PUSHING THE BOUNDARIES: INVESTIGATING PHYSIOLOGICAL LIMITS OF INVASIVE SPECIES AND eDNA DETECTION METHODS

By

Emily Rose

Lancaster

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Advisory Committee:

Markus Frederich, Professor of Marine Sciences,
Advisor
Damian C Brady, Professor of Oceanography, Advisor
Doug B Rasher, Senior Research Scientist, Advisor
Erin K Grey, Assistant Professor of Aquatic Genetics

Rhian G Waller, Senior Lecturer/Associate Professor

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Abstract Page

Invasive species are organisms moved from one region to another by humans. Although they are not always harmful to the recipient community, their lack of evolutionary history with their new community can set the stage for destruction. In a world of increasing interconnectivity and warming waters, we expect invasive species will continue to be introduced and that their ranges will expand as more areas become suitable habitats. At this critical point in our planet's natural history, the need to understand where invasive species can survive and how to detect them are important. Here, I begin with a review of invasive species physiology measurements using species identified as invasive through the Marine Invader Monitoring and Information Collaborative. These data points highlight inconsistencies in measurement technique as well as the importance that acclimation temperature and life stage play on thermal thresholds. Based on the noise in the data, I recommend laboratory experiments to understand the absolute maximum and minimum survivable temperatures for each species, followed by field observations of temperatures needed to grow and reproduce. Then, using a newer invader to Maine *Hemigrapsus sanguineus*, I measured thermal thresholds for summer and winter-acclimated crabs and found shifts in thermal thresholds as well as evidence that winter temperatures are stressful for these crabs. Lastly, to effectively detect invasive species early, I tested and designed assays for environmental DNA (eDNA) detection of 9 invasive or nuisance species in the Gulf of Maine. Using laboratory experiments and a two-year time series in a local tide pool, I found that not all of the studied invertebrate species can be detected equally. Some organisms with soft, exposed tissues shed eDNA consistently with their abundance, while organisms with exoskeletons or shells do not. This trend does not hold true for all of the studied taxa, but this premise alongside an understanding of natural history and morphology helps clarify the observed trends. Thus, eDNA techniques should not be applied equally across all taxa for management purposes without a clear understanding of the message of the signal. Overall, I made recommendations to better predict suitable habitats for invasive species, characterized thresholds for an understudied invasive species in New England, and continued building upon the challenges of detecting invertebrates with eDNA.

DEDICATION

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Introduction

Invasive species impacts in the Gulf of Maine

The Gulf of Maine (GoM) is a highly productive body of water with diverse ecosystems, oceanography, a growing aquaculture industry, and proximity to several large shipping ports. Due to climate change, the GoM is warming, with record high temperatures in 2012, 2016, and 2018 (Pershing et al., 2015). 2015-2020 was the warmest 5-year period for the Gulf, a trend that is predicted to continue under climate change projections (Pershing et al., 2021). These oceanographic differences between regions in the gulf likely serve as some protection towards invasive species northward progress, but the strength of the currents varies seasonally and annually (Pettigrew et al., 2005). The GoM is dominated by two strong current systems, the Eastern Maine Coastal Current and the Western Maine Coastal Current. The northern GoM is cooler and well mixed due to nutrient rich, cool waters from the Scotian shelf and continental slope (Goode et al., 2019; Pettigrew et al., 2005; Townsend et al., 2006; Townsend et al., 2015). To the south, the Western Maine Coastal Current is warm and stratified, driven by freshwater input and wind.

The overall warming temperatures, together with overfishing, are affecting some of the most profitable fisheries in the GoM, such as lobster (Mountain and Kane, 2010; Mills et al., 2013). Alongside climate change and the rate of warming in the Gulf of Maine, marine heat waves are events that "last for five or more days, with temperatures warmer than the 90th percentile based on a 30-year historical baseline period" (Hobday et al., 2016). The frequency of marine heatwaves is increasing and could have disastrous impacts on the already stressed native communities in Maine (Oliver et al., 2018). Warming temperatures are partially blamed for the decline of the cod fishery and changes in distribution and abundances of lobsters (Mills et al., 2013; Nye et al., 2009; Pinsky et al., 2013; Pershing et al., 2015). While here we discuss the overall implications of warming, it is important to note that warming is not affecting the oceans at a constant velocity.

Invasive species in the GoM compound other stressors, putting endemic communities at risk. An invasive species is defined here as a species transported by humans, directly or indirectly, which establishes in a new environment. Although invasive species are not always harmful to their recipient communities, they usually do not share an evolutionary history with recipient community members, leading to novel interactions which may affect either species

negatively (Sorte et al., 2010). Recently, the rate of invasion has increased dramatically due to increased global connectivity, and our increased ability to detect these species (Lockwood et al., 2013; Richardson and Pyšek, 2008). In marine ecosystems, many invasive species arrive in an area with ballast, on the hulls of boats, with the aquarium trade, or through aquaculture practices (Ruiz et al., 1999; Dijkstra et al., 2007; Lord and Williams, 2017; Marraffini et al., 2017; Weigle, 2007).

Ocean temperatures generally decrease at higher latitude along the coast of Maine and higher densities of invasive species have been observed in southern Maine due to temperature, currents (see above), and higher introduction rate (McNaught and Norden, 2011). Due to climate change, the intensity and scale of these biotic invasions are shifting. For example, between 1979 and 2005, the average cover of invasive ascidians on settlement plates deployed in southwestern Maine increased from 6% to 11% (Dijkstra et al., 2007). Ascidians are not the only non-native species proliferating; European green crab, *Carcinus maenas*, expanded their range from New Jersey to Newfoundland (1800 km) in just over 100 years (Klassen and Locke, 2007). An even newer invader, *Hemigrapsus sanguineus* has outcompeted *C. maenas* in the southern GoM due to its aggressive nature, higher feeding rates, and stronger claws (Jensen et al., 2002; DeGraaf and Tyrrell, 2004; Payne and Kraemer, 2013; Lord and Williams, 2017). There are at least fifteen readily identifiable invasive invertebrate species of concern in New England, though more are likely present and undetected (MIMIC, Rouget et al., 2016).

While understudied, the impacts of some invasive species on Northwest Atlantic ecosystems have been described. The European green crab, *C. maenas*, is one species whose impacts (such as habitat destruction and aggression) have been well classified in the GoM. For example, foraging by *C. maenas* triggered a decline of eelgrass beds in the Gulf of Saint Lawrence, leading to changes in food web dynamics and decreases in Canadian geese staging for migration (Garbary et al., 2014). Eelgrass meadows are predicted to decline in density with anthropogenic climate change, so *C. maenas* may exacerbate the loss of this habitat in the GoM (see Duarte, 2002; Goode et al., 2019). Similar observations of *C. maenas* and eelgrass interactions have been made on the Pacific coast of the United States (Howard et al., 2019). Eelgrass beds are important ecosystems for coastline protection, a source of blue carbon, and can serve as nursery habitats for young fish such as Atlantic cod (*Gadus morhua*) (Duarte, 2002; Gotceitas et al.,1997). *C. maenas* are also known to be aggressive towards juvenile American

lobster, *Homarus americanus* (Rossong et al., 2006). Lastly, *C. maenas* are potentially voracious predators of juvenile blue mussels, *Mytilus edulis*, and littorinid snails, both of which are important members of intertidal and subtidal Atlantic communities (Ebling et al., 1964; Lubchenco and Menge, 1978). The impacts of *C. maenas* are well studied due to the time since invasion and the pervasive nearshore effects on fisheries and habitats.

Despite fewer studies focusing on their impacts, several other invasive species have also been documented negatively affecting the GoM. *Didemnum vexillum*, the carpet tunicate, was introduced in the Damariscotta River estuary through aquaculture in the 1970s (Dijkstra et al., 2007). Since its introduction, it has spread rapidly throughout the GoM and now covers 230 km^2 of George's Bank, an important fishing ground and nursery habitat in the Northwest Atlantic (Valentine et al., 2007). This colonization is impacting the scallop fishery by decreasing available space for juvenile and adult scallops on gravel habitat and overgrowth of the scallops by the tunicate (Dijkstra and Nolan, 2011; Kaplan et al., 2017). Changes to benthic substrate may also affect survival of juvenile cod, *Gadus morhua* and haddock, *Melanogrammus aeglefinus* (Lengyel et al., 2009) by decreasing food availability as only about 10% of *D. vexillum* tissue contains nutritional value (Valentine et al., 2007).

Clearly, invasive species are already impacting the GoM and we are just beginning to understand their impacts on native ecosystems. As temperatures continue to rise, invasive species will continue to spread and native species will shift poleward (Goode et al., 2019; Parmesan and Yohe, 2003). These species shifts could lead to more novel species interactions, exacerbating the effects of the invasive species and creating novel species assemblages (Aronson et al., 2015; Goode et al., 2019; Sorte et al., 2010). To detect and respond to these shifts, effective monitoring strategies will be crucial.

Environmental DNA to detect invasive invertebrates

Ficetola and colleagues in 2008 detected DNA from American bullfrogs in the lab and field through the extraction of DNA from environmental samples. This DNA, referred to as environmental DNA or eDNA, opened the door for detection of species without capture of the organism. Environmental DNA has been used in a variety of applications including freshwater (Biggs et al., 2015 [newt detection with community science]; Dejean et al., 2011 [American bull frog and Siberian sturgeon]; Eichmiller et al., 2016 [common carp]; Eiler et al., 2018 [pool frog]; Laramie et al., 2015 [Chinook salmon]; Pilliod et al., 2014 [Idaho giant salamanders]; Pont et al.,

2018 [comparing electrofishing surveys with eDNA metabarcoding]; Stoeckle et al., 2015 [fish commmunity metabarcoding]; Takahara et al., 2012 [common carp]; Turner et al., 2015 [bigheaded Asian carp]), ice (Willerslev et al., 2004 [ancient bacterial DNA]), air (Banchi et al., 2020 [airborne fungi and plant DNA]; Lynggaard et al., 2022 [zoo animal detection]), soil (Levy-Booth et al., 2007 [DNA cycling]; Pietramellara et al., 2009 [review of microbial impact on soil DNA]), and salt water (Ardura et al., 2015 [European mudsnail ballast transfer; Kelly et al., 2014 [metabarcoding of a mesocosm]; Kelly et al., 2018 [tidal effect on eDNA detection]; Thomsen et al., 2016 [deepwater fish detection versus trawl data]). These studies demonstrate that species detection using eDNA is possible, even if we do not directly capture or observe the animal. In cryptic species such as amphibians, eDNA provides scientists a glimpse into population sizes and distribution, which can be critical for monitoring (Biggs et al., 2015).

The sensitivity of eDNA methods allows for the detection of invasive species as well. Invasive species are easier to manage when they are in low abundance, so early detection may be the key to eradication (see, for example, Vander Zanden et al., 2010). The Laurentian Great Lakes are now home to several damaging invasive species, including several carp species and zebra mussels. Environmental DNA was able to detect the presence of bighead and silver carp (*Hypophthalmichthys nobilis* and *Hypophthalmichthys molitrix*, respectively) before the fish were caught upstream of electrical barriers to keep the fish out of the lakes (Jerde et al., 2011; Jerde et al., 2013). Zebra mussels, *Dreissena polymorpha*, and quagga mussels, *D. rostriformis*, have been detected using eDNA in the Great Lakes and in Europe, where eDNA was used to quantify the level of infestation in lakes where traditional survey methods, such as larval counts, failed (Clusa et al., 2017; Klymus et al., 2017; Peñarrubia et al., 2016; Williams et al., 2017). These examples highlight the strength of eDNA in detecting invasive species early, which is critical to decrease the risk of establishment.

Some suggest interpreting eDNA with caution; that we cannot trust the results if we cannot see the animal and that the DNA could be coming from other sources (Jerde, 2021). Despite pushback from managers and the public, eDNA methods for some species were found to be more reliable and less expensive than traditional methods. Asian carp species are challenging to detect with traditional methods, especially in low density, so eDNA survey methods are likely more precise (Jerde et al., 2011). Invasive species management in the United States costs more than $120 billion annually and false positive detections could lead to an inefficient distribution of

limited resources (Pimentel et al., 2005). All survey methods are subject to error, and error in invasive species detection can have costly consequences, whether those methods are traditional survey methods or eDNA based methods. For example, conventional capture methods can suffer from misidentification, low detection probabilities, insufficient effort, or temporal mismatch in sampling effort (Jerde, 2021). Sources of error in eDNA studies can come from genetic misidentification, hybridization, contamination, DNA persistence, seasonal or temporal mismatch, inhibitors, or molecular failure. Some of these error sources are similar across methods so proper background information (i.e. when is the animal present in the ecosystem?) can reduce error across methodologies.

Although eDNA methods are constantly improving, guidelines have been developed to ensure that eDNA assays (primer and probe set) have been sufficiently validated for management purposes. One of these guidelines is the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments" (MIQE). An assay designed following the MIQE guidelines will have information on the limit of detection, primer specificity, inhibition testing, and other factors that can lead to differences between laboratories and locations (Bustin et al., 2009). The overall goal of these guidelines were to increase the value of reported qPCR experiments, allow editors and reviewers to assess the quality of submitted qPCR publications, and to help experiments be replicable to reduce redundancy in assay design. Thalinger et al. (2021) built upon the MIQE guidelines to create a set of guidelines specific to eDNA assays. Many of the requirements from MIQE remain in place, but they include validating eDNA samples at multiple sites and quantifying detection probabilities (Thalinger et al., 2021). The use of these guidelines as well as strong scientific communication should increase confidence in eDNA techniques for monitoring invasive species (Jerde et al., 2021).

An area of eDNA study that requires more attention is the relationship between abundance and detectable eDNA. Factors such as mixing and flowing of water (Foote et al., 2012; Stoeckle et al., 2015), degradation caused by UV, temperature, or bacteria (Pilliod et al., 2014; Strickler et al., 2015; Tsuji et al., 2017), and potentially, body plan of the animal. Studies in fish and some amphibians have found a correlation between biomass and detected eDNA in the laboratory and in field or mesocosm studies (Kelley et al., 2014; Klymus et al., 2015; Lacoursière-Roussel et al., 2016; Maruyama et al., 2014; Pont et al., 2018; Takahara et al., 2012; Thomsen et al., 2016). Both single species and metabarcoding studies have questioned whether

eDNA reads correlate with the amount of organisms present depending on primer set and morphology (Crane et al., 2021; Danziger et al., 2022; Grey et al., 2018; Thomas et al., 2016).

Here, I use eDNA to detect nine species of invasive invertebrates. These species range in body plan from fleshy, exposed tissues (squishy), to organisms covered in a shell or exoskeleton (crunchy). I predict that there will be a difference in effectiveness of quantitative eDNA detection between these groups of species, where more exposed organisms will shed more eDNA and move covered organisms will shed less eDNA.

Physiology of invasive invertebrates

Physiological plasticity is a leading hypothesis for the success of invasive species. Ability to survive in a variety of environments has allowed European green crab, *C. maenas*, to colonize every continent except Antarctica (Compton et al., 2010, Darling et al., 2008). For example, *C. maenas* can live in a wide range of temperatures, from lower than 0 ℃ to nearly 36 ℃ (Jost et al., 2012; Tepolt and Somero, 2014; Frederich and Pierce, 2024). They can live in estuaries with low salinity by increasing their urine output (Binns, 1969). As generalists, they can eat most foods they encounter and some populations have strengthened their claws to more effectively prey upon littorinid snails (Edgell and Hollander, 2011; Seeley, 1986). Finally, their larvae can develop at a variety of temperatures by increasing planktonic duration, allowing them to disperse further (deRivera et al., 2007). These characteristics have allowed *C. maenas* to spread far from its home range and outcompete native species to become a dominant part of many nearshore ecosystems. These characteristics of plasticity are likely true of other invasive species in the GoM, but extensive studies of *C. maenas* have occurred due to the prevalence of this invasive species around the world. Chapters 1 and 2 will explore what is known about physiological tolerance for other GoM invasive invertebrates and measure physiological frameworks for Asian shore crab, *H. sanguineus*, in greater detail, thereby enhancing our understanding of the role of physiological plasticity in invasion success.

Physiology of an animal becomes increasingly important as the climate changes; to predict where organisms will spread based on their physiological limits will allow for appropriate implementation of eDNA monitoring methods. Furthermore, our ability to detect an organism using eDNA methods may change under stress. Green crabs running on a treadmill increased the amount of detectable DNA in a laboratory setting, likely due to an increased metabolic rate (Danziger et al., 2022). Similar results have been seen in bluegill sunfish, *Lepomis macrochirus*,

where juvenile fish released more eDNA than adults due to activity level (Maruyama et al., 2014). One study found unexplained, individual variation in eDNA shedding rates for Idaho giant salamanders; so much variation that a salamander who produced more than four times the amount of eDNA as the others had to be excluded from analysis (Pilliod et al., 2014). Organisms may also reproduce once water temperature reaches a certain level, so these factors must be taken into consideration for field eDNA studies with goals of quantification (see, for example, Peñarrubia et al., 2016). Due to the potential interaction between temperature and eDNA detection, these topics are important to examine before usage of eDNA in monitoring practices.

Study system and species of interest

In the GoM, there are fifteen invasive invertebrate species currently being monitored by the Marine Invader Monitoring and Information Collaborative (MIMIC), which are already known to be established in New England. The purpose of MIMIC is to study the presence of easily-identifiable invasive species in the New England area through volunteer efforts. These invaders are representatives of multiple phyla from around the world. There are both solitary (*Ascidiella aspersa, Styela clava*) and colonial tunicates (*Botrylloides violaceus, Botryllus schlosseri, Didemnum vexillum,* and *Diplosoma listerianum*), bushy (*Bugula neritina* and *Tricellaria inopinata*) and encrusting bryozoans (*Membranipora membranacea*), a variety of crustaceans (*Carcinus maenas, Hemigrapsus sanguineus, Caprella mutica, Palaemon elegans*), the orange striped anemone (*Diadumene lineata*), and the European oyster (*Ostrea edulis*). These species were chosen because they are easy to identify with little guidance for trained volunteers, not because they are necessarily the most damaging. While not currently monitored by MIMIC, *Ciona intestinalis* is considered exotic or cryptogenic in the GoM and will be considered as an invasive species in this study (Leblanc et al., 2020; Martin et al., 2011; McNaught and Norden, 2011; Ramsay et al., 2008).

These species have been introduced to the GoM in a variety of ways. Green crab, *C. maenas*, was one of the first recognized invasive species in the GoM, likely first traveling in the early 1800s to New Jersey in ballast rocks, gravel, and sand (Darling et al., 2008; Edgell and Hollander, 2011). The tunicate *B. schlosseri* also arrived in the 1800s when it was described in a book, but there is no information about transportation vectors (Gould, 1870). *B. violaceus* and *D. vexillum* were transported through oyster aquaculture to the Damariscotta river in the 1970s (Dijkstra et al., 2007). *H. sanguineus* likely arrived via ballast water and due to the genetic

diversity of the invasive populations, has been introduced more than once (Epifanio, 2013; Lord and Williams, 2017). *M. membranacea* first appeared on kelp in New Hampshire from Europe, though the vector of transport is also not known (Lambert et al., 1992). *C. mutica* rapidly invaded Europe through hull fouling and was likely transported to the GoM in a similar fashion (Cooke et al., 2007). The last species with a clear path and timeline for invasion of the species studied here is *O. edulis*, which was intentionally introduced in the 1950s in a failed aquaculture attempt (Loosanoff, 1955). Each of these species, and more, are well established in the GoM and moving northward with climate change. Detection using eDNA can help us better understand their distribution and mitigate the effects of shifting invasive species on native communities.

This study primarily took place in the laboratory and at Biddeford Pool, Maine (43.44203° N, 70.34096° W). Biddeford Pool is home to more than nineteen species of shorebirds, both migratory and resident, which utilize the diverse habitats from saltmarsh to rocky intertidal in the area (Humphrey et al., 1995). The University of New England Crustacean Research Laboratory has a longstanding dataset including abundances of invasive crabs in this area and it is also a MIMIC site which is sampled visually monthly. Together with eDNA sampling, this long-term dataset will help assess seasonal variation in species presence, and perhaps abundance, in this ecologically diverse ecosystem.

Dissertation structure

In this dissertation I link physiology with ecology of invasive species through environmental DNA detection. The first chapter will be a review of invasive species thermal tolerances in the GoM. Chapter two will focus on *H. sanguineus* as a model organism for measuring different thermal tolerance frameworks and discovering which framework best predicts species spread. I will also determine whether that framework can be appropriately measured for other invasive species in the GoM. Chapter three will tie together the physiology and the eDNA, investigating eDNA shedding rates with different biomass and under physiological stress to better understand what an eDNA signal confers. Chapter four will bring eDNA detection to the field, comparing detectable eDNA from invasive species over three field seasons at Biddeford Pool to see whether biomass and seasonal variability correlate with detectability. This dissertation will provide guidance for the use of eDNA to detect invasive invertebrate species by enhancing species distribution predictions, clarifying the meaning of an eDNA signal, and providing long term monitoring data.

Chapter 1 | In Hot Water: Current Thermal Threshold Methods Unlikely to Predict Invasive Species Shifts in the Northwest Atlantic

Abstract

As global temperatures continue to rise, accurate predicted species distribution models will be important for forecasting the movement of range-shifting species. These predictions rely on measurements of organismal thermal tolerance, which can be measured using classical threshold concepts such as Arrhenius Break Temperatures and Critical Thermal Temperatures, or through ecologically relevant measurements–such as the temperature at which reproduction and growth occur. Many species, including invasive species, exhibit thermal plasticity, so these thresholds may change based on ambient temperature, life stage, and measurement techniques. Here, we review thermal thresholds for 15 invertebrate species invasive to the Gulf of Maine. The high degree of variability within a species and between applied conceptual frameworks suggests that modeling the future distribution of these species in all ecosystems, but especially in the rapidly warming Northwest Atlantic and Gulf of Maine, will be challenging. We suggest a standardization of measurements to increase the applicability of physiological thermal tolerances in order to address real world problems.

Introduction

Anthropogenic climate change driven by greenhouse gas emissions has led to unprecedented rates of warming (IPCC, 2022). Marine heat waves, which occur when temperatures reach the 90th percentile for five or more days, are also increasing in frequency as climate change continues (Hobday et al., 2016; Laufkötter et al., 2020; Oliver et al., 2018). Warming temperatures are causing species to shift deeper in the water or poleward to find favorable thermal conditions (Perry et al., 2005; Sunday et al., 2012). Many marine organisms are ectothermic, whose temperature rely on external sources of body heat, and/or poikilothermic, whose temperature varies with environmental temperature. For these organisms, temperature changes alter their physiology and increase their metabolic rate, typically at a ratio of 2-3 times base metabolic rate for every 10℃ change (Cossins and Bowler, 1987). At thermal extremes, metabolic rates begin to limit an organism's ability to survive. In order to understand how

climate change will affect ectothermic animals, accurate measurements of thermal tolerance thresholds must be made.

Several metrics are commonly used to measure thermal tolerance thresholds across the animal kingdom. One classical measurement is lethal dose 50 or LD50 (or LT50 for lethal temperature), which is derived from toxicology and is the temperature at which 50% of individuals perish (see Nagabhushanam and Krishnamoorthy, 1992). Arrhenius break temperatures (ABT) seek a break from linearity and, while originally described for enzymatic reactions (Arrhenius, 1889), are usually measured as heart rates at increasing or decreasing temperatures (Harrington and Hamlin, 2019). The concept of the Oxygen and Capacity Limited Thermal Tolerance (OCLTT) hypothesis describes two thresholds, the pejus temperature Tp, the temperature at which the animal's condition worsens, and the aerobic scope is limited (Frederich & Portner 2000), and the critical temperature, Tc, the temperature at which the animal's oxygen demand exceeds the oxygen supply due to failing circulatory and/or ventilatory systems, and the subsequent buildup of anaerobic end-products (Pörtner et al., 2017). Critical thermal maxima and minima (CT_{max} and CT_{min}) measure the point at which the animal loses controlled motion (Brett, 1956; Cowles and Bogart, 1944; Jost et al., 2012; Kelty and Lee, 2001). Lastly, the framework of Multiple Performances, Multiple Optima (MPMO) posits that organ systems, ion transport, and other processes within the animal fail at different temperatures for different species and avoids defining one general mechanism responsible for system failure at thermal thresholds (Städele et al., 2015; Clark et al., 2017).

Each of these frameworks outline valid thermal thresholds, but some are potentially more ecologically relevant than others. For example, temperatures measured in CT_{max} are so high that they are rarely experienced in the field, except perhaps for intertidal organisms which are exposed to the air (Stillman and Somero, 2000). If a threshold falls outside of water temperatures found in nature, it is not a helpful threshold for predicting species behavior. For modeling, the mechanisms behind MPMO vary widely between species, so it is unlikely to be useful for widespread use in modeling. Because MPMO is based on organ systems and other underlying mechanisms, there is no continuous variable that could drive species range shifts. Furthermore, not all invertebrate organisms have well defined organ systems, so MPMO would not broadly apply. Outside of ecological relevance, measurements vary greatly due to acclimation temperature, measuring styles, differences in populations, and even misinterpretation of

framework measurements (see for example McGaw and Whitley, 2012). These inconsistencies lead to challenges in interpreting the data in meaningful ways; a meta-analysis is impossible with invertebrate thermal tolerances. Meta-analyses are suited to data collected in a similar way to be analyzed together quantitatively by pulling the effect size and variance from a variety of studies, but the high level of variation between measurement techniques introduces too much noise and uncertainty for anything quantitative to be inferred. With different starting temperatures, acclimation temperatures, rates of temperature change, population differences, and no basis for statistical modeling (Forero et al., 2019).

Plasticity in thermal thresholds has been observed in many taxa at every life stage (see, for example, Padilla and Savedo, 2013). Geographic location can also influence thermal plasticity, which has been well documented for *C. maenas* whose CT_{max} values have a range of nearly 10℃ based on acclimation temperature and location (Tepolt and Somero, 2014). Some life stages have different energy requirements, so this plasticity may be limited for developing larvae and reproducing females, among others (deRivera et al., 2007). Thus, any thermal threshold measured should contain records of where the organisms were collected, life stage, and acclimation temperature, at a minimum, to understand the validity of those measurements.

Despite the development of these frameworks, many studies instead focus on the temperature effects on factors such as larval development, survival, reproduction, or presence of a species. Although these measurements may help inform local abundance of organisms, accurate species distribution modeling will require broad physiological understanding of organisms to be applied over a broader scale, measured in precise and replicable ways. Thus, without the mechanistic understanding of physiological limitations, models may fall short. This is increasingly important as climate change and general warming move organisms towards the poles or deeper in the water column (Sunday et al., 2012). Furthermore, some studies use survival at the minimum and maximum regional temperature in the species range as temperature thresholds, which are likely underestimating the true limits of potential invaders (see for example Willis et al., 2009).

Invasive species (defined here as organisms moved from one area to another by humans) will only have a chance at success if the temperatures in the recipient community fall within the thermal thresholds of the species. In general, many marine invasive species have a wide range of thermal tolerance and are able to live in many areas they are introduced to. Diet generalism,

salinity tolerance, and high fecundity are also predictors of invasion success. For example, European green crab (*Carcinus maenas)* is native to Europe and Northern Africa, but has established invasive populations nearly worldwide, on every continent except Antarctica (Carlton and Cohen, 2003; Compton et al., 2010; Frederich and Lancaster, 2024). Fortunately, *C. maenas* is well studied in regard to thermal tolerance, so we can predict their future range expansion (see Frederich and Lancaster, 2023). Due to warming ocean temperatures and their extreme thermal tolerance (down to -1.8℃), there are few thermal limits to where *C. maenas* could spread (Tepolt and Somero, 2014). One area of particular concern is the Antarctic shelf, which is under threat of encroaching lithoid crabs and currently has no crushing predators (Aronson et al., 2015). Due to anticipated climate changes and increased connectivity between continents, knowledge of thermal tolerance for invasive species is important to generate species distribution models, which could inform management strategies.

Fouling communities are one of the most common habitats for benthic invasive species in harbors and on boats. Assemblages of fouling communities compared in the Great Bay Estuary in New Hampshire have shown a 33% difference in community members since the late 1970s (Harris and Dijkstra, 2007). Furthermore, increases in marine heatwave frequency will lead to more erratic temperature changes, which could alter invasive species communities (Sorte et al., 2010). This difference is likely due to newly introduced species and warming waters. Not all invasive species are transported through fouling species or ballast, other common methods of introduction are the pet trade and seafood industry (Rius et al., 2014; Weigel, 2007).

Here, we review thermal tolerances of fifteen ecologically important Gulf of Maine invasive species around the world to highlight the need for more consistent measurements to inform predictive species distribution models (Table 1). These studies report thermal thresholds from field observations and laboratory experiments from all continents, including Africa (4), Antarctica (3), Asia (28), Australia (24), Europe (127), North America (146), South America (5), and Worldwide (21), with the rest being either unlisted or multi-continent. The species list for this review was chosen from the Marine Invaders Monitoring and Information Collaborative (MIMIC), a community science project based in New England hosted by the Massachusetts Office of Coastal Zone Management. While these species are not necessarily the most harmful, they are the most easily identifiable, allowing trained volunteers to make observations across the area. These observations began in 2008 and continue to be collected, creating a distribution of

species over time. One species, *Ciona intestinalis*, is not listed on MIMIC surveys, but is considered cryptogenic in Maine (Hewitt et al., 2002) and thus was added to this analysis. The fifteen species represented here span five phyla (urochordata, arthropoda, bryozoa, mollusca, and cnidaria) and consist of a variety of bauplans and metabolic rates.

Table 1. A list of invasive species including their native and invasive ranges. Information compiled from the global invasive species database (GBIF) and the Smithsonian Marine Invasions Lab.

Species name	**Phylum**	**Native range**	**Temperature in native range**	**Invasive range**
Ascidiella aspersa	Chordata	Europe	Up to 26℃	Australia, Japan, New Zealand, North America, South America; possibly also India and South Africa
Botrylloides violaceus		Asia	-0.6 - 27.4℃	Australia, Europe, North America
Botryllus schlosseri		Europe	-1 - 30℃	Asia, Australia, New Zealand, North America, South America
Ciona intestinalis		Cryptogenic	0 - 27℃	Asia, Africa, Australia, Europe, New Zealand, North America, South America
Didemnum vexillum		Asia	-2 - 24℃	Australia, Europe, New Zealand, North America
Diplosoma listerianum		Europe	2.2 - 30℃	Asia, Australia, Europe, Madagascar, New Zealand, North America, South America

Styela clava		Asia	-2 - 26.6℃	Australia, Europe, New Zealand, North America; possibly also in Africa.
Caprella mutica	Arthropoda	Asia	-2 - 28℃	North America, Europe
Carcinus maenas		Europe	-1 - 35℃	Asia, Australia, North America, South America
Hemigrapsus sanguineus		Asia	1.8 - 30℃	Europe, North America; possibly also Australia and India.
Palaemon elegans		Europe, Africa	2 - 25℃	North America
Bugula neritina	Bryozoa	Europe	2.2 - 30℃	Africa, Asia, Australia, New Zealand, North America, South America; possibly also Antarctica; present on several islands including the Galapagos and Vanuatu
Membranipora membranacea		Europe	-1.8 - 27℃	Africa, Asia, Australia, New Zealand, North America
Diadumene lineata	Cnidaria	Asia	0 - 27.5℃	Australia, Europe, New Zealand, North America, South America
Ostrea edulis	Mollusca	Europe	5 - 25℃	Africa, Australia, New Zealand, North America,

Studying these species in the Gulf of Maine is of particular importance due to the unprecedented rate of warming in this region (Pershing et al., 2021). This warming has

temporarily, positively affected the lobster fishery, but warmer temperatures may facilitate poleward movement of invasive species from lower latitudes (Duffy et al., 2017; Goode et al., 2019; Sorte et al., 2010). Depending on the rate of species spread, which is influenced by larval duration and transport, lifecycle, and bathymetric barriers (amongst other factors), high latitude ecosystems may face invasions sooner rather than later, especially as temperatures continue to climb. Due to the rate of warming in the Gulf of Maine, as well as the latitudinal gradient, this area is ideal for projecting how species distribution might change in other regions. While the rate of warming will affect species success, the extreme rate of warming in the Gulf of Maine serves as a "worst case scenario"; if organisms can survive this, they will likely be successful in other, less drastically changing areas. The implication of this study can be applied elsewhere, especially for forecasting studies which presently use field distribution to determine thermal tolerance (see, for example, Holland et al., 2021). In this context, we provide suggestions for future physiological studies to increase their applicability to species modeling.

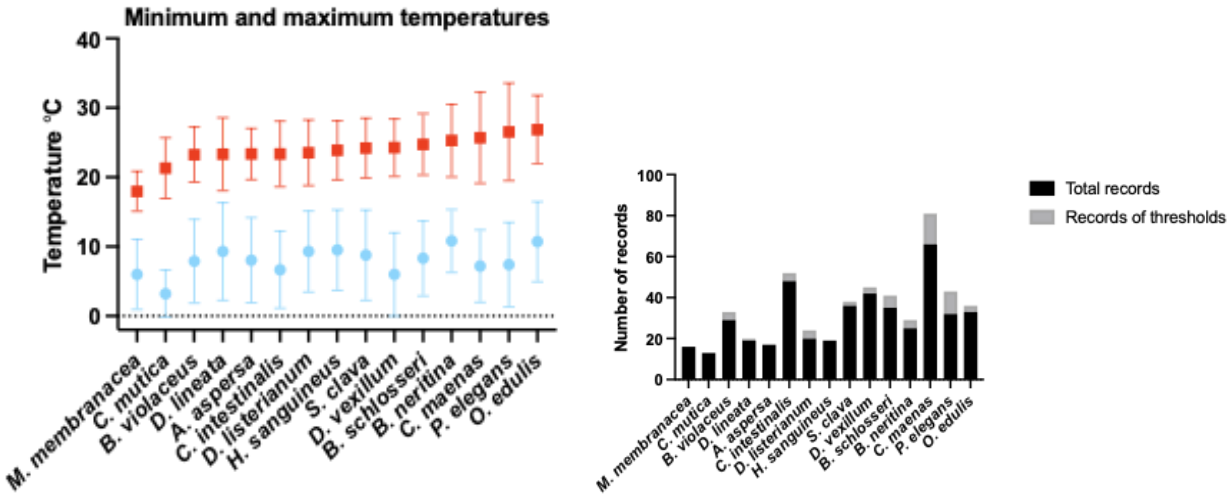

Figure 1: A) Summary of 450 measured thermal thresholds for all of the study species represented by means and standard deviations of the high (square) and low (circle) temperature measurements. Species are organized from lowest maximum high temperature to highest maximum high temperature on the x axis. B) Total number of records for each species and how many of those records measured classical thresholds (lighter gray). Most of the records analyzed studied the more ecologically meaningful measurements which lack a mechanistic understanding.

Characterization of thermal thresholds

Using a variety of search queries (supplementary table 1), we collected records of thermal thresholds for the 15 species of interest. Although the search queries were based on the mechanistic frameworks, they also captured a variety of measurement techniques outside of the frameworks. Indeed, most of the studies included did not measure traditional thermal threshold metrics, but instead investigated factors such as survival, larval development, reproduction, or growth. Information recorded from each publication included the maximum or minimum temperature threshold and exactly what was measured. Per the discretion of the authors, many frameworks could be assigned to data if the framework was not explicitly listed in the publication. A table including which frameworks were assigned can be found in the supplementary material. Furthermore, some publications used a different title for a framework that was previously established and was reassigned for the purpose of this review. For example, in 2014 Tepolt and Somero studied cardiac function of *C. maenas* and measured CT_{max}, whereas by our definition this measurement might be ABT or MPMO (Tepolt and Somero, 2014). Other publications did not specify a framework but did measure relevant values and were captured in the "general" search queries, so a descriptive term was chosen from those studies for what was being measured (i.e., development, survival, reproduction).

This literature review scanned 450 thermal threshold records for the species of interest. Most of the records did not align with any of the classical thermal physiology models, but instead focused on what temperatures the organisms were reproducing in the field, the temperatures at which they grew, and general records of survival in an area with specific temperatures. A summary of the average upper and lower measurements can be found in Figure 1.

Physiology by phylum

Ascidian thermal thresholds

The largest group of organisms in our analysis are the ascidians. *A. aspersa, B. violaceus, B. schlosseri, C. intestinalis, D. vexillum, D. listerianum,* and *S. clava*. *A. aspersa, C. intestinalis,* and *S. clava* are solitary tunicates, whereas the others live as thin layer colonies. A breakdown of all measured thresholds can be found in Figure 2. This group is fairly well studied,

as *C. intestinalis* and others are used as model organisms. They are generally suspension feeders and are dominant members of fouling communities. In the Gulf of Maine, they have been introduced through equipment fouling and aquaculture (Carman et al., 2014; Lambert, 2009). They frequently outcompete other species in fouling communities and grow over native bivalves such as mussels, oysters, and scallops as well as eelgrass (Fletcher, 2013; Gittenberger, 2007; Long and Grosholz, 2015). *Didemnum vexillum* is particularly harmful to Gulf of Maine fisheries and ecosystems. At George's Bank, located in the center of the Gulf of Maine, over two hundred square kilometers have been colonized by *D. vexillum*, which is damaging nursery habitat for commercially valuable fish like cod (Valentine et al., 2007). Unfortunately, dredging, scraping, and trawling fragments ascidians, and many of those fragments can settle and start new colonies; so the issue is made worse by traditional fishing practices.

The invasive ascidians in Maine have wide temperature tolerances and are able to reproduce early in the year, allowing them to quickly dominate fouling communities in the spring. They inhabit a variety of ecosystems, from harbors and tide pools to large swaths of benthic area (Dijkstra et al., 2007; Sorte and Stachowicz, 2011; Valentine et al., 2007). Many species exhibit a lower thermal threshold that is below the temperatures required for reproduction. For example, *S. clava* requires a temperature above 15℃ to reproduce, so it is not feasible for populations to exist if the maximum temperature does not exceed 15 even though the species can survive down to -2℃ (Davis et al., 2007; Davis and Davis, 2008). Many species exhibit population level variation, which leads to differences in thermal maxima and minima. For example, larvae of *B. schlosseri* have been reared at 10℃ (which took nearly 66 days), while other studies did not have successful reproduction at 13℃ (Brunetti et al., 1984; Sabbadin et al., 1955). Whether this difference is from laboratory versus field observations or interspecific variation is unknown.

For *C. intestinalis* and others, temperature may affect life cycle length. Individuals growing in cooler temperatures live longer (2-3 years), while individuals in warmer waters may reproduce several times per year or produce up to four generations in one year and live shorter lives (Berrill, 1947; Dybern, 1965; Yamaguchi, 1975). *Botrylloides violaceus* in the Great Bay Estuary in New Hampshire now experience more than one reproductive cycle in a year, compared to the 1970s where cooler temperatures and shorter heat extremes limited their reproductive cycles to 0.7 annual reproductive cycles (Dijkstra et al., 2011). Multiple generations

per year could extend the impacted area and allow for increased genetic diversity, so understanding how temperature impacts the reproductive capacity of each species is important for modeling.

For each species studied in this group, there was a high amount of variability within measurements. Indeed, many studies only measured up to a certain temperature (usually 25-30℃) before ending an experiment prematurely, labeling the maximum temperature measured as a thermal maximum. Studies using the frameworks above, such as LD50 and CTmax, which elicit the maximum survivable temperatures, show that the maximum temperatures for all ascidians studied here are above 27℃, some falling well above 27.

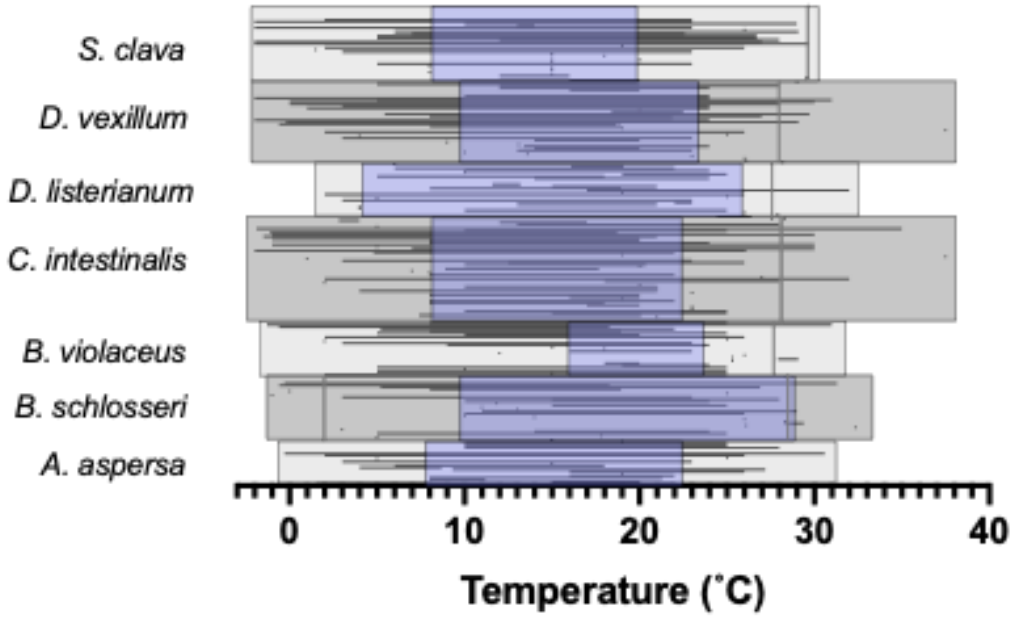

Figure 2: Measured thermal thresholds (dots) and ranges between thresholds (lines) for the ascidian species included in this study. Vertical lines in each gray rectangle indicate measured LD50 temperatures. For *A. aspersa*, none of the traditional thermal threshold measurements were taken, *C. intestinalis* has an ABT (21℃) and LD50 (27℃), *D. listerianum* has an LD50 (26.6±1.40℃), *D. vexillum* only has an LD50 (26.77±1.01℃), and lastly, *S. clava* has an LD50 of 29.5℃. Gray boxes indicate maximum and minimum measured thermal tolerance, purple boxes indicate a reproductive threshold.

Bryozoan thermal thresholds

The invasive bryozoans *Membranipora membranacea* and *Bugula neritina* can be found in fouling communities, but also exist in ecosystems that are less directly influenced by humans. A summary of their thermal thresholds can be found in figure 3. *Membranipora membranacea* is frequently found as a biofouler in kelp forests, where it grows over the kelp and leads to decreased flexibility, which causes breakage and increased mortality (Dixon et al., 1981; Førde et al., 2016; Saunders and Metaxas, 2008). Kelps are an excellent aquaculture food source that could be threatened by invasive bryozoans, and kelp forest composition in the Gulf of Maine is changing from tall canopies of brown kelps to short, dense, red algae (Witman and Lamb, 2018). This species composition may change habitat function, as kelp forests are usually considered nursery habitats due to their complex structure and wave-damping properties. Furthermore, kelp aquaculture is an emerging field in the Gulf of Maine in the winter, but warming temperatures may increase biofouling risk at this time of year (Forbord et al., 2020; Førde et al., 2016).

These two species usually inhabit different parts of nearshore ecosystems. Whereas *B. neritina* is an upright bryozoan that attaches to hard structures nearshore, *M. membranacea* is found where kelps are found in subtidal regions. According to the measurements, *M. membranacea* is able to reproduce across most of its thermal range, whereas *B. neritina* has a narrower range of reproduction closer to the bottom of its thermal range. Importantly, *M. membranacea* reproduces below the minimum threshold measured by some species, suggesting either population-level variation or vastly different measurement techniques.

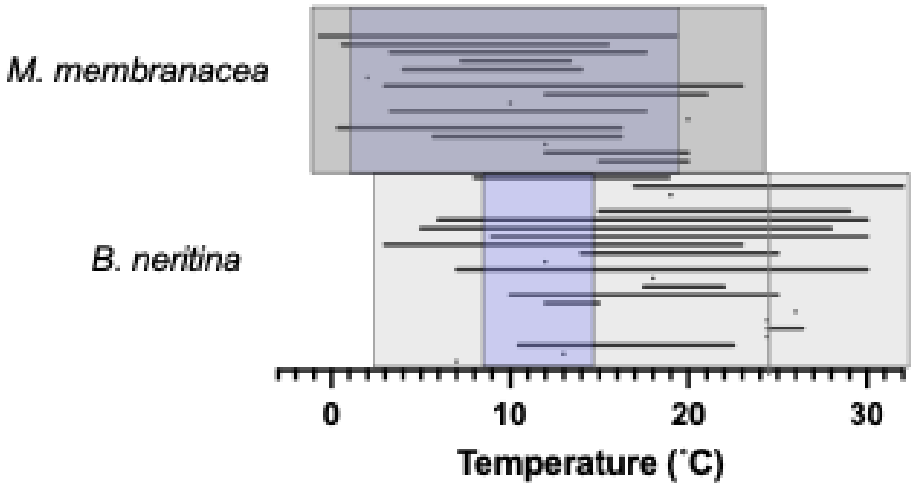

Figure 3: Measured thermal thresholds (dots) and ranges between thresholds (lines) for the bryozoan species included in this study. *B. neritina* has a measured LD50 value (25.12±0.89℃) and *M. membranacea* has no classical measured threshold values in the literature. Vertical lines in each gray rectangle indicate measured LD50 temperatures. Gray boxes indicate maximum and minimum measured thermal tolerance, purple boxes indicate a reproductive threshold.

Arthropod thermal thresholds

Invasive arthropods in the Gulf of Maine include *Carcinus maenas, Caprella mutica, Hemigrapsus sanguineus,* and *Palaemon elegans*. They arrived here through ballast water or rocks, aquaculture, and fouled equipment (Ashton et al., 2007; Edgell and Hollander, 2011; McDermott, 1998). *Carcinus maenas* is one of the most damaging invasive species in the Gulf of Maine, but in southern regions such as New Jersey *H. sanguineus* has become the dominant invasive arthropod in the tide pools. These invasive species have documented impacts on nearshore ecosystems and prediction models must take thermal preferences into account for accuracy.

While arthropods have some of the widest thermal tolerances of the studied species, reproduction is a limiting factor at low temperatures, despite surviving at near freezing temperatures. One example of this is *C. mutica*, which survives down to 0℃ in its native range, but in Scotland juveniles were not present in the winter at some sampling sites despite the low temperature only reaching 7.4℃ (Ashton et al., 2010). Some research suggests that marine species ranges conform to their thermal tolerance; if they can survive the temperatures in an area, they likely inhabit it (Sunday et al., 2012). However, other oceanographic factors, such as wave intensity, may limit the distribution of certain arthropods, despite temperatures well within their survivable ranges (Hampton and Griffiths, 2007).

C. maenas is a worldwide invader with high thermal tolerance. Despite the survival of these crabs at exceptionally high and low temperatures, different populations of crabs may struggle at middling temperatures on a physiological level. For example, *C. maenas* from Helgoland, Germany, had lower oxygen consumption rates at medium temperatures (12-21℃) compared to crabs from Cadiz, Spain (Laspoumaderes et al., 2022). However, even with the differences in oxygen consumption rates, the crabs continued to eat and grow at similar rates at increasing temperatures. As one of the better studied organisms here (66 measured temperature

thresholds), population-level variation in thermal tolerance is well documented for *C. maenas*. Critical thermal thresholds range from water temperatures of 29.7 to 38.3℃ based on haplotype and acclimation temperature.

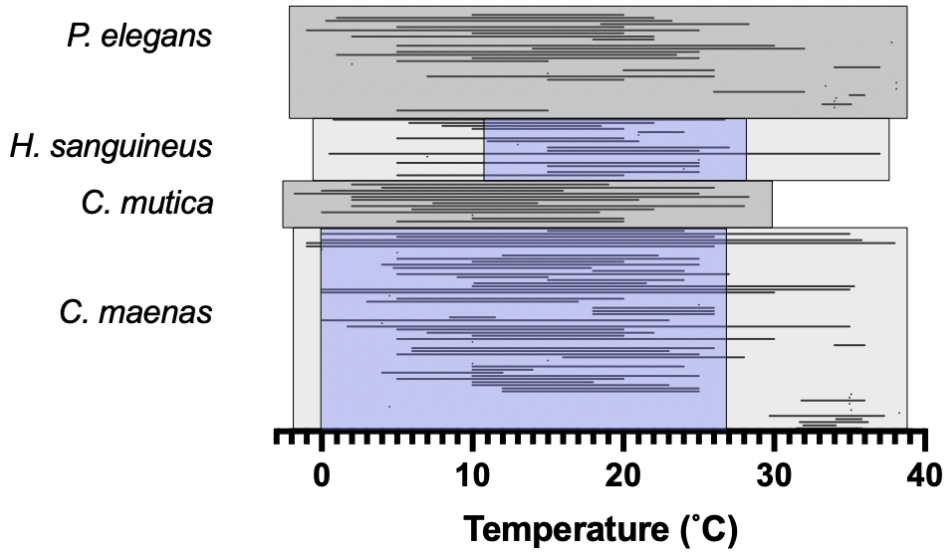

Figure 4: Measured thermal thresholds (dots) and ranges between thresholds (lines) for the arthropod species included in this study. *P elegans* CTmax (34.08±3.01℃). Gray boxes indicate maximum and minimum measured thermal tolerance, periwinkle boxes indicate an estimated reproductive threshold, if one exists.

Cnidarian thermal thresholds

The only cnidarian included in this study is *D. lineata*, who has thermal thresholds ranging from -0.6℃ to 30ºC at the extremes. Peak reproduction falls in the middle of this thermal range. Although they can survive low temperatures, they do not start growing or reproducing asexually until water temperatures reach above 10℃ (Ryan, 2017).

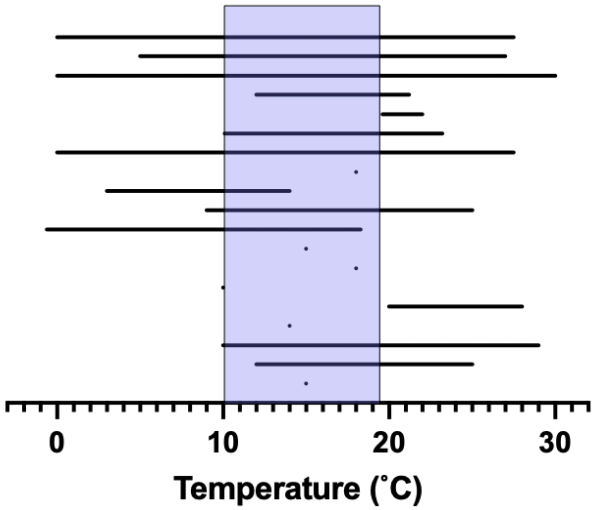

Figure 5: Measured thermal thresholds (dots) and ranges between thresholds (lines) and ranges for *D. lineata*. Box indicates temperature range over which reproduction is possible.

Molluscan thermal thresholds

For the only invasive mollusc in this study, acclimation temperature seems to have an impact on minimum reproductive temperatures for *O. edulis* across its native range. In Spain, oysters begin reproducing around 12℃, however, in Norway, spawning onset does not occur until water temperatures reach 14℃ (Bromley et al., 2016; Colsoul et al., 2021). For adult oysters reaching sexual maturity, temperature has an effect on sex ratios, where the first gametogenesis usually produces sperm, but sequential reproductions can switch between egg and sperm production and are affected by temperature (Zapata-Restrepo et al., 2019). Thus, while certain temperatures may not prove lethal to the oysters, raised temperatures may affect spawning viability for certain populations.

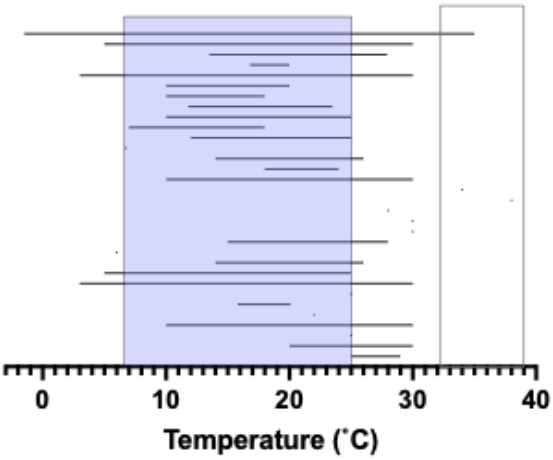

Figure 6: Measured thermal thresholds (dots) and ranges between thresholds (lines) for *O. edulis* included LD50 measurements (34-38℃) and measurement of HSP70 expression, which begins at 25℃. Gray box indicates LD50 range, purple box indicates temperature range over which reproduction is possible.

Many of the species studied had well defined thermal reproductive ranges for sexual reproduction and growth (Figure 7). The widest reproductive range belongs to *C. maenas*, which reproduces year-round in the Gulf of Maine (Frederich and Lancaster, 2023). The organism requiring the highest temperature for reproduction is *B. violaceus*, whereas *M. membranacea* and *C. maenas* have the lowest reproductive temperatures, just above freezing. Of importance, colony growth through budding and asexual reproduction was not included as reproduction for the purpose of this study, so all of the reproduction here is from sexual reproduction. In the growth specific graph, *S. clava* has the narrowest range for growth and *O. edulis* has the widest. When comparing these ranges to the ranges in Figure 1, which ranked organisms from lowest to highest upper mean thermal threshold, there is no similar pattern in reproductive or growth thresholds. In other words, understanding just the temperature range over which an organism reproduces or grows in the field does not indicate what their maximum and minimum survivable temperature is.

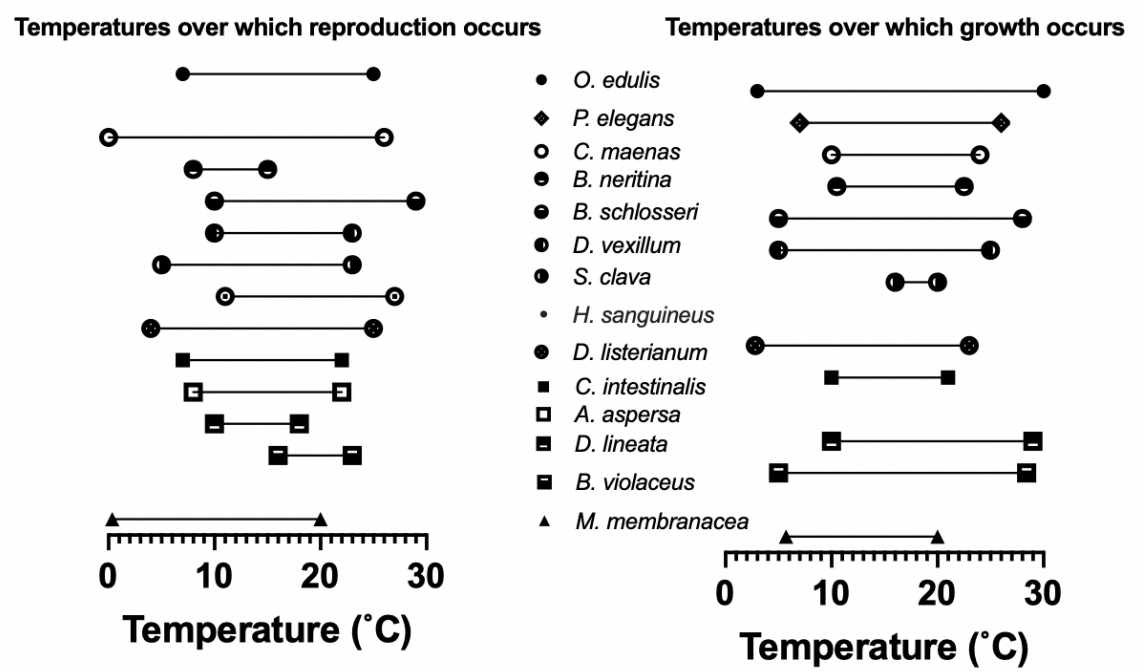

Figure 7: A summary of the temperatures over which the organisms in this study reproduce and grow. The order species in this figure are the same as figure 1, which ranks species from low to high mean high temperature. *Membranipora membranacea* has the lowest mean high thermal thresholds and *O. edulis* has the highest mean high thermal thresholds. Based on the width of reproductive and growth thermal thresholds, there is no correlation between reproductive window and measured thermal thresholds. Thus, maximum, minimum, reproductive, and growth temperatures are all important in species survival in an area.

Which metric is most reliable and recommendations for the future

No matter what measurement method was used, all examples show high variance in thermal tolerance measurements within the same species. From a functional perspective, these data should only be considered useful in the region and season in which the measurement was taken. This lack of continuity has potentially alarming consequences for species distribution modeling in worldwide, dynamic ecosystems.

Frequent examples of genetic variation and local adaptation to acclimation temperatures cause different temperature thresholds in different regions. Some of the variation comes from acclimation temperature, the temperature at which the organism in question is used to in its environment. For example, an animal that lives in temperate and tropical regions has different

acclimation temperatures along the gradient of its distribution. Even in different temperate regions, *P. elegans* experience different osmoregulation capabilities at low temperatures, with Baltic Sea populations being better adapted to colder temperatures than populations near the UK (Janas and Spicer, 2010).

One point for further study is air exposure, at which organisms may experience warmer temperatures than in the water. Many of these species survive in the intertidal zone, and several studies have looked at the impact of dry heat exposure on survival (see for example, Helmuth et al., 2010). One example for *S. clava* found that exposure at warmer temperatures (15-29℃) was more damaging than exposure at 10℃ and that body size played a role in survival under these conditions (Hillock and Costello, 2013). Asian shore crab *H. sanguineus* has a 4x higher metabolic rate out of the water at similar temperatures (Fletcher et al., 2022). Air temperatures in some regions may even reach lethal levels; in Australia coastal temperatures reach above 40 degrees, which is the LT50 for *C. maenas* (Garside and Bishop, 2014). Despite these temperatures, *C. maenas* are capable of evading unfavorable temperatures by moving into the shade, something that tunicates are unable to achieve. The same is true for Asian shore crabs, which can change their distribution in tide pools in Long Island Sound to escape air temperatures reaching above 40 °C (Kraemer et al., 2007). These extreme temperatures are usually temporary, but the length of time can also affect species survival at high temperatures, which is used to prevent spread of biofoulers (Piola and Hopkins, 2012).

Acknowledging that climate change is a very present threat, lower thermal thresholds should also not be overlooked. *Bugula neritina*, *C. intestinalis,* and *M. membranacea* have been seen near Antarctica or are predicted to invade (Avila et al., 2020; Convey and Peck, 2019). Though they have not established populations, invasions of Antarctica become more likely with climate change (Convey and Peck, 2019, Holland et al., 2021; McCarthy et al., 2019). These invasions are especially alarming when considering the current food web of the Antarctic Shelf, which currently has no durophagous, crushing predators, so the introduction of a crab such as *C. maenas* would be highly destructive (Aronson et al., 2015). Without the evolutionary history of a shell-crushing predator, many organisms evolved to have soft bodies or thin shells. Their ability to survive at very low temperatures pose *C. maenas* as an excellent candidate to negatively impact the existing ecosystem (Frederich and Lancaster, 2023). Lithoid crabs are already found

on the Antarctic slope and shelf, so this whole ecosystem will likely be threatened by crustaceans in the near future (Smith et al., 2012).

It is important in future studies to use consistent measurement strategies with larval and juvenile forms as well. In Maine and worldwide, invasive species are impacting the livelihood of fishermen, decreasing harvestable food from the oceans, and disturbing natural habitats. With climate change predicted to increase sea surface temperatures, an understanding of thermal tolerance for all species will be important for management and mitigation at a variety of developmental stages. At the same temperature, 21℃, larvae of *H. sanguineus* have different oxygen consumption rates as they develop (Marsh et al., 2001). Under different temperatures, larvae of *C. maenas* and other species take longer to develop in colder conditions (deRivera et al., 2007). For organisms able to reproduce sexually and asexually through fragmentation or budding, winter temperatures may limit larval development but still allow settlement of fragmented adults (VKM, 2023).

Of note, temperature isn't the only factor that dictates species presence. For example, in Nova Scotia, it was found that neither temperature nor salinity was predictive in *C. intestinalis* distribution. Some species, such as *H. sanguineus*, may also rely on metamorphic cues from nearby adult populations, dictating metamorphosis outside of temperature cues (Anderson and Epifanio, 2010). For *C. mutica* living in Scotland, some populations do not reproduce year round despite mild temperatures, suggesting another factor limiting their reproductive success (Ashton et al., 2010). Other factors, such as heavy metal pollutants or pesticides, may influence settlement or development success for certain species in anthropogenically impacted areas (Lange and Marshall, 2017; Rodrigues et al., 2015). These factors are important, as some invasions begin in harbors whose water quality is usually poor (Carlton, 1996; Schiff et al., 2007). So, even for studies that include temperature, species distribution models should never negate visual surveys (Murphy et al., 2019).

Based on the present study, there is so much inconsistency in the data that it is challenging to put species' thermal tolerance into perspective from an ecological and future projections standpoint. The first is understanding how growth and development are affected by temperature through in-field observations of gamete development, larval supply, settlement, and proliferation. The presence of these three parameters ensures population growth, cell turnover, and dispersal. Due to changing temperatures and local adaptations, simply reporting that an

organism can survive in a region is not enough for modeling their future spread. The second important factor requires a laboratory study that pinpoints the maximum and minimum survivable temperatures for multiple populations, which may be outside of the bounds of ecological relevance but can inform a true upper limit to survival. Rius and colleagues in 2014 do an excellent example of this, showing field abundances and investigating larval development, metamorphosis, and settlement in the laboratory. We summarize this recommendation in Figure 8. While physiological and metabolic signs of stress are important to understanding the mechanistic causes of organisms struggling at certain temperatures, these thresholds only provide limited information to predicting invasive range with climate change. Thus, focusing on the range in which an organism thrives enough to reproduce, grow, and remain healthy, as well as maximum and minimum survivable temperatures, reduces the minimum amount of critical information. Of course, traditional thermal thresholds do have importance on the cellular and molecular levels. Solely measuring survival in the field may gloss over physiological mechanisms and limitations that may hinder organism success. For example, at 20℃, both *H. sanguineus* and *C. maenas* survive easily in the field, but respirometry showed that *H. sanguineus* respiratory rate per gram is twofold higher at higher temperatures, suggesting a greater energetic cost to fill basic survival needs (Jungblut et al., 2018).

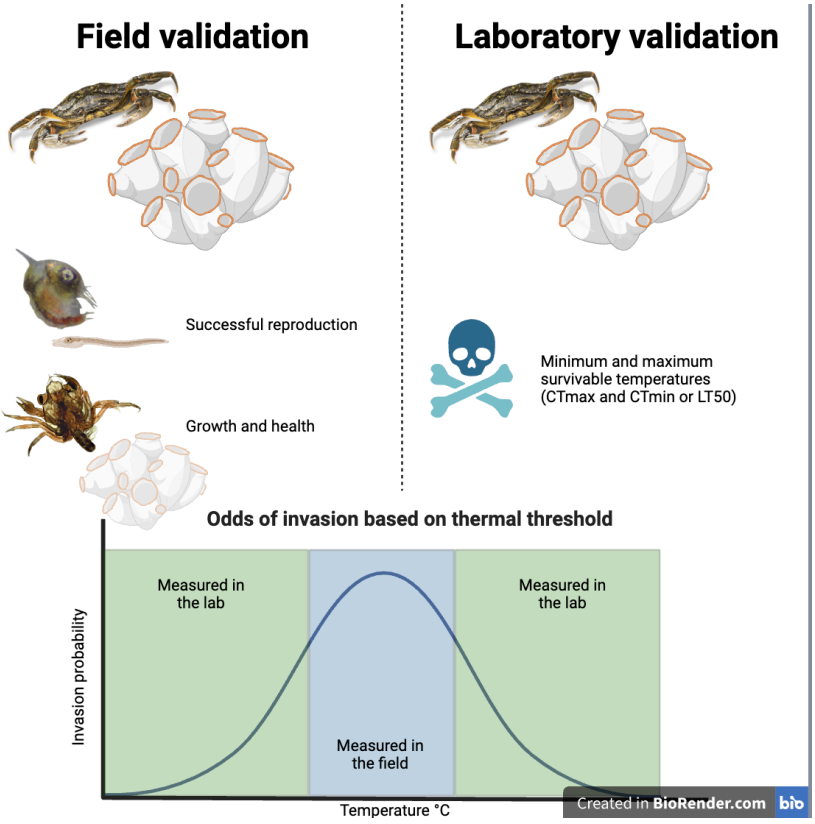

Figure 8: Conceptual model outlining the recommended minimum information for modeling species distribution based solely on thermal thresholds. While use of the traditional thresholds is important for understanding mechanisms, for basic species distribution predictions this model outlines the fewest measurements needed to generate a thermal performance curve. We suggest measurements in the laboratory to deduce the maximum and minimum survivable temperatures and in the field for important milestones such as reproduction and growth.

Here, we highlight inconsistencies in thermal performance measurements and propose a solution to some of the noise within the data. A more integrative approach combining field and laboratory studies to capture the physiology as well as the ecology is required to forecast invasion probability. This study highlights the complexities of thermal thresholds and underscore the pivotal role of acclimation temperatures and consistent measurement techniques. By visualizing the existing data together, we pave the way for more accurate predictions of species distributions, ensuring we don't just scratch the surface but delve deep into the nuanced intricacies of potential invasive species spread in a changing climate.

Chapter 2 | **Thermal Tolerance and Ecological Resilience: Investigating thermal thresholds in *Hemigrapsus sanguineus***

Emily R Lancaster, Damian C. Brady, Markus Frederich

Abstract

Hemigrapsus sanguineus is native to the western Pacific but was introduced to the United States east coast in the late 1900s. Along with other invasive species like *Carcinus maenas*, this grapsid crab has caused changes to nearshore ecosystems by outcompeting native species, eating a variety of prey, and disrupting food chains. In the southern reaches of their invasive habitat, *H. sanguineus* have outcompeted *C. maenas* due to their habitat utilization strategy, strength, and foraging strategies, but further north in the Gulf of Maine, *H. sanguineus* have not become dominant yet. Here, we describe the thermal thresholds of *H. sanguineus* in both the winter and the summer to assess physiological conditions which could limit their poleward spread. We describe a shift in thermal thresholds between summer and winter with higher heart rates, more HSP70 protein expression, and lower reaction times in the winter, coupled with behavioral modifications such as hiding under rocks enabling them to survive. Cold-acclimated crabs shifted their optimum temperatures in response to ambient temperatures. We discuss this metabolic cold adaptation in context of this species' ability to expand its invasive range further north and highlight the importance of measuring thermal thresholds at a variety of acclimation temperatures to understand physiological plasticity.

Introduction

The Asian shore crab, *Hemigrapsus sanguineus*, is native to southeast Asia, and was introduced to New Jersey as an invasive species in 1988 (McDermott, 1998). It has become a common tide pool crab in New England since its arrival; it was first detected at Woods Hole, Massachusetts by 1993 (Epifanio, 2013; McDermott, 1998). Similar to another invasive crab in New England, the European green crab (*Carcinus maenas)*, Asian shore crabs utilize nearshore ecosystems and reproduce successfully in a variety of climates, making them a significant threat to fisheries and general diversity in New England (Bourdeau and Conner, 2003; Epifanio, 2013). *Hemigrapsus sanguineus* are generalist foragers with strong claws; for the first year of life male

H. sanguineus have stronger crushing strength than *C. maenas* (Payne and Kraemer, 2013). According to laboratory studies, they are capable of opening mussels and some clams (*Mercinaria mercenaria*) and will feed on macroalgae species (Bourdeau and O'Connor, 2003). Population genetics studies suggest that New England *H. sanguineus* are continuing to diversify with eight total haplotypes having been identified, 2 of which are newly described haplotypes and the other 6 are other introduced Asian haplotypes (Lord and Williams, 2017). From New Jersey to New Hampshire Asian shore crabs have surpassed green crabs as the dominant intertidal crab through effective habitat utilization (Lord and Williams, 2017). Green crabs were introduced nearly 200 years earlier than *Hemigrapsus sanguineus*, so this drastic shift in tide pool communities suggests that *H. sanguineus* is a strong competitor, whose impacts may overshadow those of *C. maenas* in other regions. At one field site in Biddeford Pool, Maine, USA, this shift from a green crab dominated ecosystem to an Asian shore crab dominated ecosystem has not yet happened (Frederich and Lancaster, 2024). Summer mean sea surface temperatures in their native range are between 13-30℃ (Stephenson et al., 2009). Summer mean sea surface temperatures in the invasive range where *H sanguineus* is the dominant intertidal crab are above 20℃, whereas in northern New England summer mean temperatures drop to below 20℃. This leads to the question whether the Asian shore crab's poleward migration is limited by temperature in the Gulf of Maine.

The Gulf of Maine (GoM) is not only a highly dynamic ecosystem, it is also changing rapidly; the GoM warmed faster than 99% of the world's oceans between 2005-2020 (Pershing et al., 2015; Pershing et al., 2021). This warming has led to shifting kelp forest ecosystems, changes in lobster fishing grounds, and, amongst other factors, contributed to the collapse of the cod and haddock fisheries (Fogarty et al., 2008; Friedland et al., 2015; Pershing et al., 2015; Witman and Lamb, 2018). Though the GoM is warming, it is still relatively cold compared to other habitats that *H. sanguineus* experiences. In its native range in the western Pacific, temperatures range from 2 to 30℃. From New Jersey where they have become the dominant species, ocean temperatures range from 1 to 27℃ (McDermott, 1998). Historically, the influence of the Gulf Stream has been limited due to its divergence offshore, but recently the Gulf Stream has been encroaching on the Tail of the Grand Banks, bringing warm and saline water into the GoM (Neto et al., 2021). This too is decreasing the influx of water from the cold Labrador Current into the GoM. The GoM is dominated by the Gulf of Maine Coastal Current in two parts,

the western Maine coastal current (WMCC) and the eastern Maine coastal current (EMCC) (Pettigrew et al., 2005). Furthermore, there is freshwater input from several rivers along the coast, leading to a highly variable water temperature in this region (Pettigrew et al., 2005; Townsend et al., 2015).

Temperature physiology for green crabs has been extensively studied (for review see Frederich and Lancaster, 2023, Tepolt, 2024). Part of the reason that these and other crustacean species are so successful is due to their extreme temperature tolerance; they survive temperatures below freezing and above 30℃, and are known to reproduce year-round in the GoM (Frederich and Lancaster, 2023). As ectotherms, a crustacean's energy budget is heavily reliant on ambient water temperatures. Thus, their bodily and cellular functions are dependent on their environment. Some of these processes can be modeled by the principle of Q10 (Van't Hoff rule), wherein with every 10℃ temperature increase, the rate of biological processes increases by 2-3 at mid-range temperatures. At both ends of the temperature spectrum, the crabs may reallocate their energy expenditure from reproduction or growth to survival, lose motor function, and, at a point, they may die. Previous studies on green crabs have used a variety of physiological thresholds to measure temperature tolerance such as Arrhenius break temperatures (ABT), critical thermal maxima and minima (CT_{max} and CT_{min}), and the oxygen and capacity limited thermal tolerance (OCLTT) models (Cuculescu et al., 1998; Jost et al., 2012; Kelley et al., 2011). These thresholds are useful because they help standardize methods across studies, but they may not always measure an ecologically relevant threshold (for example, the measured threshold temperature may be well outside the range of expected values for the GoM).

These thresholds are derived from different measurement techniques and calculations measured in living individuals in experiments involving acute temperature stress. The concept of critical thermal maxima and minima applies to organisms across the animal kingdom, with the earliest examples being reptiles whose movement became haphazard at extreme temperatures (Cowles and Bogert, 1944). Generally, a short term exposure to these temperatures is non-lethal; fish exposed to their CT_{max} often remain alive when brought back to ambient temperatures (Brett, 1956). For many crustaceans, a popular measurement technique is to flip an organism on its back and look at righting time (Dayananda et al., 2017; MacMillan, 2019). Arrhenius break temperatures seek a deviation from linearity in mathematically transformed heart rate data, when a line plotting the natural log of the heart rate and the inverse of the temperatures in Kelvin

(multiplied by 10,000 to reach reasonable numbers) changes slope. This concept was first used for enzymes, looking at the thermal limits of their function (Arrhenius, 1889). Heart rates for crustaceans can be measured using electrical probes implanted into the animal, infrared detecting photodiodes, or visually for transparent organisms (Braby and Somero, 2006; Harrington et al., 2020; Depledge, 1984). Although ABTs are easy to measure, some have criticized their usefulness as crustaceans have an open circulatory system and an ABT measurement provides no mechanistic understanding of organismal failure at these temperatures (Frederich and Lancaster, 2024).

The Oxygen and Capacity Limited Thermal Tolerance hypothesis is more mechanistic and assumes that organisms have an optimal range of temperatures where they have enough energy to grow, reproduce, and function (see for review Pörtner et al., 2017). Outside of this range their scope for activity narrows and the animal's condition begins to worsen; this range is called the pejus temperature (Tp) (Frederich and Pörtner, 2000). Outside of the Tp is the critical temperature, Tc, where the animal switches to anaerobiosis–as it is unable to meet its oxygen requirements despite ample oxygen in the environment. This switch to anaerobiosis can be measured through anaerobic byproducts such as succinate in *Laternula elliptica* or lactate in many crustaceans (Frederich and Pörtner, 2000; Jost et al., 2012; Paul et al., 2004; Peck et al., 2004). As anaerobic metabolism ramps up, the organism may also experience an increase in gene or protein expression of heat shock protein 70 (HSP70) and/or AMP activated protein kinase to ensure a supply of ATP (Herzig and Shaw, 2017; Frederich et al. 2009; Jost et al., 2012). Lastly, the denaturation temperature or Td is likely closer to CT_{max}, where the proteins begin to denature (Pörtner et al., 2017).

For less well-studied animals, these measurements have not been performed. The current study has two objectives: First, to define thermal thresholds in the invasive *H. sanguineus* adapted to summer and to winter conditions, and second, to use the identified thresholds to estimate *H. sanguineus*' potential to expand its invasive range further north into colder waters. From these objectives, we hypothesize that using classical physiological measurements, we can explain the slowed dominance of *H. sanguineus* to the north. Furthermore, we hope to identify one traditional metric which best describes the current distribution of *H. sanguineus*. Though these measurements were taken from crabs in the Gulf of Maine, we hope that these findings can be applied to their invasive range elsewhere.

Methods

Animal collection and care

Hemigrapsus sanguineus individuals were collected between 2020 and 2023 from the intertidal zone in Biddeford Pool, Maine, USA (43.44207° N, 70.34098° W) in both the summer and winter. Ambient water temperatures ranged from 15-19℃ or 3.5 to 6.5℃ in the summer and winter, respectively. Crabs were held in a flow-through seawater system at the University of New England and fed a diet of mussels and mackerel *ad libitum* until use in experiments. Both male and female medium-sized (13-38 mm carapace width) hard shelled crabs were used. Gravid females were not used as *H. sanguineus* does not reproduce in the winter in Maine, and reproduction may impact thermal tolerance.

CT_{max} *and* CT_{min}

Crabs were placed in a temperature-controlled seawater tank seawater and temperature was increased or decreased at 5ºC per hour. Every 0.5℃, crabs were flipped onto their backs and their righting time was recorded. Animals were recorded as non-responsive if their righting time exceeded 120 seconds, and the recorded times were used for calculating means. The temperature at which the crab response began to slow down per breakpoint analysis was recorded as the critical thermal maximum or minimum temperature.

ABT

Arrhenius break temperature was measured using heart rates from crabs acutely warmed or cooled over the course of 4 hours. Photoplethysmographs (AMP03, Newshift, Leiria, Portugal were attached to the carapace using dental wax and super glue covering the heart and crab claws were immobilized using electrical tape to prevent wire damage (for more detailed methods, see Depledge, 1984 and Frederich & Pörtner, 2000). Voltages were read by a Pico Technologies oscilloscope (Scope 6404D 4 channel, 8-bit 500 MHz bandwidth) and analyzed using PicoScope 7 T&M software. Every 0.5℃, heart rate was recorded and averaged over 30 seconds of measurements in the winter. For summer acclimated crabs, measurements were taken every 1.5℃. To calculate the ABT, heart rates were transformed as the natural log and the inverse of the temperature in Kelvin was multiplied by 10,000, then used the breakpoint calculator described below to ascertain the ABT.

OCLTT

Oxygen consumption-

Oxygen consumption was measured using an intermittent flow closed-system respirometer (Qubit, Kingston, ON, Canada) with a volume of 250 mL. Animals were heated or cooled from ambient temperature to either 40℃ or 0℃, respectively, over approximately 4 hours and oxygen consumption was recorded continuously. For heating experiments, the system was closed and water circulated for 10 minutes, followed by a 7 minute flush time. For cooling experiments, flow through the chamber was reduced and water was circulated for 8 minutes followed by a 3 minute flush time. Oxygen concentration never fell below 80% during the measurement cycles. Heating experiments continued until death, whereas cooling experiments ceased around 0℃ due to ice buildup in the system. VO_2 was calculated from the difference between inflowing and outflowing oxygen concentration, the chamber volume, and the animal wet weight. Seawater oxygen content was adjusted for temperature and salinity.

Lactate-

Approximately 100 uL of hemolymph was collected from the arthropodial membrane of the last and second to last set of legs using a sterile 1 mL syringe l. Hemolymph was sampled from crabs at temperatures between 0 and 35ºC every 0.5ºC. Once a hemolymph sample was collected from a crab, the same crab was not used again for collection. Samples were stored in a -80℃ freezer until extraction using perchloric acid (PCA) and neutralizing buffer following the spectrophotometric lactate measurement in duplicates by Bergmeyer (1985) described in detail by Frederich & Pörtner 2000.

HSP70-

Heat shock protein 70 protein expression in heart tissue of *H. sanguineus* was quantified by western blots. Hearts were excised quickly from the crabs and flash frozen in liquid nitrogen, then stored in the -80℃ until protein extraction. Tissues were homogenized using a bullet blender (NextAdvance Inc. Troy, NY) and cubic zirconia in a phosphatase inhibiting buffer as outlined in Frederich et al., 2009. Proteins were separated on a 7% polyacrylamide-SDS gel for 2 hours at 120 V. Proteins were transferred to a nitrocellulose membrane on a semi dry transfer chamber (BioRad) at 20 V for 20 minutes before the membrane was blocked with bovine serum albumin blocker diluted with Tris-buffered saline with Tween 20 (TBST), following an overnight incubation in the primary antibodies (Millipore Sigma (H5147). GAPDH was used as a loading

control (Invitrogen, 437000). The membranes were rinsed in TBST before the addition of secondary antibodies for goat anti-rabbit (IRDye 680LT) and donkey anti-mouse (IRDye 800CW). The membranes were scanned on a LI-COR Odyssey infrared laser imager and TIFF files of the scans were analyzed using ImageJ.

Citrate synthase-

To estimate the number of mitochondria from winter and summer crabs, we quantified citrate synthase activity in *H. sanguineus* hearts. Starting with a potassium phosphate buffer (pH 7.4), we added 2 µg of the homogenized protein, 3 µL DTNB solution (Millipore Sigma, D8130-1G), Triton X-100 (Millipore Sigma, T8787) and 10 µL acetyl coenzyme A (Millipore Sigma, A2181), followed by 10 µL oxaloacetate solution (Millipore Sigma, O4126) to catalyze the colorimetric reaction. As a standard, we used citrate synthase from porcine heart (Millipore Sigma, C3260). Immediately upon the addition of oxaloacetate, samples were placed into a spectrophotometer reading every 10 seconds at 412 nm for 2 minutes. Citrate synthase activity was calculated as units/mg protein from change in absorbance, the extinction coefficient for DTNB (13.6 $mM^{-1}cm^{-1}$), the pathlength and the protein content.

Breakpoint analysis

To analyze differences in intercept or breakpoints for physiological data, we used the R package RespR, which calculates deviations from linearity and was designed for aquatic respirometry (Harianto et al., 2019, Carey and Harianto, 2023). RespR runs a rolling regression and rolling average on the data, seeking out the breakpoints at high resolution, following the method described by Yeager and Ultsch (1989). The regression was generated using the broken stick regression model, which seeks the intersection of two linear regressions with the smallest sum of residual sum of squares when looking at two lines fitted to the data. This was used to calculate breakpoints for CT_{min} and Arrhenius break temperatures.

Reproductive timing-

We quantified *H. sanguineus* and *C. maenas* for the past 12 years in a year-round monthly 40 m2 transect from the high- to the low water mark at Biddeford Pool (43.44207° N, 70.34098° W). Within the transect, rocks and macroalgae are moved and all crabs are measured, sexed, and it is recorded whether a crab is newly molted (soft shelled) or gravid. Both *C. maenas* and *H. sanguineus* are collected, but here we present data only for *H. sanguineus*.

Results

Reaction times of crabs were consistently low between about 10 and 30℃ (Figure 1). At the tail end of the temperatures, both summer and winter acclimated crabs slow dramatically. Solely focusing on the lower threshold for understanding potential for poleward migration, the CT_{min} shifted from 9.8℃ in summer acclimated crabs to 8.6℃ in winter acclimated crabs. Evidence for metabolic cold adaptation suggests that cold acclimated crabs shift their thermal thresholds down in accordance with lower ambient temperatures.

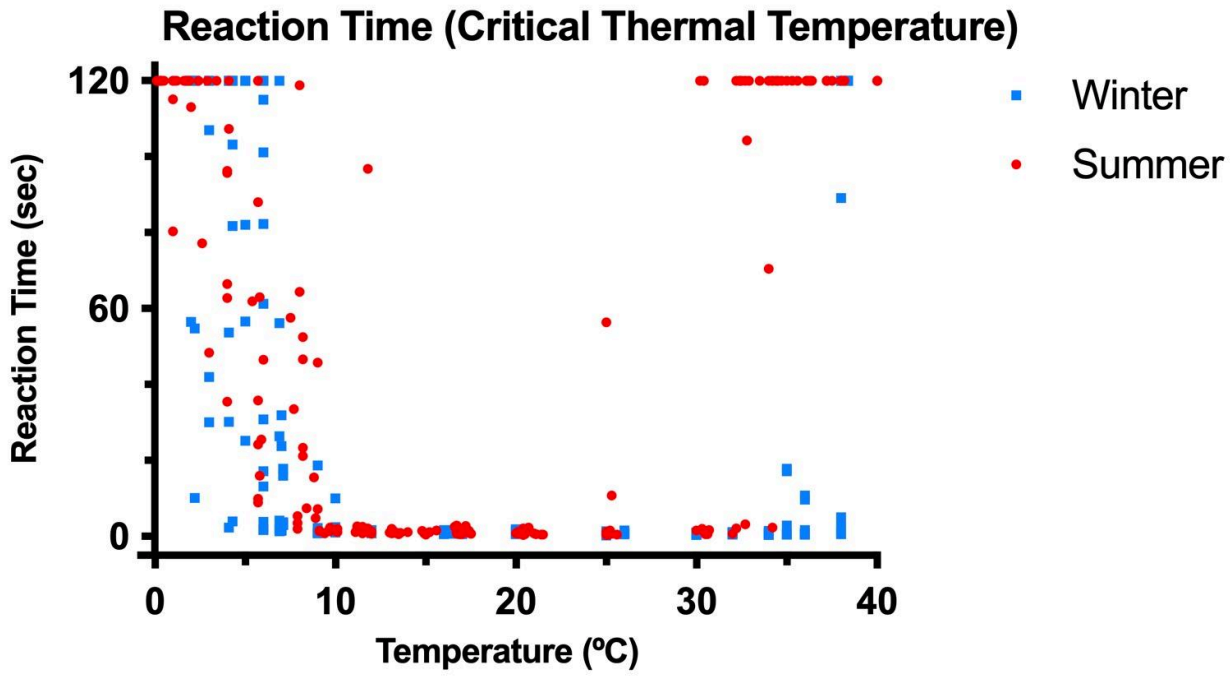

Figure 1: Reaction time of *H. sanguineus* under acute cold and warm stress in summer and winter. Winter acclimated crabs (blue) were cooled and heated from an ambient temperature of between 3.5-6.5℃, summer acclimated crabs (red) were heated and cooled from an ambient temperature between 15-19℃. In the middle of this temperature range, reaction time was fast, slowing down dramatically at the more extreme temperatures. Breakpoint analysis identified where reaction time began to slow. At the colder end of the spectrum, CT_{min} shifted from 9.8 to 8.6℃ for cold acclimated crabs, suggesting a small shift across seasons.

Heart rates were similar for summer and winter acclimated crabs between about 0-20℃ before separating as temperatures increased. Between 20-30℃, winter heart rates were higher than summer heart rates. For heart rates analyzed using Arrhenius break temperatures, the ABT for

winter-acclimated crabs (32.5℃) was lower than for summer acclimated crabs (36.6℃) (Figure 2).

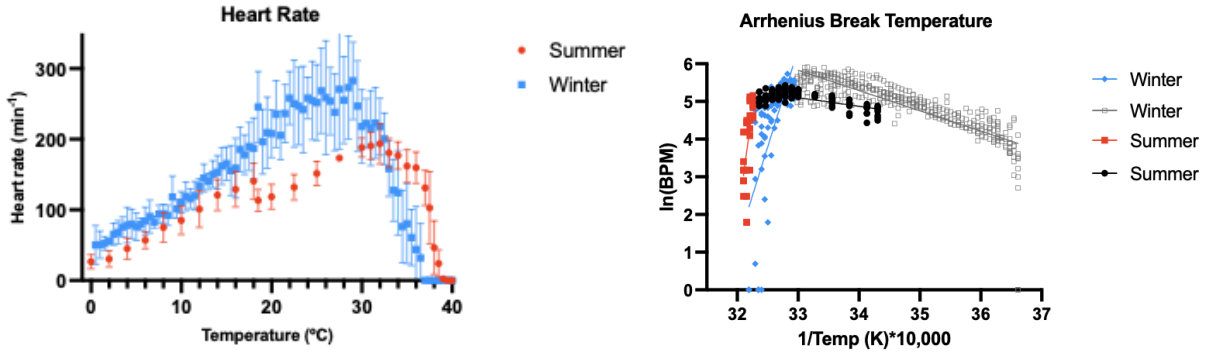

Figure 2. A. Heart rate data from winter acclimated crabs (blue) and summer acclimated crabs (red) are similar at lower temperatures. At warmer temperatures, there is a distinctive shift upward in winter acclimated crabs, suggesting physiological stress at warmer temperatures. B. Arrhenius plot for summer and winter-acclimated crabs, where black dots indicate measurements after the breakpoint for summer acclimated crabs, and open circles represent the measurements after the breakpoint for winter acclimated crabs. For winter acclimated crabs, their cardiac function is disrupted at 32.5℃, for summer acclimated crabs, this occurs at 36.6℃. There was no lower ABT, suggesting that cold temperatures are not leading to collapse in cardiac function for *H. sanguineus*.

Winter-acclimated crabs had lower oxygen consumption than summer-acclimated crabs in the upper temperatures. Between 0-20℃, oxygen consumption ranges were similar for both sets of crabs, as well as above 35℃. Summer-acclimated crabs had peak oxygen consumption around 30℃ and winter-acclimated crabs peaked around 34℃, though not as drastically. This mismatch between heart rate and oxygen consumption in winter acclimated crabs led us to quantify mitochondria using a citrate synthase assay. There was no difference in citrate synthase activity for summer versus winter crab heart tissue (Figure 3).

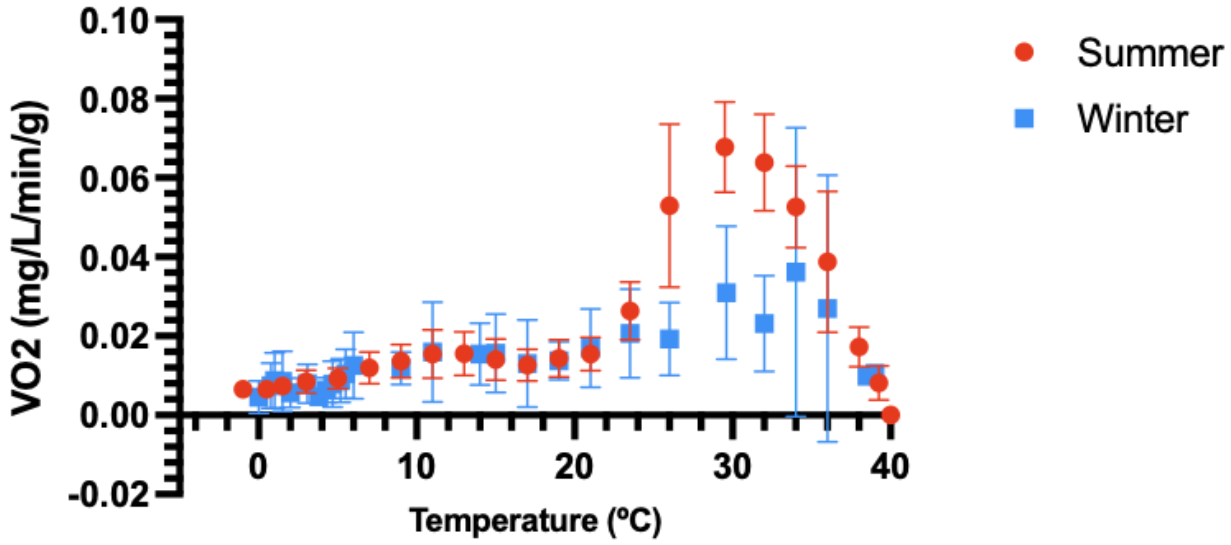

Figure 3: Oxygen consumption measured in summer versus winter-acclimated crabs. Winter acclimated crabs (blue) had generally lower oxygen consumption rates at high temperatures compared to summer acclimated crabs (red). In the lower range, there was no difference in oxygen consumption between warm and cold acclimated crabs.

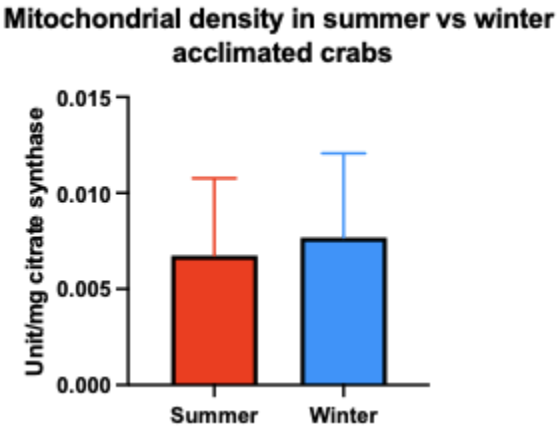

Figure 4: Measured citrate synthase activity in summer versus winter-acclimated crabs from cardiac tissue. Summer acclimated crab's citrate synthase density is marked in red, winter acclimated crab's citrate synthase density is marked in blue. There was no significant difference in citrate synthase concentration in cardiac tissue from these crabs ($t(10)=0.3861$, $p=0.71$).

Summer and winter-acclimated crabs exhibit differences in lactate accumulation due to temperature stress. For example, they switch to anaerobiosis at different temperatures based on the season, suggesting that they are well adapted to the season they are in (Figure 5).

Winter-acclimated crabs do not increase their lactate concentration with lower temperatures, but it gradually increases with warmer temperatures. Thus, this one peak resulting from a gradual slope upward as temperature increases shows thermal stress only at the upper end of the gradient. Summer-acclimated crabs appear to have a thermal optimum around 18℃ but enter anaerobiosis at colder and warmer temperatures, suggesting less plasticity in temperature tolerance in the summer. Summer and winter acclimated crabs accumulate lactate in very different patterns depending on their acclimation season.

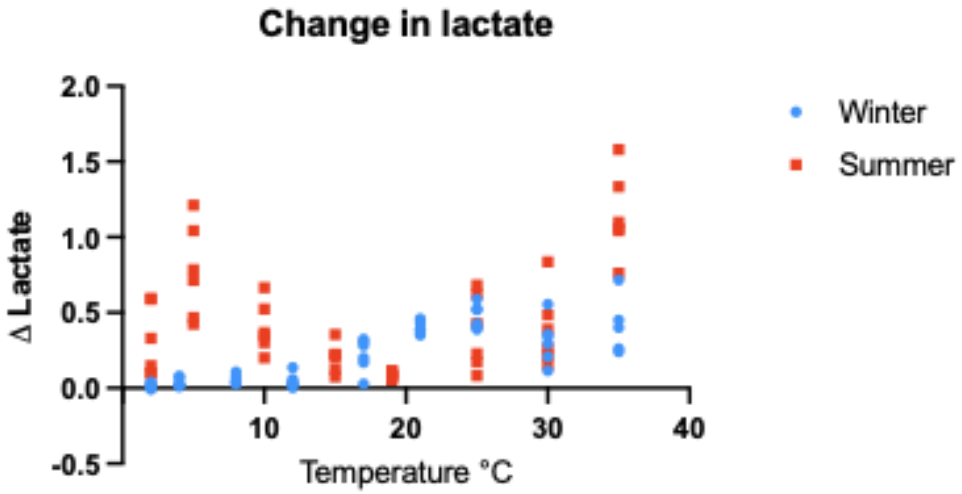

Figure 5: Difference in hemolymph lactate concentrations for summer versus winter acclimated crabs. Winter acclimated crabs (blue) have an increase in lactate as temperature increases. Summer acclimated crabs increase their lactate concentration at high and low temperatures, suggesting that they experience anaerobiosis at most temperatures outside of their range.

Heat shock protein 70 protein expression was low at all temperatures for summer-acclimated crabs (Figure 6). Furthermore, there was little variation in HSP70 protein expression amongst all summer acclimated crabs. For winter-acclimated crabs, there are increases in HSP70 protein expression at low and high temperatures under acute thermal stress. Even at the ambient cold temperature in the winter, crabs were creating HSP70 in response to physiological stress, but this stress was not irreversible as protein expression decreased at milder temperatures. Heat shock protein 70 protein expression was much more variable for winter acclimated crabs, especially at more extreme temperatures.

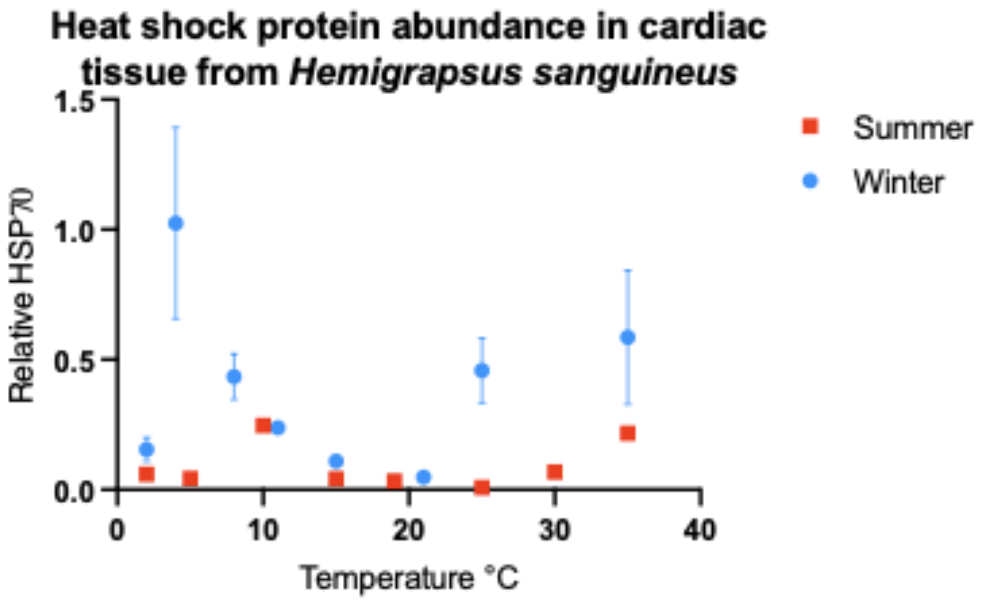

Figure 6: Protein expression of HSP70 measured with western blot for summer and winter acclimated crabs. Winter acclimated crabs not only had higher HSP70 protein concentration, they also had higher variability in HSP70 concentration than summer acclimated crabs, who varied very little. For winter acclimated crabs, the highest HSP70 protein concentrations were at 5 and 35℃. There were slight increases to HSP70 concentration in summer acclimated crabs at 10 and 35℃. Error bars are covered up by the red dots due to their size.

Asian shore crabs do not reproduce year-round in the Gulf of Maine (Figure 7). While green crabs can be found gravid in the Gulf of Maine in all seasons, Asian shore crabs do not become gravid until May, and instances of gravid crabs tapers off in the fall (Frederich and Lancaster, 2023).

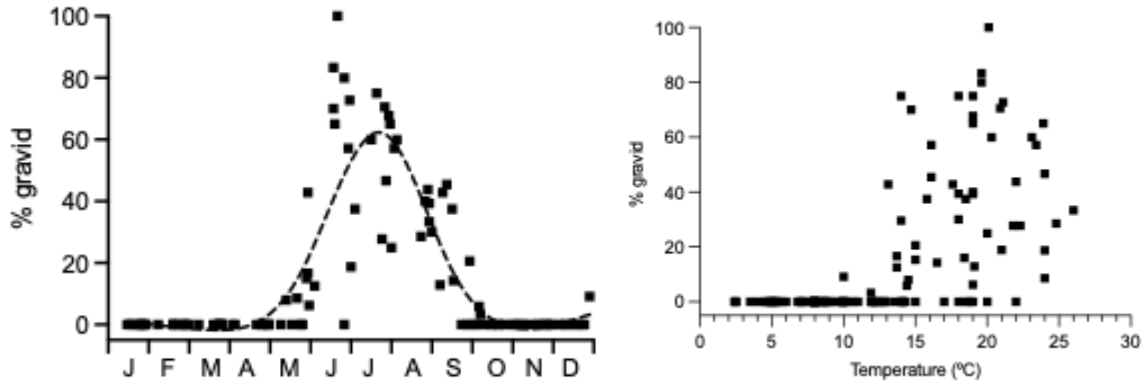

Figure 7: Percentage of female *H. sanguineus* found gravid at Biddeford Pool from 2012-2023. On the left is the percent of female crabs found gravid for each season from 2012-2023 where

gravid crabs are found between April and October. On the right are the same data but with seawater temperature on the x-axis, gravid crabs are usually only seen above 12℃.

Combining these data, we can generate thermal performance curves for summer and winter-acclimated crabs, where the pejus temperature (Tp) is indicated by declining motor activity and increasing HSP70 protein expression and critical temperature (Tc) is where lactate accumulates (a switch to anaerobic respiration) and the heart rate hits a maximum (Figure 8). For winter-acclimated crabs, the Tp_{min} was around 8.6℃ where motor function declines and HSP70 protein concentration begins increasing. Tp_{max} for winter-acclimated crabs was estimated to be 25℃, an average temperature between CTmax, lactate accumulation, and HSP70 protein expression maxima. Lactate did not increase with cold temperature for winter-acclimated crabs, so no Tc_{min} was determined, but a Tc_{max} was estimated as 32.5℃, consistent with the maximum heart rate. Using the same metrics for summer-acclimated crabs, Tp_{min} was 10℃, Tp_{max} was 30℃, Tc_{min} was 5℃ (due to an increase in lactate concentrations at low temperatures) and Tc_{max} was between 36.6 and 40℃.

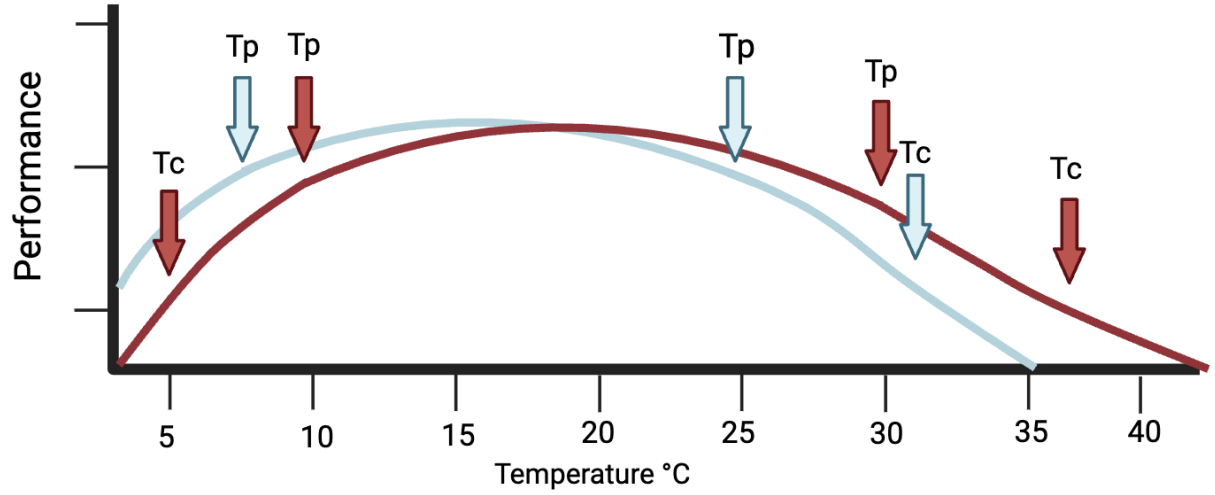

Figure 8: Thermal performance curve based on measured OCLTT values such as heart rate, lactate accumulation, HSP70 protein expression, and oxygen consumption. For summer acclimated crabs (red), the thermal performance curve shifts and Tc and Tp values fall higher than they do for winter-acclimated crabs. For winter acclimated crabs, there was no measurable Tc_{min} and the Tc_{max} was similar to the Tp_{max} of summer acclimated crabs. The y axis is scaled for maximum potential scope for performance for summer and winter acclimated crabs.

We found seasonal shifts in thermal tolerance between summer and winter-acclimated crabs for all measured thresholds. Generally, summer-acclimated crabs performed better than winter-acclimated crabs at higher temperatures, and winter acclimated crabs performed better at lower temperatures.

Discussion

We found seasonal shifts in thermal tolerance between summer and winter-acclimated crabs for all measured thresholds. Generally, summer-acclimated crabs performed better than winter-acclimated crabs at higher temperatures, and winter-acclimated crabs performed better at lower temperatures. Our evidence suggests that *H. sanguineus* acclimates to different seasons at the cellular and system levels to survive in a variety of climates. This trait is common amongst invasive species, which generally exhibit thermal plasticity (Kelley, 2014). *Hemigrapsus sanguineus* has already established invasive populations in Europe and North America and these results suggest that other areas may be at risk of invasion.

Reaction time (CT_{max} and CT_{min}) shows a shift in threshold by over 1℃ in cold acclimated crabs. The shift in CT_{min} for cold acclimated crabs suggests a physiological change that allows the crabs to survive in colder environments. While that is not the minimum temperature the crabs will experience in the Gulf of Maine, the shift in thermal threshold, as well as their behavior, allow them to overcome a lack of mobility. It is worth noting that *H. sanguineus* usually hide under rocks where they are protected from predators. Furthermore, within our crab transect, many *H. sanguineus* disappear in the winter, likely moving subtidally to avoid lower air temperatures (Frederich and Lancaster, 2024).

Shifts in CT_{max} have been seen in a variety of species in short term acclimation studies or when studying different populations. For another invasive crab in the GoM, *Carcinus maenas*, CT_{max} falls between 34 and 36℃ depending on location and acclimation temperature (Jost et al., 2012; Madeira et al., 2014). For native species such as *Cancer irroratus*, CT_{max} is far lower, around 20℃ (Jost et al., 2012). Of course, temperatures as high as 36℃ are far outside of the range of temperatures these organisms experience in water in the Gulf of Maine, but in other regions and when considering air exposure, knowing the absolute limits may help predict species spread into new territories. While we had no discernible difference in CT_{max} for summer and

winter acclimated crabs, further research on short term acclimation and population genetics and their effects on response time for *H. sanguineus* is warranted.

We also found a shift in ABT with cold and warm acclimated crabs. This time, winter-acclimated crabs had a decline in cardiac function 4℃ lower than summer-acclimated crabs. From an ecological perspective, this is acceptable because sudden changes in water temperature near the ABT values seldom occur and the crabs have time to shift between seasons. However, this shift indicates some physiological tradeoff in cardiac function between summer and winter acclimated crabs. Heart rates in the winter were also generally higher than heart rates in the summer. This is in contrast to oxygen consumption, which is lower in the winter and higher in the summer. Both winter and summer-acclimated crabs showed higher oxygen consumption rates at temperatures between 20 and 30℃. Arrhenius break temperatures have been measured for *C. maenas* ranging from 33.7℃ to 37.3℃ based on acclimation temperature and habitat range (Tepolt and Somero, 2014). This ABT is much higher than has been measured for native species such as the American lobster, *Homarus americanus*, with an ABT ranging from 25.2-26.3℃. In some cases, a lower ABT can be measured, such as in *Daphnia sp.* water fleas, where it was 6.5℃ (Frederich and Lancaster, 2024).

Framing the findings in context of OCLTT for *H. sanguineus*, there are definite differences between summer and winter acclimated crabs, suggesting a shift in the thermal optima and scope for activity for these crabs (Figure 8). We were unable to identify a Tc_{min} for winter-acclimated crabs, likely because the ambient water temperature was near the summer Tc_{min}. This is an important observation and may explain the year-round presence of *H. sanguineus* in the Gulf of Maine, despite the fact that summer-acclimated crabs are challenged physiologically and enter anaerobiosis at those temperatures. The animals must shift their energetic stores to basic survival needs rather than growth and reproduction at these temperatures. Thus, it is not surprising that we do not find gravid *H. sanguineus* year-round in the Gulf of Maine.

The concept of metabolic cold adaptation has been used to describe physiological changes that occur to allow polar species to survive in cold water such as slow movement and changing oxygen consumption (Clarke, 1991; Hodkinson, 2003). For some cold-water species, mitochondrial density may increase in colder water to meet their physiological needs. One excellent example of this is described in the lugworm *Arenicola marina* whose distribution

across the North and White Seas creates a natural experiment (Sommer and Pörtner, 2002). The White Sea is colder on average than the North Sea (lower mean annual temperatures of 4 vs 10℃, respectively), and worms in the White Sea had 2.4 times higher mitochondrial respiration and higher rates of succinate oxidation than worms in the naturally warmer environment. Physiologically, the increasing mitochondrial density led to shifts of critical temperature to lower temperatures for worms originating from the White Sea. Lower temperatures lead to slower rates of enzymatic reactions, so increased mitochondrial density helps to overcome that, but has increased maintenance cost. Due to the shifts seen in summer vs winter acclimated *H. sanguineus*, we suspected a similar increase in mitochondrial density to survive the colder temperatures, but surprisingly found no evidence of a change (Figure 4).

Here we focused on adult, non-gravid animals only, but every life stage is important to species survival. A study in Europe compared the larval development of *H. sanguineus* and *C. maenas* under a range of temperatures and food limitations. While *C. maenas* consistently survived all treatments, *H. sanguineus* larvae showed an ability to thrive at the higher temperatures even under food limited conditions (Espinosa-Novo et al., 2023). Rate of larval development is dependent on temperature; Asian shore crabs raised at 15℃ took 55 days to undergo full metamorphosis, whereas at 25℃, crabs completed this development in 16 days (Epifanio et al., 1998). Salinity also has an effect on larval development. The longer *H. sanguineus* remains planktonic, the more risk of floating into unfavorable conditions or being preyed upon, as many larvae do not survive to adulthood (Pederson et al., 2008). We understand that by leaving larvae out of this study we may have overestimated the physiological success of these crabs over their whole lifespan and urge further research on larval thermal tolerance.

Hemigrapsus sanguineus have been slowly moving northward into even colder temperatures in Canada. They were first reported in Canada in southwest Nova Scotia in 2017, and subsequently surveyed throughout southwest Nova Scotia and southwest New Brunswick in 2020 and 2021, with reproduction occurring late spring through early fall (Claudio DiBacco, personal communication). Our data suggest that in the winter in southern Maine, the crabs are physiologically challenged, but not enough to restrict their survival with the help of behavior, so it is reasonable that the crabs have continued their poleward spread. With expected warming temperatures, we anticipate that the northward spread will continue as long as the water temperature hits the threshold for reproduction long enough for larval development to occur.

Although *H. sanguineus* population numbers are limited temporally by a small reproductive window, which could be holding the dynamic between *C. maenas* and *H. sanguineus* relatively constant, behavior and thermal tolerances suggest that the Asian shore crab can continue its northward march, impacting intertidal communities as it goes.

Acknowledgements

Thank you to Aubrey Jane for help troubleshooting western blot protocol. Also, thank you to University of New England undergraduate students and staff for their help in collecting crabs and taking some measurements during the first year of this study, specifically Melissa Butler, Kai Alger, Benjamin Rico, Emma Parish, Tyler Ferrin, Lindsay Forrette, and JJ Custer.

Chapter 3 | Detecting Squishy and Crunchy Invasive Invertebrates: environmental DNA is not shed equally.

Emily R. Lancaster, Erin K Grey, Damian Brady, Markus Frederich

Abstract

Environmental DNA (eDNA) is a powerful tool for detecting organisms in low abundance and can be crucial for early invasive species detection. Despite its potential, the body plan diversity of invertebrates can pose significant challenges, notably arthropods with exoskeletons like the European green crab which can be particularly difficult to detect. In this study, we validated nine single-species quantitative PCR assays targeting invasive and nuisance species in the Gulf of Maine using a two-year eDNA time series. Combining visual surveys and molecular analyses, we successfully detected eight of nine target species with qPCR; however, quantitative assessment was not feasible for all species. [DB1] Our findings demonstrated the effectiveness of eDNA for early invasive species detection but emphasized the need for long-term field and laboratory validation, informed by species' natural histories. It is imperative to recognize that while eDNA is a valuable tool, its applicability varies across taxa. Therefore, interpreting eDNA results requires careful consideration of its limitations and the specific characteristics of the target organisms.

Introduction

Environmental DNA (eDNA) consists of nucleic acids shed by organisms into their environments and can originate from shed cells, waste, gametes, or free DNA from degraded cells (Ficetola et al., 2008; Rees et al., 2014). Environmental DNA with molecular techniques can be a useful tool for species detection and has been used to detect species in air, soil, ice, and water (Ariza et al., 2023; Clare et al., 2021; Ruppert et al., 2019; Willerslev et al., 2004). There are two major methods of detection when using eDNA: a broader community approach and a single species approach. For analyzing the broader community, metabarcoding, amplification and sequencing of a barcode locus (such as cytochrome c I oxidase (CO1), ribosomal genes 18S, 16S, or 12S) can provide insight into a broad range of taxa present in an area (van der Loos and Nijland, 2021). Metabarcoding can detect multiple species in one sample, rather than multiple

tests, so it can be a faster method for detecting invasive species semi-quantitatively (****). The alternative is a single species assay, using techniques such as quantitative PCR (qPCR), light mediated isothermal amplification (LAMP), or digital droplet PCR (ddPCR), which use primers specific to a species to provide a quantitative measure of eDNA abundance for the taxa of interest (Baudry et al., 2023; Kageyama et al., 2022). These species-specific methods are more precise and give more quantitative results but take more time as they have to be processed separately in many cases. Depending on the research objective, one or both techniques may be used to detect the species of interest.

Abiotic factors can influence the fate of eDNA. For example, fish eDNA tends to accumulate in sediment, which may over-represent species if disturbed during water sampling (Turner et al., 2015). Furthermore, many studies have investigated the longevity of eDNA in water, finding that factors such as temperature, bacteria, and UV can lead to degradation of samples (Eichmiller et al., 2015; Tsuji et al., 2017). Tides and other water movements may influence detection probabilities, though one study found that eDNA from benthic species and plankton stayed consistent across tidal cycles (Kelly et al., 2018). Thus, there are many factors that must be considered when designing an eDNA experiment that can be used in decision making contexts. Although eDNA is ephemeral, it is useful for detecting species within a certain amount of time.

In aquatic ecosystems, eDNA has been used to detect fish, mammals, microbes, plants, algae, and invertebrates (Ruppert et al., 2019). Fish communities in aquariums are studied to ensure that all species are represented in the sequencing data (Kelly et al., 2014; Silverbrand, 2021). In smaller-scale mesocosms, single species assays have shown agreement between fish biomass and magnitude of eDNA detection (Dejean et al., 2011; Lacoursière-Roussel et al., 2016; Takahara et al., 2015; Tsuji et al., 2017). For fish species, there is generally good agreement between eDNA detection and fish abundance, even in systems with moving water (Kelly et al., 2018; Pont et al., 2018). Even in ecosystems like the deep sea, eDNA detection levels are correlated to fish caught by trawls and can detect species that are usually not caught in trawls, such as the Greenland shark, *Somniosus microcephalus* (Thomsen et al., 2016). In lakes, eDNA has been similarly used to compare catch data to qPCR eDNA concentrations and found similar quantitative results using metabarcoding (Hänfling et al., 2016; Valentini et al., 2016).

Due to the precision of qPCR, it is generally considered to be more reliable for biomass assessments than metabarcoding.

Environmental DNA has also been used to detect and monitor invasive species. There are various definitions for invasive species, but for the purpose of this study we define invasive species as organisms which establish a population in a recipient community after being moved using humans as a vector (Molnar et al., 2008). These species are not necessarily harmful to humans or the environment, but due to a lack of evolutionary history with members of the recipient community, they can cause damage (Edgell and Hollander, 2011). Early detection of invasive species is important to reduce the spread of these species to new regions. As the effect size of invasive species increases with time, eradication of invasive species becomes more difficult, and management strategies may switch to control and adaptation rather than removal (see, for example, Haubrock et al., 2022). Thus, detection of invasive species early in their invasion history is critical if the overall goal is eradication and eDNA techniques could assist in this aim.

Many invasive species eDNA studies focus on presence/absence of invasive species rather than attempting to quantify the number of animals, despite using single species assays (Kim et al., 2018; Takahara et al., 2013). Many eDNA studies focus on Asian carps, which are invasive in many waterways and cause damage to ecosystems by eating and outcompeting other fish species; they cause harm to other organisms by decreasing water quality; and they cause harm to humans by jumping out of the water (Kolar et al., 2007). Although they had not been detected or caught in the Laurentian Great Lakes in the early 2000's, an eDNA survey beginning in 2009 found their DNA present nearby in 2010 (Jerde et al., 2011). This detection was met with scrutiny, but bighead and silver carps are challenging to catch with traditional surveys. Eventually, the fish were caught where eDNA was detected, validating this method for detecting hard-to-catch invasive species (Jerde, 2021). Laboratory studies using the same species (*Hypophthalmichthys nobilis* and *Hypophthalmichthys molitrix*) and other carp species have found correlations between biomass and amount of eDNA detected (Klymus et al., 2015; Takahara et al., 2016).

Some invasive species are harmful to ecosystems and their inhabitants. These impacts can be direct, through direct aggression and space utilization (Bullard et al., 2004; Macdonald et al., 2007; Rius et al., 2009;), or indirect, through resource sharing and allelopathy (Davis et al.,

1991; Schenk, 2006). The impacts of these species can change community dynamics and may be exacerbated by climate change and other disturbances, which further stresses native species (Altman et al., 2007; Mack et al., 1998). Furthermore, invasive species can cost millions of dollars annually in damaged structures, fisheries losses, and effects on human health (Finnoff et al., 2005; Pejchar and Mooney, 2008; Pimentel et al., 2004). Therefore, from a management and policy perspective, early detection of these species not only helps local ecology, it also helps reduce the costs of managing potential invaders. Traditional species monitoring methods include fishing, trapping, or visual surveys, but eDNA could assist or replace these methods.

Despite numerous success stories, eDNA methods cannot be applied to all systems equally. Mammalian species are notoriously challenging to detect with traditional visual surveys due to their elusive nature. Qu and Stewart detected the endangered Yangtze finless porpoise, *Neophocaena asiaeorientalis asiaeorientalis*, with qPCR, a method that is more repeatable, efficient, and cost effective than visual surveys (Qu and Stewart, 2017). This study compared cost for visual, PCR, and qPCR-based detection of the porpoise; it found that qPCR was the best option for detection, as the method was highly sensitive and less expensive than widespread visual surveys. Due to the low density of mammals and the ephemeral nature of eDNA, false negatives are a serious concern for species detection. In a stream, eDNA was used to detect a freshwater pearl mussel (*Margaritifera margaritifera*) where they concluded that eDNA can be used as a non-destructive complement to traditional surveys (Stoeckle et al 2015). They noted degradation of the eDNA downstream and detection of an extinct population, either due to missed individuals or shells shedding eDNA. So, while there is a lot of potential in using eDNA to detect species, there are still uncertainties about the source and reliability of the method. Whether these difficulties are related to the body plan of the species being studied or species level variation is unclear.

Although there are challenges to detection using eDNA signals, there is also considerable promise for the widespread use of eDNA methods to detect species. Due to the cost-effective nature and ease of taking a water sample over traditional survey methods, using eDNA techniques would be preferable if the molecular signal is properly understood (i.e. biomass estimations versus presence/absence). To test whether species-specific eDNA signals can be used to detect presence or absence, and even biomass, of invasive invertebrates in a highly dynamic marine intertidal system we compared qPCR-based eDNA data to visual surveys in a tide pool

over two years. The species detected included seven species identified as invasive through the Marine Invader Monitoring and Information Collaborative, as well as one cryptogenic species and a nuisance species. Based on previous eDNA literature, we hypothesized that the species with soft and exposed tissues would shed eDNA consistently with their abundance, whereas organisms covered with shells or exoskeletons would be more challenging to detect.

Methods

Field molecular methods

Environmental DNA samples were collected monthly from a tide pool in Biddeford Pool, Maine, USA (43.44207° N, 70.34098° W) from June 2021 through July 2023, excluding October-December 2021 (Figure 1). The samples were taken at six sites, four of which were fully disconnected from the ocean at low tide (tide pools) and two of which were on the ocean-side of the rocks with constant flowing water. The tide pool area was approximately 40 m wide and consisted of rocky sided pools where water depth did not exceed 1 meter. Samples were always collected at slack low tide using an extendable 8 m pole fitted with a water bottle holder which could hold bleach-sterilized 500 mL Nalgene bottles. 500 mL seawater samples were collected from 6 sites along a 40 m long area in the tide pool. Water samples were stored in the dark and on ice until filtered later the same day in the laboratory. A field control consisting of a 500 mL Nalgene bottle of deionized water was opened for one minute at the site. Field controls were analyzed through each of the following steps to ensure no contamination.

Figure 1: The field site location in Biddeford, Maine, USA in Southern Maine in Saco Bay. Inlaid map of Maine with black star indicates the general location of the tide pools relative to the state. An aerial view of the tide pools is shown, with the pools sampled visually and with eDNA outlined in white. The open ocean side of the pools is in the top of the photos and two eDNA samples were also taken there, though visual surveys were not conducted.

Field visual methods

Visual surveys were used to detect nine invertebrate species common to New England: *Botrylloides violaceus*, *Botryllus schlosseri, Ciona intestinalis, Didemnum vexillum, Diplosoma listerianum, Hemigrapsus sanguineus, Membranipora membranacea, Ostrea edulis*, and *Semibalanus balanoides*. The only species studied here that is not an invasive species is the northern acorn barnacle, *Semibalanus balanoides*, which is a common intertidal organism and belongs to a group of organisms known as biofouling species for aquaculture (Zazzaro et al., 2018). The Marine Invader Monitoring and Information Collaborative (MIMIC) is an organization using visual surveys performed by trained volunteers in northern New England studying over 100 sites in Massachusetts and Maine since 2008 to detect marine invasive

species. The nine invasive and nuisance species in this study were categorized as such by MIMIC or have been identified by other parties as cryptogenic (*Ciona intestinalis*) (Dewitt, 2002). Immediately after sampling, we conducted a visual survey for invasive species using the MIMIC protocol. The MIMIC protocol is based on the Puget Sound Expedition and generates a relative abundance of invasive species, whereas the photographic data provided a quantitative measure of changing abundances (Cohen et al., 1998; Pappal and Baker, 2011). The amount of each organism across the tide pool area was categorized as abundant (present everywhere), common (present in more than half of surveyed sites), few (present in less than half the surveyed sites), or rare (one or two individuals across the tide pool). These rankings were coded for visualization, where 4 was abundant, 3 was common, 2 was few, 1 was rare, and 0 was not present at the time of the survey. Visual surveys only occurred for the four sites that were isolated at low tide; due to the flux of water on the oceanic side of the transect, visual surveys were not used. Temperature was measured in the tide pools during each sampling event.

Laboratory methods

eDNA filtration and extraction

All eDNA filtration occurred in the laboratory on the same day as samples were collected. All 500 mL of each sample were vacuum filtered through 0.45 μm cellulose nitrate filters (Sartorius Stedim Biotech GmbH). Before pouring the samples onto the filter, the water was swirled in the bottle to suspend any particulate matter that may have fallen to the bottom of the bottle. The vacuum manifold was housed in a hood, which was always sanitized before use with a 10% bleach solution and an 8W UV light for at least 10 minutes. All removable filtration equipment was soaked in a 10% bleach solution for 10 minutes prior to use and then rinsed with water until no bleach smell remained.

Filters were rolled using bleach cleaned forceps and placed into a labeled 1.5 mL Eppendorf tube and stored at -80℃ until DNA extraction using the Qiagen DNeasy Blood and Tissue Kit. The extraction protocol was slightly modified from the manufacturers recommendation to increase eDNA yield. First, after the addition of lysis buffer ATL and proteinase K, the filters were incubated at 56℃ for three hours and vortexed once every hour to ensure the buffer was reaching all parts of the filter. Final elution of DNA was performed in 80 μL of elution buffer AE to increase eDNA yield.

Laboratory based experiments

To ground-truth some of the trends observed in the field, we conducted controlled lab experiments. These experiments were only performed for organisms that could be harvested without risk of fragmentation, which could lead to the spread of more invasive species (Valentine et al., 2009), or that were commonly found alive in the tide pool. Organisms were placed in 19 L buckets containing sterile artificial seawater (Instant Ocean SeaSalt mixed with deionized water, UV sterilized) with a sterilized bubbler for oxygen. Organisms were distributed amongst the buckets in groups of small, medium, or large (by weight or surface area) to assess how biomass impacted eDNA shedding rate. Buckets were sealed and set into a tank with flowing seawater to maintain ambient seawater temperatures between 14 and 18℃ within the mesocosms. After 24 hours, 500 mL of water was collected from each bucket for filtering and DNA extraction using the same protocol as the field samples. Organism abundance was measured in two ways, either surface area was calculated and the eDNA concentration was analyzed using a linear regression, or wet weight was used, and organisms were classified as small, medium, and large for comparison.

Table 1: Small, medium, and large groupings of three of the species studied in laboratory experiments. Numbers are listed in grams of wet weight.

Species	**Small**	**Medium**	**Large**
Botrylloides violaceus	10.24±5.03 g	34.22±2.78 g	78.03±3.01 g
Hemigrapsus sanguineus	8.45±1.23 g	30.55±1.61 g	71.60±5.20 g
Ostrea edulis	49.67±6.32 g	203.35±29.00 g	444.36±37.77 g

qPCR qPCR assays were used to identify some of the invasive invertebrate species common to New England: *Botrylloides violaceus*, *Botryllus schlosseri, Ciona intestinalis, Didemnum vexillum, Diplosoma listerianum, Hemigrapsus sanguineus, Membranipora membranacea, Ostrea edulis*, and *Semibalanus balanoides* (not an invasive species, but a nuisance species) (Table 2). Assays were generated either with Primer3 or IDT PrimerQuest. Most of the qPCR assays were performed on a Stratagene MX3005P qPCR thermocycler aside from the *O. edulis* assay, which used a fluorophore not detectable with the filters installed on the MX3005P, so that assay was

developed on a BioRad qPCR CFX Opus 96 Real-Time PCR System. The supermix for all qPCR reactions was identical: 10 µL of Applied Biosystems TaqMan FastAdvanced Master Mix, 0.3 µL of each primer and probe (all 10 µM stock solution), 7 µL of water, and 1 µL of DNA. For one qPCR plate of each sample, the mixture was also spiked with an exogenous internal positive control (ThermoFisher) to test for PCR inhibition. Most assays were performed for 3 minutes at 95℃ to activate the polymerase, followed by 40 cycles of 15 seconds at 95℃ and 30 seconds at 60℃. The only assay that varied from this was *D. vexillum*, where we followed the qPCR protocol from Matejusova et al., (2021) which started with 2 minutes of 50℃, 10 minutes at 95℃, then 45 cycles of 95℃ for 15 seconds and 55℃ for one minute.

Table 2: Primers, probes, and their sources for species specific qPCR of invasive Gulf of Maine species. F represents the forward primer, R represents the reverse primer, and the probes were labeled with 6-FAM and MGB. All oligos are listed 5'-3'. Limit of detection (LOD) and limit of quantification (LOQ) are calculated based on concentrations determined in a Nanodrop spectrophotometer of genomic DNA extracted from the organisms.

Species	**Primer**	**Source**	**Length (bp)**	**LOD*/LOQ*/efficiency** *(copies/µL)
Botrylloides violaceus	F-GGACAATGTTGGTAACTACTG R-CGAAGAAAGACGTATTGAAATTAC Probe- CAGCAGCCATTACA	This study	105	
Botryllus schlosseri	F-TGAACTGTTTATCCTCCCCTTTCTAGA	LeBlanc et al., 2020	179	0.0002 / 0.02 / 0.89

	R- CAAAACAAAGATATAGAAAA RAGTCCCCA Probe- TCATTCTAGAGCTGCTTTG			
Ciona intestinalis	F - ACTTTTTTTGATCCTAACAGAA GAAGGG R- CACACTAGAAATCTAAGAAAC CTAATTCCTCTT Probe- TTGATCCTACCAAGATTTAGAA	LeBlanc et al., 2020	212	0.000018 / 0.0018 / 1.1
Didemnum vexillum	F- CGACTAATCATAAAGATATTAG AACA R- TTCTTGTAGAACTTAATTCTATT CG Probe- ATAGT{T}{A}GAGCT{A}G{A}T TTAGT{A}TA{A}	Matejusov a et al., 2021	111	0.00015 / 0.0015 / 0.88
Diplosoma listerianum	F- CTAGGCAATTGATTAGAAAT AGAC R- GCTCTTAGTATTAAAGGTAAT AACC	This study	119	0.0003 / 0.03 / 0.94
Hemigrapsus sanguineus	F- CCTGGGCCGGTATAGTAGGT	Knudsen et al., 2020	136	0.00056 / 0.056 / 1

	R-GGGGCTCCGAGTATAAGTGG Probe- CGAGCAGAATTAAGACAACCAGGAAGC			
Membranipora membranacea	See citation for details	Greenlee et al., in prep		0.0017 / 0.17 / 0.92
Ostrea edulis	F- GGTAGTTTCTGCATTTGTTG R- TGCACATTCCATGATATGAA Probe- ACTGGCTGAACTGTCT	This study	89	0.000045 / 0.0045 / 0.89
Semibalanus balanoides	F - TGCCACCAGCTTTAATACTTCTA R - GATCTACAGAGGCTCCAGAATG	This study	120	0.00025 / 0.025 / 0.99

All qPCR assays were validated by comparing the primers and probe to DNA extracted from tissues of many intertidal organisms common to the Gulf of Maine (all species included in this study as well as the American lobster *Homarus americanus,* green sea urchin *Strongylocentrotus droebachiensis,* green crab *Carcinus maenas,* Jonah crab *Cancer borealis,* European grass shrimp *Palaemon elegans,* common periwinkle *Littorina littorea,* flat periwinkle *Littorina obtusata,* European sea squirt *Ascidiella aspersa,* knotted wrack *Ascophyllum nodosum,* bladderwrack *Fucus spp.,* northern sea star *Asterias rubens,*forbes sea star *Asterias forbesii,* and American oyster *Crassostrea virginica*). All primers were validated *in silico* against these species as well as using a SYBR green qPCR and melt curve analysis to assess specificity. Only assays with no amplification of other products were used. To calculate the limit of detection (LOD), limit of quantification (LOQ), efficiency, and r^2, three replicates of standard curves ranging over

eight orders of magnitude were performed simultaneously (Klymus et al., 2019). In addition to this standard curve, a standard curve consisting of two replicates of at least four orders of magnitude concentration was included with each plate to ensure optimum efficiency and r^2 values of those curves.

Results

qPCR

For many species, we found seasonal oscillations in detectable eDNA consistent with the life cycles and abundances of each organism. For some of the colonial tunicates (e.g., *B. violaceus* and *B. schlosseri*), their presence in the tide pools was highest in the spring and summer and they nearly disappeared in visual surveys during the winter (Figure 2). We found higher eDNA concentrations [DB1] when tunicate biomasses were at the highest in the tide pools and in laboratory experiments (Figures 2 and 3). The lowest eDNA concentrations for each of these species occurred when water temperatures dropped below 15℃, especially when the species became dormant in the coldest winter temperatures.

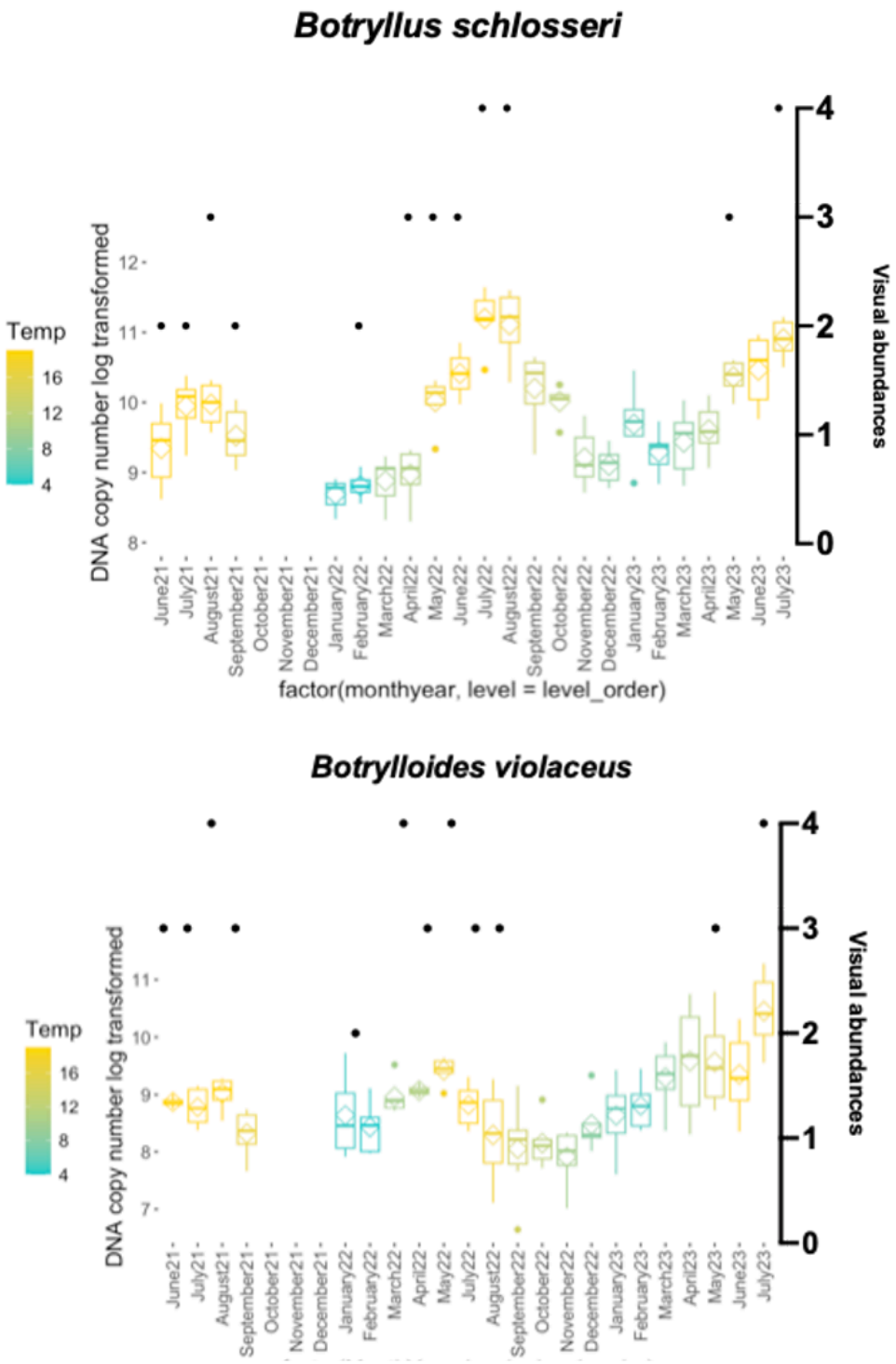

Figure 2: The amount of eDNA shed by two species of colonial tunicates, *Botryllus schlosseri* and *Botrylloides violaceus*. Black dots indicate visual abundance. These invasive species visually had their highest abundance in the summer when water temperatures were warmer. Both species

nearly disappeared from the tide pools in the winter. These patterns were especially strong for *B. schlosseri*, and *B. violaceus* increased in abundance over time.

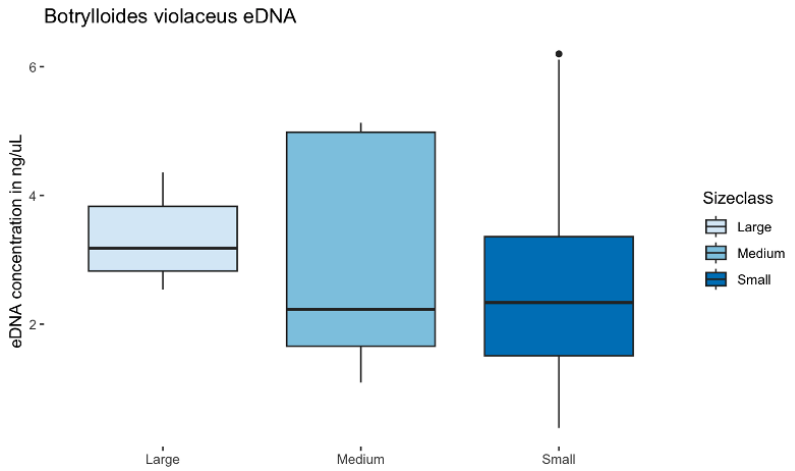

Figure 3: eDNA shedding for *Botrylloides violaceus* in controlled lab conditions. While the means appear to increase with increasing size, there is no difference in the amount of eDNA shed amongst the different size tunicate colonies (ANOVA $F_{2,27}$=0.3, p=0.743).

For the other two colonial tunicate species, these trends were not as consistent. *Didemnum vexillum* was visually present year-round in the tide pools, though the colonies degraded and fragmented in the winter (Figure 4). They were also subject to snail predation year-round, but it was especially noticeable in the winter because *D. vexillum* was the most common sessile invertebrate present in the winter.[DB3]

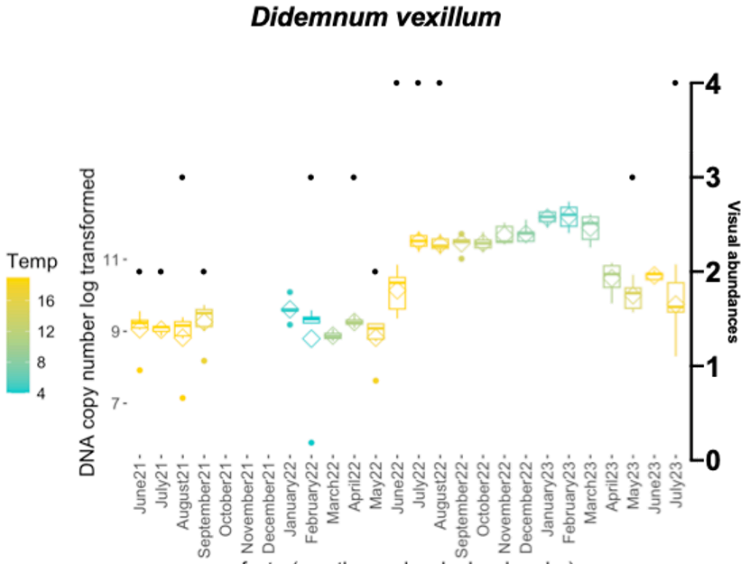

Figure 4: Amount of detectable eDNA shed by *Didemnum vexillum* at Biddeford Pool between June of 2021 and July of 2023. Black dots indicate visual abundance. This species was visually present year-round and detected with eDNA even when temperatures were low.

The colonial tunicate, *D. listerianum*, was not detected using qPCR, despite being observed in the summer and fall in the tide pools (Figure 5). Similar to other soft bodied tunicates, their presence increased in the summer.

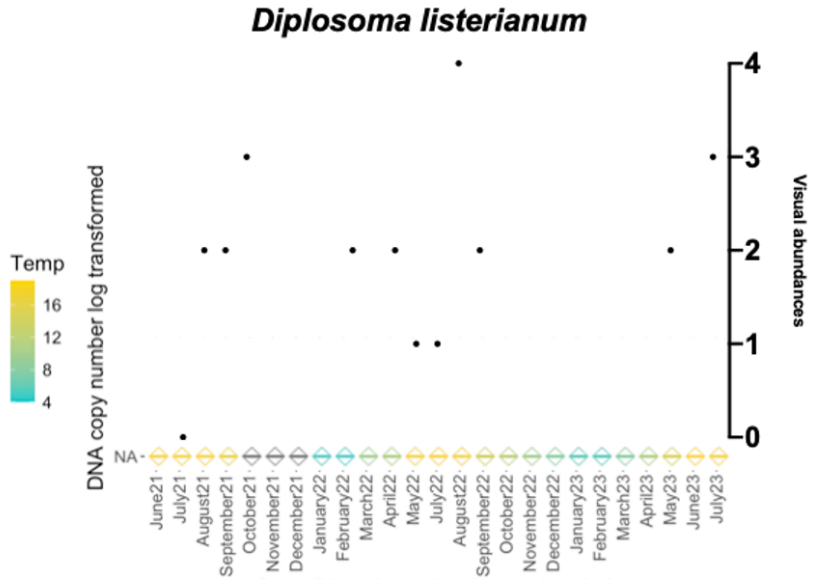

Figure 5: Amount of detectable eDNA shed by *Diplosoma listerianum* at Biddeford Pool between June of 2021 and July of 2023. Black dots indicate visual abundance. Environmental DNA from this species was not detected at any time during this study, despite visual confirmation of its presence.

In April of 2022, we identified one adult sea vase tunicate, *C. intestinalis* individual in the tide pool, which had not been observed previously in 5 years of monthly summer surveys of this tidepool (Figure 6). Using eDNA, this solitary tunicate was detected beginning in January, four months before it was observable in the visual survey. No other individuals were visually detected in the tide pool over this time series.

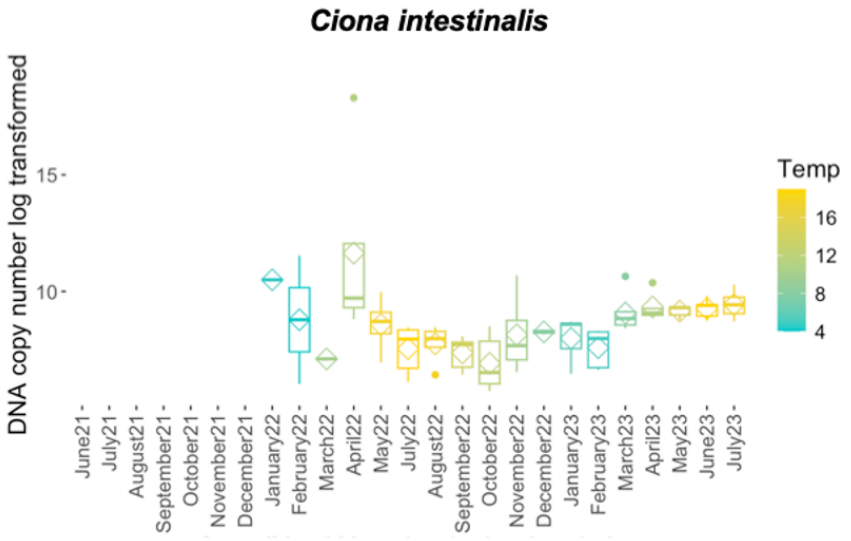

Figure 6: Amount of detectable eDNA shed by *Ciona intestinalis* at Biddeford Pool between June of 2021 and July of 2023. Abundance was not measured in the same way, as this species is not recorded by the Marine Invader Monitoring and Information Collaborative, so no visual abundance is graphed. This species was first seen in visual surveys in April of 2022 and only that individual was seen throughout the course of this survey.

In a laboratory study, we found a linear increase in detectable eDNA with increasing amounts of *C. intestinalis* (r^2=0.658) (Figure 7).

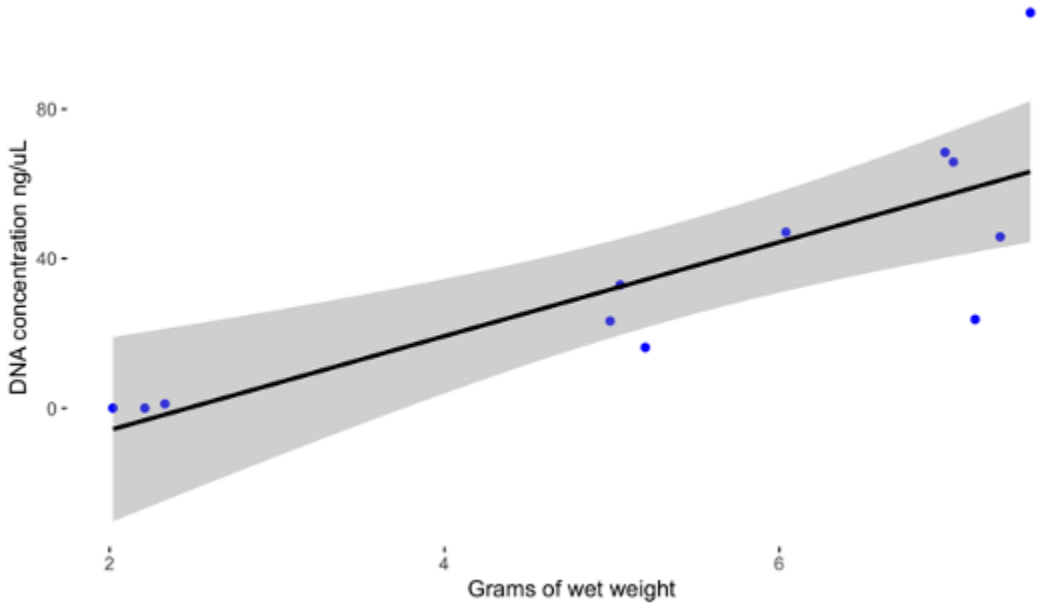

Figure 7: A comparison between biomass of *C. intestinalis* and detected eDNA concentration. There is a positive correlation between shed eDNA and wet biomass of *C. intestinalis* in laboratory experiments (r^2=0.658). The gray area indicates the 95% confidence interval around the linear regression which is the black line.

Several species displayed no pattern of eDNA in the tide pools. One of those species, *M. membranacea* was usually only found on wrack which washed into the tide pool as there were not many macroalgae surfaces conducive to their growth, and colonies were mostly dead. Thus, there were no seasonal patterns in eDNA detection over time for this species, but the second year of the time series generally had more detectable eDNA (Figure 8).

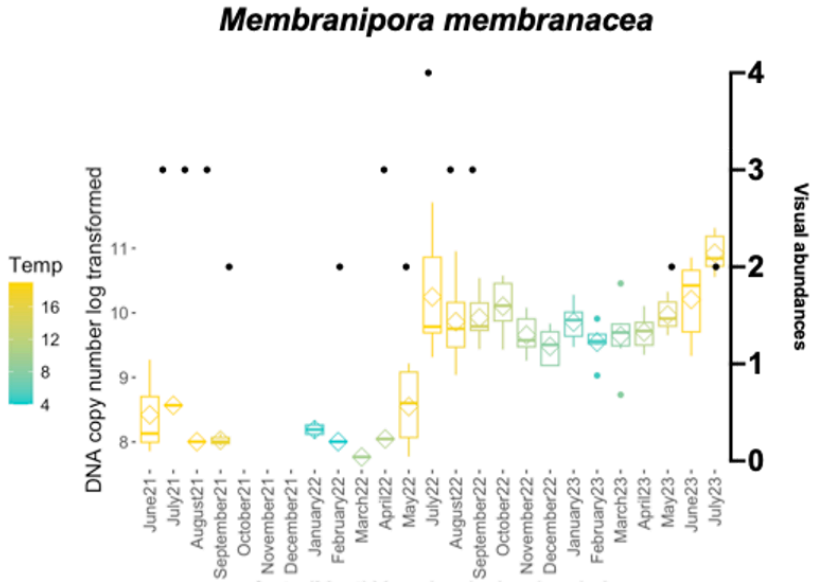

Figure 8: Amount of detected eDNA shed by *Membranipora membranacea* at Biddeford Pool between June of 2021 and July of 2023. Black dots indicate visual abundance. Most of the *M. membranacea* seen in the tide pools came in on wrack that washed in from offshore.

The Asian shore crab, *H. sanguineus,* was rarely observed in the tidepools themselves, but rather inhabited the exposed intertidal cobble above the tide pools (Figure 9). Of the 186 samples, 91 were negative for the eDNA of *H. sanguineus*. Their detection was also sporadic, similar to *M. membranacea*. Laboratory experiments confirmed that there is no relationship between number of crabs and eDNA shedding (Figure 10).

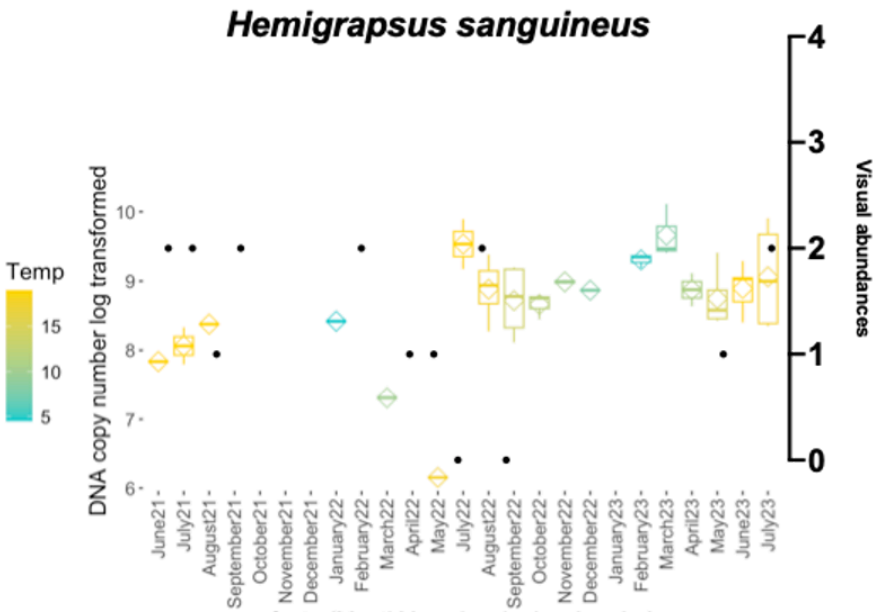

Figure 9: Amount of detected eDNA shed by *Hemigrapsus sanguineus* at Biddeford Pool between June of 2021 and July of 2023. Black dots indicate visual abundance. Many of the crabs seen in this habitat were out of the water under rocks; Asian shore crabs were infrequently seen in the water, so they would not be actively emanating eDNA into the water.

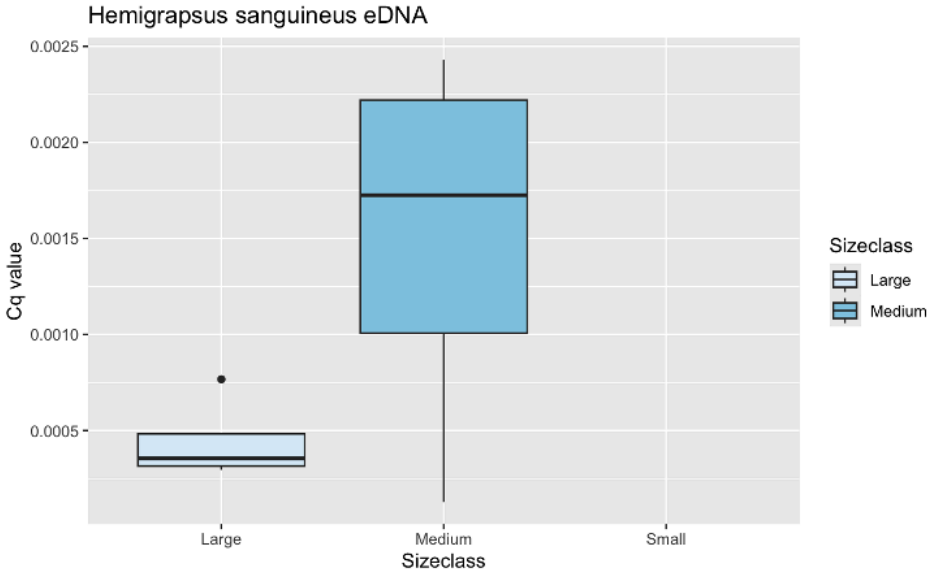

Figure 10: Laboratory eDNA shedding experiment for *H. sanguineus*. There was no correlation between wet weight and eDNA shedding rate. No eDNA was detected for the small group.

The final invasive species analyzed with qPCR was the European flat oyster, *O. edulis* (Figure 11). This long-lived species experienced a mass mortality event for reasons not identified in this study in the middle of this sampling period (April 2022), but their bottom shells were left behind. The laboratory study showed that the shells have DNA entombed in the shells, so eDNA was being shed even if the animal was not alive. We also did not find a significant relationship between biomass and shed eDNA in mesocosm experiments (Figure 12).

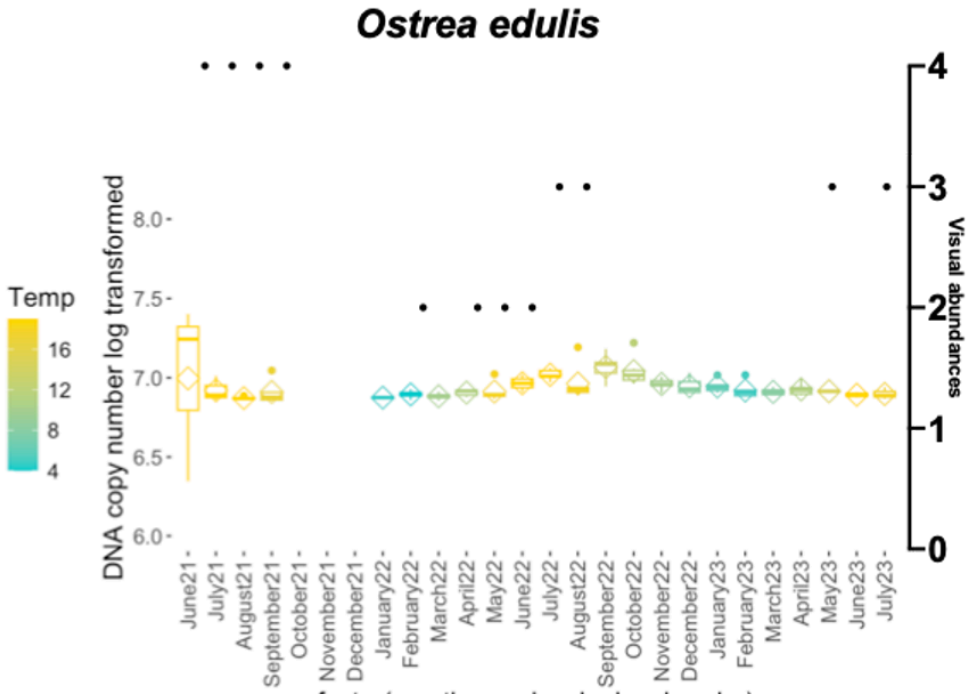

Figure 11: Amount of detected eDNA shed by *Ostrea edulis* at Biddeford Pool between June of 2021 and July of 2023. Black dots indicate visual abundance, which does not include dead shells. In spring of the first year of sampling, a mass mortality event affected most of the adult *O. edulis* in the tide pool, but despite the loss of many individuals, the amount of eDNA detection did not change.

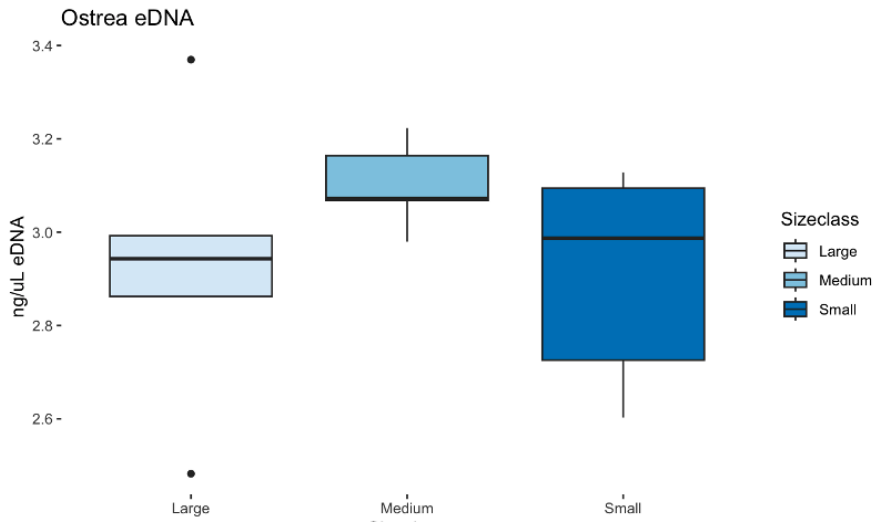

Figure 12: Laboratory eDNA shedding experiment for *Ostrea edulis*. There was no correlation between wet weight and eDNA shedding rate. Of all metrics taken from *O. edulis*, wet weight of the whole organism was most correlated with the amount of soft tissue over other factors such as maximum shell diameter.

The nuisance species, *S. balanoides*, had a similar oscillating pattern to some of the tunicates but with the highest eDNA concentrations in early spring and declining concentrations through the summer and to the early winter (Figure 13).

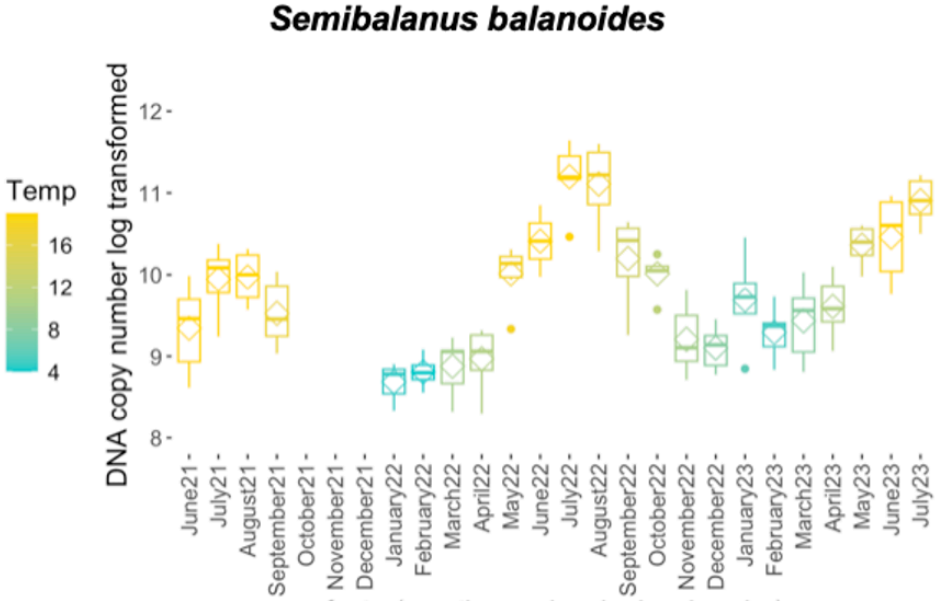

Figure 13: Amount of detected eDNA shed by *Semibalanus balanoides* at Biddeford Pool between June of 2021 and July of 2023. This species is not an invasive species, so we did not

measure visual abundance over time, thus it is not graphed. This species is present throughout the tide pools and while it was not directly measured, there was no visual change in abundance over time.

All samples were tested for inhibition, which was defined as a deviation from Cq values of 3 between samples and controls. None of the samples exhibited any level of inhibition.

Discussion

Four of five species adhered to the initial expectation that soft, exposed organisms would shed eDNA consistent with their body mass. Furthermore, five species followed general trends based on known life history or visual abundance over time. The only species that did not follow the anticipated pattern was the gray tunicate, *D. listerianum*. This species is gelatinous and semi-transparent–more so than the other sea squirt species studied here. Frequently in visual surveys, *D. listerianum* could be overlooked because the rock under the colonies would show through. The best way to identify the colonies was to feel over the rock surface for the distinct slime feeling that differed from the rock. The high water concentration in the body of this species likely led to the lack of eDNA detected; they have high surface area but low body mass which means that despite having a lot of biological material to interface with the water, they shed little eDNA.

For the other tunicate species, there was agreement with eDNA concentrations and visual biomass in the tide pools. *Botryllus schlosseri* and *B. violaceus* showed oscillations between high and low concentrations of eDNA in agreement with visual surveys, which showed that in the late spring into summer these tunicates grew to be a dominant intertidal species. They began disappearing in the early fall and were nearly undetectable in the winter, save for a few colonies scattered throughout the tide pool. These tunicates begin growing and reproducing as seawater temperatures get above 12 or 15℃ for *B. schlosseri* and *B. violaceus* respectively and the increases in tide pool coverage are consistent with these expected temperatures (Brunetti, 1974; Takeuchi, 1980). The in-laboratory experiments did not produce a significant correlation between biomass and eDNA concentration, likely due to the challenges of keeping *B. violaceus* and *B. schlosseri* alive in the flowing seawater system. An attempt to replicate this experiment was

made, but colony health quickly declined each time individuals were brought into the laboratory, so the eDNA results shown here are likely influenced by high levels of tissue degradation. The results for *D. vexillum* are particularly interesting because this species does not disappear as the water gets colder (< XX C). *Didemnum vexillum* was often the visibly dominant sessile invertebrate in the tide pool when water temperatures dropped. Furthermore, when we reflect on previous MIMIC surveys from this area, we see that the detectable eDNA from *D. vexillum* has been increasing over time; summer 2022 and 2023 had the highest visual record of *D. vexillum* over the time series, which is consistent with our eDNA results (Figure 4). *Didemnum vexillum* is frequently seen being eaten by periwinkles in the winter, so the high eDNA signal in the winter may also be influenced by the feeding of the snails as they fragment colonies. The last tunicate meeting this expectation that soft bodied organisms shed eDNA consistently with their abundance is *C. intestinalis*, a surprising addition to the tide pool whose eDNA was detected three months before an individual was observed in visual surveys. Following the discovery of this individual, we used cameras to search under cracks in the rocks but found that the tunicate was the only one to appear during this time series, which is consistent with the unchanging eDNA concentration over time. In-laboratory experiments showed a positive correlation between biomass and eDNA concentrations for *C. intestinalis*, which have a life expectancy of 2-3 years, we assume that no other individuals arrived as eDNA concentration remained consistent. Furthermore, the short larval period for *C. intestinalis* (less than 24 hours from egg and sperm release to potential settlement) would make capturing the spawning event of one individual in the field particularly challenging if only sampling once per month (Dybern, 1965). This result also shows that this technique was sensitive enough to detect a single individual living in the tide pool, highlighting how powerful this tool can be for some species.

Based on the body plans of organisms studied here, we can group them into two large categories. Organisms that have an exoskeleton or outer shell can be described by their texture as ‘crunchy’. Organisms with exposed soft tissue and no exoskeleton can be grouped together as ‘squishy’. The crunchy organisms studied here were *H. sanguineus*, *S. balanoides, O. edulis,* and *M. membranacea*, which either have a chitin and calcium carbonate exoskeleton or a shell. During a molt, those with exoskeletons have more exposed soft tissues, but this has not been shown to increase eDNA shedding in green crabs *C. maenas* (Crane et al., 2021). The squishy species included *B. schlosseri, B. violaceus, C. intestinalis, D. listerianum,* and *D. vexillum*. We

assumed that squishy organisms might shed eDNA consistently with their abundance due to the increased surface for fluid exchange with the surrounding environment. While environmental DNA shedding rates were not completely consistent with the squishy versus crunchy hypothesis, we use these groups to contextualize the results further.

Generally, the crunchy species were more unpredictable in their eDNA release than the squishy species. Several of the eDNA detection patterns can be explained by behavior; for example, *H. sanguineus* was rarely spotted in the water, but rather lives under rocks in the mid and upper intertidal. While some of the peaks in eDNA detection for this crab occurred during the reproductive season (May - October), this trend was not consistent across all months, suggesting that this detection was haphazard. Due to their general abundance out of the water and the ephemeral nature of eDNA, it is not necessarily surprising that there was not a lot of agreement between nearby *H. sanguineus* crabs and eDNA detected in the pool. Another example of behavior explaining eDNA detection is *S. balanoides*, which is a common intertidal organism and showed an interesting seasonal oscillation potentially due to reproduction rather than settlement. These barnacles brood their larvae in the late fall and winter, and release them from February to April (King et al., 1993). The larvae develop and grow as plankton before returning to shore in the summer for settlement. This life history is consistent with eDNA trends, with increasing eDNA concentrations as brooded larvae are released from the barnacles and captured in the water sample. It is difficult to say whether there is an increase in eDNA concentrations for settlement as the largest signal came from larval release, so further study should investigate an eDNA signal of settling barnacle larvae.

The signals that came from *O. edulis* and *M. membranacea* may not be attributable to living organisms established in the tide pools. For *M. membranacea*, individuals were detected throughout the year, especially in the 2022-2023 season when more frequent storms brought encrusted kelps into the tide pool. However, frequently these colonies did not contain live zooids. Similar to *O. edulis*, following a mass mortality event in early 2022, the detectable eDNA did not change despite fewer live individuals. When the oysters die, the individuals attached to the rocks leave behind half of their shell. In-laboratory experiments confirmed that oyster shells shed eDNA. In both of these cases, the presence of eDNA did not confirm the living presence of invasive species. Importantly, without visual surveys, it would be impossible to assess invasion severity by eDNA alone for these species.

Through this two-year time series, we uncovered inconsistencies in the utility of eDNA for detecting invasive species in tide pools. Environmental DNA was not shed equally for all species and thus, an eDNA signal cannot be interpreted equally across all taxa. This finding suggests that laboratory validation of eDNA shedding rates or visual surveys are required for organisms with diverse body plans. One of the challenges of studying invertebrates is that their diverse phylogeny goes hand in hand with diverse body plans. That is, soft exposed bodies or covering with an exoskeleton or shell could lead to large differences in exuded mucus or other bodily fluids. There are multiple studies investigating the amount of eDNA shed by fish into a controlled environment and in nature, validating that in general, fish biomass is correlated with eDNA shedding. Studies with Asian carps in man-made ponds, buckets, and mesocosms indicate that with more fish, the amount of eDNA shed also increases (Klymus et al., 2015; Takahara et al., 2012). For lake trout, catch per unit effort is significantly correlated with eDNA concentration, suggesting that molecular methods can save time and money in the management of this species (Lacoursière-Roussel et al., 2016). These trends appear to extend to other vertebrates such as amphibians (Pilliod et al., 2013; Thomsen et al., 2012). So, while there may be outlier species, eDNA can generally be applied quantitatively to fish and amphibians in a variety of systems.

A few studies have captured the challenges of eDNA research for invertebrates. Crane et al. (2021) investigated eDNA shedding of the green crab *Carcinus maenas* at different life phases such as soft shell, ovigerous, male, female, and at high density. Another study found no correlation between biomass and eDNA detection in mesocosm experiments for *C. maenas* (Danziger and Frederich, 2022). Detection was generally low in all treatments, except for ovigerous females who shed more eDNA, especially when zoea were present (Crane et al., 2021). These trends were corroborated in a 2022 study, which also found an increase in eDNA shedding of *C. maenas* for crabs running on a treadmill (Danziger et al., 2022). For the freshwater crayfish *Procambarus clarkii*, eDNA was not detected in high enough concentrations to overcome the limit of detection and trapping methods were more effective at detecting the freshwater crustaceans (Tréguier et al., 2014). Outside of arthropods, the potential for shells that shed eDNA was identified with *Margaritifera margaritifera*, the freshwater pearl mussel, when eDNA was detected from an extinct population (Stoeckle et al 2015). More studies are needed to continue looking at the challenges of studying invertebrate animals with eDNA.

The implications of this research indicate that eDNA should not be used equally for all species and systems. We understand that these findings may bring into question the applicability of eDNA in widespread species monitoring;- however, we argue that the key finding here is variable success, rather than no success. Environmental DNA should still be trusted for many applications, especially following rigorous testing. For species studied here, the results from *B. schlosseri*, *B. violaceus*, *S. balanoides*, and *C. intestinalis*, and *D. vexillum* show that the eDNA can be analyzed quantitatively as the results show good agreement with visual abundances[DB21] . With enough sampling, species such as *O. edulis, H. sanguineus,* and *M. membranacea* can also be detected for presence, if not quantitatively. While we show inconsistencies in the tool, with proper validation, many species can successfully be detected by eDNA. These findings expand beyond the nine species studied here; the variety of body plans for invertebrates challenge eDNA detection success. One could speculate that crunchy organisms could be more challenging to detect than squishy organisms across the board, which is particularly problematic considering the number of arthropod species and insect pests (Ezcurra et al., 1978). Environmental DNA techniques can be used quantitatively for many species, but without a full understanding of the eDNA signal, it cannot be applied to all systems equally.

Conclusion

Shedding rates of eDNA for invertebrate taxa vary. Some of this variation can be attributed to body plan of the invertebrates; many of the soft bodied, squishy animals shed eDNA in a manner consistent with their seasonal variation. For these species, eDNA can be used quantitatively to assess invasion severity in similar ecosystems with low flow. Some soft bodied organisms, such as *Diplosoma listerianum*, do not shed much eDNA, likely due to the general lack of organic material making up their bodies. In general, organisms with exoskeletons or other hard surfaces do not shed eDNA consistently with abundance, but the barnacle *S. balanoides* showed seasonal variation consistent with spawning. The generalizations of body plan for eDNA shedding rates of invertebrates are not consistent enough to make a broad statement about the reliability of an eDNA signal. This highlights the need for visual surveys or laboratory experiments to understand the meaning of an eDNA signal before it should be used for broader management perspectives.

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Conclusion

Sea surface temperatures are continuing to rise globally, so these issues apply further than the GoM, impacting marine ecosystems worldwide (Johnson and Lyman, 2020; Pershing et al., 2021). By extrapolating findings from invasive invertebrate studies in the GoM, we can anticipate similar ecological challenges in other regions experiencing temperature shifts. Therefore, comprehending the nuances of invasive species detection methodologies becomes paramount not only for regional conservation efforts but also for broader ecological management strategies. As we navigate these environmental changes, integrating insights from physiology and molecular biology into adaptive management frameworks will be essential for safeguarding marine biodiversity and sustaining valuable marine resources beyond the GoM.

From a physiological perspective, I demonstrated the variety in measurements in the literature and highlighted one example of a species which acclimates to different seasons, shifting its thermal thresholds. For the review, there were inconsistent measurement techniques, with few studies using the classical frameworks and more leaning towards observational measurements in the field which, while ecologically relevant, do not elucidate a mechanism for survival. Furthermore, these observational methods were susceptible to local adaptations, as are the framework measurements, but with little context to why these changes matter and how they affect the animal. Despite the lack of underlying causal mechanisms, measurements in the field such as growth or reproductive thresholds do serve some purpose in species distribution modeling but should not be the only measurements taken. Without understanding the ecologically relevant temperatures at which an animal thrives, rather than just survives, modeling may overestimate invasive range. Thus, by measuring the maximum and minimum survivable temperatures, plus the temperatures at which the animal thrives, modeling will consider many thresholds required for the animal to persist.

Of course, these measurements alone are not helpful without context. Many of the analyzed studies showed variation based on acclimation temperature and local adaptation. For example, the reproductive temperature for *Carcinus maenas* ranged from 0 to 27℃ based on location (Himes et al., 2017; Thresher et al., 2003; Yamada and Kosro, 2010; Young and Elliott, 2020). If we were to assume that its reproductive temperature was only 4-26℃ as it was reported in Australia, that would underestimate its potential invasive range (Thresher et al., 2003). Thresholds can vary for a variety of reasons, including life stage, acclimation temperature,

population variation/local adaptation, and exposure to other abiotic factors, so reporting all metadata about animal rearing conditions is important. Overall warming ocean temperatures may also lead to shifting thermal thresholds for organisms with thermal plasticity, so it should also be acknowledged that thresholds may continue to change worldwide. Warming sea surface temperatures will also lead to less ice cover, decreasing winter hypoxia, and changing flow patterns, which can open up new areas to invasion (Rahel and Olden, 2008). The best thing individual studies can do is list all metadata in order to contextualize these measurements on a broader scale.

For *H. sanguineus* specifically, I found seasonal shifts in temperature tolerance likely due to acclimation. This species only reproduces in the summer months and spends much of its time in the colder months huddled under rocks, moving seldomly, or deeper in the water (Frederich and Lancaster, 2024). Despite the lack of year-round reproduction, this species is present and becoming a dominant force in the intertidal in southern New England, though its dominance over *C. maenas* has not come to fruition in southern Maine. It continues to spread northward where it is found on both sides of the Bay of Fundy (C. DiBacco, personal communication).

Lastly, in order to understand the distribution of these species over time, I validated 9 qPCR assays for several of the MIMIC species and other local pests to compare against a 2 year time series of eDNA collected at Biddeford Pool. I found that some species, especially those with exposed soft tissue (squishy), shed eDNA in a manner consistent with their abundance. This was not true for all squishy species, as *D. listerianum* was not detected using eDNA using qPCR nor metabarcoding. For crunchier species, organisms with an exoskeleton or less exposed soft tissue, generally there was no correlation between visual abundance and eDNA concentration. Interestingly, at least one crunchy species did show seasonal variation in eDNA shedding, not necessarily in relation to visual abundance, but with regard to reproduction. This species broods its larvae until January when they begin to release through April. Settlement occurs as waters warm through the spring, and I did not find a notable increase in eDNA at this time. The last interesting trend was for eDNA from *O. edulis*, which exhibited consistent eDNA detection throughout the year despite mortality and changing abundances over time. Laboratory experiments showed no trend in eDNA shedding rate and amount of oysters as well as shed eDNA from cleaned shells containing no live oysters.

These results show varied detection of invasive species using eDNA from an abundance standpoint. With enough samples, all species but one was detected, suggesting that if the only research goal is to detect the presence of an invasive species, eDNA is still a powerfully useful tool. This chapter highlights the importance of validation and comparison to traditional survey methods before blindly applying eDNA methods to all systems equally. Although these findings may raise doubts as to the validity of eDNA results, they also show great promise for quantitative results for many species.

Overall, this dissertation has filled in information gaps regarding invasive species physiology and detection using eDNA. As the world becomes more interconnected, we expect incidence of invasive species to continue to increase (Hulme, 2009). This, paired with global warming, will lead to drastic changes in all ecosystems which will affect humans in a variety of ways. By understanding the temperatures which could allow species to continue spreading, we can predict where they might spread, and use eDNA to detect them as early as possible. Through these three chapters, I've turned a lot of traditional understandings of physiology and eDNA on their heads but hope that the findings here will continue to strengthen these fields for the betterment of our planet's beloved oceans.

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Appendix A

Appendix A. Two way ANOVA tables for Biddeford Pool qPCR runs comparing location in the tide pool and sampling date.

Botryllus schlosseri

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Location	9	16.96	1.885	1.265	0.265	
monthyear	22	109.00	4.954	3.325	2.08e-05	***
Residuals	102	152.00	1.490			

Botrylloides violaceus

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Location	7	4.57	0.6524	2.115	0.0493	*
MonthYear	21	44.66	2.1265	6.895	1.68e-11	***
Residuals	94	28.99	0.3084			

Ciona intestinalis

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Location	5	3.69	0.738	0.349	0.88053	
MonthYear	17	96.24	5.661	2.680	0.00291	**
Residuals	56	118.31	2.113			

Didemnum vexillum

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Location	11	139.32	12.666	48.08	< 2e-16	***
monthyear	21	52.61	2.505	9.51	2.87e-15	***
Residuals	94	24.76	0.263			

Hemigrapsus sanguineus

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Location	5	3.883	0.7765	4.888	0.004027	**
MonthYear	17	14.423	0.8484	5.340	0.000224	***
Residuals	21	3.336	0.1589			

Membranipora membranacea

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Location	11	43.57	3.961	18.417	1.31e-15	***
MonthYear	20	12.02	0.601	2.794	0.00105	**
Residuals	62	13.34	0.215			

Ostrea edulis

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Location	9	1.469	0.16327	2.609	0.00987	**
monthyear	22	2.421	0.11006	1.759	0.03301	*
Residuals	93	5.820	0.06258			

Semibalanus balanoides

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Location	5	0.97	0.194	0.477	0.793	
monthyear	22	106.72	4.851	11.924	<2e-16	***
Residuals	107	43.53	0.407			

Appendix B

Metabarcoding and bioinformatics

PCR was run in triplicate using the Leray COI primers. All PCR results were visualized on a 1% agarose gel with SYBR Green to ensure PCR success. Following PCR, and if the gel indicated success, PCRs were pooled and cleaned using Ampure beads in a 1.4x concentration. Following binding to Ampure beads, the samples were washed three times using a 70% ethanol solution before being eluted into 10 µL of nuclease free water. All samples were then measured with a NanoDrop spectrophotometer to ensure that DNA concentrations were between 10 ng/µL and 200 ng/µL. Sequencing was performed on an Illumina MiSeq using either a 2x300 or 2x200 sequencing kit at the discretion of the sequencing facility (Integrated Microbiome Resource, Dalhousie University).

The returned FASTQ files were processed with a pipeline designed by collaborators at the NSF EPSCoR Maine-eDNA consortium using dada2 and phyloseq. Due to the two sequencing runs being processed with different kits, the reads of half of the sequences were not able to be merged. So, we moved through the pipeline using only the forward reads. Amplicon sequence variants (ASVs) were compared to MIDORI and an internal Maine-eDNA database containing whole mitogenomes and generated using a species list of Maine-specific species. Visualizations in phyloseq allowed us to generate nMDS and diversity index plots.

Metabarcoding

Using the DADA2 pipeline, we only analyzed the forward reads due to the differences in sequencing kits leaving the sequences too short to merge paired reads. Approximately 70% of sequences passed the filters of the pipeline and were analyzed to generate amplicon sequence variants (ASVs). Amplicon sequence variants were compared against a modified MIDORI database to assign taxonomy to the sequences (Leray et al., 2022).

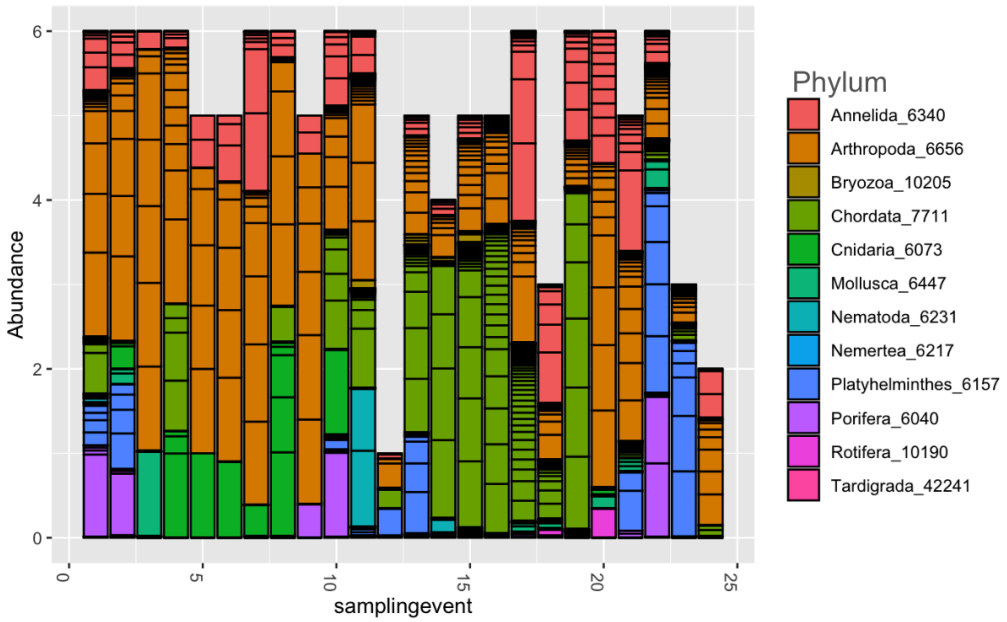

Figure 14: Amplicon sequence variants that were successfully assigned to a variety of phyla. Each color represents a different phylum. These metabarcoding data exclude any unassigned ASVs.

Despite the broad range of phyla observed above, most ASVs did not assign to any taxa in the database. The resolution of the matched phyla decreases when placed in the context of the entire dataset.

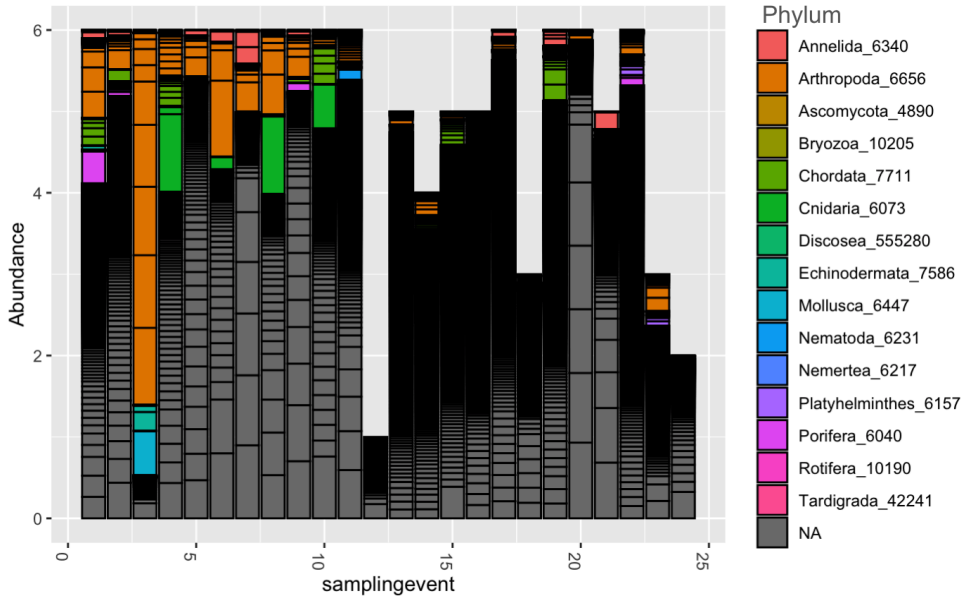

Figure 15: Total ASVs and matched phyla for Biddeford Pool metabarcoding data. Each color represents a different phylum. Gray bars indicate unassigned ASVs.

BIOGRAPHY OF THE AUTHOR

Emily Lancaster (nee Pierce) was born in Murrieta, California on September 10, 1994. She was raised in Reno, Nevada and graduated from Sage Ridge School in 2012. She attended Pepperdine University for a bachelor's degree in biology where she graduated in 2016. She attended Moss Landing Marine Laboratories through California State University, Monterey Bay and received a master's in marine science in 2020. Then she moved to Maine to pursue a Ph.D. in marine biology. Emily is a candidate for the Doctor of Philosophy degree in Marine Biology from the University of Maine in August 2024.