Climate Change and an Evolving Fishery: Do Declining Maternal Size and Planktonic Foods Affect Lobster Larval Survival in the Gulf of Maine?

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CLIMATE CHANGE AND AN EVOLVING FISHERY: DO DECLINING MATERNAL SIZE AND PLANKTONIC FOODS AFFECT LOBSTER LARVAL SURVIVAL IN THE GULF OF MAINE?

By

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B.A. Boston University, 2017

A Dissertation

Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (in Marine Biology)

The Graduate School
The University of Maine
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Many marine organisms exhibit bipartite life-cycles whereby reproductive adults dwell on the benthos while the larvae are pelagic. The pelagic stage is subject to mortality rates which far exceed those experienced by the organism during its benthic existence. The larval phase therefore represents an important bottleneck to larval recruitment. Small changes to survivorship in the plankton can have large consequences for subsequent year-class strength. Understanding the factors influencing successful larval recruitment is an important step towards predicting future stock abundances and maintaining healthy fisheries.

The American lobster (*Homarus americanus*) is a large benthic crustacean which is economically and culturally important in the Gulf of Maine. In the past few decades lobster broodstock abundance and egg production has soared, but young-of-year recruitment to benthic nurseries has declined. This suggests a breakdown in the spawner-recruit relationship due to changes in the factors that control larval survival and recruitment success. In this dissertation, I have investigated potential intrinsic and extrinsic sources of variation to larval survival, which may be responsible for declining lobster benthic recruitment.
Body size is an important intrinsic trait that affects both larval production and survival. Ongoing warming within the Gulf of Maine has led to earlier maturation of female lobsters at smaller sizes. Smaller females were found to produce fewer, lower quality larvae. Meanwhile, food-limitation is an important extrinsic source of larval mortality. Warming-induced changes to zooplankton regimes could put planktivorous lobster larvae at increased risk of food-limitation, as suggested by recent correlative evidence. Results presented here suggest that risk of food-limitation coupled with declining maternal size interact to lead to lower recruitment success for lobster larvae.

Larval ecology is frequently termed a ‘black box’ due to the difficulties associated with studying small dispersive larvae. This dissertation seeks to surmount these difficulties through the use of mixed-methodologies and a combination of laboratory and field-based approaches. The overarching goal of this research is to further understanding of lobster larval recruitment and ecology while demonstrating the efficacy of new tools that, when paired with traditional approaches, help to shine a brighter light on the larval ‘black box.’
ACKNOWLEDGEMENTS

Firstly, and mostly, I would like to thank my wife Ana and my daughter Penelope. Ana has been my biggest fan and greatest supporter, even as I pushed back my defense date (twice). Penelope- I won’t pretend that you made it easier to finish my dissertation, but you certainly made it more fun.

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In the last 2 years or so of my degree, Pete Countway acted more like a third advisor rather than a committee member. I joined the Maine eDNA program with no knowledge of eDNA or genetic approaches in general. Thanks to Pete’s tutelage I can now frequently trick people into thinking I know what I’m talking about.

A medley of students assisted me on projects through the years. So thank you to Grace Andrews, Donaven Baughman, Tahnee-Rae Buckelew, Jess Capista, Curtis Morris, Emily Patrick, and Caitlin Haley for all your hard work.
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Finally, thank you to my in-laws, my sisters, and my parents for years of support. Thank you also to my committee members and my fellow students, and the lobstermen who provided research specimens. Lastly, thank you again to Ana and Penelope, who deserve twice the thanks.
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CHAPTER 1 - INTRODUCTION

1.1 The bipartite life histories of marine invertebrates

The life history of most benthic marine organisms includes a dispersive pelagic larval phase (Strathmann et al., 2002). The prevalence of these bipartite life histories across taxa suggests they have a number of benefits. For one, having larvae which utilize a separate habitat from their parents ensures offspring do not compete with their parents for resources (Strathmann et al., 2002). Additionally, pelagic larvae broadcast widely, effectively hedging bets against the likelihood of extinction in any one habitat, and allowing gene flow among subpopulations, despite the risk of export from suitable benthic nurseries (Jablonski and Hunt, 2006). Once the larval phase is completed, the next step in their development is a metamorphosis to a postlarval form that enables them to settle from a pelagic existence to the seabed. Postlarvae then recruit to the benthos and become juveniles, and eventually join the reproductive adult population in a matter of weeks or years. Successful recruitment to adulthood is therefore governed by both pre- and post-settlement processes (e.g., Pineda 2000). Pre-settlement barriers to larval recruitment most commonly include advection (away from suitable settlement habitat) (Hudon and Fradette, 1993), predation (Thorsson, 1949; Pepin et al., 2002), and food-limitation (Hjort, 1914; Cushing, 1969; 1990; Olson and Olson, 1989). Entirely different agents of mortality operate during the post-settlement phase: they frequently are density-dependent and are governed by habitat/shelter availability, resource availability, and predation pressure (Ricker, 1954; Wahle, 2003). Fisheries biologists seek an understanding of the factors governing the recruitment process because they provide insight into the stock-recruit relationship (SRR). The stock-recruit relationship, also referred to as the spawner-recruit relationship, describes the quantitative relationship between the
adult stock and its resulting offspring at a defined life stage, age, or size. A firm grasp of the
stock-recruit relationship can provide insights into the drivers of population dynamics and be an
important predictor of subsequent year class success. In the benthos, strong density-dependent,
compensatory or depensatory, processes may lead to non-linear stock-recruit relationships
(Wahle, 2003). The SRR can be elusive because of factors that cause the relationship to
decouple. For example, in the pelagic realm, significant larval export or mortality from density
independent processes can mask the stock-recruit relationship (Grosberg, 1992). The causes of
such disconnects can be difficult to pinpoint due to pre- and post-settlement processes operating
prior to recruitment. For this reason, understanding the extrinsic and intrinsic sources of variation
in larval production and mortality is an important goal for understanding population dynamics.

1.2 Intrinsic sources of larval mortality; body size

Intrinsic sources of larval mortality are those that arise from the species-specific traits of
an organism. Endogenous energy stores, swimming speed, prey preference, and body size are all
effects of intrinsic traits that may affect the survival of a pelagic larva. Body size, in
particular, is noteworthy as it has a pronounced effect on production by the adults and the
survival of pelagic larvae. Maternal body size is a significant predictor of reproductive output in
a number of taxa: e.g. fish (Barneche et al., 2018) and stomatopods (Reaka et al., 2008),
including lobsters (Attard and Hudon, 1987). Reproductive output frequently scales
disproportionately with maternal size (Barneche et al., 2018; Attard and Hudon, 1987), allowing
larger mothers to not only produce more offspring, but sometimes also produce larger, more
competent, offspring (Trippel et al., 1997; Moland et al., 2010). Larval size has been found to be
a significant predictor of survival in the pelagic realm (Ware, 1975; Peterson and Wroblewski,
1984). In fish, the positive relationship between body size and larval survival is attributed largely to lower predation risk (Ware, 1975; Shepherd and Cushing, 1980; Anderson, 1988), but in herring, in particular, greater endogenous stores imbued by the mother have also been linked to longer survival during starvation (Blaxter and Hempel, 1963). In short, greater maternal size not only results in greater fecundity, but may also result in larger, more robust, larvae with a greater likelihood of survival. Maternal body size therefore has the potential to have a pronounced effect on recruitment success and to be a significant predictor of speciation/extinction risk (Cooper et al., 2008; Reaka et al., 2008). The broad effects of body size on both larval production and survival can be tied in part to metabolic theory (Henneman, 1983; Pettersen et al., 2022). Theory predicts, and empirical evidence confirms, that while whole-body energy usage (metabolic rate) increases approximately as the 0.75 power of an organism's mass, the mass-specific rate of energy consumption declines as the -0.25 power of body mass (Peters, 1986). For the mother, this means a greater amount of energy may be invested into reproduction (Henneman, 1983). For larvae, greater size may result in a greater length of time before critical energy reserves are depleted (Peters, 1986; Pettersen et al., 2022). However, metabolism is also affected strongly by environmental factors, particularly temperature. This can relate back to body size, as increased temperature can lead to smaller maximum sizes in fish and invertebrates. This phenomenon is potentially explained by the Gill Oxygen Limitation Theory (GOLT) where growth rate is defined as the sum of constructive metabolic processes (anabolism) and destructive metabolic processes (catabolism) (Pauly et al. 2022). When the two are equal, growth ceases. Increased temperatures increase the rate of catabolism through the destruction of proteins, while anabolic processes are less affected, as they scale more closely with oxygen saturation rather than temperature. So in warmer temperatures growth rate approaches zero more rapidly, and adults
attain smaller max body sizes. Thus, a warming ocean may have cascading effects leading to smaller females producing fewer, less robust larvae that in turn, may have consequences for larval recruitment.

1.3 Extrinsic sources of larval mortality; food-limitation

Extrinsic sources of mortality are factors external to the individual larva. Assuming favorable physical/chemical conditions, the most common extrinsic sources of larval mortality include advection (Hudon and Fradette, 1993), predation (Thorsson, 1949; Pepin et al., 2002), and food-limitation (Hjort, 1914; Cushing, 1969; 1990; Olson and Olson, 1989). Out of these three common sources of mortality, advection risk is governed most by abiotic forces. Most larvae are passively transported by currents, although there is much evidence that many can control their dispersal by swimming or vertical migration (Miller and Morgan, 2013; Buston et al., 2012). Typically, advection risk can be modeled based on easily monitored environmental conditions resulting in dispersal models for species of interest (e.g. barnacles: Bertness et al., 1996; Shkedy and Roughgarden, 1997; limpet: Viard et al., 2006; lobster: Xue et al., 2008; coral reef fishes: Almany et al., 2017). Predation on the other hand is a function of the density of pelagic predators, and has been cited by some authors as the most significant source of larval mortality (Thorsson, 1949; Pepin et al., 2002). In fact, the observation that increased size, or growth rate, decreases mortality rates in planktonic larvae is typically attributed to lower levels of predation on larger larvae (Ware, 1975; Shepherd and Cushing, 1980; Anderson, 1988). This has been termed the growth/mortality hypothesis, and though mortality is directly linked to predation in this framework, differences in body size are also inexorably tied to food-limitation. Hjort (1914) and later Cushing (1969; 1990) provided some of the most influential theories relating recruitment success to food-limitation. Yet despite observations connecting year-class
strength to food supply in the pelagic realm, direct evidence of starvation is typically difficult to obtain (Thorsson, 1949; Anderson, 1988). Still, correlations and laboratory experiments relating food-limitation to larval mortality still abound (Olson and Olson, 1989). A possible explanation could be a unique feature of food-limitation as a mortality source: sublethal effects. While predation and advection have immediate and direct effects on a larva’s likelihood of successful recruitment, food-limitation also influences growth rates. Limited nutrition extends the larval period, increasing the probability of mortality through other sources, even if the larva does not experience mortality as a direct cause of starvation (Thorsson, 1949). Therefore, the true influence of food-limitation may be related largely to sublethal effects, and thus is underestimated as a source of larval mortality.

1.4 The American lobster (*Homarus americanus*) as a model system

The American lobster (*Homarus americanus*) is a large-bodied benthic crustacean with pelagic larvae. It supports economically and culturally important fisheries along the Northwest Atlantic coast, particularly in the Gulf of Maine (GoM). Lobsters undergo three pelagic larval instars (SI-SIII), and a final pelagic postlarval stage (SIV). Lobster larvae are planktrophic, and rely on a sufficient density of zooplankton prey in order to progress through their developmental stages (Abrunhosa and Kittaka, 1997). In their final pelagic stage, SIV, lobster postlarvae begin diving to search for suitable settlement habitat. In total, the pelagic stages of lobster last roughly 30-40 days (Mackenzie, 1988; Harrington, 2019). Interestingly, although the abundance of broodstock has risen steeply within the GoM over the past few decades (ASMFC, 2020), a long running time series called the American Lobster Setlement Index (ALSI) has recorded widespread declines over the past decade in the number of newly settled young-of-year lobster successfully recruiting to benthic nurseries (Carloni et al., 2018). This reflects a breakdown in
the normal stock-recruit relationship, and provides an opportunity to study the factors that govern larval survival and subsequent recruitment in an economically important and experimentally tractable benthic crustacean. Concurrent with the increase in lobster broodstock and the decline in larval settlement, over the past 20 years the GoM has been warming faster than 99% of the world’s oceans (Pershing et al., 2015). The result of this warming is earlier maturation at smaller sizes for female lobster (Waller et al., 2021), likely coupled with a decrease in the average/max size of mature lobsters (Aiken and Waddy, 1976; Pauly et al., 2022). Increasing temperatures have also led to declining summer-time abundance of a foundational copepod prey species in the GoM: *Calanus finmarchicus* (Record et al., 2019). Correlative analysis has revealed that while indices of predation and advection do not trend with declining larval recruitment, *C. finmarchicus* abundance does (Carloni et al., 2018). Carloni and coworkers also found no other zooplankton taxon to correlate as strongly with the lobster postlarval or young-of-year time trends as *C. finmarchicus*. This correlation has led to the hypothesis that lobster larvae are experiencing the effects of food-limitation. Therefore, interactive effects of intrinsic (body size) and extrinsic (food-limitation) mortality sources may be the root cause of falling larval recruitment.

1.5 Dissertation goals and overview

The goal of this dissertation is to examine the factors responsible for declining lobster larval recruitment in the GoM. Climate induced changes to maternal size at maturity and the pelagic food-web will be the main focus. The dissertation will begin with an investigation of maternal size effects and transition into the diet of lobster larvae and evidence of food-limitation. This structure serves to follow potential sources of recruitment variation from intrinsic to extrinsic factors, but also ontogenetically from embryo production to pelagic larval survival. The
hypotheses being tested are (1) the declining size of female lobsters in a warming climate has adverse effects on the quality and quantity of embryos and resultant larvae produced. (2) Lobster larval growth and survival is food-limited. As such, CH. 2 begins with a discussion of maternal size effects on embryo/larval production and quality in three lobster populations with contrasting thermal regimes. The next two chapters concern the larval diet. CH. 3 is a review of the methodologies employed in our study of the larval diet, both microscopy, as well as genetic techniques. This includes development and testing of a novel lobster blocking primer, employed to allow the sequencing of the larval diet while reducing the amplification of lobster DNA. CH. 4 then presents the results of our lobster larval diet study. To our knowledge this is the first larval diet study comparing different regions within the GoM, and the first to employ genetic techniques alongside conventional microscopy. Finally, CH. 5 assesses the possibility of food limitation in lobster larvae using laboratory-based experiments on starvation sensitivity and through a direct comparison of field-caught larval fatty-acid profiles to lab-reared benchmarks.
CHAPTER 2 - CLIMATE INDUCED DECLINES IN MATERNAL SIZE MAY COME AT A COST TO EMBRYONIC INVESTMENT AND LARVAL PERFORMANCE IN THE AMERICAN LOBSTER.

2.1 Abstract

As the Gulf of Maine warms, female American lobster (Homarus americanus) are maturing at significantly smaller sizes. In addition to the lower fecundity associated with smaller adult size, it is hypothesized that small females do not invest as much energy per larva as large females, thereby compromising post-hatch performance and survival. This study assessed the maternal size effect on these important traits, while also utilizing an existing thermal gradient for a space-for-time comparison. This allows us to assess how cooler northern lobster populations may react to future warming by studying a warmer southern population. Maternal size was found to have significant effects on both embryo and larval quality, as well as embryo quantity. However, there was also evidence of counter-gradient adaptation, whereby small females living in a warmer thermal regime are more fecund and produce larger eggs than females of the same size from colder regimes. These results help to reveal the cascading effects warming can have on important reproductive and larval survival traits on an economically important species.

2.2 Introduction

To understand how marine invertebrates will react to climate warming over their native range, it is imperative to understand how temperature affects key life-history traits. Since 1980, NW Atlantic seawater temperature has increased 0.3°C per decade (Nixon et al. 2004; Mills et al. 2013; Seidov et al., 2021). Depending on future emissions of greenhouse gasses, conservative estimates of ocean temperature above a depth of 100 m predict increases from 0.6° to 2.4°C by
Current atmospheric CO₂ levels of ~419 ppm are ~50% higher than pre-industrial levels (climate.nasa.gov) and are predicted to double by 2100, driving continued anthropogenic temperature rise in the coming decades (Andrews et al., 2014; IPCC 2021). As a result of this warming, marine organisms are forced to adapt (Calosi et al. 2016; Palumbi et al. 2019), migrate (Pinsky et al. 2013, Allyn et al., 2020), or face extinction (Barnosky et al. 2011; Dulvy et al. 2003).

The body size at onset of maturity is one trait that has broad implications for survival and reproduction (Peters 1983; Attard and Hudon 1987; Moland et al. 2010; Garrido et al. 2015; Barneche et al. 2018). It is well-known that maturation in ectotherms tends to occur at smaller sizes in warmer regimes (Deevey, 1960; Precht et al., 1973). This “Temperature Size Rule” is a predictable phenotypic response to a warmer environment (Forster and Hirst 2012). Less well understood are the fitness consequences of temperature induced phenotypic shifts in maternal size for the investment in and performance of their offspring. “Space-for-time” comparisons of geographically separated subpopulations residing in differing thermal regimes can be an informative way of evaluating how populations will respond to a changing thermal (Blois et al, 2013).

The native range of the American lobster (Homarus americanus) spans one of the steepest known latitudinal thermal gradients for a marine species. This creates a natural laboratory and the potential to conduct a space-for-time analysis; comparing how range-related temperature differences affect life history traits, such as size at maturity and fecundity, embryo size and energy investment that ultimately influence reproductive success. While it is well known that lobsters in warmer, southern zones mature at a smaller size (Estrella and McKiernan,
1989), recent surveys have revealed that sustained warming over the species’ range has induced a downward trend in female size at maturity in all locations (Waller et al. 2021). For example, Long Island Sound (Landers et al., 2001), the Gulf of Maine (ASMFC 2015; Pugh et al. 2013), and the Bay of Fundy (Gaudette et al. 2014) all report steady declines in size at maturity since the 1990s. In Maine, the mean size at maturity of reproductive females has decreased by nearly 20% over the past two decades (Waller et al. 2021). Despite initially faster growth rates in warmer water, the earlier maturation of gonadal tissues taxes somatic growth, causing lobsters in warmer water to be smaller than those in colder regimes (Aiken and Waddy, 1976).

Maternal size is widely regarded as a governing factor in embryonic and larval development, typically through greater investment in embryo size and quality (Mousseau and Fox, 1998). Greater investment in embryo quality is broadly linked to greater larval survival rates (Vance, 1973), which can be observed across many different taxa such as, polychaetes (Smith and Bolton, 2007) lobsters (Moland et al., 2010), sea urchins (McAlister and Moran, 2013), and fish (Hixon et al., 2014). From a fishery management standpoint, the implications of smaller reproducing females on recruitment to the lobster fishery is not fully understood. In lobsters, maternal body size is significantly correlated to fecundity (Attard and Hudon 1987, Waddy et al. 1995, Koopman et al. 2015, Goldstein et al., 2022), and may limit embryo production under future warming conditions. What is less well known is how maternal body size specifically relates to embryo and larval quality and performance. Past research has documented a general positive relationship between maternal body size and caloric content of embryos (Attard and Hudon, 1987), which holds true for lobsters (Attard and Hudon, 1987, Ouellet and Plante, 2004). However, to our knowledge, there is no evidence indicating if maternal body size relates to the investment of particular essential fatty acids such as Eicosapentaenoic acid (EPA),
or Docosahexaenoic acid (DHA), both of which have been shown to be crucial for larval crustacean growth (Sui et al. 2007, Andres et al. 2010, Beder et al. 2018). And importantly, there is also no evidence that such investment relates to larval duration or ability to survive spells without planktonic food, which often occurs in the patchy and temporally variable pelagic environment (Winemiller and Rose, 1992).

This study investigated the quantity and quality of embryos, as well as the performance of larvae originating from female lobsters collected over a naturally occurring range of sizes from three locations along the east coast of the United States spanning a steep latitudinal gradient in summer temperatures. In southern New England, the warmer extent of the species’ range, females mature earlier and at smaller sizes than those in the cooler, northeastern Gulf of Maine and Bay of Fundy (Estrella and McKiernan 1989, Waller et al., 2021). This latitudinal gradient allowed the comparison between sexually mature lobsters from sub-populations residing in different thermal regimes to determine how maternal body size relates to fecundity, embryo size, energy content, fatty acid profile, and total lipid content. Furthermore, laboratory experiments were used to assess growth and survival under starvation of larvae from females collected from near the midpoint of the geographic domain of the study, where there was access to reproductive females that spanned a relatively large range of sizes. From these observations, inferences were made surrounding the effects of future warming on key life-history traits in the American lobster.

2.3 Methods

2.3.1 Study areas and lobster collection

The relationship between maternal body size and embryo metrics such as fecundity, embryo volume, mass, and energy content (maternal investment) was investigated at three study
regions: Downeast, ME (DE), Midcoast, ME (MC), and Rhode Island (RI). These regions differ in their thermal regime, with RI, the southernmost study area, having the warmest summer temperatures and becoming deeply stratified during the warm growing season with average summer temperatures exceeding 20 °C. DE in the eastern Gulf of Maine, by contrast, has the coldest summer temperatures where proximity to the Bay of Fundy and the Eastern Maine Coastal Current ensures the water column remains well mixed throughout the year, and average summer temperatures tend not to exceed ~12 °C. MC represents the intermediate conditions typical of the western Gulf of Maine (GoM), which is separated from the eastern GoM by the Penobscot River discharge, and is not as strongly influenced by the Eastern Maine Coastal Current. A lower degree of mixing allows for the formation of a relatively shallow thermocline in the MC during the summer where average surface temperatures reach ~16-18 °C (Fig. 2.1). Although RI once had a thriving lobster fishery, rising temperatures coupled with a high rate of shell disease resulted in the lobster population center shifting northward and offshore (Le Bris et al., 2018, Casey et al. 2022), leading to lower rates of recruitment and a decline in lobster landings (Wahle et al. 2009, Oppenheim et al. 2019).
Figure 2.1 Seasonal climatology of thermal depth profiles to 100 m averaged from 2011-2020 for A) Downeast Maine, B) Midcoast Maine, C) Rhode Island. The full 100 m of depth is not available at Rhode Island due to a shallower bathymetry. Data source: FVCOM (https://www.fvcom.org)
2.3.2 *Fecundity and embryo size.*

Ovigerous female lobsters were collected at these three study sites along the New England coast of the USA: RI (n=65), MC (n=60), and DE (n=60). Lobsters from RI were collected via the state’s inshore trawl survey, while those from the MC and DE were supplied by local harvesters. The carapace length (CL) of each female was recorded and the total number of embryos carried by the female was estimated using a non-invasive technique to calculate the volume of the brooded clutch (Currie et al., 2010). The clutch of embryos is assumed to approximate the shape of a half cylinder. Briefly, the length and depth (averaged from five measurements along the length of the embryo mass) of each clutch was measured and the volume calculated using the equation for a half cylinder: \[ \frac{\pi D^2 L}{2} \times 0.535 \] where \( D \) is the average depth (cylinder radius), \( L \) is the length of the clutch, and 0.535 is the packing efficiency of loosely packed spheres which accounts for interstitial space between individual embryos (Currie et al., 2010).

The average volume of an individual embryo was estimated from a subsample of embryos collected from each female by stripping them from one pleopod. Embryo volume was calculated as a sphere with the equation \[ \frac{4\pi r^3}{3} \] where \( r \) is the average of the short and long radius of the embryo. Embryo radius was determined for each lobster by measuring the diameter of the embryo and dividing by two. Diameters were measured on the short and long axis of 10 embryos using a bespoke plugin for ImageJ software (Schindelin et al., 2012). The number of embryos, or fecundity, was calculated by dividing the volume of the clutch by the average embryo volume for each female.
All statistical analyses were conducted in JMP Pro statistical software (v.15.2.0). Each variable (embryo quantity, and embryo volume) was first plotted against the mother’s carapace length for each region, and 50% density ellipses fitted to the data. This allows for easy visualization of the data as the ellipse shape can be indicative of relationships between variables. A thinner ellipse indicates low variability, and horizontal stretching is typically indicative of a significant linear relationship (note that x-axis scale may also affect ellipse shape). A simple one-way ANOVA was then conducted for each dependent variable in order to determine whether a regional effect existed independent of maternal CL. To determine whether there was an interactive effect of region and maternal CL, that is to say, whether the relationship between CL and the response variable differed by region, a homogeneity of regression test was performed. This was accomplished by fitting a model for each response variable against a full factorial of region and CL (i.e., construct model effects were region, CL, and region*CL). If the independent variables were significant, a separate regression for each region was performed in order to assess the relationship between maternal CL and the response variable. If no interactive effect was present (as in estimated embryo quantity) an ANCOVA with region as the single categorical factor and maternal CL as the covariate on a continuous scale was performed.

### 2.3.3 Embryo dry weight, lipid and fatty acid content

Embryos were collected from 10 lobsters (30 embryos per lobster) from each of the three regions. Embryo-bearing females were chosen to represent the full size spectrum of lobsters sampled in the region. A single, extra-large female was included in the DE dataset, this female had the largest CL of lobsters sampled in any region (CL=165 mm). This individual was included to investigate whether a significantly larger female produced embryos of significantly
higher quality, but the single animal was removed from analyses where it exerted too much leverage on regression results. Embryos were placed into tins and left in a drying oven for a minimum of 48h at 60°C and then weighed (Mettler Toledo ML204 scale) to determine dry mass. The same embryos were then combusted in a mini-bomb calorimeter (Parr 6200 Isoperibol Calorimeter) to determine caloric content (MJ). The total amount of energy a female invests (EI) in her brood was calculated as:

\[ EI = \# \text{Embryos} \times (MJ/\text{embryo}) \]

An additional subset of embryos was collected from each region for fatty acid profiling. Samples from multiple females were pooled for three non-overlapping female size classes: small (80 – 90 mm CL), medium (100 – 110 mm CL), and large (130-140 mm CL). For each size class and region, seven females were selected. Then, 30 embryos were selected from each female and subdivided into three pooled subsamples. Each of the three pooled samples then contained 70 embryos (7 females*10 embryos in each pooled sample), with a total of 210 embryos being represented for each region and size class (70 embryos*3 pooled samples). The Midcoast and Downeast regions each had only one female within the large size class, so all the necessary embryos were taken from those individuals; but still subsampled in the method described above. Rhode Island had no females large enough to provide samples for the medium or large size classes.

Total lipid content and FA profiles were run by Bigelow Analytical Services (Bigelow Laboratory for Ocean Sciences, East Boothbay, ME). Briefly, embryos were oven dried (60°C), weighed to the nearest microgram, and then the lipids were extracted using a slightly modified Folch et al. (1957) method, as described in McMeans et al. (2012). Samples were extracted 3X
using 2 mL of 2:1 (v/v) chloroform:methanol and pooled, after which polar impurities were removed by adding 1.6 mL NaCl solution (0.9% w/v) and discarded following centrifugation. The resulting lipid-containing solvent was concentrated to 2 mL and 2 aliquots (100 μL each) were removed and evaporated to dryness to quantify total lipid gravimetrically. Fatty acids in the lipid extracts were derivatized to fatty acid methyl esters (FAME) using sulfuric acid as the catalyst (Christie and Han, 2012). The FAME were then extracted 2X using hexanes: diethyl ether (1:1; v/v), after which they were dried under a gentle stream of extra dry nitrogen gas. The FAME were separated and analyzed using a gas chromatograph (GC) (Shimadzu-2010 Plus, Nakagyo-ku, Kyoto, Japan) equipped with an SP-2560 column (Sigma-Aldrich, St. Louis, Missouri). All solvents used in the extraction and FAME derivatization procedures were of high purity HPLC grade (>99%). The FAME were identified and quantified by retention time matching and a 5-point calibration curve, respectively, using a reference standard (GLC-463, Nu-Chek Prep, Inc., Waterville, Minnesota). A known concentration of 5 alpha-cholestane (C8003, Sigma-Aldrich, St. Louis, Missouri) was added to each sample prior to extraction to act as a surrogate internal standard to estimate extraction and instrument recovery efficiency. Individual fatty acid contents were expressed as percent (molar) of total quantified fatty acid methyl esters (FAME).

Statistical methods for this dataset followed the same basic methods outlined above, with some slight differences; it is impossible to perform a homogeneity of regression or ANCOVA while including RI, as RI only had females in the small size class. For this reason an ANOVA between each region and variable in this dataset was conducted with only the small females, for which there was data from each region. As no regional effect was detected Tukey’s HSD was
then conducted to determine whether significant differences existed between small, medium, and large-sized females, but only for DE and MC (Tables 2.1-2.3).
Table 2.1 Sampling strategy by region, sample size, and data collected.

<table>
<thead>
<tr>
<th>Region</th>
<th>Sample; CL variable type</th>
<th>Subsample: 10 females per region representative of full size spectrum. Continuous variable</th>
<th>Females collected from each region DE (n=60), MC (n=60), RI (n=65). Continuous variable</th>
<th>Subsample: small (85mm CL ± 5 mm), medium (105mm CL ± 5 mm), and large (135 mm CL ± 5 mm) Categorical variable</th>
<th>Small (81-93 mm CL) large (107-136 mm CL) Categorical variable</th>
<th>Small (80-92 mm CL) large (124-132 mm CL) Categorical variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Downeast</td>
<td>embryo mass, calories, TEI</td>
<td>embryo quantity estimate, embryo volume</td>
<td>Lipids, EPA, DHA, DHA:EPA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Midcoast</td>
<td>embryo mass, MI/embryo, TEI</td>
<td>embryo quantity estimate, embryo volume</td>
<td>Lipids, EPA, DHA, DHA:EPA</td>
<td>Larval length (SI, SII), Larval mass (SI, SII), Larval starvation mass (SI)</td>
<td></td>
<td>Larval starvation duration (SI)</td>
</tr>
<tr>
<td>Rhode Island</td>
<td>embryo mass, MI/embryo, TEI</td>
<td>embryo quantity estimate, embryo volume</td>
<td>Lipids, EPA, DHA, DHA:EPA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2 Summary of results for measured egg metrics in RI, MC, and DE. Significant relationships are marked with *, relationships which are marginally significant, i.e. $\alpha=0.1$, are marked with ^.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Simple Region effect 1Way ANOVA</th>
<th>Homogeneity of Regression Analysis (Region x CL interactive effect)</th>
<th>If no signif Reg x CL interaction- Do ANCOVA to assess Reg and CL effects</th>
</tr>
</thead>
</table>
| Embryo number                       | F= 2.86 ; p = 0.06 ^            | Region x CL Interaction F= 0.36 ; p = 0.70 $\rightarrow$ Go to ANCOVA | Region: F= 11.02 ; p<0.0001 *  
CL: F= 194.30 ; p<0.0001 *  
Significant Region effect; significant Maternal size effect |
| Embryo volume (ul)                  | F=1.874 ; p =0.157              | Region x CL Interaction F= 2.89 ; p =0.06 ^ $\rightarrow$ Go to ANCOVA | Region: F= 3.85 ; p= 0.02 *  
CL: F= 25.71 ; p<0.0001 *  
Significant Region effect; significant Maternal size effect |
| Embryo mass (mg)                    | F=0.24 ; p = 0.79               | Region x CL Interaction F=0.42 ; p = 0.66 $\rightarrow$ Go to ANCOVA | Region: F=0.27 ; p=0.77  
CL: F=6.00 ; p=0.02 *  
No Region effect; significant Maternal size effect |
| Calories (J)                        | F= 0.52 ; p = 0.60              | Region x CL Interaction F= 0.24 ; p = 0.787 $\rightarrow$ Go to ANCOVA | Region: F= 0.251 ; p= 0.78  
CL: F= 9.90 ; p= 0.004 *  
No Region effect; significant Maternal size effect |
| Mass-specific calories (J/mg)       | F= 2.46 ; p = 0.1               | Region x CL Interaction F=0.67 ; p = 0.52 $\rightarrow$ Go to ANCOVA | Region: F= 1.62 ; p= 0.21  
CL: F= 6.02 ; p= 0.02 *  
No Region effect; significant Maternal size effect |
| Total Energetic Investment (J x 1000)| F=2.02 ; p = 0.15              | Region x CL Interaction F= 0.638 ; p = 0.54 $\rightarrow$ Go to ANCOVA | Region: F=2.45 ; p= 0.11  
CL: F=148.87 ; p<0.001 *  
No Region effect; significant Maternal size effect |
2.3.4 Larval growth experiment

Experiments to determine maternal size effects on larval quality under well-fed and starved conditions were carried out at the Bigelow Laboratory for Ocean Sciences (BLOS).

Lobsters carrying embryos near-hatch were collected from Midcoast Maine, by local harvesters,
and held in a flow through tank at ambient temperature in the hatchery at the University of Maine’s Darling Marine Center (DMC). Lobsters were divided into small (81-93 mm) and the large (107-136 mm) size categories. Offspring from at least three mothers in each size category were combined. In the well-fed treatment 1000 larvae from small and 1000 larvae from large mothers were separated into four communal rearing tanks per size treatment, with 250 larvae in each tank. Rearing tanks were maintained at 16° C and kept well-aerated and the water well-circulated to reduce the probability of cannibalism among larvae. Each day larvae were fed to saturation with (1-3 dph) nauplii of *Artemia salina*. During larval stages I and II, five larvae from each rearing tank were removed daily and photographed (Canon EOS Rebel T3i, Japan) under a dissecting microscope (Olympus SX 61). Carapace length was measured in profile from the back of the eye to the posterior edge of the carapace using NIH-ImageJ software (NIH, USA). For dry weights an additional five larvae from each rearing tank were removed, rinsed three times in deionized water to remove salt and then placed into pre-weighted tin boats. Samples were desiccated at 40°C for 48+ hours and weighed (dry weight). In order to determine whether the mean length and mass differed significantly between maternal size classes within larval stages, t-tests were conducted for each group.

2.3.5 Larval starvation experiments

Larval starvation experiments were conducted at the University of Maine’s Darling Marine Center flowing seawater facility to determine how maternal size affects the rate of weight loss during starvation. A sample of 25 newly hatched larvae from each of the same two maternal size classes was used in the larval growth experiment: small (81-93 mm) and large (107-136 mm) were placed individually into 1 L Mason jars in filtered seawater. The jars were partially
immersed in a temperature bath maintained at 16° C for 5 days with no food supply. Salinity and temperature were measured daily to ensure consistency. Each day, five larvae were randomly removed from each treatment to transfer to a drying oven for dry mass measurements.

An ANOVA was used to determine whether mass differed between larvae from maternal size classes, followed by a homogeneity of regression in order to determine whether the rate of mass loss was significantly different between the two groups.

An additional starvation experiment was carried out to determine whether a larva’s ability to endure starvation depended on maternal size. This experiment used larvae hatched from a separate group of mothers that were also divided into small (80-92 mm) and large (124-132 mm) size classes. The purpose of this experiment was to directly test whether maternal size affects the length of time larvae survive starvation. The progeny of three mothers in each size class were used in the experiment. From each female, 30 SI larvae were placed individually into starvation chambers maintained at 18 C for a total of n=90 larvae per treatment. These larvae were monitored daily for changes in behavior related to starvation: cessation of active swimming followed by sinking to the bottom of their enclosure and remaining in a state of immobility. If larvae remained inactive after a gentle stirring of the chamber, the trial was discontinued for that individual and the time to starvation recorded.

JMP Pro statistical software (v. 15.2.0) was used to perform a survival analysis for this dataset. This analysis compares time to inactivity among our two maternal size treatments using a log-rank test in order to determine whether inactivity is reached after a significantly different amount of time between these treatments.
2.4 Results

The data show that in each study region larger female *Homarus americanus* are not only more fecund, but the individual embryos from larger females also tend to be larger, heavier and have a higher total and mass-specific caloric content, resulting in considerably greater energy investment per egg. The larger embryos have significantly higher total, but not mass-specific, lipid content; and total amounts of the key essential fatty acid DHA also scale with maternal size. Finally, the larvae of larger mothers were larger on average, and less vulnerable to starvation than larvae of smaller females. In short, larger maternal size results in the production of more embryos and a greater energy investment per embryo that confers a higher probability that larvae will survive longer periods of starvation.

Regional effects were evident in some metrics of reproductive investment. Most noteworthy is the smaller size of sexually mature lobsters in the warmer thermal regime of southern New England: mature females rarely exceeded 90 mm CL, whereas those from the Gulf of Maine measured greater than 130 mm. This phenomenon limited the degree to which it is possible to compare maternal size effects across regions. Nonetheless, differences in the regression intercepts suggest that, size-for-size, female lobsters in RI have higher fecundity and larger individual embryos than those in the Gulf of Maine. With the possible exception of specific lipid levels, no interactive effects were found between maternal size and region, suggesting that the scaling relationship between maternal size and embryo metrics were the same, even when a regional effect was detected.

*Embryo number (Fig. 2.2A) (F₃, 180 =68.71; p<0.001, R²=0.53):* The data show a significant relationship between maternal size and clutch volume with an average clutch comprised of
approximately 8768 embryos. For each additional millimeter of carapace length a female lobster was capable of producing roughly 411 more embryos. A regional effect was also detected such that the x-intercept of each region increased with latitude (i.e. RI had the smallest intercept and DE the largest), however the difference between MC and the RI was not significant.

*Embryo volume (Fig. 2.2B) \( (F_{3, 180}=9.96; p<0.001, R^2=0.14)\):* Maternal size and embryo volume varied significantly by carapace length, with the average embryo having a volume of 2.18 µl and each additional millimeter of carapace length resulted in an increase of 0.011 µl of volume. There was also a significant effect of region where RI differed significantly from MC. RI had the highest y-intercept with the regression lines for DE and MC grouped closely together, though there was no significant difference detected between DE and RI.
Figure 2.2 A) The number of embryos, and B) average volume of individual embryos (μl) within a clutch of Homarus americanus as a function of female size. Colored lines represent the linear regression for each region.
Embryo dry weight (Fig. 2.3A) \( (F_{1, 29}=6.36; p<0.017, R^2=0.18) \): Embryo mass increased significantly with maternal size. The average embryo had a dry weight of 1.11 mg, with each millimeter of carapace length resulting in an increase of 0.003 mg. Also, despite the significant regional effect on embryo volume (Fig. 2.2B), there was no such effect on the embryo mass (Fig. 2.3A).

Total caloric content (Fig. 2.3B) \( (F_{1, 29}=11.36; p<0.002, R^2=0.28) \): As maternal size increased, so did the caloric content in joules per embryo (J/embryo). The caloric content of an average embryo was 28.7 J, with an increase of 0.09 J for each millimeter of carapace length in maternal size.

Mass-specific caloric content (Fig. 2.3C) \( (F_{1, 29}=8.44; p<0.007, R^2=0.23) \): Similar to total caloric content, mass-specific caloric content also increased significantly with maternal size. The average embryo contained 25.7 J of calories per mg of embryo dry mass with a 0.02 J increase in calories per mg with each added millimeter of maternal carapace length.

Energetic investment (Fig. 2.3D) \( (F_{1, 29}=153.96; p<0.001, R^2=0.84) \): Energetic investment, a function of clutch volume (fecundity) and embryo caloric content as described above, significantly scaled with maternal size. On average females invested roughly 360,000 J into a clutch with an increase of 14,476 J for each added millimeter of maternal carapace length.
Total lipid content (Fig. 2.4A) \( (F_{2,15}=7.53; p=0.006, R^2=0.50) \): Total lipid content of embryos was found to relate significantly to female size class. Embryos of medium (Tukey’s HSD, \( p = 0.026 \)) and large (Tukey’s HSD, \( p = 0.006 \)) sized lobsters contained significantly more...
micrograms of lipids than small lobsters, but no difference could be detected between medium and large sized females (Tukey’s HSD, $p = 0.757$). On average, embryos of small lobsters contained $192.17 \mu g$ of lipids, medium $227.82 \mu g$, and large $236.53 \mu g$.

**Mass-specific lipid content (Fig. 2.4B)** ($F_{2,15}=2.70; p=0.099, R^2=0.26$): Considered with a confidence of $\alpha=0.05$, the relationship between maternal size and mass specific lipid content of embryos was not significant. On average embryos contained $175.24 \mu g$ of lipids per mg of embryo dry mass.

Figure 2.4 Lipid content of *Homarus americanus* embryos as a function of maternal size class small (80 – 90 mm CL), medium (100 – 110 mm CL), and large (130-140 mm CL), each point represents one pooled sample containing 70 embryos each- A) Total lipid content of an average embryo (µg). B) Total lipid content in µg normalized to average egg mass. The upper edge of the box is the 75th quantile, the lower edge the 25th quantile, and the center line is the median. Whiskers represent the max/min values of data. The black horizontal line is the grand mean of the combined data. Groups with different letters are significantly different.

**Total EPA content (Fig. 2.5A)** ($F_{2,15}=3.15; p=0.072, R^2=0.30$): Considered with a confidence of $\alpha=0.05$ the relationship between maternal size and total eicosapentaenoic acid (EPA) content of embryos was not significant. On average embryos contained $41.29 \mu g$ EPA.

**Total DHA content (Fig. 2.5B)** ($F_{2,15}=6.07; p=0.012, R^2=0.45$): Maternal size had a significant effect on total docosahexaenoic acid content of embryos. Embryos from large lobsters contained
significantly more DHA than either small (Tukey’s HSD, p=0.017) or medium (Tukey’s HSD, p=0.030) sized lobsters. On average, small lobsters contained 39.33 µg of DHA, medium 40.11 µg, and large 47.60 µg.

Mass-specific EPA content (Fig. 2.5C) \((F_{2, 15}=2.11; p=0.156, R^2=0.22)\): There was no significant relationship between maternal size and mass-specific EPA content. On average embryos contained 33.12 µg of EPA per mg of embryo dry mass.

Mass-specific DHA content (Fig. 2.5D) \((F_{2, 15}=0.92; p=0.419, R^2=0.11)\): There was no significant relationship between mass-specific DHA content and maternal size; however, further analysis revealed a significant effect of region between DE and MC \((F_{1, 16}=6.31; p=0.023, R^2=0.28)\). On average embryos from DE contained 31.73 µg of DHA per mg of embryo mass, while the average embryo from MC contained 36.00 µg of DHA per mg of embryo dry mass.

DHA:EPA ratio (Fig. 2.5E) \((F_{2, 15}=1.76; p=0.205, R^2=0.19)\): There was no significant relationship between maternal size and the ratio of total DHA to EPA content. On average embryos had a DHA:EPA ratio of 1.14.
Figure 2.5 Essential fatty-acid content of *Homarus americanus* embryos as a function of maternal size class (Small=80-90, Medium=100-110, Large=130-140). A) Total Eicosapentaenoic acid content of an average embryo in µg. B) Total Docosahexaenoic acid content of an average embryo in µg. C) Eicosapentaenoic acid content normalized to average embryo mass. D) Docosahexaenoic acid content normalized to average embryo mass. E) Ratio of docosahexaenoic to eicosapentaenoic acid content. The upper edge of the box is the 75th quantile, the lower edge the 25th quantile, and the center line is the median. Whiskers represent the max/min values of data. The black line across the graph is the grand mean of the combined data. Groups with different letters are significantly different.
Larval mass (Fig. 2.6A) \( (F_{2, 525}=30.67; p<0.001, R^2=0.10) \): Both maternal size and developmental stage had a significant effect. The average SI larva had a dry mass of 1.27 mg, and SI larvae from large mothers were 17.8% heavier than those from small mothers. The average SII larva had a dry mass of 1.58, with SII larvae from large mothers outweighing those from small mothers by 26.8%.

Larval carapace length (Fig 2.6B) \( (F_{2, 517}=1193.53; p<0.001, R^2=0.82) \): Again, maternal size class and developmental stage each significantly affected larval carapace length. On average SI larvae had a carapace length of 2.44 mm, with SI larvae of large mothers being 8.3% longer than SI larvae from small mothers. SII larvae were 3.30 mm on average, with SII larvae from large mothers being 7.5% longer than those from small mothers.
Figure 2.6 A) Mass and B) Carapace length of *Homarus americanus* larvae by maternal size class (Small=81-93 mm, Large=107-136mm) and developmental stage. The upper edge of the box is the 75th quantile, the lower edge the 25th quantile, and the center line is the median. Whiskers represent 1.5X the IQR. The black line across the graph is the grand mean of the combined data. Groups with different letters are significantly different.
Larval starvation mass loss (Fig. 2.7A) \((F_{2, 56}=15.22; \ p<0.001, \ R^2=0.35)\): There was no evidence for a differential rate of mass loss during starvation between larvae from large vs. small mothers. However, in agreement with previously discussed results larvae from larger mothers started with a greater mass before starvation began. The average initial mass of larvae from small mothers was 1.00 mg, and 1.49 mg for larvae from large mothers. Average mass loss for both treatments was 0.08 mg.

Larval starvation behavior (Fig. 2.7B) \((X^2_{1, 180}=3.86; \ p=0.049)\): Larvae from larger mothers took significantly longer to reach a state of starvation. Larvae from smaller mothers had an \(LD_{50}\) (time until half of larvae were starved) of 7.39 days, while those from larger mothers had an \(LD_{50}\) of 8.08 days. Additionally, the larva which remained in treatment the longest from small mothers lasted 14 days, while its counterpart from large mothers lasted 15 days.
Figure 2.7 A) Mass of larvae from large (107-136 mm; n=90) and small (81-93 mm; n=90) *Homarus americanus* over the course of five days of starvation. B) Proportion of larvae from large (124-132 mm) and small (80-92 mm) *Homarus americanus* still active after consecutive days of starvation.
2.5 Discussion

Larvae die for a number of reasons: predation, starvation, disease, physiological stress etc. Species responses to these pressures often involve adaptive trade-offs. For example, starvation and predation may be overcome through production of larger larvae with greater energy reserves, the trade-off being reduced fecundity or diminished dispersal potential. A widely held explanation for why a large diversity of marine invertebrates and fish tend to produce large numbers of planktonic offspring relate to bet-hedging - the advantage of spreading risk over several potential outcomes in a variable environment difficult to predict (Winemiller and Rose, 1992). Within its own phyletic constraints, there is evidence that the life history traits of *Homarus americanus* represent adaptations consistent with that hypothesis. For example, among clawed lobsters and crayfishes (clade Astacidea) the shallow coastal dwelling lobsters (e.g., *Homarus* spp.) have relatively high fecundity and planktotrophic larvae, whereas their freshwater (e.g., *Orconectes* spp.) and deep sea (e.g., *Metanephrops* spp.) counterparts have lecithotrophic larvae and abbreviated development (Wahle et al. 2012). Still, geographic differences in these traits among subpopulations (e.g., southern and northern) reveal plasticity in the response to environmental variability, providing a natural experiment in the form of a space-for-time comparison that is useful in the context of understanding the impacts of a climate that is changing rapidly relative to the generation time of the species.

This study demonstrates that maternal body size has a significant positive impact not only on fecundity, but also on the energy content of the individual embryo, and in turn, the ability of the larva to endure starvation. By way of overview, the strongest relationships were between carapace length and fecundity (embryo number) (Fig. 2.2A), and the collective energy index of
the clutch of embryos (EI; Fig. 2.3D). As in most invertebrates and fishes (Peters, 1983; Barneche et al., 2018) greater brood size with increased female size has been reported in clawed lobster (e.g., Attard and Hudon 1987; Lizárraga-Cubedo et al. 2003; Ouellet and Plante, 2004; Koopman et al., 2014; H. gammarus - Moland et al., 2011), but these previous studies included a much smaller size range of females, typically only up to 110 mm. Our data set includes females exceeding 140 mm CL. Koopman et al represents another recent study which investigated the effect of maternal size on reproductive measures such as fecundity and fatty-acid investment over a broad size-range in lobster from the Bay of Fundy (2014). The greater northern extent of this study allows us to superficially extend the range of our comparison of regional effects on reproductive output. Our size-fecundity curves show a mean of ~ 17,000 embryos at a CL of 110 mm, a value significantly higher than was found in lobster from the Bay of Fundy (Koopman et al 2014). This suggests that when corrected for size, Maine lobsters carry more embryos. This follows a latitudinal trend in our own data that when compared at size, females from southern populations (RI) carry more embryos. Additionally, energetic investment in individual embryos in the Maine populations was greater, resulting in a significantly larger energy expenditure per female, with Bay of Fundy lobsters investing in a smaller number of less well-provisioned embryos. This follows Koopman et al.’s observation that fecundity, and perhaps embryo quality metrics, have declined in the Bay of Fundy lobster population; this suggests that colder northern populations may suffer greater effects of warming waters.

Producing larger embryos with higher caloric value and larger larvae at hatching enhances the probability the larvae will survive longer in the plankton, or survive low food concentrations during early development (Anderson, 1988). However, while variables related to embryo quality, such as embryo volume (Fig. 2.2B), mass (Fig. 2.3A), caloric content (Fig.
2.3B+C) and lipid profile (Figs. 2.4+2.5) also showed significant relationships with maternal size; the correlations were significantly weaker than the relationship between maternal size and embryo number. Therefore, the data suggest that embryo size is more constrained than fecundity. In other words, it appears that resources are partitioned preferentially towards producing a greater quantity of moderately provisioned embryos, rather than a moderate quantity of well provisioned embryos.

2.5.1 Maternal size effects on embryo quality

In general, larger females produced embryos with a greater amount of lipids and a larger amount of some essential fatty acids (FA) (Figs. 2.4, 2.5). Lipids and FA provide high-density bioenergetic fuel and the structural components for growth of larval and juvenile crustaceans (Watanabe, 1993; Bascur et al., 2018). As discussed above, the larger lipid content provides an energetic buffer during times of low food supply or high metabolic cost. Of the suite of fatty acids measured, the omega-3 fatty acids EPA (eicosapentaenoic acid, 20:5w3) and DHA (docosahexaenoic acid, 22:6w3) are of particular importance (Masuda 2003). The significant increase in total DHA within the embryos with increased maternal size suggests that larger females produce higher quality embryos (Fig. 2.5). These specific long-chain polyunsaturated fatty acids (LC-PUFAs) molecules must be obtained from dietary sources, although there is some small amount of chain elongation from the omega-3 parent ALA (a-linolenic acid, 18:3w3), but this pathway represents only a small percentage of the total LC-PUFAs available to the animal (Arts et al. 2001). Previous studies on American lobster larvae (*Homarus americanus*) have found them to be reliant on LC-PUFAs in the diet (Tlusty et al. 2005; Beltz et al. 2007). Tlusty et al. (2005) observed higher survival and growth rates in lobster larvae fed diets enriched with
omega-3 fatty acids compared with those fed unenriched diets. Deficiencies in DHA and EPA were linked to a change in the level and temporal pattern of neuronal proliferation in the brains of lobsters (Beltz et al. 2007; van de Meeren et al., 2010) and early development (Siu et al., 2007; Andres et al., 2010; Beder et al., 2018). Interestingly, there was no difference in DHA:EPA ratio across larval size classes or regions (averaging ~1.125) (Fig. 2.5E). The constancy of the ratio highlights the importance of these PUFAs. An imbalance in the ratio of these essential FAs would create an excess of some compounds and a dearth of others, while also compromising the integrity of the lipid membrane (Watanabe, 1993). When normalized for mass of the embryos, mass-specific lipid, EPA, and DHA quantities also did not scale significantly to maternal size (Figs. 2.4.+2.5.). Since maternal size had a significant effect on total quantities, the lack of significance in these mass-specific values means that relative fatty acid investment into embryos remains roughly constant across female size classes. So, larger females are able to maintain mass-specific investment in embryo quality, even while producing a greater quantity of embryos. One exception to this rule is the significant relationship between mass-specific calories and maternal size (Fig. 2.3C). Lipid content and calories are both ways to estimate the energetic content of the embryos; lipids take into account only the quantity of fatty acids, which are commonly used for energy storage, while calories are a more all-encompassing measure of total energy. This difference between lipid and caloric content may mean that lobsters store a significant amount of energy in molecules other than fatty acids, such as proteins, carbohydrates, and glycogen, which have been demonstrated to be important energy stores in other crustaceans (Sanchez-paz et al., 2006).
2.5.2 Maternal effects on larval quality

Larvae hatched from larger mothers were themselves larger, both in mass and length, at stages I and II of development (Fig. 2.6). Stages III and IV (postlarvae) were not examined in this study. This result follows the same trends found for embryo mass and volume (Figs. 2.2.B+2.3.A). Factors such as length and mass can have important implications to the survival of larvae (Anderson, 1988; Garrido et al., 2015). Greater size is related to movement efficiency. Larger animals expend a lower amount of energy to move the same distance (speed being held constant) as a smaller animal (Peters, 1983). Size can also impact the breadth of prey a planktotrophic predator such as lobster larvae can handle (Vucic-pestic et al., 2010). Conversely, greater size serves as a refuge from predation by other planktotrophs, as a larger larva may have outgrown their predator’s preferred prey size (Nowlin et al., 2006, Almeida et al., 2011, Zhang et al., 2017).

2.5.3 Maternal size effects on starvation resistance

The ability to withstand starvation is important for the survival of lobster larvae. The reliance of recruitment on the correlation between the timing of larvae hatching and the timing of peak abundance of their prey suggests that food limitation may play an important role in recruitment for this species (Carloni et al., 2018). Larger larvae with more energy stores are more likely to survive starvation better than smaller ones. This was supported by our starvation experiments showing that larvae from smaller mothers reach a critically low mass earlier than larvae from larger mothers during starvation (Fig 2.7A). During starvation, lobster larvae reach a “point of no return” beyond which they will be unable to survive even if fed amply (Abrunhosa and Kittaka, 1997). Results from behavioral experiments showed that after just a few days of
starvation, active swimming stops and the larva sinks to the bottom of their enclosure. Larvae from larger mothers maintained active swimming for a greater amount of time than larvae from smaller mothers, though the difference was slight (Fig. 2.7B). Still, with such high mortality rates during planktonic stages, even small changes in survival can have major impacts on a population (Thorson, 1950; Grosberg and Levitan, 1992).

2.5.4 Regional effects

The regions used in this study reflect the broad thermal regime occupied by this species. In the process of conducting this study, the widely recognized trend that lobsters in southern warmer regions tend to become sexually mature at a smaller size compared to their counterparts in cooler thermal regimes to the north was confirmed (Estrella and McKiernan 1989, Goldstein et al. 2022). Warming over the recent decades has also been linked to a decrease in the size at maturity of adult females (Waller et al., 2021; Aiken and Waddy, 1976). Because of their smaller size, lobsters in southern regions tend not to be as fecund as their northern counterparts. Controlling for body size, however, lobsters in RI tended to be more fecund than those in the north (DE) (Fig. 2.2A). Moreover, the individual embryos from mothers of the same size were larger in RI than those in MC (Fig. 2.2B). These regional effects suggest that there may be counter-gradient adaptation in lobster- wherein smaller females from warmer populations are genetically pre-disposed to out-produce similarly sized females from cooler populations (Levins, 1968; Conover et al. 1995). Maternal size therefore only plays a partial role in embryo size and fecundity, and there are other important factors that determine these traits. Another potential source for the greater reproductive capacity of RI females could be age and reproductive experience. As maturity induces a decrease in growth-rate for female lobster, small females from
an early-maturing population (RI) may in fact be older and have more reproductive experience than females from later-maturing populations of a similar size (MC+DE) (Aiken and Waddy, 1976). In American lobster (Ouellet and Plante, 2004) and fish (Trippel et al., 1997) primiparous (first time breeder) females have been shown to produce undersized larvae, and maternal age has been linked to offspring quality in many other species (Benton et al., 2008; Plaistow et al., 2015; Coakley et al., 2018). This phenomenon may also help to explain why ovigerous females from DE and MC had a much greater size range than those from the warmer RI population.

2.6 Conclusion

With the rapid warming of the Gulf of Maine it is important to understand species’ responses to the changing environment. For commercially exploited species such as the American lobster, understanding how climate change affects life history traits is especially important because changes in these traits can affect the sustainability of the current exploitation rates. The impact of thermal regime on lobster size of maturity has long been evident in geographic comparisons among subpopulations along New England’s steep thermal gradient (Estrella and McKiernan 1989). Warming since the 1980s has also measurably reduced the size at maturity for female lobsters in the Gulf of Maine by about 20% (Waller et al. 2021). These biological changes may have important demographic consequences. Our results suggest that larger maternal size confers reproductive benefits not only in terms of fecundity, but also to individual embryos and their resulting larvae. However, there is also evidence that while mothers in southern, warmer regimes may be smaller, they are detectably more fecund and embryos are larger than those from mothers of comparable size in the coolest regime at the northern extreme of the geographic domain of our study. The larger fecundity and embryo size of southern
mothers may, therefore, offset the adverse effects of warmer regime on the size at maturity.

Whether this is evidence of counter-gradient variation (Levins, 1968; Conover et al. 1995) in the American lobster remains a question for further study. Therefore, when considering how other marine crustaceans may adapt to future warming conditions, it is important to consider the effect of warming waters on body size, maturation and their cascading effect on important traits related to reproduction and larval survival.
CHAPTER 3 - A COMPARISON OF METHODOLOGIES IN LARVAL DIET STUDIES:  
CONVENTIONAL MICROSCOPY VERSUS CONTEMPORARY eDNA METHODS

3.1 Abstract

The diet of pelagic marine larvae can prove difficult to analyze due to their small size, the even smaller size of their prey, and their occasional elusiveness due to their brief existence in the plankton and their dispersal in nature. Conventionally, diet studies of marine larvae rely on visual identification of gut contents through dissection and microscopy. While this method has its benefits, fine scale identification of gut contents can suffer when already small prey items are masticated prior to or during digestion. Here the utility of conventional microscopy in larval diet studies is compared to two contemporary eDNA methods: DNA metabarcode sequencing (metabarcoding) and real-time PCR (rtPCR). Further, the development of new eDNA methods as applied to studies on the diet of American lobster larvae are described in order to illustrate the utility and challenges of these methods. Metabarcoding represents a promising technique for identifying broad portions of the larval diet down to a finer taxonomic scale compared to microscopy. However, eDNA approaches like metabarcoding are not without their own challenges, including how to block PCR amplification of highly-abundant predator DNA (e.g., lobster) while permitting amplification of prey DNA with ‘universal’ PCR primers, but without masking prey that are genetically similar to the predator (e.g., other crustaceans). Here, the novel development of a blocking primer to better reveal the prey taxa consumed by larval lobster is described. In contrast, rtPCR is a molecular technique allowing the identification of particular prey taxa with high fidelity - but involves \textit{a priori} decisions regarding which prey to target. This technique is best employed when a particular prey taxon is of interest, but it is not efficient to
broadly characterize the diversity of prey taxa. An rtPCR assay was thus developed to target the copepod *Calanus finmarchicus*, a species of interest in the diet of larval lobster. Based on our review and comparison of these three methods, it is recommended that researchers consider using a mixture of these methodologies in future larval diet studies.

### 3.2 Introduction

Diet analysis is a tool that can reveal important insights into the trophic interactions and energy requirements of a target organism. Knowledge of a predator’s diet is fundamental to the understanding of its ecology, and may impact such varied traits as: range, carrying capacity, intra/interspecific competition, feeding rate, and sensitivity to food-limitation (Olson and Olson, 1989; Svanbäck and Bolnick, 2008; Nielsen et al., 2017). Analysis of diet can prove difficult though as diet material is degraded through digestive processes (Blankenship and Yayanos, 2005). Furthermore, as the size of the target predator decreases, the traditional tools used for diet analysis become increasingly difficult to employ.

The pelagic realm is home to some of the smallest metazoan predators, such as zooplankton and the larvae of benthic organisms. These creatures exist within a complex food-web, and much thought has been given to how to best characterize their diets (Blankenship and Yayanos, 2005; Pepin et al., 2007; Chow et al., 2011; Nielsen et al., 2017). Novel molecular techniques using eDNA approaches have become more common to surmount common diet analysis difficulties (e.g. **copepods**: Durbin et al., 2008; **spiny lobsters**: O’Rorke et al., 2012; **spiders**: Krehenwinkel et al., 2017; **fishes**: Su et al., 2018; **turtles**: Diaz-abad et al., 2021; **mussels**: Weber et al., 2022), but these carry their own limitations and biases.
In this chapter, three different diet analysis techniques will be compared and contrasted. These include microscopy and visual identification, as well as two molecular eDNA approaches: metabarcoding and real-time PCR (rtPCR). This will be illustrated through applying each technique to the study of the American lobster (*Homarus americanus*) larval diet. Lobster larvae are small predatory meroplankton, and so the methods reviewed here could be applied towards further detangling pelagic food-webs, and particularly the diets of other pelagic larvae.

### 3.2.1 Microscopy

Gut dissection and subsequent visual identification with the unaided eye or through microscopy is perhaps the most well established and classic methodology in diet studies where observation is not possible or practical. Microscopy and visual identification of gut contents has been used to study the diet of fishes (e.g. **trout**: Ball, 1961; **walleye**: Houde, 1967; **pompano**: Bellinger and Avault, 1971; **lutefish**: Arawomo, 1976), crustaceans (e.g. **mysid**: Bremer and Vijverberg, 1982; **shrimp**: Wahle R.A., 1985; **lobster larvae**: Juinio and Cobb, 1992), and even **marine mammals** (Gimenez et al., 2017). Data collected with this method can be recorded simply as presence/absence, the total number of individuals for each diet constituent can be recorded, or the amount of food may even be recorded volumetrically or gravimetrically (Hyslop, 1980; Nielsen et al., 2017). Depending on the size of the organism being studied, dissection may be possible with the naked eye, or may necessitate the use of a microscope; either for gut content identification alone, or for actual gut removal. The level of difficulty associated with gut content identification may also vary depending on the size of the study organism and its feeding ecology. The diet of a large fish which captures its prey whole would be much easier to discern than that of a small crustacean which tears its prey into smaller pieces. Microscopy is
certainly the simplest of the three methods discussed here, and beyond the taxonomic expertise necessary to distinguish different diet constituents, very little specialized knowledge or tools are required.

3.2.2 DNA tools

Environmental DNA (eDNA) approaches have been gaining increasing use in contemporary diet analysis as the technology has developed and become more widely recognized. Analysis of eDNA is a broad topic with a rapidly developing range of techniques. It has also spawned a sometimes opaque jargon that can be an impediment to communicating with the uninitiated. The aim of this paper is to describe two widely used methods of DNA analysis and to translate the associated terminology to a broader scientific audience, and to illustrate the comparison from a limited sample of larvae. For the purposes of this paper eDNA refers to the analysis of DNA not isolated directly from a tissue sample. In this case, the ‘environment’ being sampled is the contents of the lobster larval gut. Two specific eDNA techniques are discussed in this paper: metabarcoding, and real-time Polymerase Chain Reaction (rtPCR).

Metabarcoding is a technique that can be used to identify the genetic diversity within a sample for a selected gene or set of genes (Creer et al., 2016; Nielsen et al., 2017). It makes use of universal primers that bind to highly conserved DNA regions flanking more variable regions. Typically these are areas of the genetic code associated with metabolic or other basic pathways that are more likely to be conserved across taxa. Because these regions are highly conserved, universal primers are capable of amplifying a very broad range of taxa with a single PCR. The variable region that is amplified by a universal primer set acts as the barcode allowing for identification of the genetic diversity within the sample through Next-Gen Sequencing. During
sequencing, the base-pairs constituting the fragments (reads) of DNA amplified from the sample are identified. However, as amplification through PCR can introduce errors that may be misinterpreted as biological variation, reads must be assessed for quality using bioinformatics tools. This is typically accomplished through algorithms designed to filter the raw reads, managing error rates and inferring true biological diversity through clustering reads into Operational Taxonomic Units (OTUs), or Amplicon Sequence Variants (ASVs) (Callahan et al., 2016; 2017). An OTU is a cluster of reads based either by similarity (typically 3%), or through comparison to a reference library. An ASV method infers biological reality through the expectation that “true” sequences are more likely to be repeated within a sample, and can distinguish unique ASVs based on a single nucleotide difference (Callahan et al., 2017). This technique, utilizing the DADA2 (Divisive Amplicon Denoising Algorithm) bioinformatics pipeline, has been found to be a better representation of true biological diversity, and performs as well or better than similar OTU methods (Callahan et al., 2016; 2017).

Through this process, a properly designed metabarcoding assay can be used to assess the diet of a predator by amplifying and sequencing the DNA of undigested prey species. After clustering metabarcode sequences into ASVs or OTUs, their taxonomies can then be assigned by comparison to a reference database to identify the prey taxa. However, the vast majority of genetic material in a predator’s gut belongs to the predator itself, and so it is imperative that the amplification of this ‘host’ DNA be delayed or halted. Failure to do so will result in DNA from the predator being preferentially amplified during the PCR leading to an effect known as species masking (Skelton et al., 2022), causing the less abundant prey DNA to remain unidentified (Chow et al., 2011; Liu et al., 2019).
To minimize amplification of the host/predator DNA, a blocking primer must be employed. There are two main varieties of blocking primers: Annealing inhibiting (AI), and elongation arresting (EA). Annealing inhibiting blocking primers work by taking advantage of a variable sequence specific to the predator near the universal priming site. The annealing step takes place after double-stranded DNA is split into its single-strand components. In a typical metabarcoding reaction, universal primers would then attach or “anneal,” to the single-stranded DNA at the universal priming site. An AI blocker anneals to a variable site specific to the predator, but stretches into the universal priming site in order to stop the universal primer from annealing, halting amplification before it happens. An elongation arresting blocking primer is typically only used when there is no variable site specific to the predator that is close enough to the priming site (Vestheim and Jarman, 2008). Elongation is the step after annealing, where DNA polymerase attaches to the primer and begins to add nucleotides to the single-stranded DNA to create a copy of the original double-stranded DNA. EA blockers are capped with a hydrocarbon chain that interferes with DNA polymerase, halting the elongation process. Typically AI blockers are preferred, since they halt PCR at an earlier step (primer annealing) than EA blockers do (elongation), and so tend to be more efficient and successful blockers (Vestheim and Jarman, 2008).

Lobster larvae provide a particularly challenging case study as their diet is composed chiefly of other crustaceans (Harding et al., 1983; Juinio and Cobb, 1992), leading to a high degree of genetic similarity between predator and prey, even within the variable barcode region. In preliminary tests, standard blocking primers were ineffective, and had the tendency either to allow amplification of lobster DNA, or hinder the amplification of both host and prey DNA. To surmount this issue a Peptide Nucleic Acid (PNA) was utilized for the blocking primer. A PNA
is a synthesized molecule similar in structure to DNA, but lacking a charged phosphate group (Nielsen and Egholm, 1999). Because of this, a PNA-DNA dimer has a stronger bond than a DNA-DNA dimer, as it lacks an opposing electrostatic force. This quality makes it possible to design very short blocking primers that 1) bond strongly and accurately to the targeted DNA region, and 2) have a greater degree of specificity as there will be fewer opportunities for sequences to match up with non-target DNA. This PNA was developed to work as an EA blocker within the V4 region of the 18S rRNA gene, delaying the amplification of lobster DNA, while allowing the amplification of prey DNA.

The second eDNA technique discussed is real-time PCR. One advantage of rtPCR over metabarcoding is that no sequencing is required, and it therefore costs less and has a faster turnaround time. However, each rtPCR assay must be developed for a particular taxon of interest making it impractical for broad identification of diet constituents. Instead, an rtPCR assay can be used to assess the presence of a particular taxa of interest. This technique makes use of primers and a probe specifically designed to target a DNA sequence unique to the taxa of interest. This means that no blocking primer is required, as the primers being used would not have an affinity for the host DNA. In an rtPCR, primer and probe each bind to the target DNA. The probe molecule contains a fluorescent reporter as well as a quencher to suppress fluorescence while the probe is intact. During PCR, DNA polymerase binds to the priming site, and subsequently cleaves the probe through exonuclease activity, freeing the reporter molecule from the effects of the quencher and allowing the reporter to fluoresce. As target DNA amplification continues, the level of fluorescence increases. Fluorescence is measured by a camera at the end of every amplification cycle, and a level of fluorescence measuring above a threshold is evidence of the presence of the target taxa within a sample. Declining larval recruitment of lobster has been
linked to a decrease in the abundance of a foundational copepod species in the Gulf of Maine, *Calanus finmarchicus*, suggesting a potential predator-prey relationship. In order to discern whether *C. finmarchicus* is an important diet constituent of larval lobster, an rtPCR primer-probe assay was designed to detect its presence in larval lobster gut samples.

3.3 Methods

3.3.1 Larval collection

Larvae for this study were collected live aboard the University of Maine’s research vessel R/V Ira C. through a combination of horizontal tows at 30, 20, and 10 meters of depth as well as neuston tows at the surface. These tows were conducted at several stations near the mouth of the Damariscotta River in Midcoast Maine. Horizontal tows were conducted with 1 m diameter ring nets with a mesh size of 1000 µm and a cod end mesh size of 150 µm. Neuston tows were conducted with a 1 m X 2 m rectangular neuston net with a mesh size of 500 µm and cod end size of 1000 µm. Once brought aboard larvae were sorted by developmental stage and placed in separate vials in a cooler, then transferred to a -80 °C freezer when brought ashore. Additional lobster larvae were provided by Maine Department of Marine Resources (DMR) collected through their annual lobster larval survey, and Normandeau Associates, Inc. supplied larvae captured during annual ecosystem monitoring tows in New Hampshire. Larvae supplied by Normandeau Associates and Maine DMR were stored in 95% ethanol and refrigerated. While larvae were collected at all 4 pelagic stages of development, only SIV larvae were used in this study due to their greater abundance in tows.
3.3.2 Dissection and light microscopy

Larvae which were stored in ethanol were first rinsed in distilled water. Once sufficiently rinsed the gut can be removed from the animal. To do so the head of the larva was removed from the body just behind the eyestalks with a pair of fine dissecting scissors in order to sever the esophagus and mouthparts from the cardiac stomach, as well as to remove some of the connective tissue of the carapace. After this was accomplished, the carapace was gently removed from the body using a pair of forceps, revealing the gut. The gut (cardiac and pyloric stomach) was then removed carefully with forceps and placed in seawater so that any extraneous tissue could be removed and rinsed off. Seawater rather than distilled water was used at this step, otherwise the osmotic gradient causes stomach tissue to swell and flake off, mixing with the stomach contents. This can make identification of gut contents more difficult at later stages of analysis.

Once sufficiently cleaned off, the stomach was placed on a 1 x 1 mm gridded Sedgewick rafter slide. The percent fullness of the gut was ranked and recorded. The gut was then pulled open with a pair of forceps, and the contents rinsed out with Lugol’s solution (deionized water mixed with iodine) onto the slide. Lugol’s solution is used in order to increase contrast, which aids in gut content identification. Once the gut was fully rinsed out, all pieces of the gut were removed and the slide was flooded with Lugol’s solution. The gut contents were then distributed evenly across the slide using a pair of forceps. At this point identification of gut contents through a high powered stereo microscope (Olympus SZ40) began.

It can take time to build up competency with gut content identification. Some methods used in this study included consultation with taxonomic keys and experts, survey of potential diet
constituents through net tows, and dissection of potential diet constituents to gain familiarity with identification through body parts rather than a whole organism. Using a gridded slide can also aid in the identification of gut contents, as the grid can be used as a size reference.

Presence/absence of diet constituents was ranked on a gut-to-gut basis and recorded down to the lowest possible taxonomy, and then also at each broader taxonomic level, e.g. when a copepod of the genus *Calanus* was identified, the genus *Calanus* was marked present, as was the family: Calanidae, the order: Calanoida, the class: Copepoda, and finally the phylum: Arthropoda. This method allows for easy comparison of gut contents at different taxonomic levels. When unable to identify a diet constituent immediately, an image was taken and stored for later identification.

### 3.3.3 DNA extraction

Larvae for analysis using eDNA methods were dissected with a similar technique to the one described above, with a few alterations to ensure no cross-contamination between samples. All tools used for dissection were dipped in ethanol and sterilized over a bunsen burner in between samples. Larvae were dissected over a fresh, sterile petri dish. Once the gut was removed it was placed directly into a 1.5 mL DNA LoBind microcentrifuge tube (Eppendorf). If the gut was not extracted for DNA immediately, it was submerged in DNA/RNA Shield (Zymo Research), and then placed in a -20 °C freezer until extraction.

The Invitrogen ChargeSwitch Forensic DNA extraction kit was used for DNA extraction as this kit has been demonstrated to be adept at recovering degraded or rare DNA, which fits the profile of prey DNA recovered from a predator’s stomach (O’Rorke et al., 2012). Extractions were conducted per the manufacturer’s directions with one alteration: digestion of the sample with Proteinase K was conducted for 2 hours rather than 1 h. Additionally, after the first hour of
lysis the sample was ground with a sterile pestle to ensure complete lysis of all gut contents.

After DNA extraction, a Qubit high sensitivity DNA concentration assay was conducted to determine whether extraction was successful. At this stage, most samples would be considered ready to be sent to a laboratory for amplification and sequencing, however as the vast majority of DNA in these particular samples belongs to the lobster larvae rather than its diet constituents, a pre-amplification step using a lobster blocking primer was conducted.

3.3.4 Development and testing of lobster blocking primer

In order to reduce the quantity of predator DNA present in the samples prior to sequencing, samples used in diet studies must first be amplified using a predator specific blocking primer. An EA blocking primer constructed from a PNA oligomer was designed to target lobster DNA near the 18S V4 universal metabarcde priming site.

In order to ensure optimization of our PCR reaction with regards to the blocking primer, lobster DNA was isolated for use in testing the PNA blocker. Lobster DNA was extracted using the methods described above, and then amplified using 18S V4 universal metabarcde primers (Comeau et al., 2011). The PCR product was then isolated in a gel for cloning. Next, the resulting DNA product was diluted into a 10 ng/µL stock solution that was used for all testing needs.

**Blocker concentration:** First, the effect of blocker concentration on its efficacy was tested. The blocker was tested at concentrations of 0.25, 0.5, 2, 5, 10, 15, and 20 mM per reaction across three different trials. Each concentration was tested in triplicate, and a blank No Template Control (NTC) was included for each concentration. A sample containing lobster DNA but no PNA (0 mM) was also included in triplicate for each trial as a point of comparison. The samples
with 0 mM blocker served as a benchmark in order to determine how many cycles each blocker concentration delayed amplification. Reactions were run as an rtPCR using a Bio-Rad CFX96 thermocycler in order to immediately visualize results. Each reaction was comprised of 23 µL of Quanta Perfecta Sybr Green FastMix mastermix with 2 µL of either lobster DNA (10 ng/µL) or DNA-free water for the NTC for a total reaction volume of 25 µL.

**Cross-reactivity:** It is necessary to ensure the blocker does not also block amplification of prey DNA, especially where predator and prey are closely related. Therefore, the impact of the blocker on amplification of one common zooplankton and one common algal species was tested: *Calanus finmarchicus* (copepod) and *Pseudo-nitzschia sp.* (diatom). DNA was isolated from each of these organisms in the method described above and mixed with lobster DNA in order to determine whether the blocker would be successful in blocking the lobster DNA from amplification while allowing the ‘prey’ DNA to amplify. These mixtures were then tested in triplicate to see how well they amplified, with and without the blocking primer in comparison to pure lobster DNA. Additionally, DNA was extracted from the gut of a lab-reared lobster larva known to have exclusively grazed on *C. finmarchicus* to provide a more natural test of the blocker. A field collected larval gut sample was also included for comparison.

**Annealing temperature:** The annealing temperature of the blocker was calculated to be 70 °C when synthesized by PNA Bio Inc. (Thousand Oaks, CA). It is recommended to use an annealing temperature 5 °C above the calculated value for greater annealing specificity. In order to determine whether 75 °C was indeed the optimal temperature, a gradient PCR test was performed with annealing temperatures of 70, 75, and 78 °C. At each temperature, controls
without any blocker were run in triplicate, as well as triplicate 25 µL reactions containing 2 mM blocker plus an NTC reaction.

3.3.5 Metabarcoding

Universal metabarcoding primers targeting the 18S V4 region were used, as described in Comeau et al. (2011), due to the broad coverage of eukaryotic taxa exhibited by these primers. The exact volume and concentration of each reactant for a single 25 µL PCR can be seen in Table 3.1, and the thermal protocol in Table 3.2. The PNA concentration in each reaction was chosen to be 2 mM based on the previously described testing. After pre-amplification with the PNA lobster blocking primer using the high-fidelity Phusion Hot Start 2X mastermix (New England Biolabs), the PCR product was concentrated with the Zymo-5 Clean and Concentrator kit (Zymo Research) and the concentration of DNA present in each sample was determined using a Qubit high sensitivity DNA concentration kit (ThermoFisher Scientific). Samples were then sent to Dalhousie University’s Integrated Microbiome Resource (IMR) laboratory for sequencing. Amplicon Sequence Variants (ASVs) were inferred from the sequence reads using the DADA2 bioinformatics pipeline (Callahan et al., 2016), and taxonomy assignments were produced using the SILVA reference database (Yilmaz et al., 2014). Any ASVs attributed to lobster were removed in order to focus solely on inferred prey ASVs. It should be noted that prey originating from larval lobster cannibalism would be undetectable using the eDNA metabarcoding approach.
Calanus finmarchicus specific primers were designed to target a species-specific region of the cytochrome oxidase subunit-I marker gene (COI). Tables 3.1 and 3.2 show the reaction mixture and thermal protocol for the rtPCR respectively. A lab-reared larval lobster, fed a known diet of Calanus, was tested to prove that the assay was capable of detecting Calanus within the gut of a larva. A total of 48 fieldcaught larvae were assessed using the rtPCR assay. All rtPCR reactions were run in triplicate on a Bio-Rad CFX96 thermocycler. Any samples which amplified above a fluorescence threshold that was determined automatically by CFX Manager software were considered to have tested positive for the presence of C. finmarchicus.

3.4 Results

The results described here will focus on the efficiency and metadata for each technique presented. For a full discussion of the actual lobster larval prey discovered through these
methods, please see Chapter 4, “Revealing the diet of larval lobsters by microscopy and DNA sequencing”.

3.4.1 Microscopy

In total, 5 genera, 5 families, 5 orders, 9 classes, and 7 phyla of organisms were detected as larval lobster diet constituents after dissecting and investigating 112 SIV lobster postlarvae under a microscope. The greatest proportion of identifications were recorded at the class level (40%), with a steep drop off in positive identifications between class and order (13%) (Fig. 3.1). There were no unique identifications made at the family level. The only families of diet constituents recorded were inferred from the presence of a prey taxa within a representative genus.

![Figure 3.1 Proportion of unique ASVs at each phylogenetic level from metabarcoding data, along with proportion of microscopy data in each associated phylogenetic level. Note that these data are not directly comparable as the microscopy data is ranked on a presence absence basis; but instead serves as a general illustration of each technique's taxonomic resolution.](image)

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3.4.2 Development and testing of lobster blocking primer

**Blocker concentration:** Blocker concentrations were tested across three different trials, with some concentrations being tested more than once, reflected in the sample sizes reported in Table 3.3. When amplified without any blocking primer, lobster DNA amplified after an average of 15.44 cycles (Table 3.3, Fig. 3.2). This value can be used to calculate the effectiveness of the blocker as the delta Cq (▲Cq) - the number of cycles amplification was delayed from this average. At 0.25 mM and 0.5 mM the efficacy was roughly similar, with an average ▲Cq of 7.73 and 7.39 respectively. Increasing concentration to 2 mM provided a jump in blocking efficiency with a ▲Cq of 9.21. Increasing concentration to 5 mM, 15 mM, and 20 mM actually decreased blocking efficiency slightly. However, a concentration of 10 mM provided a greater average ▲Cq of 12.48 (Table 3.3). Significant variation was observed at this concentration though, with a minimum ▲Cq of 4.21, and a maximum of 22.76 resulting in a significantly higher standard deviation than other blocker concentrations of 6.29 (Table 3.3).

Table 3.3 Sample size as well as the mean Cq, ▲Cq and standard deviation for each PNA blocker concentration tested.

<table>
<thead>
<tr>
<th>PNA concentration</th>
<th>n</th>
<th>Mean Cq</th>
<th>Mean ▲Cq</th>
<th>Std Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>0mM</td>
<td>9</td>
<td>15.44</td>
<td>NA</td>
<td>2.18</td>
</tr>
<tr>
<td>0.25mM</td>
<td>3</td>
<td>26.03</td>
<td>7.73</td>
<td>0.26</td>
</tr>
<tr>
<td>0.5mM</td>
<td>3</td>
<td>25.69</td>
<td>7.39</td>
<td>0.29</td>
</tr>
<tr>
<td>2mM</td>
<td>6</td>
<td>25.48</td>
<td>9.21</td>
<td>0.88</td>
</tr>
<tr>
<td>5mM</td>
<td>3</td>
<td>22.36</td>
<td>8.56</td>
<td>0.07</td>
</tr>
<tr>
<td>10mM</td>
<td>6</td>
<td>26.50</td>
<td>12.48</td>
<td>6.49</td>
</tr>
<tr>
<td>15mM</td>
<td>6</td>
<td>22.68</td>
<td>8.67</td>
<td>1.16</td>
</tr>
<tr>
<td>20mM</td>
<td>3</td>
<td>21.90</td>
<td>7.66</td>
<td>0.39</td>
</tr>
</tbody>
</table>
Cross reactivity: In this trial, various combinations of DNA samples were mixed and amplified with universal eukaryote metabarcoding primers to assess the effect of multiple DNA templates on PCR amplification, both with and without the lobster blocking primer. Lobster DNA alone amplified after an average of 15.00 cycles, similar to prior results. Samples containing a mixture of lobster and prey DNA amplified after an approximately similar amount of time; while the larva of the lab-reared lobster larva fed on *Calanus* and the field-caught larva each amplified later, with average Cq's of 19.92 and 23.21, respectively. Our algal sample amplified the latest with a Cq of 24.47, while the Cq of *Calanus* was roughly similar to that of lobster at 16.19 (Fig. 3.3). Addition of the blocker had no significant effect on *Calanus* or algal samples alone – demonstrating that the lobster-specific blocker should not inhibit prey amplification. However, the reactions with the lobster blocker did delay amplification in samples containing a mixture of lobster and prey,
presumably impacting the amount of lobster DNA amplified. In this trial the blocker at a concentration of 2 mM delayed amplification of lobster DNA alone by 9.97 cycles, again similar to prior results. Lobster mixed with algal DNA and the field-caught lobster larva were delayed by similar amounts, with ▲Cqs of 10.43 and 10.64 respectively. There was a limited effect of the blocker on lobster DNA mixed with *Calanus* DNA with a ▲Cq of 2.98, as well as on the lab reared larva fed on *Calanus* which had a ▲Cq of 1.24 (Table 3.4).

Figure 3.3 Cycle number (Cq) at which different samples amplified with and without the PNA blocker present. Samples included isolated as well as mixtures of predator (lobster) and prey (*Calanus* or *Pseudo-nitzschia*) DNA in order to test the cross-reactivity of the PNA blocker. A lab-reared larva fed *Calanus* as well as a field-caught lobster larval sample were also tested.
Annealing temperature: In general, there were very few differences among blocking primer efficiency at the three annealing temperatures tested. An ANOVA found there was no effect of temperature on Cq without the blocker present ($F_{2,9}=0.081; \ p=0.924, R^2=0.03$), with an average Cq of 14.58. Though with the blocker present, Cq did seem to increase slightly with annealing temperature, however this result also was not significant ($F_{2,9}=3.68; \ p=0.091, R^2=0.55$), and the average Cq was 22.82 cycles (Fig. 3.4). Correspondingly, ▲Cq appeared to increase slightly with increased temperature, but not to a significant degree ($F_{2,9}=1.43; \ p=0.310, R^2=0.32$), with an average ▲Cq of 8.24 (Table 3.5).

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>Mean ▲Cq</th>
<th>Std Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calanus</td>
<td>3</td>
<td>0.34</td>
<td>1.09</td>
</tr>
<tr>
<td>Pseudonitzschia</td>
<td>3</td>
<td>-0.04</td>
<td>0.41</td>
</tr>
<tr>
<td>Lobster</td>
<td>3</td>
<td>9.97</td>
<td>1.68</td>
</tr>
<tr>
<td>Lobster+Calanus</td>
<td>3</td>
<td>2.98</td>
<td>1.18</td>
</tr>
<tr>
<td>Lobster+Pseudonitzschia</td>
<td>3</td>
<td>10.43</td>
<td>0.54</td>
</tr>
<tr>
<td>Lobster fed Calanus</td>
<td>2</td>
<td>1.24</td>
<td>0.48</td>
</tr>
<tr>
<td>Lobster field sample</td>
<td>2</td>
<td>10.64</td>
<td>1.85</td>
</tr>
</tbody>
</table>

Table 3.4 Sample size as well as the mean ▲Cq and standard deviation for each sample tested for cross-reactivity.
3.4.3 Metabarcoding

A total of 925 distinct ASVs were inferred from 1,275,772 reads, split among three SIV postlarval gut samples. Of these, 667 ASVs were attributed to either *Homarus americanus*, or the Norwegian lobster *Nephrops norvegicus*. As the Norwegian lobster is not present in the Gulf of Maine, these ASVs are almost certainly misidentified lobster DNA due to *Nephrops* and *Homarus* being nearly identical at this genetic locus. These ASVs were removed from further consideration as they most likely represent lobster larval DNA that was not fully blocked.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>n</th>
<th>Mean ▲Cq</th>
<th>Std Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>3</td>
<td>7.85</td>
<td>0.71</td>
</tr>
<tr>
<td>75</td>
<td>3</td>
<td>8.29</td>
<td>0.54</td>
</tr>
<tr>
<td>78</td>
<td>3</td>
<td>8.59</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Figure 3.4 Cycle number (Cq) at which lobster DNA amplified using 18s18S V4 metabarcoding primers at 3 different annealing temperatures with and without PNA blocker present. An ANOVA found no significant effect of temperature on Cq value with (F₂,₉=3.68; p=0.091, R²=0.55) or without (F₂,₉=0.081; p=0.924, R²=0.03) the blocker present.
leaving 258 prey ASVs inferred from 219,360 reads. Divided by sample this leaves 48 ASVs and 12,566 reads in sample 1, 161 ASVs and 201,483 reads in sample 2, and 55 ASVs and 5311 reads in sample 3 (Table 3.6). The majority of ASVs were identified all the way to the species level, with the next greatest proportion of ASVs identified to their family (Fig. 3.1). Relative Read Abundance (RRA) was calculated for each ASV in order to determine which ASVs may be significant contributors to the larva’s diet. Most ASV’s had very low RRAs, with only 7 having RRAs greater than 0.05: 3 from sample 1, 2 from sample 2, and 2 from sample 3 (Fig. 3.5).

Table 3.6 Total number of reads and ASVs in each sample and in total after removal of ASVs attributed to lobster.

<table>
<thead>
<tr>
<th></th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASVs</td>
<td>48</td>
<td>161</td>
<td>55</td>
<td>258</td>
</tr>
<tr>
<td>Reads</td>
<td>12,566</td>
<td>201,483</td>
<td>5311</td>
<td>219,360</td>
</tr>
</tbody>
</table>
3.4.4 rtPCR

Testing on a lab-reared larva which had grazed on *Calanus* was successful in proving that the rtPCR assay was capable of amplifying *Calanus* DNA within a larval lobster gut (Fig. 3.6). In testing on field-caught SIV lobster larval samples, 10 out of 48 samples amplified, though this amplification occurred relatively late. Of the 10 samples with *Calanus* present an average of 1.9/3 technical replicates amplified. Amplification also occurred relatively late, with the average successful amplification occurring after 38.54 cycles (Table 3.7). Despite the late amplification it
was found that 20.8% of the larvae had consumed *Calanus*, and demonstrated that the rtPCR primers and probe designed for this study were effective on field-caught larval samples.

Figure 3.6 Amplification graph showing positive detection using the rtPCR analysis on DNA extracted from the gut of a lab-reared larva fed on *Calanus finmarchicus*.

Table 3.7 Metadata for the 10 larval guts which showed presence of *Calanus finmarchicus*. The Cq value represents the cycle number at which a sample amplified beyond a baseline threshold, indicating presence of Calanus.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Average Cq</th>
<th>Lowest Cq</th>
<th>Highest Cq</th>
<th># of amplified replicates</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42.00</td>
<td>42.00</td>
<td>42.00</td>
<td>1</td>
<td>NH</td>
</tr>
<tr>
<td>2</td>
<td>36.88</td>
<td>27.92</td>
<td>41.67</td>
<td>3</td>
<td>NH</td>
</tr>
<tr>
<td>3</td>
<td>34.06</td>
<td>25.54</td>
<td>38.75</td>
<td>3</td>
<td>NH</td>
</tr>
<tr>
<td>4</td>
<td>39.46</td>
<td>35.69</td>
<td>42.66</td>
<td>3</td>
<td>NH</td>
</tr>
<tr>
<td>5</td>
<td>37.21</td>
<td>35.52</td>
<td>38.90</td>
<td>2</td>
<td>NH</td>
</tr>
<tr>
<td>6</td>
<td>39.19</td>
<td>36.03</td>
<td>42.34</td>
<td>2</td>
<td>MC</td>
</tr>
<tr>
<td>7</td>
<td>37.52</td>
<td>37.52</td>
<td>37.52</td>
<td>1</td>
<td>MC</td>
</tr>
<tr>
<td>8</td>
<td>39.49</td>
<td>39.49</td>
<td>39.49</td>
<td>1</td>
<td>MC</td>
</tr>
<tr>
<td>9</td>
<td>39.21</td>
<td>37.34</td>
<td>41.07</td>
<td>2</td>
<td>MC</td>
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<tr>
<td>10</td>
<td>40.40</td>
<td>40.40</td>
<td>40.40</td>
<td>1</td>
<td>MC</td>
</tr>
<tr>
<td>Average</td>
<td>38.54</td>
<td>35.75</td>
<td>40.48</td>
<td>1.9</td>
<td></td>
</tr>
</tbody>
</table>
3.5 Discussion

Each technique evaluated here demonstrated some amount of success in revealing a portion of the lobster larval diet: 1) Microscopy proved most capable of identifying which classes of organisms were present, and required the fewest specialized materials or techniques. However, the lack of more specific identifications illustrates the need for a high degree of taxonomic expertise using this technique. 2) Metabarcoding was successful in providing some of those more specific identifications, however this technique was also the most expensive, and required development and testing of a novel blocking primer as well. 3) Finally, rtPCR successfully targeted a prey species of interest, and demonstrated that there is more predation on *Calanus* by lobster larvae than was apparent from either microscopy or metabarcoding, however it may not be practical for revealing the full range of the lobster larval diet.

3.5.1 Microscopy

In general, microscopy has the lowest bar for entry, with few specialized tools or skills required, and is the most well-established technique discussed here. It also affords the opportunity to record more information on the disposition of gut contents than either of the eDNA techniques discussed. For example: 1) Many marine invertebrates have a variety of larval and adult forms, some of which may be more energy dense, and so provide a better prey item for the predator. With enough expertise, a researcher may be able to determine the larval form present in the gut, while eDNA would only be able to provide a taxonomic identification. 2) Some organisms found in the gut are not prey- a freshly dissected larval gut in this study revealed a nematode which was presumed to be a parasite rather than prey as it was observed to still be living while in the larval gut. 3) Level of digestion of a prey item could be crucial
information revealing how recently it was consumed (Juinio and Cobb, 1992). Finally, 4) volumetric, gravimetric, or abundance counts can help to reveal important information about the relative abundance of prey in the diet (Hyslop, 1980).

In this study, presence/absence by microscopy were recorded, rather than using one of the quantitative methods. Crustaceans tend to tear up their food rather than consuming prey whole, making it very difficult to record the abundance of prey items, especially when you may be unable to determine which appendages belong to which individual prey item (Chow et al., 2011). This creates other issues, namely the difficulty associated with identifying a prey item solely on the basis of the body parts present in the gut (Campos et al., 2020). Approximately 40% of the microscopy data in this study could only be identified to the class level, and no prey were identified to the species level. In this study prey could only be identified as low as the genus level, and of the 20 prey items for which genus level data were recorded, 12 belonged to a single copepod genus, *Centropages*. This is worth noting as *Centropages* is a genus of copepod with a notable diagnostic feature, the last prosome segment has characteristic hook-like protrusions making it easy to identify even when fragmented. This draws attention to a possible bias using the microscopy method; it is difficult to determine whether *Centropages* was identified more often than other genera because of its abundance as a prey species, or the ease with which it is identified. What’s more, using microscopy constrains the researcher to recording only that portion of the diet with identifiable hard parts. If the predator were to consume soft-bodied organisms, such as ctenophores or salps, they would be unlikely to be identified visually (Campos et al., 2020).
3.5.2 Metabarcoding

Metabarcoding with universal eukaryote PCR primers is one way to skirt some of the biases associated with microscopy. Where microscopy may be open to significant bias based upon what the researcher is most capable of identifying, metabarcoding is a technique where, executed properly, any researcher should be able to obtain similar data from the same sample. Additionally, samples are not destroyed after analysis as they typically are during microscopy, allowing for multiple replicates from the same sample to increase confidence. However, time must first be spent perfecting the technique to obtain that data. For a diet study such as this one, that means blocking amplification of the predator DNA (the lobster larva) in order to allow for amplification of the target (prey DNA), but not blocking the amplification of taxa closely related to the predator. This can be singularly difficult, as predator DNA will be significantly more abundant, and prey DNA is likely to be degraded during the digestive process (Vestheim and Jarman, 2008; Su et al., 2018). Additionally, initial microscopy of lobster larvae showed that the most common prey items for lobster larvae were other crustaceans, making the larvae’s prey their close relatives. This adds a degree of difficulty, as a blocking primer needs to both adequately suppress amplification of predator DNA, without also stymieing the amplification of prey DNA. The solution to this problem was to develop a blocking primer using a Peptide Nucleic Acid (PNA).

PNA molecules have a high bonding affinity for DNA, allowing these oligonucleotides to be shorter than typical blocking primers (12-21, rather than 20-33 nucleotides) (Wu et al., 1991; Nielsen and Egholm, 1999; PNAbio, 2022). A shorter sequence can lead to greater specificity as there is less opportunity for an off-target match between the blocking primer and prey.
Additionally, PNA molecules have higher melt temperatures than standard oligonucleotides, allowing these shorter sequences to have higher melt temperatures (Tm) than the metabarcoding primers. This is important as the Tm is the temperature at which an oligonucleotide will most efficiently bind to single stranded DNA. A blocking primer with a higher Tm allows for a PCR with a 2-step annealing step following the high temperature denaturing step, which breaks double stranded DNA into single stranded DNA. A higher Tm for the first annealing step allows the blocking primer to attach to the single stranded predator DNA before the metabarcoding primers begin to anneal, leading to better specificity of the blocking primer and greater suppression of the predator DNA. One challenge with development of the lobster-blocking PNA for this study was trying to find a unique lobster-specific sequence within the relatively short V4 region of the 18S rRNA gene targeted by our DNA metabarcoding approach. Newer ‘long-read’ metabarcoding approaches may alleviate this current challenge, by providing more options for developing alternative PNAs.

Despite these challenges, our blocking primer was capable of significantly suppressing the amplification of lobster DNA while having only a mild effect on potential prey species (Fig. 3.3, Table 3.4). However, no change to concentration or annealing temperature could suppress the amplification of lobster DNA fully (Figs. 3.2, 3.4, Tables 3.3, 3.5).

The recommended annealing temperature for our blocking primer was 75° C. A higher temperature could theoretically allow for greater specificity in annealing of the blocking primer, and a lower temperature may allow for the blocking primer to anneal more efficiently (Wu et al., 1991). In testing, very little difference between suppression and specificity over a reasonable range of annealing temperatures was found (Fig. 3.4, Table 3.5).
While increased concentration of a blocking primer is typically associated with greater blocking efficacy, increased concentration did not have a particularly dramatic effect on the efficacy of the blocking primer tested here (Fig. 3.2, Table 3.3) (Vestheim and Jarman, 2008; Su et al., 2018). This is likely because at higher concentrations (>5 mM) PNA oligonucleotides tend to have difficulty remaining in solution, and so the increased concentration at this point is doing little to influence the PCR (PNAbio, 2022). As a result, 2 mM was chosen as the best blocker concentration, despite the greater average blocking power of the 10 mM concentration. Blocking primers need to be as uniformly reliable as possible; adding the blocking primer at a concentration of 10 mM led to significant variability in efficiency, likely due to differential solubility among samples (Table 3.3). Additionally, though the cross-reactivity of the blocking primer was examined, it is impossible to rule out the possibility that it would inhibit amplification of some prey species without extensive testing. Therefore, it is best to use the lowest effective concentration of blocking primer (Vestheim and Jarman, 2008). As a result, 2 mM as the next most efficient blocking concentration is more preferable to 10 mM.

Using the PNA blocker at a concentration of 2 mM, the blocker was capable of suppressing lobster DNA while prey DNA was present, without unduly affecting the amplification of the prey. Alone, isolated *Calanus finmarchicus* and *Pseudo-nitzschia sp.* DNA was unaffected by addition of the blocker. When mixed with lobster DNA there appears to be some slight effect on *Calanus finmarchicus*, however that is more than likely due to the lobster and *Calanus* DNA competing for primers; rather than an actual impact of the PNA blocker. This explains the slight delay in amplification seen in the lobster+*Calanus* sample (Fig. 3.3, Table 3.4). The lobster+*Pseudo-nitzschia sp.* sample seems to be significantly delayed by the addition of the PNA blocker, however this is also misleading. From the isolated *Pseudo-nitzschia sp.*
sample it can be observed that the 18S V4 primers tend to amplify this algal sample later than others. So in the mixed lobster+*Pseudo-nitzschia sp.* sample earlier amplification in the unblocked trial is due to the lobster DNA amplifying, and the later amplification in the blocked trial is likely a mixture of both lobster and *Pseudo-nitzschia sp.* amplification. Since no effect of the blocker on the pure *Pseudo-nitzschia sp.* sample was detected, it can be deduced that the PNA blocker is not significantly impacting its amplification. The small change in Cq between the blocked and unblocked trial for the lab-reared larva fed on *Calanus* is also promising, as it shows that the consumed *Calanus* is most likely amplifying prior to significant larval amplification. However, the ▲Cq for the field-caught larval sample is similar to that of the pure lobster DNA, which could mean that diet DNA from field specimens may be more dilute or degraded when compared to the lab-reared larva (Fig. 3.3, Table 3.4). Variable concentration or quality of dietary DNA from field-caught larvae could lead to inconsistent blocker efficiency, as low concentrations of poor quality DNA may require longer to amplify.

While ideally the blocking primer would fully suppress predator DNA amplification, it was able to suppress amplification of lobster DNA by an average of 9.21 cycles at the preferred concentration, which translates to around a 600X lower concentration of predator DNA in the final PCR product. Although neither manipulating annealing temperature nor blocking primer concentration could increase that suppression, this also means that the ability of this blocking primer to suppress amplification is robust. Though a greater amount of suppression would be ideal, a moderate amount of suppression could be expected from this blocking primer in a variety of PCR environments, at a variety of concentrations. This quality could make the PNA blocking primer protocol developed here a good example for other labs and studies to use as a jumping off point for their own diet studies, as it demonstrates consistent results.
Despite use of our blocking primer, 72% of ASVs inferred from our three metabarcoding samples were still identified as lobster. Additionally, many of the ASVs identified after removal of those ASVs were identified simply as malacostracan, which may still represent lobster DNA; so the actual amount of lobster ASVs remaining after manual removal is likely being underestimated. Regardless of these difficulties, the number of ASVs identified down to specific taxonomic levels far outstripped the microscopy data. Where the majority of microscopy data were identified at the class level, 51.0% of ASVs were identified down to the species level (Fig. 3.1); demonstrating the capability of metabarcoding to produce gut content data at levels more specific than microscopy. However, most ASVs had very low RRAs (Fig. 3.5). RRA is calculated as $\frac{\text{# of reads for an ASV}}{\text{Total # of reads in a sample}}$ and can be used as a measure of an ASV’s relative importance to a predator’s diet. A low RRA could represent environmental contamination, secondary-consumption, or less common diet items (King et al., 2008; Deagle et al., 2018). While environmental contamination in particular is to be guarded against, rare diet items may still be consequential, so researchers must examine ASV and RRA data critically with respect to the studied predator’s ecology. Samples in this study contained either two or three ASVs with a significantly higher than average RRA (Fig. 3.5). Anecdotally, this number is relatively consistent with the number of unique organisms ID’d per sample during the microscopy study.

3.5.3 rtPCR

Development of a blocking primer is one method for overcoming the imbalance in the ratio of predator to prey DNA concentration. A second method is to instead utilize a species-specific real-time PCR (rtPCR) assay in order to exclusively amplify DNA from a targeted prey item. The *Calanus* rtPCR assay was capable of amplifying a DNA target from larval lobster gut
samples fed a known diet of *C. finmarchicus* (Fig. 3.6), as well as field-caught larval lobster gut samples; providing evidence that lobster larvae in the field feed on lipid rich *Calanus* species (Table 3.7). Furthermore, one of the field samples, which amplified with the *Calanus* rtPCR, was sample 1 from the metabarcoding study. Using the species-specific rtPCR primers, evidence of *Calanus* predation by this larva was found, despite not identifying *Calanus* as present using the universal metabarcoding primers. This demonstrates the importance of utilizing species-specific primers when specific species are of interest.

Unfortunately, a review of *C. finmarchicus* sequences found in GenBank revealed many sequences that are indistinct from the closely related congener *Calanus glacialis*. There are a few potential causes for two distinct species generating indistinct DNA sequences. One possibility is that the two species are simply very similar at the chosen genetic marker, cytochrome oxidase subunit I (COI). Choice of genetic marker can be influential for a number of reasons to metabarcoding and rtPCR assays alike, including differences in, “...taxonomic coverage, taxonomic resolution, and correspondence between morphology and DNA-based identification” (Clarke et al., 2016). It has also been theorized that *C. finmarchicus* and *C. glacialis* may hybridize in their natural environment, which could cause difficulties in distinguishing the two species genetically through promoting gene-flow between the two (Parent et al., 2012). Finally, it has also been noted that the two species are so similar in morphology that genetic testing may be the only reliable way of distinguishing the two species (Choquet et al., 2018). This suggests that *Calanus sp.* samples could have been misidentified prior to sequencing, meaning sequences labeled as *C. finmarchicus* could in fact have been isolated from *C. glacialis* and vice-versa.
Though the *Calanus* rtPCR was designed to amplify *Calanus finmarchicus*, there remains the possibility that *Calanus glacialis* hybrids with a *C. finmarchicus* mother would also amplify given the maternal inheritance of the mitochondrial genes including COI. Importantly, our novel rtPCR assay successfully detected the presence of *Calanus sp.* in field-caught larval lobster guts. Ecologically, the two *Calanus* species that could have been detected by the assay fulfill similar roles and are each significantly lipid rich, making either one a potentially important prey species to larval lobsters. In this case it may even be sensible to use an rtPCR which is species-agnostic for *Calanus*, as the lobster larva likely derives the same benefit from the two. However, researchers looking to produce a more specific rtPCR will need to keep in mind that efficacy of the assay may be dependent on marker choice and target species ecology/morphology.

### 3.6 Conclusion

As eDNA technology becomes more common and papers referencing eDNA surge (Garlapati et al., 2019), there could be a tendency to adopt a “new is always better” attitude. However, what was made clear in this study is that each technique, established microscopy and contemporary eDNA methods alike, had their own strengths and weaknesses. While individual gut dissection and microscopy may be time consuming and biased due to disparate ease in prey identification, this technique is also the cheapest and has the potential to reveal specific information regarding quantity and disposition of prey. This makes microscopy a good candidate for a pilot study prior to or in the beginning stages of a diet study. After microscopy provides a general idea of the predator’s diet, eDNA techniques can be used to augment the analysis. Metabarcoding studies can have significant benefits in providing a relatively unbiased picture of the diversity of prey taxa in the diet. Still, initial costs can be significant, and necessitate time
spent developing a PCR protocol and blocking primer. Additionally, metabarcoding is sensitive to marker choice and completeness of reference libraries, but initial microscopy data could be used to better inform marker choice for targeted prey species. Nonetheless, once a protocol is developed and optimized, metabarcoding can be used to quickly generate large amounts of reproducible diet data not subject to individual researcher biases. Utilizing microscopy and metabarcoding together have the added benefit of allowing the researcher to cross-reference and validate each data set; a diet constituent which appears in both is more likely to represent a true prey item, rather than environmental contamination or secondary-predation. Finally, rtPCR, unlike the other two methodologies discussed, while not adept at revealing the entirety of a predator’s diet, can be particularly valuable in identifying particular species of interest. A species-specific rtPCR assay is the best way to ascertain how frequently that prey taxon is ingested. It is the recommendation of this study, that a combination of both microscopy and eDNA techniques are likely to produce the most accurate picture of a planktonic larval diet.
CHAPTER 4 - REVEALING THE DIET OF LARVAL LOBSTERS BY MICROSCOPY AND DNA SEQUENCING

4.1 Abstract

The diet of larval American lobster (*Homarus americanus*) has been understudied. Now with declining young-of-year recruitment being potentially linked to food-limitation, it is crucial to understand this fundamental trait. This study represents the first diet analysis of larval *Homarus americanus* on three counts: (1) it is the first to incorporate both molecular eDNA techniques (metabarcoding and rtPCR) and conventional microscopy; (2) it is the first larval lobster diet study conducted within the nearshore Gulf of Maine, the only other having been conducted in offshore waters of Browns Bank; and (3) it is also the first to do a spatial comparison of larval diets in two study areas at the same time. The diet of SI larvae proved difficult to resolve with microscopy, although copepods appeared to be a common prey item. Copepods were also common prey items for SIV, with the addition of malacostracans, in particular decapod larvae. Differences in diet between two study areas approximately 125 km apart, Midcoast ME (MC) and coastal New Hampshire (NH), were slight, although larvae from NH were found to have guts significantly less full than larvae from MC at both SI and SIV. A taxon-specific real-time Polymerase Chain Reaction (rtPCR) assay additionally uncovered significant predation by SIV postlarvae on the lipid-rich foundational copepod *Calanus finmarchicus*. Although the sample size for our metabarcoding assay was limited, the detection of additional prey taxa within just a few samples suggests metabarcoding could prove a powerful tool in future diet studies. Results from this study open a window on the trophic interactions of
lobster larvae with their pelagic environment, while also demonstrating the effectiveness of mixed-methodologies in larval diet studies.

4.2 Introduction

Food-limitation is a significant source of mortality to pelagic larvae of marine invertebrates, particularly crustacean larvae, and fishes, affecting subsequent recruitment and year-class strength (Hjort, 1914; Cushing, 1969; 1990; Olson and Olson, 1989). A first step in testing the food-limitation hypothesis in pelagic larvae is to gain an understanding of their natural diet. Yet for many species this represents a crucial knowledge gap. This is certainly true of the American lobster (*Homarus americanus*), which produces large-bodied raptorial larvae that theory predicts would be significantly at risk for food-limitation, as large-bodied crustacean predators show more evidence for reliance on an ample food supply (Olson and Olson, 1989).

The American lobster is culturally and economically important in the Northwest Atlantic. The collapse of the fishery in southern New England and its meteoric rise as the population shifted to more northern waters in the Gulf of Maine (GoM; Le Bris et al., 2018), has motivated increased research into the factors affecting the distribution and abundance of this iconic species. In particular, while much attention has been given to the role of egg production and larval advection as a determinant of subsequent recruitment (e.g., Xue et al. 2008, Incze et al. 2010), a number of authors have recognized lobster larval trophic dynamics as a potentially important factor influencing recruitment (Harding et al., 1983; Juinio and Cobb, 1992; Carloni et al., 2018), as well as one of the least well understood segments of lobster life history.

The American lobster has three planktonic larval instars (SI-III) and a final postlarval stage (SIV) that is initially pelagic but settles to the seabed before the end of the instar. While the
earliest pelagic stage (SI) has some maternal reserves, they do not progress to the next stage of development without feeding and obtaining exogenous energy (Abrunhosa and Kittaka, 1997). Analysis of a three-decade larval time series in the Gulf of Maine by Carloni et al. (2018) has revealed that while egg production and SI larval abundance remains high, postlarval SIV abundance has substantially declined. In turn, Carloni et al (2018) found young-of-year (YoY) recruitment has also declined over a large area of the Gulf of Maine, as measured by the American Lobster Settlement Index (ALSI). These observations are not only consistent with the widely observed phenomenon that benthic populations are sensitive to fluctuations in larval survival, (Thorsson, 1949; Vance, 1973), but the study also revealed a noteworthy disconnect in the normal stock-recruit relationship (Carloni et al., 2018). Their analysis of potential correlates of variability in postlarval supply, including predator and prey abundance, and offshore transport revealed postlarval and YoY lobster declines to be most significantly correlated with changes in the abundance of a key GoM prey species *Calanus finmarchicus*, an energy-rich, pelagic marine copepod. This analysis suggested that larval lobsters are food-limited prior to reaching the postlarval stage due to the low abundance of important food sources. To investigate the food limitation hypothesis, a logical next step was to examine the natural diet of larval lobsters. While larval diet studies of the American lobster have been conducted within the Gulf of St. Lawrence (Varma, 1979) and southern New England (Williams, 1907; Herrick, 1911; Juinio and Cobb, 1992), no data are available on larval lobster diets within the coastal Gulf of Maine. Additionally, as climate change alters species assemblages, updated datasets are necessary to maintain a current knowledge base.

Past dietary studies on larval lobster focused on the inspection of gut content by microscopy. This technique requires finding readily identifiable features of specific prey items
within the undigested material (Varma, 1979; Harding et al. 1983; Juinio and Cobb, 1992). While this technique can provide quantitative and qualitative assessment of the diet, visual identification of gut contents is dependent on the taxonomic expertise of the researcher.

This study sought to document the diet of lobster larvae collected in the Gulf of Maine both by traditional microscopy and through genetic techniques. Developing molecular genetic techniques offer a complementary method for diet studies that are not reliant on visual identification. Two genetic techniques were employed and reviewed here: Metabarcoding, and real-time PCR (rtPCR). Metabarcoding is an eDNA technique in which genetic material from a broad range of taxa are all amplified and subsequently sequenced from a single sample. This technique can be used to discover diet constituents within a larval gut without the need for visually identifiable components. While rtPCR (commonly referred to as qPCR when utilized as a quantitative technique) is a targeted eDNA assay used to determine the presence of a single taxon within a sample. A complementary lab-based experiment was also conducted in which the survival and time to first molt were recorded for SI larvae fed different diets in order to determine the impact these dietary items may have on larval development.

Given the strong correlation between postlarval abundance and *Calanus finmarchicus* observed by Carloni et al. (2018), a specific objective was to determine whether *C. finmarchicus* was an important diet constituent. Therefore, in addition to the broader microscopy and metabarcoding methods, a *Calanus finmarchicus*-specific real-time Polymerase Chain Reaction (rtPCR) assay developed for this project was also employed. Each technique has its own strengths and weaknesses. Using a mixture of methodologies was intended to help cover data
gaps and compensate for the biases associated with each technique, and in doing so produce a more complete and nuanced view of the larval lobster diet.

4.3 Methods

4.3.1 Collection

Larvae were collected from sampling stations in New Hampshire and mid-coast Maine between 2019 and 2022 roughly 125 km apart (Fig. 4.1). Normandeau Associates, Inc., provided larvae caught in neuston nets during annual ecological surveys in New Hampshire (NH). Larvae were preserved in 95% ethanol and refrigerated. Additional larvae were caught in Midcoast Maine (MC) at four discrete depths using a rectangular (1 m X 2 m – 500 um mesh) neuston net at the surface and a round closing net (1 m – 1000 um mesh) towed at 10, 20, and 30 m depth. Both nets were equipped with flow meters. Samples were sorted on deck and larvae were immediately frozen at -80 °C. The remainder of the net tow was preserved in 10% ethanol and refrigerated for taxonomic identification in the laboratory. Additional larvae were provided by Maine Department of Marine Resources (DMR), which were caught during their summer lobster larval tows.
Figure 4.1 Map of the sampling area. Insets show detailed views of each region with 10 m isobaths, blue markers are sampling sites.
4.3.2 Gut dissection

The intact foreguts (cardiac and pyloric stomachs) of SIV postlarval lobsters were removed and placed on a 1mm gridded slide. The fullness of the gut (≥0, 25, 50, 75, ≤100 percent) was estimated visually using a dissecting microscope (Olympus SZ40). The gut was then dissected with forceps and the contents rinsed into droplets of Lugol’s solution and placed on the slide for identification. SI larval gut dissections were performed similarly, however due to the more delicate nature of the gut in this early stage it was impossible to remove the gut intact. Instead, visible pieces of the digestive tract were pulled from the thoracic cavity and placed in Lugol’s solution where they were macerated using forceps to release their contents. Gut contents were then scored on a presence-absence basis, down to the lowest taxonomic level distinguishable, as well as the appropriate higher taxonomic levels; e.g. presence of a copepod in the genus *Acartia* would lead *Acartiidae* (family), *Calanoida* (order), *Copepoda* (class), and *Arthropoda* (phylum) to also be marked present in that sample.

4.3.3 Metabarcoding

Metabarcoding is an eDNA ‘next-gen’ sequencing technique which can be used to broadly describe the genetic diversity within a sample. DNA was extracted from 3 SIV larval guts using the Invitrogen ChargeSwitch Forensic DNA extraction kit (ThermoFisher Scientific). The DNA concentration was quantified using the Qubit high sensitivity DNA concentration assay, and then amplified using 18S V4 eukaryote primers (Comeau et al., 2011). To prevent the larger quantity of the lobster DNA in samples from overwhelming the much rarer prey DNA, a lobster blocking primer targeting the V4 amplification region was developed (Ascher et al., CH3). A pre-amplification step was run using the lobster blocker prior to sequencing reactions.
The reaction mixture for the pre-amplification included 1.25 µL of the forward and reverse primers, 2.5 µL of the blocking primer (a concentration of 2 µM), 12.5 µL of IDT Prime Mastermix, 2 µL of sample DNA, and 5.5 µL of water to bring the total reaction volume to 25 µL. The PCR consisted of a 2-step annealing protocol: an initial denaturing step at 98 °C for 30 seconds, followed by 40 cycles of denaturation at 98 °C for 10 seconds, a lobster blocking primer anneal step at 75 °C for 30 seconds, a metabarcoding primer anneal step at 55 °C for 30 seconds, and an elongation step at 72 °C for 30 seconds. After pre-amplification with the lobster blocking primer, DNA products were purified and concentrated using the DNA Clean and Concentrator-5 kit (Zymo Research), and the final DNA concentration was measured using the Qubit fluorometer. The purified DNA products were sent to the Integrated Microbiome Resource (IMR) facility at Dalhousie University (Halifax, Nova Scotia) for Illumina MiSeq sequencing (Comeau et al. 2017). Although MiSeq sequencing is highly accurate, the PCR process can introduce errors which may be misinterpreted as biological variation, requiring quality control (QC) of the sequences through a filtering and trimming process on the raw reads followed by clustering of sequences by similarity. To accomplish this the DADA2 bioinformatics pipeline was utilized, as it has been shown to accurately represent true biological variation through its unique method of inferring Amplicon Sequence Variants (ASVs). An ASV represents a unique sequence of nucleotides, which may be shared by multiple reads within a sample, and has been shown to better represent biological variation within a sample than other common methods, while also controlling error rates (Callahan et al., 2016; 2017). Inferred ASVs were classified using the SILVA reference database of 18S rRNA gene sequences (Quast et al., 2013). Different ASVs can still correspond to the same taxon, due to genetic variation within a community or individual and the extent to which this diversity is represented in the reference database. Taxa
present within a sample can then be sorted by the number of ASVs and the sequences with which they are associated. A prey taxon with a large number of associated ASVs and/or sequence reads was likely abundant within a particular gut sample, and so represents a more substantial portion of that larva’s diet.

4.3.4 Real-time PCR assays (rtPCR)

Unlike metabarcoding, rtPCR is a genetic technique that can be used to amplify genetic material belonging to a single taxon of interest in real time without the need for sequencing to determine presence or absence. There was specific interest in whether the copepod, *Calanus finmarchicus*, could be detected in the larval lobster gut contents. A *Calanus finmarchicus* specific rtPCR assay was developed and tested first *in silico*, then in the lab for specificity. Unfortunately, due to potential hybridization in the field with a closely related congener, *Calanus glacialis*, some sequences attributed *C. glacialis* in GenBank were indistinguishable from *C. finmarchicus* at our chosen locus. This means that our assay may also amplify samples containing *Calanus glacialis x finmarchicus* hybrids as well as our target *C. finmarchicus*. However, functionally these copepods are both large-bodied lipid-rich prey items, and so could serve the same dietary role for lobster larvae. Additionally, *C. glacialis* is much more polar in its distribution, and very rare within the Gulf of Maine. Once its efficacy was determined, the assay was used to determine the presence of *Calanus* within the guts of field-caught lobster larvae.

Each reaction contained 1.25 µL of the forward and reverse primers, 0.625 µL of the probe, 12.5 µL of IDT Prime Mastermix, 2 µL of sample DNA, and 7.375 µL of water to bring the total volume of the reaction mixture to 25 µL. The protocol for the rtPCR was an initial 3 min long denaturing step at 95° C, followed by 45 cycles of a 95° C denaturing step for 15 seconds and a
60° C dual annealing/extension step for 30 seconds. Amplification of *Calanus* DNA causes the sample to fluoresce, as a sample continues to amplify, the Relative Fluorescence Units (RFUs) of the sample increase. A total of 48 SIV larval guts were tested for the presence of *C. finmarchicus* using this primer and probe-based rtPCR assay. Each sample was tested in triplicate, and an RFU above the baseline threshold was taken as proof of presence of the taxon of interest. The baseline threshold is automatically calculated through BioRad CFX manager 3.1 software, and the cycle number at which a sample’s RFUs exceed the threshold is called the Cq value. Though this assay was not intended to be quantitative, a higher Cq (e.g. 35-40) typically indicates a lower concentration of the target.

4.3.5 *Effect of diet on larval development and survival*

SI lobster larvae were reared in individual glass jars maintained at ambient temperature and gently aerated. Larvae were reared on one of four potential dietary regimes: crustacean (*Artemia salina*), algae (*Tetraselmis* sp.), mixed (½ crustacean, ½ algae), and starved (control). Each treatment consisted of 20 larvae, other than the algae treatment which included 30 larvae. The proportion of larvae still surviving for each treatment was recorded over the course of 9 days and a survival analysis was performed using JMP V.16 statistical software. For larvae which successfully molted to SII, the time to molt was also recorded. A chi-square analysis was used to test whether molt probability varied by treatment, and an ANOVA was used to test whether treatment had an effect on time to molt.
4.4 Results

4.4.1 Microscopy

In total, 170 larval and postlarval guts were dissected: 54 SI, 1 SII, 3 SIII and 112 SIV. Due to the low sample size of the intermediate stages (SII and III), our results and subsequent discussion will focus on the SI and IV samples. The SI and SIV larval samples can be further broken down based on region: 13 SI guts from MC, 41 from NH; 38 SIV guts from MC, 74 from NH.

Larvae from MC were found to be ~35% more full than those from NH, and that SIV postlarvae were 91% more full than SI larvae, with no interaction between location and larval stage effects (2-Factor ANOVA: Location effect: $F_{162,1}=17.62$, $p<0.001$; Stage effect: $F_{162,1}=70.70$, $p<0.001$; Interaction $F_{162,1}=0.05$, $p=0.832$). Empty guts were rare in either stage or region. The greatest proportion of SIV guts were found to be full (65.4%), while the greatest proportion of SI guts were one-quarter full (63.0%). Additionally, SIV postlarvae from MC never had guts less than 50% full, regardless of stage, while one postlarva from NH even had a completely empty gut (Fig.4.2).
Figure 4.2 Percent of SI (A) and SIV (B) guts categorized into each gut fullness category divided by region.
Noteworthy differences were observed in the diets of SI larvae and SIV postlarvae. SIV guts contained a greater overall diversity of prey at each taxonomic level with 7 phyla, 9 classes, 5 orders, 5 families, and 5 genera identified; compared to SI guts, which contained 1 phylum, 3 classes, 2 orders, 1 family, and 1 genus (Fig. 4.3). In SIV guts the greatest number of identifications were recorded at the class level (n=149), compared to SI guts where the greatest number of identifications were recorded at the phylum level (n=50). No species level identifications were made by microscopy, as gut content material was too degraded for fine-scale identifications. The vast majority of prey items identified for both stages were arthropods, particularly crustaceans; although a small number of larval guts also contained insect parts. The next most commonly identified prey phylum was annelida, due to the occasional ingestion of polychaete larvae. The most abundant crustacean prey in SIV guts were malacostracans and copepods, whereas the vast majority in SI guts were copepods, with just a few guts containing malacostracan material.
Figure 4.3 The proportion of SI (A) and SIV (B) larval guts in which individual prey items were present- separated by region and recorded at 5 different taxonomic levels. Phylogenetic levels are separated by colored overlays, the y-axis represents the proportion of larval guts containing the specified prey.
Also noteworthy is the similarity of the larval diets among larvae collected at stations some 125 km apart (Fig. 4.1). Some slight regional differences were detected in the larval diet. In SIV guts the classes gymnolaemata, hydrozoa, and foraminifera (bryozoa, cnidaria, and retaria respectively, at the level of phyla) were detected in larvae from NH, but not MC, potentially indicating a greater diversity in diet for NH larvae. A few SI guts from MC contained decapod material, where NH SIs did not, and insect material was identified in an NH SI, but not MC. There was a notably greater proportion of larvae from MC with guts containing remnants of the calanoid copepod *Centropages* sp. This additionally is reflected in the greater proportion of MC guts containing calanoid copepods at the level of class. Finally, only one *Calanus* was ever positively identified through microscopy; found within the gut of a SIV from MC.

### 4.4.2 Metabarcoding

From the DNA extracted from the three postlarval lobster guts a total of 1,275,772 reads were recovered and 925 ASVs inferred. However, despite the use of our lobster blocking primer (Ascher et al., in prep.), some ASVs were still identified as belonging to lobster. After filtering these out 219,360 reads were left, and 258 ASVs. Table 4.1 displays how these reads and ASVs were distributed among the three samples. The most common prey taxa identified in each sample are detailed in Table 4.2. Common prey taxa are defined as those with at least one associated ASV that has over 100 reads. This helps to narrow down the dataset into prey taxa which are present in appreciable amounts (Table 4.2). The Relative Read Abundance (RRA), the number of reads associated with a prey taxon divided by the total number of reads in the sample, can give a further estimate of each prey taxa’s relative importance within an individual larval gut sample (Table 4.2). Significant prey items identified through metabarcoding included *Islandinium*
tricingulatum (dinoflagellate), Carcinus maenas (Green crab), the neustonic copepod Anomalocera patersoni, and Bivalvia. Using NCBI’s Basic Local Alignment Search Tool (BLAST), ASVs identified as Bivalvia were found to most likely be Placopecten magellanicus (Atlantic Sea Scallop). Other common prey taxa tended to have low RRAs, which may indicate they represent environmental contamination, secondary consumption, or less significant/rare portions of the larval diet (Table 4.2).

Table 4.1 Distribution of reads, ASVs, and the ratio of the two among samples after filtering out those belonging to lobster.

<table>
<thead>
<tr>
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<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
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<td>5311</td>
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</table>

Table 4.2 Most common taxa present in postlarval gut samples identified through metabarcoding. Taxa names are displayed with the finest level of taxonomic resolution the SILVA database was capable of assigning.

*A BLAST of the associated ASVs for these taxa reveal that the dinoflagellate is most likely Karlodinium veneficum, while the Bivalve is most likely Placopecten magellanicus, the Atlantic Sea Scallop.

<table>
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<tr>
<th>Taxa</th>
<th># reads for top ASV</th>
<th># ASVs associated with taxa</th>
<th>Total reads</th>
<th>RRA</th>
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<td>0.013</td>
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<tr>
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<table>
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<td>Biechealeria sp.</td>
<td>2166</td>
<td>1</td>
<td>2166</td>
<td>0.011</td>
</tr>
<tr>
<td>Scrippsiella acuminata</td>
<td>322</td>
<td>1</td>
<td>322</td>
<td>0.002</td>
</tr>
<tr>
<td>Dinoflagellate*</td>
<td>182</td>
<td>4</td>
<td>314</td>
<td>0.002</td>
</tr>
<tr>
<td>Dasysiphonia japonica</td>
<td>128</td>
<td>2</td>
<td>161</td>
<td>0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Taxa</th>
<th># reads for top ASV</th>
<th># ASVs associated with taxa</th>
<th>Total reads</th>
<th>RRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bivalvia*</td>
<td>2200</td>
<td>12</td>
<td>3106</td>
<td>0.578</td>
</tr>
<tr>
<td>Anomalocera patersoni</td>
<td>701</td>
<td>6</td>
<td>877</td>
<td>0.163</td>
</tr>
</tbody>
</table>
4.4.3 Real time PCR assays (rtPCR)

Of the 48 larval guts, 11 were from NH and 37 from MC. Of these samples, 20.8% (n=10) revealed the presence of *C. finmarchicus*, including 5 larvae from NH and 5 from MC. This contrasts with <1% reported from the microscopy analysis and zero from metabarcoding. Table 4.3 displays the average, max, and min Quantification Cycle (Cq) value for each sample, as well as the number of replicates amplifying for each sample and its region of origin. Each replicate represents a subsample of DNA isolated from the same larval gut sample. Out of the 10 samples which amplified, typically only one or two technical replicates (of three total) showed amplification, rather than all three (Table 4.3), which is characteristic of low-abundance gene targets analyzed in this manner. The highest Cq of any replicate was 42.00, while the lowest was 25.54 (representing a difference in gene copies spanning 5 orders of magnitude). The average Cq for each sample is also reported, however this measurement can be biased as it is impossible to quantify a non-amplification. This results in the average Cq of samples with replicates which did not amplify appearing more favorable (lower). Each trial included both a positive control and a negative “No Template Control” (NTC) which can be seen in the example amplification graph (Fig. 4.4). As expected, the NTC did not amplify in any trial, while the positive control rendered the most robust response.
Table 4.3 Metadata for the 10 larval guts which showed presence of *Calanus finmarchicus* in MC and RI. The Cq value represents the cycle number at which a sample amplified beyond a baseline threshold, indicating presence of *Calanus*.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Average Cq</th>
<th>Lowest Cq</th>
<th>Highest Cq</th>
<th># of amplified replicates</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42.00</td>
<td>42.00</td>
<td>42.00</td>
<td>1</td>
<td>NH</td>
</tr>
<tr>
<td>2</td>
<td>36.88</td>
<td>27.92</td>
<td>41.67</td>
<td>3</td>
<td>NH</td>
</tr>
<tr>
<td>3</td>
<td>34.06</td>
<td>25.54</td>
<td>38.75</td>
<td>3</td>
<td>NH</td>
</tr>
<tr>
<td>4</td>
<td>39.46</td>
<td>35.69</td>
<td>42.66</td>
<td>3</td>
<td>NH</td>
</tr>
<tr>
<td>5</td>
<td>37.21</td>
<td>35.52</td>
<td>38.90</td>
<td>2</td>
<td>NH</td>
</tr>
<tr>
<td>6</td>
<td>39.19</td>
<td>36.03</td>
<td>42.34</td>
<td>2</td>
<td>MC</td>
</tr>
<tr>
<td>7</td>
<td>37.52</td>
<td>37.52</td>
<td>37.52</td>
<td>1</td>
<td>MC</td>
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<td>39.49</td>
<td>39.49</td>
<td>39.49</td>
<td>1</td>
<td>MC</td>
</tr>
<tr>
<td>9</td>
<td>39.21</td>
<td>37.34</td>
<td>41.07</td>
<td>2</td>
<td>MC</td>
</tr>
<tr>
<td>10</td>
<td>40.40</td>
<td>40.40</td>
<td>40.40</td>
<td>1</td>
<td>MC</td>
</tr>
<tr>
<td>Average</td>
<td>38.54</td>
<td>35.75</td>
<td>40.48</td>
<td>1.9</td>
<td></td>
</tr>
</tbody>
</table>
Subdivided by region, 13.5% of MC (5/37) and 45.5% (5/11) of NH larvae contained *Calanus*. Though zooplankton data corresponding to the spatial and temporal domain where the NH larval samples were collected could not be obtained, these data do exist for the MC larvae as vertical zooplankton tows were conducted alongside larval sampling. Analysis of these zooplankton samples reveals that the average percent composition of *Calanus* as a proportion of the total zooplankton regime never rose far above 1.5%, and was most commonly between 0 – 1.0% over 3 years of larval sampling (Fig. 4.5). This contrasts with the greater percentage of MC larvae containing *Calanus*.
SIV larvae found to contain this prey item. The average abundance of *C. finmarchicus* as a proportion of the total zooplankton regime over the entirety of our larval sampling period was found to be 0.005, whereas the proportion of MC postlarvae containing *C. finmarchicus* in their gut was 0.135. These proportions can be used to calculate selectivity for this prey item using Ivlev’s electivity index: \( E = \frac{r_i - p_i}{r_i + p_i} \) where \( r_i \) is the relative abundance of the prey item within the predators gut, and \( p_i \) is the relative abundance of the prey item in the field. Electivity varies between -1 and +1, where 0 shows random consumption, values close to -1 reflect a prey item which is inaccessible or avoided, and values close to +1 reflect a prey item which is preferentially consumed (Ivlev, 1961). So for predation of *C. finmarchicus* by lobster postlarvae:

\[
\frac{0.135 - 0.005}{0.135 + 0.005} = 0.93.
\]

As this value falls near +1 it can be inferred that postlarvae show high preference for this prey item.
4.4.4 Effect of diet on larval development and survival

Only lobster larvae fed the crustacean or mixed (crustacean and algae) diet successfully molted from SI to SII. Larvae fed on the crustacean only diet also showed greater survival than any of the three other dietary treatments by the log-rank test ($X^2_{3, 90}=12.87; p=0.005$) (Fig. 4.6). Additionally, larvae fed a crustacean only diet had a significantly lower time to molt ($F_{1, 25}=13.78; p=0.001$) and a significantly greater proportion of successful molts ($X^2_{1, 39}=8.15; p=0.004$) when compared to the mixed diet (Fig. 4.7)
Figure 4.6 Proportion of larvae surviving over the course of 9 days when reared on one of four different dietary treatments
Figure 4.7 A) Mean number of days to first molt for larvae reared on two different dietary regimes, error bars show standard error. B) Mosaic plot showing the percent of larvae which failed or succeeded to molt for two different dietary regimes.
4.5 Discussion

This lobster larval diet analysis represents the first to encompass two separate regions: MC and NH, and has one of the highest sample sizes of both SI and SIV larvae (Table 4.4). This is also the first diet study of larval American lobster to incorporate a significant amount of both molecular and visual microscopy data.

Table 4.4 A brief review of the literature available on natural American lobster larval diets, including the current study.

<table>
<thead>
<tr>
<th>Study</th>
<th>Year published</th>
<th>Stages included</th>
<th>Sample size</th>
<th>Region(s)</th>
<th>Gut fullness</th>
<th>Top 2 diet constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascher et al</td>
<td>2023</td>
<td>I, II, III, IV</td>
<td>54, 1, 3, 112</td>
<td>MC, NH</td>
<td>Y</td>
<td>Copepoda, Malacostraca</td>
</tr>
<tr>
<td>Juiño and Cobb</td>
<td>1992</td>
<td>IV</td>
<td>802</td>
<td>RI</td>
<td>Y</td>
<td>Copepoda, Malacostraca</td>
</tr>
<tr>
<td>Varma</td>
<td>1979</td>
<td>I, II, III</td>
<td>60, 12, 3</td>
<td>New Brunswick</td>
<td>Y</td>
<td>Copepoda</td>
</tr>
<tr>
<td>Herrick</td>
<td>1911</td>
<td>IV</td>
<td>2</td>
<td>MA</td>
<td>N</td>
<td>Crustacea</td>
</tr>
<tr>
<td>Williams*</td>
<td>1907</td>
<td>I, II, III, IV</td>
<td>25, 25, 25, 25</td>
<td>RI</td>
<td>N</td>
<td>Copepoda</td>
</tr>
</tbody>
</table>

*Larvae were lab-reared and suspended off a dock in a bait bag supplied with clam meat.

Larval American lobsters become increasingly capable predators as they develop, particularly as postlarvae. In the present analysis, most larvae, regardless of stage or region, had guts that contained at least some prey, and often were estimated to be completely full (Fig. 4.2). The significant fullness of SIV guts suggests that this final pelagic stage is least likely among the four stages to be food-limited. SI larvae had significantly less full guts (Fig. 4.2) and may be more likely to be food limited, though a faster relative gut clearance rate could also account for the less full SI stomachs. SI and SIV from NH also had significantly less full guts when compared to larvae of the same stage from MC, and may provide evidence of the geographic variability in the pelagic food supply as well as vulnerability to food limitation.

Compared to SI larvae, SIV had relatively broad diets, feeding on prey such as copepods, decapod larvae, amphipods, mysids, polychaetes, and even insects. SII and SII larvae were also dissected for this study, but low sample sizes precludes an in-depth analysis of the diets of these intermediate stages. Anecdotally though, the SII larva was similar to SI in diet and gut fullness,
and SIII larvae were similar to SIV. Of the 48 postlarval guts tested with the *Calanus* rtPCR assay, 20.8% showed evidence of *C. finmarchicus* predation despite the relatively low abundance of this copepod in plankton samples during the larval period, suggesting a potential preference for *Calanus* (Fig. 4.5). Feeding on crustacean prey in general may satisfy larval lobster nutritional requirements. Crab larvae and lobster larvae share a need for Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), essential fatty acids which must be acquired through the diet (Arts et al., 2001; Kaur et al., 2014). Copepods, and *C. finmarchicus* in particular, are also highly enriched in EPA and DHA (Scott et al., 2002).

Observations of larvae reared on different dietary regimes in the lab reinforced this assertion that crustaceans are vital prey components for lobster larvae in the field. Larvae fed a diet of crustacean prey (*Artemia salina*) showed significantly better survival and molt success than larvae fed diets with a lower proportion of crustacean prey, or absent of crustacean prey altogether (Figs 4.6+4.7).

4.5.1 Microscopy

Traditional microscopy provides a valuable tool for identifying broad taxonomic groups within the diet of lobster larvae. One diagnostic feature found to be useful for identifying copepods down to more specific taxonomic level was the mandibular gnathobase (Fig. 4.8). This allowed for a handful of genus-level identifications even in SI lobster larvae.
Prey items in SI guts were difficult to identify due to two main issues. Firstly, the larval gut at this stage is not well developed and its removal is more complicated (Factor et al., 1981). Secondly, SI larvae are less than half the size of postlarvae, and so the prey fragments found in SI guts were typically much smaller than those in SIV.

The diet of NH and MC larvae were generally similar, though NH SIVs contained a higher diversity of prey at the phylum and class level, and MC SIVs contained a greater diversity of prey at the genus level (Fig. 4.3). However, these unique diet constituents were present only in very small amounts; so dietary differences may have more to do with sample size or detection biases rather than any significant difference in diet composition. Also, as these additional prey were rare within larval gut samples they are unlikely to be significant parts of the diet. The SI

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**Figure 4.8** Comparison of the mandibular gnathobases of 4 different copepods found within larval guts to anatomical drawings of the same, demonstrating the efficacy of this feature as a tool for copepod identification. Mandibular gnathobase illustrations taken from: *Acartia* (Schnack, 1989), *Centropages+Calanus* (Anraku and Omori, 1963), *Metridia* (Bradford Grieve, 1999).

Prey items in SI guts were difficult to identify due to two main issues. Firstly, the larval gut at this stage is not well developed and its removal is more complicated (Factor et al., 1981). Secondly, SI larvae are less than half the size of postlarvae, and so the prey fragments found in SI guts were typically much smaller than those in SIV.
larval diet from NH and MC also appears similar, although there is less to compare due to the previously noted difficulty in prey identification in these samples. All in all, the relative similarity in diets between NH and MC larvae points to either a similarity in the available prey items, or a more generalist approach to feeding for lobster larvae.

Other recent studies of the natural diet of larval lobsters are few and far between (Table 4.4). Most recently Juinio and Cobb (1992), focused on the diet of postlarvae in Rhode Island. Varma (1979) by contrast, focused on SI larvae in the Northumberland Strait in the southern Gulf of St. Lawrence. As in the present study, Juinio and Cobb recorded malacostracans and copepods to be most common diet constituents; malacostracans (primarily decapod larvae) were found in 62.3% of guts and copepods in 53.9% of guts, compared to 55.4% and 50.0% respectively in our study. However, the next most abundant prey items in their study were insect parts and fish eggs; insect parts were rarely encountered, and never identified any fish eggs. A total of five different malacostracan genera were identified by Juinio and Cobb, including Cancer spp; while Cancer spp. was the only decapod genus identified in this study, though it is likely other genera were present and remained unidentified due to difficulties with specific identification. Juinio and Cobb also identified 11 different genera of copepod, compared to only four in this study, although again that could be due to the lower amount of genus level identifications, in general. Of the four copepod genera identified in our study, three, Calanus, Acartia, Centropages, were also identified by Juinio and Cobb, while the last, Metridia, was not. Finally, Juinio and Cobb noted similar trends in gut fullness as those recorded here; namely that postlarval guts were rarely empty, and tended to be 50% full or more. One notable difference though is that they found gut contents to frequently contain more intact prey items, such as whole or nearly whole copepods, whereas this study encountered whole prey items extremely
rarely. Whether this is due to differences in sampling times (sampling during a period of
digestion, rather than ingestion) or preservation is unclear, but could be the reason Juinio and
Cobb were able to record the postlarval diet at a greater resolution. Alternatively, larvae from
Juinio and Cobb’s study may have engaged in feeding activity more frequently, leading to a
greater incidence of newly ingested prey.

Varma et al. (1979) also found it difficult to identify individual prey components in SI
larvae, noting that many of the guts contained largely amorphous, inorganic, or otherwise
unidentifiable material (1979). The material that was identified belonged to algal cells, or were
identified as fragments of copepods. The gut fullness of larvae from Varma’s study also followed
trends similar to those in this study, with the majority of SI larvae having guts that were less than
half- to one quarter full. Interestingly, Varma concluded in their survey of the SI diet that the low
degree of gut fullness may indicate starvation or food limitation at this stage, while Juinio and
Cobb reported just the opposite for postlarvae; conclusions which reflect the comments made
earlier in the discussion of our own results.

4.5.2 Metabarcoding

Metabarcoding is a promising technique in larval diet studies. The greatest difficulty in
larval diet studies is identification of the minute portions of prey species left in the gut. Much of
the material in lobster larval guts is found to be unrecognizable or amorphous (Varma et al.,
1979; Juinio and Cobb, 1992). Metabarcoding could potentially allow us to identify even
visually unrecognizable tissue. However, there are difficulties associated with metabarcoding as
well. Primer bias occurs when the primers in use preferentially amplify one taxon over another.
Species masking may occur when already abundant genetic material is amplified exponentially,
causing less abundant prey to go unnoticed (Skelton et al., 2022). This is an especially difficult issue to tackle in a diet study, where the predator is closely related to its prey. As lobster larvae consume a large amount of other crustaceans, it is unlikely that a universal priming region for the larva’s prey could be found, which would not also amplify the larva’s much more abundant DNA. The solution to this problem is to use a blocking primer designed to limit the amplification of lobster DNA in order to mitigate the effects of species masking. While our lobster blocking primer was successful in decreasing the abundance of lobster DNA in the final product, results from metabarcoding of the three larval guts sequenced clearly show that species masking was still occurring. Less than 30% of the ASVs identified belonged to prey taxa, rather than lobster. Metabarcoding must therefore be treated as a developing, but still promising technique for the identification of lobster larval prey. As noted in chapter 3, choices for the design of the lobster blocking primer were constrained by our selection of the 18S V4 rRNA region for metabarcoding. This genetic region was selected because reference databases for the 18S rRNA gene include the broadest taxonomic coverage available and are among the most highly curated, however the amplified V4 region is relatively short (< 500 nucleotides) which limits primer design. Despite these difficulties, a number of common prey items in our three samples were able to be identified. Typically, a greater number of ASVs or reads associated with a taxon is indicative of its elevated presence within the sample. However, precisely distinguishing the relative abundance/importance of a prey item in such a way is challenging due to a number of potential biases (Deagle et al., 2018). Relative Read Abundance (RRA), the proportion of reads in a sample attributed to a particular taxon is one way to help minimize these biases and help distinguish which common taxa may represent important prey items in a sample, along with the number of ASVs and reads (Deagle et al., 2018).
The most abundant prey item within sample 1 was a dinoflagellate identified as *Islandinium tricingulatum* (Table 4.2). This dinoflagellate is most likely an example of secondary consumption, i.e. its presence is the result of the larva having fed on a zooplankton which fed on the *Islandinium*. Lobster larvae, especially the large postlarvae, are unlikely to actively feed on small phytoplankton (Herrick, 1911), and algae do not seem to provide enough nutrition alone to have significant effects on larvae (Hache et al., 2017), as was observed in laboratory feeding trials (Fig. 4.6). The inability to separate secondary consumption or environmental contamination from true prey items is one bias associated with metabarcoding; but critical evaluation using comparison to alternative methods (microscopy, rtPCR), and weighting prey taxa by the number of associated ASVs, reads, and RRA can help to substantially minimize this bias (Deagle et al., 2018). The only common taxa identified in sample 1 were various phytoplankton, rather than zooplankton. This may be a sign that this larva had not fed recently.

Sample 2 however contained a large amount of ASVs identified as the green crab *Carcinus maenas* resulting in an RRA of 0.949, and leaving other prey identified as likely inconsequential to this larva’s diet. Decapod larvae have already been found to be important diet constituents based on microscopy, and *C. maenas* is an abundant crab in the surrounding habitat. Repeated identification of a prey item through separate methods provides valuable validation for diet studies. This sample also contained a dinoflagellate, which was tentatively identified as *Karloodinium veneficum* via BLAST analysis (Altschul et al. 1990). This toxin producing dinoflagellate is capable of producing harmful algal blooms, so its presence in a larval gut is notable. An additional taxon of note identified in this gut is *Dasysiphonia japonica*, a locally abundant and invasive red algae. Metabarcoding of a benthic crustacean gut has been proposed
as a method for identifying fish diversity in a region (Siegenthaler et al., 2018); while it would likely be currently impractical, as eDNA approaches continue to become cheaper and faster it could be possible to utilize postlarvae as a natural sampler of plankton diversity. Metabarcoding of postlarval guts could then help reveal plankton diversity, but may also help to specifically reveal the presence of harmful or invasive algae.

Sample 3 contained just two common prey items- a bivalve and a copepod. Using BLAST, the bivalve was tentatively identified as *Placopecten magellanicus* - the Atlantic sea scallop. No bivalves were identified using microscopy, so its presence in the metabarcoding sample represents a significant contribution to knowledge of the larval diet. *Anomalocera patersoni*, the other common prey item, is a copepod. Copepods, of course, were already commonly identified in microscopy samples, but never this particular species.

The typically low number of reads and ASVs associated with each prey taxa (Table 4.2), as well as the continued presence of lobster DNA in samples, limited the power of our metabarcoding analysis. However, even with only 3 samples and the described difficulties, novel prey items not detected in the 112 SIV guts analyzed with microscopy were still able to be identified. Thus, continuing to refine this technique could yield significant results in the future. This holds especially true for identifying soft-bodied prey, or prey which otherwise may not leave behind recognizable features for visual identification.

### 4.5.3 Real time PCR assays (rtPCR)

Results from microscopy and metabarcoding would suggest that *C. finmarchicus* does not represent a significant prey species for lobster larvae of any stage in either of our study regions. *C. finmarchicus* was a species of interest because of the strong correlation reported between
trends in its abundance and lobster young-of-year recruitment in the Gulf of Maine. However, due to the difficult nature of prey identification in small pelagic larvae, the results obtained are sensitive to the tools employed. Microscopy and metabarcoding are methods that lend themselves to the broad identification of diet constituents, rtPCR on the other hand is a much more powerful tool for identifying the presence of specific prey taxa. While less than 1% of microscopy samples were found to contain *Calanus*, our *Calanus finmarchicus* specific rtPCR assay found that more than 20% of the 48 guts assayed contained *C. finmarchicus*, and MC postlarvae showed high electivity (0.93) for this prey item. In addition, on average fewer than two out of three technical replicates amplified for each sample, and the high Cq values observed indicate a low concentration of the target DNA (Table 4.3). It is therefore likely that even the *Calanus* specific rtPCR may be prone to false negatives, and the actual frequency of *C. finmarchicus* ingestion by postlarval lobsters.

The late amplification coupled with non-amplification of many replicates is strong evidence that our target, a portion of the COI gene of *C. finmarchicus*, is rare and possibly also degraded through digestive processes within our samples. This could explain why metabarcoding did not detect *Calanus* in sample 1 (Table 4.2), but the rtPCR assay applied to the same sample did detect *Calanus* (Fig. 4.4). Though a powerful tool, the generic primers used for metabarcoding are subject to being biased towards high concentration, high quality DNA, as the primers are more likely to bind to those sequences (Deagle et al., 2019). So when targeting DNA which is both low quality and low concentration, the incidence of false negatives is presumably
high. The use of both rtPCR and metabarcoding in this study provides proof that tailoring your methods to your target can dramatically improve results, and provide new insights on diet analysis.

For some researchers the amplitude of our Cq values may cause hesitation, as typical rtPCRs are only allowed to run for 40 cycles, whereas our reactions ran for 45 cycles. The 40 cycle maximum is employed to reduce the incidence of false positives caused by PCR errors, however, our probe-based rtPCR assay should only indicate true amplification of a target gene. PCR is by nature a stochastic exponential reaction, and so the longer it is allowed to run, the more likely it is that off-target products may amplify. These samples were allowed an extra 5 cycles to amplify, as pilot studies had shown us that the target was easy to miss. While this may open us to the possibility of false positives, testing in triplicate, using an NTC, and using both species specific primers and probe are all ways to reduce that risk. While 40 cycles is the norm for this type of study, it is also important to recognize that this value is arbitrary, and worth increasing when there is adequate justification. Finally, of the 10 samples which amplified, only 2 did not have a replicate which amplified before 40 cycles had passed, so a false positive due to increasing the cycle number is unlikely (Table 4.3).

Out of the 10 samples that amplified, half were from MC and half from NH. Notwithstanding the small sample size, this may suggest that larvae from each region have an equal preference and capture rate of *C. finmarchicus*, however, larvae from NH comprised less than ¼ of the samples tested. So in reality 45.5% of NH larvae tested contained *C. finmarchicus*, whereas only 13.5% of MC larvae did. While this difference was found to be significant ($X^2 (1,$
N = 46) = 4.779, p = 0.029), realistically further testing with a greater sample size is necessary to truly determine whether *C. finmarchicus* predation varies between these two populations.

**4.5.4 Effect of diet on larval development and survival**

Results from the diet analysis show the prevalence of crustacean prey in the natural diet of lobster larvae (Fig. 4.3). However, metabarcoding of three SIV lobster guts also contained traces of algae (Table 4.2). While previous studies have asserted that the algal portion of the larval diet is either incidental (Herrick, 1911) or inconsequential (Hache et al., 2017) to the nutrition of the larva, dietary data alone cannot support this assertion. Results from our experiment on the effect of diet regime on larval survival and development however do show strong support for this theory. The significantly greater survival (Fig. 4.6) and faster development time (Fig. 4.7) of larvae fed on a crustacean only diet provides evidence that the fatty-acids and other organic compounds found within crustaceans are key nutritional components for lobster larvae. While the initial source of these fatty-acids within the pelagic marine realm are indeed algae (Arts et al., 2001), lobster larvae either require greater magnitudes of fatty-acids or other nutrients in their prey (e.g. through biomagnification), or algal cells may lack a key behavior/signal to induce a predatory response in lobster larvae; thus making algae a poor prey item for lobster larvae. The observation that none of the 30 larvae in the algal treatment successfully molted within the nine day timeframe of the experiment, compared to 85% of larvae molting in the crustacean treatment (Fig. 4.7), suggests that though algae may appear in the diet of lobster larvae it is not a significant source of nutrition.
4.6 Conclusion

Understanding the lobster larval diet leads to an overall greater understanding of lobster larval ecology in general, which can help us to anticipate ongoing changes to important life-history processes such as benthic recruitment success. Our analysis of the larval diet bears resemblance to past studies of the same: if gut fullness in this and other studies is an indication, SI larvae are less well-nourished than SIV, and copepods and malacostracans appear to be the primary diet constituents.

Our use of eDNA techniques in conjunction with microscopy provides new insight into both the diet of lobster larvae, and the influence that methodology has over results in diet studies. Using an rtPCR assay tailored to detect the presence of *C. finmarchicus*, a significant incidence of this lipid-rich copepod in the guts of SIV larvae was detected, despite lack of detection using metabarcoding and microscopy. While microscopy and metabarcoding both succeeded in identifying broad patterns and the more abundant prey species, a tailored method was required to identify the rarer, yet nonetheless impactful, prey.

In addition to being the first study to employ both genetic and standard microscopy techniques for a lobster larval diet study, this is also the first to compare the diet of larvae from coastal Gulf of Maine in two different study areas. Our study areas, Midcoast ME and NH, included larvae that generally had similar diets, but an analysis of gut fullness reveals some indication that NH larvae may be more food-limited at both SI and IV. Yet, there is also some indication that NH larvae have a larger incidence of *C. finmarchicus* predation, perhaps reflecting that when larvae are unable to fill their guts enough, they rely to a lesser extent on high
calorie foods. Our finding that there is high electivity for *Calanus* by MC SIVs provides additional evidence that this copepod is an important prey item.

The question of food limitation in lobster larvae is an ongoing one, however our results serve to both provide some evidence of food limitation, while hopefully inspiring other researchers to broaden the methodologies they use to interpret larval diets.
CHAPTER 5 - EVIDENCE OF FOOD LIMITATION IN LARVAE OF THE AMERICAN LOBSTER (HOMARUS AMERICANUS)

5.1 Abstract

Declining larval recruitment when broodstock abundance is otherwise high has led to the hypothesis that food-limitation mediates lobster larval survival. However, direct evidence of food-limitation in pelagic larvae is rare, as has been the case for lobster larvae. Data from a mixture of laboratory and field-based studies are presented that are mostly consistent with this hypothesis. First, a lab-based starvation sensitivity experiment found that the first two larval stages are significantly more at risk for food-limitation than the last two larval stages. To provide more direct evidence of food-limitation, lab-reared fed and starved larvae were used as a comparison benchmark for the fatty-acid profiles of field-caught larvae. Particular attention was given to two essential fatty-acids: DHA and EPA, and specifically the DHA:EPA ratio, which is a common larval nutritional index. While SIII and IV larvae compared generally favorably, SI larvae were found lacking when compared to lab-reared benchmarks. SI field-caught larvae had a lower DHA content, as well as a lower ratio of DHA:EPA when compared to fed larvae, and a mass that was similar to that of starved larvae. Taken together, these results suggest that initial larval stages are most at risk for food-limitation. Though quantifying the exact toll food-limitation may take on lobster larvae is difficult to assess, the impact is probably underestimated in many taxa as sublethal effects most likely feature prominently.

5.2 Introduction

The pelagic larvae of benthic organisms tend to experience extreme mortality prior to their settlement in the benthic realm as juveniles. It has been estimated that less than 1% of
planktotrophic marine invertebrate larvae survive their pelagic phase (Vance, 1973), and so even small changes to survival could represent a major effect, e.g. an increase from 1% to 2% survival would double the amount of successful recruits. The larval stage then represents a crucial bottleneck, which could have lasting impacts on benthic population structure.

While larval mortality is commonly high, the most significant sources of mortality are more in question. Predation (Thorsson, 1949; Pepin et al., 2002), advection/transport from suitable habitat (Hudon and Fradette, 1993), food limitation (Hjort, 1914; Cushing, 1969; 1990; Olson and Olson, 1989), and physiological stress (Sameoto and Mataxis, 2008) have all been theorized to represent significant sources of mortality, but the primary source of mortality seems to be taxon and region dependent. In the North Sea, for instance, larval sand eel (*Ammodytes sp.*) have been found to suffer heavy predation by herring (Hunter, 1981), while on the California coast stable ocean conditions promoting planktonic food aggregations were connected to larval anchovy survival (*Engraulis mordax*: Lasker, 1981). Biophysical models of larval dispersal generally predict a high degree of advection away from suitable habitat, which would result in massive mortality, however empirical data from a larval reef fish found dispersal to be much shorter than predicted (Buston, 2012). Early larval ecologists pointed to food-limitation in particular as a potential driver of larval mortality. Hjort (1914) formulated his influential critical period hypothesis when he noticed that year class strength of some North Sea fishes was highly variable, and that in the right conditions a diminished broodstock could produce an abundance of successful recruits. Later, the match-mismatch hypothesis proposed by Cushing (1969, 1990) asserted that larval survival depended on the coincident timing of larvae with their food source. Yet, direct evidence of food-limitation is still sparse, Thorsson (1949) noted that larvae captured from the field are rarely in a starved condition. Still, food limitation has the potential to be
influential through sublethal indirect effects, as decreased food supply could lead to lengthened
development times, exposing larvae to other threats such as predation and offshore transport
(Thorsson, 1949; Castonguay et al., 2008); this relationship is commonly termed the
growth/mortality hypothesis (Ware, 1975; Sheperd and Cushing, 1980; Anderson, 1988). Most
recently, in the American lobster (*Homarus americanus*) evidence has surfaced that may link
declining young-of-year recruitment to larval food-limitation.

The American lobster holds both cultural and economic value along the Northwest coast
of the Atlantic, but recently a long running time series (ALSI, 2022) has recorded declines in
benthic young-of-year recruitment in the Gulf of Maine (GoM) despite an increase in broodstock
(ASMFC, 2020). A decline in settlement when egg/larval production is otherwise high, points to
an increase in one of the factors which govern larval mortality. Additionally, correlative
evidence has suggested that a decrease in the supply of *Calanus finmarchicus*, a lipid rich prey
species, may be the cause of falling planktonic postlarval abundance and benthic young-of-year
recruitment for this species (Carloni et al., 2018). Crucially, indices of predation and offshore
transport have not increased during this time-period, and so there is a significant possibility that
food-limitation is propelling settlement declines for lobster larvae in the Gulf of Maine (Carloni
et al., 2018).

The American lobster has three pelagic larval instars (SI-III), followed by a postlarval
stage (SIV) which remains in the pelagic realm until a suitable settlement site is found. All four
pelagic stages are obligate planktivores, and are unable to progress to the next developmental
stage without food (Abrunhosa and Kittaka, 1997). Field and laboratory observations indicate
that lobster larvae are voracious predators, particularly the postlarvae (Herrick 1911, Harding et
Lobster larvae feed on other crustaceans, namely copepods and decapod larvae (Junio and Cobb, 1992; Ascher et al., in prep). These prey are rich in essential fatty-acids necessary for larval development, specifically, DHA (docosahexaenoic acid, C22:6 n-3 (4, 7, 10, 13, 16, 19)) and EPA (eicosapentaenoic acid, C20:5 n-3 (5, 8, 11, 14, 17)). Essential fatty-acids must be acquired through diet, as they cannot be synthesized within the body of animals in significant amounts; and yet they are necessary for health and function (Arts et al., 2001, Kaur et al., 2014). These two essential fatty-acids help produce compounds such as prostaglandins, involved in the immune response, as well as play necessary roles in crustacean larval development and survival (Sui et al., 2007; Beder et al., 2018). Additionally, the DHA:EPA ratio can be used as a condition index, as a higher ratio of DHA to EPA helps maintain cellular membrane fluidity and aids larval growth and survival (Watanabe T., 1993; Brett et al., 2009).

In this study, two hypotheses were tested: (1) early stage larvae are more vulnerable to starvation than late stage larvae and postlarvae, and (2) evidence of larval food limitation in the wild may be assessed through comparing the fatty-acid content of field-caught larvae to well-fed and starved benchmark larvae reared on natural foods in the laboratory.

5.3 Methods

5.3.1 Starvation sensitivity

Ovigerous lobsters were procured from local lobstermen in South Bristol, ME. Lobsters were held in hatchery tanks at the University of Maine’s Darling Marine Center (DMC) flow-through seawater lab at ambient temperature and 5 micron filtered seawater. The progeny from three different mothers were combined for each trial in order to mitigate any individual maternal
effects. Mothers were all of a similar size (80-92 mm carapace length) chosen to represent the approximate average size of ovigerous lobsters in the GoM. Larval rearing tanks were maintained at the same conditions as the hatchery tanks. Larvae were fed daily with natural zooplankton caught in tows from the estuary. Zooplankton food consisted most commonly of copepods such as *Acartia sp.*, *Centropages sp.* and *Temora sp.* as well as decapod zoea and assorted nauplii. Gastropod larvae, cladocerans, and polychaete larvae were also present, but in lesser amounts. At each developmental stage, 30 larvae from each of the three mothers for a total of n=90, were selected and placed individually into 1L starvation chambers filled with filtered seawater and maintained at ambient temperature. For SIV, only 15 individuals could be obtained from one of the mothers, and so n=75. Individual larvae were monitored daily for active swimming behavior. Larvae which exhibited a state of tonic immobility, or that continued to be immobile within 20 sec of gentle agitation, were considered dead, as active swimming is necessary to maintain position in the water column. Time to death was recorded for each individual larva, and a survival analysis was performed using JMP V.16 statistical software. A log-rank test was used to determine whether mean survival times were different between stages.

### 5.3.2 Fatty-acid analysis

Ovigerous females and larvae were held and raised under the same conditions as those described above. Larvae raised under fed and starved conditions were used to create our condition benchmarks with the following methods. At each larval stage, up to 24 larvae were placed individually into 1L starvation chambers. These larvae were starved for 5 days, or until they showed significant signs of starvation, e.g., immobility. Over the course of the 5 d trial, larvae were removed when they appeared to be dead according to the criteria described for the
starvation experiment. The starved larvae were then randomly pooled into subsamples. At the same time up to 24 larvae were removed from the hatchery tanks with plentiful food and pooled in the same way. These two treatments, starved and fed, serve as benchmarks to which field-caught larvae may be compared.

Field-caught larvae were sampled using a combination of neuston and horizontal tows at depths of 5, 10 and 30 m conducted off the R/V Ira C. These samples were also pooled into groups based on their availability. Full descriptions of the number of samples, and number of larvae pooled in each sample broken down by treatment and larval stage can be found in Table 5.1.

Table 5.1 Fatty-acid sampling scheme across different treatments and larval stages. Each cell represents a sample, and the number within represents the number of larvae pooled in that sample.

<table>
<thead>
<tr>
<th></th>
<th>STAGE I</th>
<th>STAGE III</th>
<th>STAGE IV</th>
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<tbody>
<tr>
<td></td>
<td>Fed</td>
<td>Field</td>
<td>Starved</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>4</td>
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<td>7</td>
<td>4</td>
<td>4</td>
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<tr>
<td>6</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Total lipid content and fatty-acid (FA) profiles for each pooled sample of were then run by Bigelow Analytical Services. Briefly, embryos were oven dried (60°C), weighed to the nearest microgram, and then the lipids were extracted using a slightly modified Folch et al. (1957) method, as described in McMeans et al. (2012). Samples were extracted three times using 2 mL of 2:1 (v/v) chloroform:methanol and pooled, after which polar impurities were removed by adding 1.6 mL NaCl solution (0.9% w/v) and discarded following centrifugation. The
resulting lipid-containing solvent was concentrated to 2 mL and 2 aliquots (100 μL each) were removed and evaporated to dryness to quantify total lipid gravimetrically. Fatty-acids in the lipid extracts were derivatized to fatty-acid methyl esters (FAME) using sulfuric acid as the catalyst (Christie and Han, 2012). The FAME were then extracted twice using hexanes: diethyl ether (1:1; v/v), after which they were dried under a gentle stream of extra dry nitrogen gas. The FAME were separated and analyzed using a gas chromatograph (GC) (Shimadzu-2010 Plus, Nakagyo-ku, Kyoto, Japan) equipped with an SP-2560 column (Sigma-Aldrich, St. Louis, Missouri). All solvents used in the extraction and FAME derivatization procedures were of high purity HPLC grade (>99%). The FAME were identified and quantified by retention time matching and a 5-point calibration curve, respectively, using a reference standard (GLC-463, Nu-Chek Prep, Inc., Waterville, Minnesota). A known concentration of 5 alpha-cholestane (C8003, Sigma-Aldrich, St. Louis, Missouri) was added to each sample prior to extraction to act as a surrogate internal standard to estimate extraction and instrument recovery efficiency. Individual fatty-acid contents were expressed as percent (molar) of total quantified fatty-acid methyl esters (FAME).

JMP V.16 statistical software was used for statistical analyses. A single factor ANOVA was conducted for each larval stage to determine the statistical significance of treatment effects (field, starved, or fed), on eight variables: (1) dry mass, (2) total lipid content, (3) mass-specific lipid content, (4) total EPA content, (5) mass-specific EPA content, (6) total DHA content, (7) mass-specific DHA content, and (8) DHA:EPA ratio. Where treatment effects were significant, Tukey’s HSD was used as a post-hoc test to identify which treatments differed significantly from one another.
5.4 Results

5.4.1 Starvation sensitivity

Initial developmental stages, SI and II, were found to have lower mean times to starvation and lower maximum survival times under starvation when compared to the other two developmental stages. Mean time to starvation as well as maximum survival time under starvation increased dramatically between each stage, except between SI and II (Fig. 5.1). Developmental stage was found to have a statistically significant effect on survival time by the log-rank test ($X^2_{3,345}=275.72; p<0.001$). A post-hoc analysis comparing each stage pairwise found that only SI and II failed to differ significantly in their survival time ($X^2_{1,180}=2.14; p=0.143$). Mean survival time at SI was 5.9 days, at SII 6.2 days, at SIII 11.3 days, and at SIV 19.1 days (Fig. 5.2). After SII, each successive stage nearly doubled mean survival time. Maximum survival time, that is the day on which the last larva of a particular stage succumbed to starvation, also increased after SII. Maximum survival time at SI was 14 days, at SII 13 days, at SIII 20 days, and at SIV 32 days. Over the course of ontogeny maximum survival time more than doubled.
Figure 5.1 Starvation sensitivity of larvae at each developmental stage. SI-III n=90 larvae per stage, while n=75 for SIV, a log-rank test found a significant effect of stage on survival time (Chi-square 3, 345=275.72; p<0.001). Stages I and II were the only stages for which no significant difference in survival time was found (Chi-square 1, 180=2.14; p=0.143).
5.4.2 Fatty-acid analysis

SI: For SI larvae significant treatment effects were found in all eight fatty-acid metrics (Table 5.2). The $R^2$ values were also typically high, with the highest being 0.91 in our analysis of total lipid content, and the lowest $R^2$ of 0.42 in our analysis of larval mass. Field-caught larvae were most similar to the lab-reared fed larvae. A Tukey’s HSD analysis found that field-caught SI larvae were indistinguishable from their lab-fed counterparts in 5 of 8 variables analyzed (Table 5.3, Figs. 5.3-5.7). Lab-fed larvae were 35% larger than field-caught larvae with an average mass of 1.36 mg (Fig. 5.3). They also contained 45% more DHA with an average of 11.48 µg (Fig. 5.6). Finally, fed larvae had a DHA:EPA ratio 34% higher than that of field-caught larvae, with a ratio of 1.03 (Fig. 5.7). Field-caught larvae outperformed starved larvae in every category other than average mass, where there was no significant difference (Table 5.4) (Fig. 5.3).
**SIII:** An ANOVA again found that treatment was a significant predictor of each analyzed variable. The highest $R^2$ value was 0.92 in our analysis of total EPA content, the lowest $R^2$ value was once more related to larval mass with a value of 0.78 (Table 5.2). Field-caught larvae were once more most similar to the lab-reared fed larvae; though in contrast to the SI larvae, field-caught SIII larvae were only outperformed by lab-reared fed larvae when it came to DHA:EPA ratio (Fig. 5.7) (Table 5.3). Field-caught larvae had a DHA:EPA ratio of 0.91, which was 8.1% lower than that of fed larvae (Table 5.4). Also unlike the SI larvae, field-caught SIII larvae actually outperformed the lab-reared fed larvae in four variables: Total lipid content, larval mass, total DHA content and total EPA content (Figs. 5.3-5.6) (Table 5.3). Field-caught larvae had an average lipid content of 279.01 µg, 35% more than that of fed larvae. Field-caught larvae were 33% larger than fed larvae, with an average mass of 6.48 mg. The total DHA content of field-caught larvae was 65.01 µg, 26% greater than fed larvae. Similarly, the EPA content of field-caught larvae was 31% greater than that of fed larvae, with an average of EPA content of 71.06 µg. (Figs. 5.3-5.6) (Table 5.4). Also, unlike the SI field-caught larvae, SIII field-caught larvae maintained a greater condition than the lab-reared starved larvae in every category (Figs. 5.3-5.7) (Table 5.3).

**SIV:** Once more, an ANOVA revealed a significant effect of treatment on each variable tested. The $R^2$ values in the analysis of SIV larvae were higher than any other stage, with a high of 0.98, shared by larval mass and mass-specific EPA content, with mass-specific lipid content having the lowest $R^2$ value of 0.91 (Table 5.2). While field-caught larvae were again most similar to the lab-reared fed larvae, they contained lower total and mass-specific EPA and DHA levels than fed larvae (Figs. 5.5+5.6) (Table 5.3). Similar to field-caught SIII larvae, field-caught SIV larvae had both a greater average lipid content and mass when compared to fed larvae, but an
indistinguishable mass-specific lipid content. Field-caught larvae had an average total lipid content of 2466.57 µg, 41% greater than fed larvae. Field-caught larvae weighed an average of 24.04 mg, 37% heavier than fed larvae. Again, just as in SIII larvae, field-caught SIV larvae outperformed starved larvae in every category (Figs. 5.3+5.4) (Table 5.3). However, field-caught SIV larvae were the only field-caught larvae to have a higher DHA:EPA ratio than both lab-reared fed and starved larvae (Fig. 5.7) (Table 5.3).
Table 5.2 Results for the ANOVA of Treatment (i.e. Fed, Field, Starved) effect on each variable (left-hand column) by stage.

<table>
<thead>
<tr>
<th></th>
<th>Stage I</th>
<th>Stage III</th>
<th>Stage IV</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>F-ratio</td>
<td>p-value</td>
<td>R²</td>
</tr>
<tr>
<td>Total lipid content (ug)</td>
<td>$F_{2,23} = 106.35$</td>
<td>$&lt;0.001$</td>
<td>0.91</td>
</tr>
<tr>
<td>Larval mass (mg)</td>
<td>$F_{2,23} = 7.32$</td>
<td>0.004</td>
<td>0.42</td>
</tr>
<tr>
<td>Mass-specific lipid content (ug/mg)</td>
<td>$F_{2,23} = 27.92$</td>
<td>$&lt;0.001$</td>
<td>0.74</td>
</tr>
<tr>
<td>Total EPA content (ug)</td>
<td>$F_{2,23} = 66.58$</td>
<td>$&lt;0.001$</td>
<td>0.87</td>
</tr>
<tr>
<td>Total DHA content (ug)</td>
<td>$F_{2,23} = 59.46$</td>
<td>$&lt;0.001$</td>
<td>0.86</td>
</tr>
<tr>
<td>Mass-specific EPA content (ug/mg)</td>
<td>$F_{2,23} = 19.33$</td>
<td>$&lt;0.001$</td>
<td>0.66</td>
</tr>
<tr>
<td>Mass-specific DHA content (ug/mg)</td>
<td>$F_{2,23} = 30.66$</td>
<td>$&lt;0.001$</td>
<td>0.75</td>
</tr>
<tr>
<td>DHA:EPA</td>
<td>$F_{2,23} = 73.44$</td>
<td>$&lt;0.001$</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Table 5.3 Tukey's HSD results describing significant differences between treatments for each stage and variable. Values within each cell are the relevant p-value. Each column describes the relationship between a treatment pair within a stage. Cells are color coded based on significance and relationship between treatments. White cells are not significant differences, green cells are a significant difference where the first treatment listed in the pair has a mean significantly greater than the second, red cells denote the first treatment has a significantly lower mean than the second.
Finally, a previous analysis of the fatty-acid profile of lobster embryos from Ascher et al (in prep.) found that they had a DHA:EPA ratio that was similar to that of lab-reared fed larvae at each stage. The lab-reared fed larvae, therefore, maintained their initially invested DHA:EPA ratio, while starved larvae at each stage had lower DHA:EPA ratios. Field-caught larvae also had a lower DHA:EPA ratio than embryos at SI, but at SIII field-caught larvae had a DHA:EPA ratio equal to embryos, and field-caught SIV postlarvae had a DHA:EPA ratio which exceeded that of the embryos, suggesting a greater ability of later stage larvae to satisfy their nutritional demands (Fig. 5.8).

Table 5.4 Average mean response for each variable divided by treatment.

<table>
<thead>
<tr>
<th></th>
<th>Fed</th>
<th>Field</th>
<th>Starved</th>
<th>Fed</th>
<th>Field</th>
<th>Starved</th>
<th>Fed</th>
<th>Field</th>
<th>Starved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipid content (ug)</td>
<td>52.29</td>
<td>56.27</td>
<td>14.14</td>
<td>182.58</td>
<td>279.01</td>
<td>57.09</td>
<td>1449.97</td>
<td>2466.57</td>
<td>109.51</td>
</tr>
<tr>
<td>Larval mass (mg)</td>
<td>1.56</td>
<td>1.01</td>
<td>0.87</td>
<td>4.84</td>
<td>6.48</td>
<td>3.43</td>
<td>15.22</td>
<td>24.05</td>
<td>7.92</td>
</tr>
<tr>
<td>Mass-specific lipid content (ug/mg)</td>
<td>42.75</td>
<td>56.10</td>
<td>17.45</td>
<td>42.90</td>
<td>42.88</td>
<td>17.55</td>
<td>95.24</td>
<td>102.21</td>
<td>14.09</td>
</tr>
<tr>
<td>Total EPA content (ug)</td>
<td>11.93</td>
<td>10.35</td>
<td>4.12</td>
<td>48.74</td>
<td>71.06</td>
<td>15.38</td>
<td>347.33</td>
<td>213.42</td>
<td>28.48</td>
</tr>
<tr>
<td>Total DHA content (ug)</td>
<td>11.48</td>
<td>7.92</td>
<td>2.52</td>
<td>48.29</td>
<td>65.01</td>
<td>11.98</td>
<td>358.18</td>
<td>301.28</td>
<td>23.12</td>
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<tr>
<td>Mass-specific EPA content (ug/mg)</td>
<td>8.51</td>
<td>10.33</td>
<td>5.12</td>
<td>11.42</td>
<td>10.98</td>
<td>4.72</td>
<td>22.81</td>
<td>8.86</td>
<td>3.65</td>
</tr>
<tr>
<td>Mass-specific DHA content (ug/mg)</td>
<td>8.76</td>
<td>7.93</td>
<td>3.14</td>
<td>11.20</td>
<td>9.98</td>
<td>3.68</td>
<td>23.48</td>
<td>12.52</td>
<td>2.95</td>
</tr>
<tr>
<td>DHA:EPA</td>
<td>1.93</td>
<td>0.77</td>
<td>0.61</td>
<td>0.99</td>
<td>0.91</td>
<td>0.78</td>
<td>1.03</td>
<td>1.42</td>
<td>0.81</td>
</tr>
</tbody>
</table>
Figure 5.3 Mean dry mass (mg) of larvae by treatment at each stage. Within each stage letters connect treatments with statistically similar means. Error bars are standard error.

Figure 5.4 Mean total (µg) and mass-specific (µg/mg) lipid values of larvae by treatment at each stage. Within each stage letters connect treatments with statistically similar means. Error bars are standard error.
Figure 5.5 Mean total (µg) and mass-specific (µg/mg) EPA values of larvae by treatment at each stage. Within each stage letters connect treatments with statistically similar means. Error bars are standard error.

Figure 5.6 Mean total (µg) and mass-specific (µg/mg) DHA values of larvae by treatment at each stage. Within each stage letters connect treatments with statistically similar means. Error bars are standard error.
Figure 5.7 Average ratio of DHA:EPA (µg/µg) of larvae by treatment at each stage. Within each stage letters connect treatments with statistically similar means. Error bars are standard error.

Figure 5.8 Average ratio of DHA:EPA (µg/µg) of larvae by treatment at each stage, including the DHA:EPA ratio for lobster eggs for comparison. Unlike previous figures, letters connect treatments with statistically similar means across stages. Error bars are standard error.
5.5 Discussion

The starvation sensitivity and the fatty-acid analysis each suggest that SI, and possibly SII, larvae are more likely to be affected by food-limitation in the field than the larval SIII or postlarvae (SIV). Larval SI and II succumbed to starvation in less than half the time of SIV postlarvae. In addition, SI larvae from the field weighed less and had a poorer DHA:EPA ratio than those fed in the laboratory. Additional sampling could help determine the status of SII larvae in the field; they were absent from this fatty-acid analysis as an adequate number was unable to be collected over three summers of sampling. Nonetheless, the starvation sensitivity experiment suggests that larvae remain most at risk for food-limitation until they reach larval SIII. SIII and IV larvae were significantly more robust when it came to surviving starvation.

Both stages also had fatty-acid profiles that appeared elevated compared to lab-reared fed larvae in a number of categories. Interestingly, despite appearing in otherwise good condition, field-caught SIV larvae contained lower levels of total and mass-specific essential fatty acids than the lab-fed larvae. Yet, in comparison to field-caught SIII larvae, SIV larvae improved in every respect other than their mass-specific EPA content, meaning they continued to feed and accumulate nutrients. This suggests that postlarvae either have lower essential fatty-acid requirements, or that they are utilizing essential fatty-acids at an elevated rate as they prepare to settle.

5.5.1 Starvation sensitivity

Larvae hatch with endogenous energy stores derived from their mother. Over time these stores deplete and the larvae must hunt for food, or starve. This observation is at the center of Hjort’s landmark critical period hypothesis (1914) which states year-class strength is determined
early in the larval life-cycle during the transition from endogenous to exogenous energy sources. The time of transition is known as the critical period. During this critical period, larvae are at risk of mortality if they do not eat enough to maintain energy stores. A larva’s sensitivity to starvation is therefore a product of the speed of their metabolism and the quantity/quality of the endogenous resources invested by their mother. Our finding, that SI larvae are most at risk of starvation (Fig. 5.1) match well with Hjort’s critical period hypothesis. That SII larvae also share a similar sensitivity, however, was unexpected. At ambient temperatures with plentiful food, it takes roughly one week for larvae to develop from SI to II, and an additional two weeks to go from SII to III (MacKenzie 1988; Harrington et al., 2019). So, there is roughly a three week period post-hatch where lobster larvae are at an elevated risk for starvation. During this time larvae are subjected to other forms of mortality such as predation and advection from suitable habitat as well. Periods of starvation also lengthen the time it takes larvae to molt (Abrunhosa and Kittaka, 1997; Andres et al., 2010), and so increase the risk of these other mortality events (Thorsson, 1949). Successive days of starvation may also leave the larva in such poor condition that no amount of added food will lead to a successful molt. This is called the Point of No Return (PNR), and the day at which 50% of larvae reach PNR, the PNR50, is used as a measure of starvation sensitivity for larvae. The PNR50 for SI lobster larvae has previously been documented as 6.9 days (Abrunhosa and Kittaka, 1997), very similar to our estimated mean time to starvation of 5.9 days for SI, and 6.2 days for SII (Fig. 5.2). This validates our method of using tonic immobility as a starvation endpoint, and suggests that tonic immobility may in fact be the behavioral precursor to the PNR.

On the other hand, SIII larvae and SIV postlarvae exhibited an impressive resistance to starvation (Fig. 5.1). Larvae at this point are assumed to have used up their maternally invested
endogenous energy stores, and so must rely on energy stores built up from their own feeding activity as they develop. Larvae surviving to this stage have thus demonstrated an ability to feed efficiently. They are also significantly larger, with SIII larvae being about three times heavier, and SIV postlarvae being roughly 10 times heavier than SI larvae, and are equipped with pleopods to swim actively and appendages to seize prey (Table 5.4). This additional mass represents both important energy stores, and higher metabolic efficiency, as larger organisms tend to have lower mass-specific rates of metabolism than smaller (Peters, 1983). So, it appears that larvae passing through the initial bottleneck may be better prepared to handle food-limitation and periods of starvation.

If the significant additional size of later stage larvae is indeed the crucial factor in their resistance to starvation, it stands to reason that within individual stages larger larvae are more likely to withstand starvation for greater periods. Indeed, mortality rates of planktonic larvae have long been considered to be size-dependent (Ware, 1975, Peterson and Wroblewski, 1984, Anderson, 1988). Recent evidence has shown that rising temperatures in the GoM are leading to an onset of lobster maturation at a younger age and smaller size (Waller et al., 2021; Aiken and Waddy, 1976). This has important implications for larval survivability, as smaller females tend to produce smaller larvae with a lesser amount of maternally invested energy (Attard and Hudon, 1987, Mousseau and Fox, 1998, Ouellet and Plante, 2004, Ascher et al., 2023). It can be inferred that future lobster larvae in the Gulf of Maine may be trending smaller, with lower endogenous energy stores at the time of hatch. Combined with the starvation sensitivity of early larval stages reported here, the interactive effects may lead to even greater vulnerability to food-limitation.
5.5.2 Fatty-acid analysis

Lipids are a common source of energy utilized by many organisms, including crustaceans, under starvation conditions (Arts et al., 2001, Sanchez-Paz et al., 2006). Even when removed from starvation conditions, polyunsaturated fatty-acids (PUFAs), and highly unsaturated fatty-acids (HUFAs) have special utility in the growth and development of crustacean larvae (Sui et al., 2017; Wang et al., 2020). DHA and EPA are two HUFAs in particular which have wide ranging effects on marine organisms, particularly larvae and crustaceans, from: prostaglandin production (Watanabe T., 1993, Kaur et al., 2014), neural function (Scott et al., 2002, Brett et al., 2009), phospholipid membrane fluidity (Watanabe T., 1993, Brett et al., 2009), growth rate (Sui et al., 2007, Beder et al., 2018, Wang et al., 2021), lipid uptake (Beder et al., 2018, Wang et al., 2021), osmotic stress response (Sui et al., 2007, Beder et al., 2018), and survival (Watanabe T., 1993, Sui et al., 2007). Additionally, a higher ratio of DHA to EPA has been found to be critical for proper utilization and membrane fluidity (Watanabe T., 1993, Sui et al., 2007, Brett et al., 2009, Wang et al., 2021). Therefore, while total lipid quantities in a larva may give insight into their available energy stores, specific attention can be paid to the quantities and ratio of DHA and EPA as an additional condition index. Furthermore, as these two HUFAs are also essential fatty-acids derived from dietary items, it can be inferred from their elevated presence in a larva that the larva has been actively feeding.

SI: SI field-caught larvae were the only field-caught larvae to not outperform the lab-reared fed larvae in any category. Furthermore, field-caught SIII and IV larvae maintained a significantly better condition than starved larvae in every category, while SI field-caught larvae showed no significant difference to starved larvae when it came to their average mass (Fig. 5.3) (Table 5.3).
SII larvae therefore appear to be in the worst condition out of the field-caught larvae examined here. Having a mass similar to that of starved larvae is a particularly good indicator of their diminished condition. As previously noted, mortality in the plankton is size-dependent. Body size plays roles in predation avoidance, movement speed and efficiency, foraging efficiency, and is an indicator of the energetic stores available to a larva (Ware D.M., 1975, Peters R.H., 1983, Peterson and Wroblewski, 1984, Anderson J.T., 1988). Although field-caught larvae contained a commensurate amount of total and mass-specific lipids to fed larvae (Fig. 5.4) (Table 5.3), some crustacean larvae have been found to utilize other biomolecules as their primary energy source under starvation (Sanchez-Paz et al., 2006). So while lipid levels can act as an indicator of larval condition, it may be important to consider them in the context of other variables such as larval mass and critical HUFA content. Field-caught SII larvae had the second lowest total DHA content and DHA:EPA ratio of any larva, with starved SII larvae having the lowest (Table 5.4). DHA and the DHA:EPA ratio has been demonstrated many times to be critical to the growth and survival of larval crustaceans (Nghia et al., 2007, Sui et al., 2007, Beder et al., 2018, Wang et al., 2021). The low DHA:EPA ratio demonstrated by the field-caught SII larvae could therefore be indicative of a slower growth rate which is a key sublethal effect of food-limitation. Slower growth rates increase the pelagic larval duration which puts larvae at risk of mortality through other sources (Thorsson, 1949).

**SIII:** SIII field-caught larvae demonstrated some improvement over SI larvae, however, they still had a significantly lower DHA:EPA ratio than fed larvae (Fig. 5.7) (Table 5.3). There is otherwise no clear indication that SIII larvae are food-limited. At this stage, larvae begin to develop a body plan more similar to that of an adult lobster, with well-developed pleopods, chelipeds, and a telson divided into 5 uropods. These developments could serve to increase the
movement and foraging efficiency of larvae which survive to this stage, leading to a better overall condition.

**SIV:** The average mass and total lipid quantities of field-caught larvae were particularly striking, with the average field-caught larva being ~1.6X heavier and accumulating ~1.7X the amount of total lipids than the fed benchmark larvae. These variables also increased significantly over field-caught SIII larvae, with SIVs being 3.4X heavier and accumulating 7.4X the total amount of lipids than the previous larval stage. By these two metrics alone it would appear that SIV postlarvae are not food-limited. Yet despite their larger size, field-caught postlarvae contained a significantly lower amount of EPA and DHA to fed larvae, both total and mass-specific quantities (Fig. 5.5+5.6), but still had a greater DHA:EPA ratio (Fig. 5.7). Assessing the exact nutritional status of postlarvae is therefore difficult without information on what the actual nutritional needs of the larvae are. As previously stated, the DHA:EPA ratio is significant, and an optimal ratio can lead to greater growth rates, a better stress response, etc. (Watanabe T., 1993, Sui et al., 2007, Brett et al., 2009, Wang et al., 2021). The optimal DHA:EPA ratio for growth and survival of American lobster larvae is unknown, though in other larval decapods the optimal dietary ratio of DHA:EPA has been found to range from 1.2-4.0 in a laboratory environment (Nghia et al., 2007, Sui et al., 2007, Beder et al., 2018, Wang et al., 2021). In a recent diet study of larval lobsters it was found that a significant proportion of postlarval lobsters fed on *Calanus finmarchicus* (Ascher et al., in pub.). *C. finmarchicus* is a foundational copepod species whose presence in the GoM is currently declining due to rising temperatures (Carloni et al., 2018, Record et al., 2019; Ji et al., 2021). The declining presence of *C. finmarchicus* has been linked to declining larval lobster settlement (Carloni et al 2018), and they may represent an optimal dietary component of lobster larvae due to their large size and lipid concentration. In particular,
the phospholipid membranes of this copepod contain a high proportion of DHA and EPA, with a DHA:EPA ratio of 1.7 (Scott et al., 2002; Kattner and Hagen, 2009); not too dissimilar from the average DHA:EPA ratio of field-caught postlarvae which was 1.42. Field-caught SIV postlarvae were the only group which had a significantly greater DHA:EPA ratio than that initially invested in embryos (Fig 5.8) suggesting a greater ability of later stage larvae to satisfy their nutritional demands. This may be accomplished through showing a preference for prey with elevated quantities of DHA in the field, such as *C. finmarchicus*. Furthermore, in preparation for settlement to the seafloor, postlarvae initiate bottom-seeking behavior. As a result, field-caught postlarvae would have regularly experienced colder temperatures than those raised in the lab as they submerged below the thermocline. Greater quantities of DHA relative to EPA are necessary to maintain phospholipid membrane fluidity in colder temperatures (Brett et al., 2009). The elevated DHA:EPA ratio observed in field-caught postlarvae may be in response to, as well as preparation for, colder temperatures on the seafloor. This would also explain why the DHA:EPA ratio of lab-reared larvae did not increase similarly, as they would have lacked the environmental cue to elevate their DHA:EPA ratio. Viewed in this light, the lower quantities of DHA and EPA in field-caught larvae may in fact be an attempt to regulate the ratio of these two HUFAs. The question therefore is whether the quantities of EPA and DHA found in the tissues of field-caught postlarvae represent adequate amounts. Due to its importance, the ratio of DHA:EPA has been found to be tightly regulated in many marine crustaceans (Scott et al., 2002, Brett et al., 2009, Andres et al., 2010). So, lobster larvae may either limit uptake, or utilize DHA/EPA at differential rates in order to maintain an optimal ratio. This could explain the especially low mass-specific EPA quantities in field-caught larvae, as lowering EPA levels could be a simple
method for increasing the DHA:EPA ratio. Laboratory testing of optimal HUFA quantities could help to further interpret these results.

5.6 Conclusion

Determining the sources of larval mortality is an ongoing and important debate in larval ecology. While food limitation has long been theorized to be of major importance in controlling larval recruitment, direct evidence of food limitation is rarely reported. Here, evidence of food limitation across the ontogeny of lobster larvae is given through integrating both laboratory and field based data. By quantifying the risk of food-limitation through starvation sensitivity, combined with conditional indices of field-caught larvae, theoretical results can be meaningfully connected to empirical data. Thus, it appears that the early lobster larval stages are most at risk for food-limitation, and a lower average mass and nutritional index in the field supports the inference that lobster larvae are likely to suffer from food limitation at this critical period. In addition, warming in the Gulf of Maine is leading to a decrease in the average size of ovigerous lobster; and will likely lead to a corresponding decrease in larval size and their endogenous resources at hatch. This would further increase the risk of food-limitation in early larval stages. While postlarval lobsters appear more robust both in their low sensitivity to starvation, and most nutritional indices, some evidence suggests this may be partially due to preferred predation on a rich source of both PUFAs and HUFAs: *Calanus finmarchicus*. With the abundance of this copepod on the decline, postlarval lobster may need to find other lipid rich food sources in order to maintain their hardiness. Unlike other sources of mortality, food limitation has the additional effect of lengthening development time; increasing the risk of alternative mortality sources. Because of this, the true impact of food limitation is difficult to quantify, and indeed is likely
underestimated. Additive effects, such as decreasing maternal size, or declining abundance of a key prey item, could further exacerbate risk of mortality due to food limitation for lobster larvae; as well as in other taxa.
CHAPTER 6 - CONCLUSION

6.1 Dissertation rationale

Ongoing warming of the world’s oceans has broad effects on marine life. In the Gulf of Maine (GoM), water is warming rapidly as the influence of warm, high salinity Gulf Stream water increases, and the influence of cooler, fresh Labrador Current water decreases (Gonçalves Neto et al, 2021). This has caused significant changes to the pelagic food-web and on reproductive traits of the iconic and economically important American lobster. Simultaneously, lobster larval recruitment in the GoM has been observed to be declining, despite record broodstock abundance. Lobsters are thus a superb model system in which to study sources of variation in larval recruitment mediated by a changing climate.

6.2 Major findings

The hypotheses set forth by this dissertation were: (1) the declining size of female lobsters in a warming climate has adverse effects on the quality and quantity of embryos and resultant larvae produced. (2) Lobster larval growth and survival is food-limited.

With regards to hypothesis (1) there is evidence that larger maternal size has significant positive effects on the quantity and the quality of embryos as well as the performance of the resultant larvae. Across all three study regions larger mothers produced more embryos which were larger and more energy rich. The previously reported phenomenon that lobsters in the warmer southern regime mature at a smaller size and reach a considerably smaller maximum size than their counterparts in the cooler northern region (Gulf of Maine) was also observed. Fatty-acid profiling revealed that embryos from larger mothers contain a significantly greater lipid content, as well as elevated levels of Docosahexaenoic acid (DHA), a fatty-acid particularly important to crustacean larval development. Increased embryo quality was conferred to the
resulting larvae, as larger mothers were found to produce larvae which were longer, heavier, with greater lipid stores, and more resistant to starvation than those from smaller mothers.

Maternal size had similar effects on embryo production and quality across our thermally contrasting study regions, with two exceptions: First, size-for-size, females from the warmest regime produced more embryos than females from the coldest regime. Second, size-for-size females from the warmest regime produce larger embryos than females from the intermediate regime. This result suggests counter-gradient adaptation favoring enhanced egg production, in effect compensating for the smaller adult size of lobsters in warmer regimes. While this is speculative, it warrants further investigation.

With regards to hypothesis (2) our larval diet analysis revealed that both SI and SIV larvae feed on a significant amount of copepods, with SIVs also feeding heavily on malacostracans. Genetic techniques also revealed important details about the diet not evident through conventional microscopy. Metabarcoding analysis of gut contents yielded more taxonomic resolution of the gut contents than microscopy. Importantly, targeted rtPCR revealed more evidence of predation on *Calanus* by field-caught SIVs than was evident by either of the other methods suggesting selective predation on this foundational prey species. This finding may be important in the context of food-limitation, as the abundance of *C. finmarchicus* during the larval period has been in decline over the past decades. Also, SI larvae were found to have significantly less full guts than SIV postlarvae, and larvae from NH had less full guts than larvae from MC.

Laboratory-based starvation sensitivity analysis found that initial larval stages are significantly more at risk of starvation than later stages. In addition, feeding trials with SI larvae indicated that they were more likely to survive to SII when fed on a diet consisting of
crustaceans (brine shrimp) than on a diet of phytoplankton or a mixture of the two. Larval performance may be tightly linked to the quantity and quality of fatty acids in their diet. Fatty-acid profiling largely reflected starvation sensitivity results, as SI larvae compared less favorably to lab-reared benchmarks than either SIII or SIV. In particular SI larvae from the field had dry masses similar to lab-starved larvae. Additionally, SI larvae had DHA:EPA ratios lower than lab-fed larvae and embryos. As the DHA:EPA ratio is related to growth rate among other important factors (Sui et al., 2017; Beder et al., 2018; Wang et al., 2021), this could further point to increased mortality due to food-limitation in SI larvae.

6.3 Synthesis of results and implications

The structure of this dissertation was intended to follow lobster larvae through ontogeny from embryo to each successive pelagic developmental stage in order to assess factors which may be responsible for observed declining larval recruitment rates. The past two decades have seen soaring broodstock abundance and lobster landings (ASMFC, 2020), as average water temperature within the GoM has also increased (Pershing et al., 2018). As described in CH. 2, this has led to female lobsters maturing earlier at smaller sizes, and in the long-term will lead to a smaller body size for reproductive adults (Aiken and Waddy, 1976; Pauly et al., 2022). Crucially, smaller females produce a lesser amount of lower quality embryos and larvae. The probability of larval survival is widely believed to be exceedingly low, by some estimates as few as 1% of pelagic larvae recruit successfully to the benthos (Vance, 1973). Survival is due to a slew of environmental factors such as prey availability and predator abundance, but there is also a significant element of stochasticity. Female lobsters can invest additional energy into each larva to maximize their potential to survive an unfavorable environment, but the only recourse
for dealing with the stochastic element is to produce more larvae. Smaller females fail at both aspects when compared to larger females from the same population.

The period directly post-hatch is particularly crucial. The critical-period hypothesis states that significant larval mortality occurs when newly hatched larvae exhaust remaining maternally invested energy before finding sufficient prey (Hjort, 1914). For larvae of smaller mothers, this timeframe is narrower. Crustacean larvae require significant sources of essential fatty acids to progress through developmental stages, particularly DHA+EPA (Sui et al., 2017; Beder et al., 2018; Wang et al., 2021). The larval diet as revealed through microscopy and genetic techniques reflects these requirements, as they feed on copepods and decapod larvae which are likely to provide significant amounts of essential fatty-acids DHA and EPA. Additionally, maintaining a higher DHA:EPA ratio has been shown to increase growth rate, a critical trait for planktonic survival (Ware D.M., 1975; Shepherd and Cushing, 1980; Anderson J.T., 1988). However, field-caught SI lobster larvae were found to have a DHA:EPA ratio that was lower than that found in embryos or lab-fed larvae, and so may suffer a lower growth rate. This is additionally reflected in the result that SIs from the field had a similar dry mass to lab-starved larvae. The DHA:EPA ratio increased through ontogeny for field-caught larvae, and later stages compared more favorably to lab-reared benchmarks than the initial SIs. This is mirrored in the results of lab-based starvation sensitivity experiments. SI larvae were found to be more sensitive to starvation than SIII or SIV, and SI larvae hatched from smaller mothers are additionally more at risk than those hatched from larger mothers.

Later stage larvae showed significant increases in mass and lipid values over SI larvae. The diet analysis revealed that *Calanus finmarchicus*, which maintains a high DHA:EPA ratio within its phospholipids, is preyed upon significantly by SIV postlarvae. Therefore, SIVs may be
capable of attaining large sizes and an elevated DHA:EPA ratio through feeding on rich EFA sources such as *C. finmarchicus*. If this is the case, climate mediated declines in the summertime abundance of *C. finmarchicus* could mean that future lobster larvae may be unable to achieve maximal growth rates.

Though the hypotheses laid out above were largely supported, lobsters and their larvae exist within a complicated framework of interacting biotic and abiotic influences. As such, there can be no simple explanation for the breakdown in the stock-recruit relationship observed for lobster. While maternal size was found to be widely influential on reproductive traits, a significant amount of variation was still left unaccounted for, suggesting additional important factors yet to be described. Though maternal size may decline with increased temperatures, the effect of temperature on these other factors is unknown. Starvation sensitivity and fatty-acid analysis of lobster larvae point to food-limitation increasing mortality risk for early larval stages, but an understanding of the actual nutritional needs of larvae is necessary to truly understand the impact of these results. Warming waters have also been found to increase fish predation rates (Michel et al, 2020), and risk of lobster larval loss through advection due to ovigerous females releasing larvae further offshore (Casey et al, 2022). So while evidence presented here supports the conclusion that the interactive effects of declining maternal size and increased food-limitation has led to greater lobster larval mortality; predation, advection, and other changes within the GoM cannot be ruled out as additional influences.

6.4 Concluding remarks

Results presented in this dissertation provide evidence that the interactive effects of decreased maternal size and food-limitation may explain declining lobster larval recruitment in the Gulf of Maine. The interaction of the two is an important aspect of this research. Declining
maternal size may increase the risk of poor recruitment, but provided with enough prey and otherwise optimal conditions, a lesser amount of smaller larvae could still produce a healthy year class. However, our assessment of food-limitation through lab and field based techniques shows this is not the case, as field-caught SI larvae exhibit a poorer, rather than greater nutritional condition to lab-reared fed benchmarks. However, to truly assess the degree of food-limitation in field-caught larvae, one piece of the puzzle is still missing. Although the comparison of field-caught larvae to laboratory fed and starved benchmarks provides a useful framework, the comparison is relative by necessity. Further lab-based experimentation could reveal the actual minimum nutrient requirements needed to progress from one larval stage to the next, as well as the nutrient and requirements to achieve maximum growth-rates. Additionally, while SIV diets revealed high rates of predation on *Calanus finmarchicus* relative to the copepod’s availability in nature, earlier larval stages have yet to be tested. Further analysis of larval diets at each stage combined with a comparison to natural zooplankton abundances could provide an additional method to assess risk of food-limitation.

Rapidly warming waters provide the impetus for continued monitoring of maternal size-effects and food-limitation in lobster larvae. Results presented in this dissertation provide a strong basis to further our understanding of the sources of recruitment variation and recent declines in the American lobster. Moreover, the techniques and ideas discussed here can be applied to other taxa with benthic adults and pelagic larvae. Utilizing mixed methodologies and a combination of both lab-based and field-based approaches served to provide stronger evidence in support of our hypotheses. Dispersive larvae can be particularly difficult to study, but a holistic approach considering both the intrinsic and extrinsic sources of variation throughout ontogeny can lead to better outcomes.


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