obs.). With an average summer seawater temperature of 16.9^{o}C at 1m below the surface (NERACOOS buoy at Appledore

Island, 43.02, -70.54) and average dive temperatures of 18.2ºC at 9m below the surface for the Isle of Shoals and 16.7ºC near the shore in New Castle, New Hampshire and York, Maine in 2018, it is not surprising that these species are becoming more difficult to find.

Gastropods have already been shifting ranges faster than other mollusks (Sagarin et al. 1999; Sorte et al. 2010). Nearly 80% of the 24 mollusks found by Sorte et al. (2010) and almost 50% of the 15 species found by Sagarin et al. (1999) that have shifted their geographic ranges were gastropods. Marine species are moving towards the poles at a rate nearly 20 times faster than terrestrial species (Sagarin et al. 1999; Sorte et al. 2010). Range shifts of at least 139 marine species had already been documented by 2010 (Sagarin et al. 1999; Sorte et al. 2010). Many of the species that shifted range were primary producers and primary consumers, including protists, phytoplankton, algae, sponges, hydroids, corals, bivalves, anemones, and barnacles (Sorte et al. 2010). The shift of such species to colder habitats may also influence the polar shift of other species at higher trophic levels.

Although adult sea slugs may not be good bioindicators for climate change due to the variation seen in the different species (Chapter 1), they are highly sensitive to seawater temperature. Studying larval development, larval dispersal and age to maturation at higher temperatures may answer the question as to why so many small nudibranchs were found late in the spring. Daufresne et al. (2009) suggest that along with phenology and range shifts, warmer temperatures are causing smaller body size and should be thought of as a third universal ecological response. Unlike the fish represented in the study by Daufresne et al. (2009), size-selective fishing does not influence body size of nudibranchs, thus my findings (Chapter 2) of

smaller individuals in warmer water may further support his claim. What is happening to the sea slugs in the GOM is likely happening to other snails and marine invertebrates throughout the ocean. Invertebrates make up the majority of the animal biomass on Earth and are essential to life's processes, yet are still underrepresented in the field of conservation and scientific studies as to the effect of anthropogenic change on organisms. Knowing how organisms will react to the projected changes can help inform future policies and practices.

APPENDIX A: BASIC BIOLOGY

Aeolidia papillosa

Aeolidia papillosa (Linnaeus, 1761) (Nudibranchia: Aeolidiidae) is an aeolid nudibranch, a species of sea slug, that inhabits cold Arctic waters of the North Atlantic (North America and Europe), and the North American Pacific coast, from Alaska to Mexico (Thompson & Brown, 1984; Behrens & Hermosillo, 2005). Like other nudibranchs, *A. papillosa* is a specialist predator usually found concurring with its preferred prey, anemones (Shine, 2012). In European waters, Stehower (1952) found the preferred prey to be the anemone *Metridium senile*. Although *M. senile* is found year-round in the GOM, *A. papillosa,* preferring colder water temperatures, is commonly found only from late fall through the winter and early spring (L. Harris, University of New Hampshire, pers. comm.). In the GOM, *A. papillosa* occurs along rocky shores and fouling areas, wherever *M. senile* is abundant. Aside from being found in intertidal areas, *A. papillosa* can be found at depths up to 800m (Thompson & Brown, 1984).

The life span of *A. papillosa* ranges from 12-16 months (Thompson & Brown, 1984). Most individuals found in late fall are juveniles that reach maturity within a few months. *Aeolidia papillosa* can grow to approximately 12 cm in length (Thompson & Brown, 1984) and is the largest native nudibranch found in the GOM, although it usually does not grow beyond 6cm in the GOM (Shine, 2012). When documenting heart rates, body size must be taken into account, as size is a critical factor in the heart rate of molluscs (Biering, 1929). Like other nudibranchs, the heart of *A. papillosa* is clearly visible through the translucent pericardium located dorso-medially directly behind the rhinophores, between the cerata.

The tips of the cerata of *A. papillosa* are filled with undischarged nematocysts, that are consumed from their prey (Hyman, 1967) and, in turn, are used to defend the nudibranch from predators, such as haddock (*Gadus aeglefinus*), cunner (*Tautogolabrus adspersus*), winter flounder (*Pseudopleuronectes americanus*) and the large carnivorous sea slugs *Navanax* sp. and *Pleurobranchaea* sp., in Pacific waters (Homans & Needler, 1944; Harris, 1973 & 1986; L. Harris, University of New Hampshire, pers. comm.). In addition to a toxic defense, *A. papillosa* uses crypsis to hide from predators by having the same natural body coloration and appearance of their prey. Curling up next to *M. senile* allows *A. papillosa* to passively blend with their food (Morris et al., 1980; pers. obs.).

Due to having large sized neurons, *A papillosa* are used in neurobiology studies (Farber & Grinvald, 1983; Watson & Willows, 1992; Baltzley & Lohmann, 2008). *Aeolidia papillosa* are also popular in predator-prey studies, especially for chemoreception and defensive mechanisms (Stehouwer, 1952; Edmunds et al. 1974; Edmunds et al. 1976; Howe & Harris, 1978; Harris & Howe, 1979; Hall et al. 1982; Hall et al. 1984; Harris, 1986; Seavy & Muller-Parker, 2002; Greenwood et al. 2004). Other studies using *A. papillosa* have included growth (Hall & Todd, 1986), distribution (Alder & Hancock, 1851; Franz, 1970; Thompson & Brown, 1984; Harms, 1993; Aerts, 1994; Reichert & Buchholz, 2006), taxonomy (Kienberger et al. 2016), reproduction (Longley & Longley, 1984), larval development (Averkina, 1964; Kress, 1971; Williams, 1980; Eyster, 1983; Eyster, 1986; Carr & Podolsky, 2016), ingestion of nematocysts (Greenwood & Garrity, 1991; McFarland & Muller-Parker, 1993), feeding mechanisms (Nybakken & McDonald. 1981), behavior (Verwey & van Haaften, 1960), defense capabilities

(Putz et al. 2010), and other biological studies (Harris, 1973; Clark, 1975; Porter & Rivera, 1980; Porter & Rivera, 1983; Hall, 1984; Marsden et al. 2012).

Cuthona gymnota

Like *A. papillosa*, ***Cuthona gymnota*** (Couthouy, 1838) (Nudibranchia: Cuthonidae) is a cold water aeolid nudibranch, but much smaller than other nudibranchs in the GOM, only reaching a maximum of 2.2 cm (Thompson & Brown, 1984) and has life span of only a few months (Miller, 1962). *Cuthona gymnota* can be found on both sides of the North Atlantic and the Arctic Oceans, as well as the western Mediterranean Sea (Thompson & Brown, 1984; Shine, 2012). Being specialist predators of hydroids, including *Ectopleura* spp., *Sarsia eximia*, and *Bougainvillea ramosa*, *C. gymnota* occur most often at the base of their prey (von Salvini-Plawen, 1972; pers. obs.). In the GOM, they are commonly found feeding on *Ectopleura crocea* in fouling communities: both intertidally and subtidally up to 30m (Shine, 2012; pers. obs.). Other than studies on biodiversity, taxonomy, and distribution (Alder & Hancock, 1851; Franz, 1970; Williams & Gosliner. 1979; Thompson & Brown, 1984; Harms, 1993; Aerts, 1994; Cella et al. 2016), *C. gymnota* is also studied for its eyes (Hughes, 1970), larva (Selenka, 1881; Thompson, 1959; Marshall, 2006), defense capabilities (Putz et al. 2010), chemosensory orientation in various currents (Verwey & van Haaften, 1960), and sensitivity to ultra-violet light (Klugh, 1931).

Dendronotus frondosus

Dendronotus frondosus (Ascanius, 1774) (Nudibranchia: Dendronotidae) is a cold water, hydroid specialist nudibranch in the Family Dendronotidae. Unlike aeolid nudibranchs,

dendronotids do not store nematocysts in their cerata (Thompson & Brown, 1984) and their cerata are branched, giving an arboreal appearance. Although *D. frondosus* specializes on hydroids, such as *Ectopleura* spp., *Sarsia* spp., *Hydractinia* spp., and *Obelia* spp., some have been found to feed on the tunicate *Botryllus* spp. on the west coast of the United States (Morris et al. 1980). Like *A. papillosa*, *D. frondosus* can be found in the Arctic and both sides of the North Atlantic and North Pacific coasts, both intertidally and subtidally to 400 m (Morris et al. 1980; Thompson & Brown, 1984; Shine, 2012). *D. frondosus* can grow to a maximum of 11.5cm (Morris et al. 1980) and Miller (1962) suggests they can live up to two years off the Isle of Man, but appear to only have an annual life cycle in the GOM (Harris, 1973). Additionally, larval development ceases at temperatures greater than 12^{o}C or less than 1.4^{o}C (Runnström, 1927). *Dendronotus frondosus* has large sized neurons (Sakharov, 1962), making it a popular nudibranch species to study in neurobiology (Newcomb, 2006; Newcomb et al. 2006; Newcomb & Katz, 2007). Other studies on *D. frondosus* include the species' biology (Carefoot 1967; Gionet & Aiken. 1992; Marsden et al. 2012; Ekimova & Malakhov, 2016), feeding habits and mechanisms (Nybakken & McDonald. 1981), recruitment (Lambert, 1990 & 1991), larval development and settlement (Buznikov et al. 1965; Eyster, 1986; Watt & Aiken. 2003; Sisson, 2005), defensive strategies (Thompson, 1960; Lippert et al. 2004), chemosensory and visual capabilities (Zaitseva, 2016), distribution (Alder & Hancock, 1851; Franz, 1970; Clark, 1975; Thompson & Brown, 1984; Harms, 1993; Aerts, 1994; Reichert & Buchholz, 2006; Valdés et al. 2017), and taxonomy (Thollesson, 1998; Ekimova et al. 2016; Korshunova et al. 2017).

Flabellina verrucosa

Flabellina verrucosa (M. Sars, 1829) (Nudibranchia: Flabellinidae) is a cold water, aeolid nudibranch belonging to the Family Flabellinidae (previously Coryphellidae), and is a hydroid specialist, though it also feeds on colonial ascideans (A. Kuzirian, Marine Biological Laboratory, pers. comm., pers. obs.). *Flabellina verrucosa* is often found in conjunction with *D. frondosus* and *C. gymnota* on both sides of the North Atlantic and Arctic Oceans, as well as along the coasts of northern Russia south to Japan (Thompson & Brown, 1984). Like the other nudibranchs in this study, *F. verrucosa* are found both intertidally and subtidally down to 450m, but only grow to 3.5 cm (Thompson & Brown, 1984). Studies of *F. verrucosa* have focused on predator-prey interactions (Tullrot, 1994), especially impacts on population density of *Aurelia aurita,* as *F. verrucosa* prefer to feed on the scyphistoma stage (Hernroth & Gröndahl, 1985; Östman, 1997), larval development and reproduction (Eyster, 1985), nematocyst uptake (Frick, 2003; Frick, 2005), cytochemistry (Porter & Rivera, 1980; Mikhlina et al. 2018), defense capabilities (Penney et al. 2010; Putz et al. 2010), anatomy (Porter & Rivera, 1980; Porter & Rivera, 1983; Mikhlina et al. 2015), and distribution and taxonomy (Alder & Hancock, 1851; Franz, 1970; Kuzirian, 1979; Thompson & Brown, 1984; Harms, 1993; Eriksson et al. 2006; Reichert & Buchholz, 2006).

Onchidoris bilamellata

Onchidoris bilamellata (Linnaeus, 1767) (Nudibranchia: Onchidoriddidae) is a cold water, dorid nudibranch that is a specialist predator of acorn barnacles. *Onchidoris bilamellata* can be found on both sides of the northern Atlantic Ocean, as well as on both sides of the northern Pacific Ocean, though there is some discussion that the Pacific *O. bilamellata* may be a different

species (Thompson & Brown, 1984). *Onchidoris bilamellata* can grow to 4 cm (Thompson & Brown, 1984) and are found both intertidally and subtidally up to 20m (Shine, 2012), often feeding on barnacles even in areas of lower salinity (pers. obs.). Depending on location, *O. bilamellata* can live up to two years and produce multiple egg masses per year (Miller, 1962). Unlike the aeolid nudibranchs in this study, *O. bilamellata* do not have cerata and their heart is near the posterior dorsal region of the animal, just anterior to the gill (Alder & Hancock, 1851). Studies on *O. bilamellata* include: distribution (Alder & Hancock, 1851; Franz, 1970; Clark, 1975; Thompson & Brown, 1984; Harms, 1993; Aerts, 1994; Valdés et al. 2017), molecular phylogeny (Thollesson, 1999; Wollscheid-Lengeling et al. 2001; Fahey & Valdés, 2005; Hallas & Gosliner, 2015), larval development and settlement (Todd & Doyle, 1981; Chia & Koss, 1988; Chia & Koss, 1989; Arkett et al. 1989; Marshall, 2006), ecology (Todd, 1979a & 1979b), specifically associated with chemoreception and choice experiments (Barbeau et al. 2004), respiration (Potts, 1981), feeding mechanisms (Crampton, 1977; Nybakken & McDonald. 1981), and biochemistry of secondary defense chemicals (Hellou, 1985).

Placida dendritica

Although ***Placida dendritica*** (Alder & Handcock, 1843) (Sacoglossa: Limapontiidae) has a morphology similar to aeolid nudibranchs, they instead belong to the herbivorous Order Sacoglossa and feed on the algae, *Bryopsis* spp. and *Codium* spp. (Morris et al. 1980). Unlike other sacoglossans that repurpose the chloroplasts they ingest for sustained energy production, *P. dendritica* digest the algal tissue fairly quickly and completely (McLean, 1976), thus are unable to use the photosynthetic properties of their kleptoplasts (Evertsen & Johnsen, 2009). The cerata of *P. dendritica* lack nematocysts, so their defense mechanisms, besides crypsis, include waving around, then autotomizing their cerata that contain toxic mucus (Marín & Ros, 2004), as well as

lowering their outer body pH (Trowbridge, 1994). Although *P. dendritica* are difficult to see to the untrained eye, reaching a maximum length of 1.1cm (Thompson, 1976), they occur in the waters on both sides of the Atlantic Ocean from Norway to the Mediterranean Sea and from New England to the Caribbean Sea, as far south as Curacao, as well as in the Pacific Ocean from Alaska to the Gulf of California, Japan, and Australia (Thompson, 1976; Behrens & Hermosillo, 2005). Typically, *P. dendritica* are found while examining the algae they consume. Accordingly, most studies have focused on the relationship of the sacoglossan and their prey: feeding ecology on native and invasive algae (Trowbridge, 1989, 1991a, 1991b, 1993, & 2004), effects of algal choice on anatomical evolution (Bleakney, 1990; Jensen, 1993; Trowbridge, 1997), effects of feeding on algal distribution (Trowbridge, 1992a & 2002; Harris & Jones, 2005) and chloroplast associations (Greene & Muscatine, 1972; Raven et al. 2001; Evertsen & Johnson, 2009). Other publications include phenology and distribution (Lambert, 1976; Thompson, 1976; Millen, 1980; Trowbridge, 1992b), reproduction (Gascoigne, 2013), defense (Thompson, 1960; Di Marzo et al. 1993; Marín & Ros, 2004), biochemical (Vardaro et al. 1992; Cutignano et al. 2003; Cutignano et al. 2009; Zuidema & Jones, 2005), and genetic studies (Fan et al. 2013; Han et al. 2015).

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