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Decapod crustacean larval developmental plasticity and the evolution of lecithotrophy and abbreviated development

By

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ABSTRACT

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DECAPOD CRUSTACEAN LARVAL DEVELOPMENTAL PLASTICITY AND THE EVOLUTION OF LECITHOTROPHY AND ABBREVIATED DEVELOPMENT

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Within the marine environment, the diverse development modes among marine invertebrate taxa follow a macro-ecological pattern across latitude. Generally, across the latitudinal gradient from the tropics to the poles, larval development becomes increasingly independent of external food resources. Low latitude species are fecund and produce relatively poorly provisioned eggs, which develop into swimming and feeding larvae. This mode of development becomes increasingly scarce with increasing latitude, whilst development from large eggs (produced in low numbers) into larvae, which are non-feeding, becomes increasingly prevalent. From the tropics to the poles, there is an increasing mismatch between the longer periods required for larval development (resulting from increasing cold) and the shorter periods of food availability (resulting from increasing seasonality). This macro-ecological trend in development modes results from the convergent evolution of diverse taxa, which have adapted to high latitude environments in much the same way: through producing larvae which develop independently of external food resources.

Developmental plasticity during the larval phase is a mechanism by which larvae are able to cope better with unfavourable conditions or variations in their environment. Critically, the interaction between per offspring investment (POI; the quantity and quality of resources allocated to offspring) and developmental plasticity, and the role of this interaction in the evolution of larval development modes, is little considered or studied. Experiments comprising this thesis assess the potential role of this interaction in the evolution of abbreviated and lecithotrophic development within decapod crustaceans, and the establishing of the macro-ecological trend in development outlined above.

Experiments used the palaemonine shrimp, *Palaemonetes varians*, which inhabits temperate, salt marsh, and peripheral brackish water, as a study species. Palaemonine

shrimp originated from a tropical marine clade and extant species are found in marine, brackish, and fresh water environments. The evolutionary transition from marine to fresh water has involved life history adaptations in development mode within this group. As species occupy differing habitats along this environmental gradient, this group has been used to study evolutionary adaptations along the environmental gradient from marine to fresh water.

Sampling of a wild population of *P. varians* from Lymington salt marsh (Hampshire, UK) revealed the highly variable environment that this species inhabits. POI within this population varied inter- and intra-annually, though these variations could not be correlated with variations in environmental temperatures. Larvae hatch with significant yolk reserves and can be considered facultative lecithotrophic in the first and second larval instar, and planktotrophic from the third larval instar. Larval development was successful at temperatures between 15 and 30 °C and temperature-mediated developmental plasticity was observed; at higher temperatures, larvae increasingly developed through fewer larval instars. Development through fewer larval instars resulted in more rapid development, but development to a lesser juvenile dry weight at settlement. Consequently, this developmental plasticity may have ecological implications. Developmental plasticity was also influenced by the energy content of larvae at hatching (as proxy for POI). Larvae with greater energy content developed through fewer larval instars at all temperatures, indicating that higher POI buffers larvae against poor conditions during development. Greater energy content also enabled larvae to tolerate starvation for longer and to develop to more advanced larval stages in the absence of food.

Developmental plasticity within decapod crustaceans enables larvae to tolerate unfavourable conditions during development. Interestingly, it maximises the potential fitness benefits provided by POI by enabling larvae to settle as juveniles earlier, though at a smaller size. The interaction between POI and developmental plasticity forms a ‘pre-adaptation’ for the evolution of abbreviated development. The results presented in this thesis indicate that, indeed, the evolutionary transition to abbreviated development and lecithotrophy is based on selection for increasing POI. The abbreviation of development associated with increasing POI arises through developmental plasticity in larval instar number.

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Oliphant A, Thatje S, (2013) Per Offspring Investment implications for crustacean larval development: evolutionary insights into endotrophy and abbreviated development. Marine Ecology Progress Series 493:207-217

Oliphant A, Hauton C, Thatje S, (2013) The implications of temperature-mediated plasticity in larval instar number for development within a marine invertebrate, the shrimp *Palaemonetes varians*. PLoS ONE 8(9)e75785

Other works

Oliphant A, Ichino M, Thatje S, (revision submitted) The influence of Per Offspring Investment (POI) and starvation on larval developmental plasticity within the palaemonid shrimp, *Palaemonetes varians*. Marine Biology (revision submitted)

New P, Brown A, **Oliphant A**, Burchell P, Smith A, Thatje S, (2014) The effects of temperature and pressure acclimation on the temperature and pressure tolerance of the shallow-water shrimp *Palaemonetes varians*. Marine Biology 161:697-709

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DECLARATION OF AUTHORSHIP

I, Andrew Oliphant, declare that the thesis entitled 'Decapod crustacean larval developmental plasticity and the evolution of lecithotrophy and abbreviated development' and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
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Oliphant A, Hauton C, Thatje S, (2013) The implications of temperature-mediated plasticity in larval instar number for development within a marine invertebrate, the shrimp *Palaemonetes varians*. PLoS ONE 8(9)e75785

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Signed: ……..

Date:…….

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"...the young of the same litter, sometimes differ considerably from each other, though both the young and the parents... have apparently been exposed to exactly the same conditions of life; and this shows how unimportant the direct effects of the conditions of life are in comparison with the laws of reproduction, and of growth, and of inheritance; for had the action of the conditions been direct, if any of the young had varied, all would probably have varied in the same manner. To judge how much, in the case of any variation, we should attribute to the direct action of heat, moisture, light, food, etc., is most difficult: my impression is, that with animals such agencies have produced very little direct effect..."

Charles Darwin (The Origin of Species, 1859)

1. Introduction

In 1950, Danish ecologist Gunnar Thorson's review of the 'Reproductive and Larval Ecology of Marine Bottom Invertebrates' was published in Biological Reviews (Thorson 1950). This seminal work developed hypotheses proposed in Thorson's earlier work 'The Larval Development, Growth, and Metabolism of Arctic Marine Bottom Invertebrates compared with those of other Seas' (Thorson 1936). These now classic works helped to shape marine ecological research for the next five or so decades and still influence our views on the reproductive macro-ecology of aquatic invertebrates. Thorson highlighted the trend for the quantity of resources allocated to individual offspring to increase with latitude, noting that species from the high latitude Arctic region of East Greenland have larger eggs than closely related species from the lower latitude Boreal region of Denmark (Thorson 1936). Given that egg size is [somewhat] correlated with development mode, Thorson proposed that high latitude benthic invertebrates mainly reproduce by lecithotrophic, abbreviated, aplanktic larvae. The concept that invertebrates increasingly reproduce via large, energy rich eggs, which develop into food independent, non-swimming, abbreviated larvae at higher latitudes became known as 'Thorson's Rule' (Mileikovsky 1971). Since Thorson's initial works, studies have shown that aplanktic development is less important in high latitude regions than Thorson proposed. Also, there are of course exceptions to the concept: some of the most numerous species in the Arctic and Antarctic reproduce by planktic planktotrophs (Picken 1980). Still, the macro-

ecological concept -that the quantity and quality of resources allocated to individual offspring, or per offspring investment (POI), and correlated increases in endotrophic development and abbreviation of the larval phase increase with latitude- holds true (Marshall et al. 2012).

Development via non-feeding and abbreviated larval phases also increases with depth in the oceans (Thorson 1950) and with decreasing marine connectivity within brackish and freshwater environments (see Anger 2001, Vogt 2013 and references therein). These macro-ecological trends in POI and development modes indicate that life history traits have been selected for along environmental gradients. Increases in POI are associated with increases in nutritional independence (endotrophy/lecitothrophy) and increasingly abbreviated development (Thorson 1950, Marshall & Keough 2007, Marshall et al. 2012). These reproductive and developmental traits are considered to be evolutionary adaptations to low or unpredictable food availability and/or the mis-match between short periods of primary production (resulting from high seasonality) and prolonged development (resulting from low temperatures) at high latitudes (Thorson 1950, Anger 2001, Thatje et al. 2003).

Interestingly, Thorson (1936) observed intra-specific variation in POI along a latitudinal gradient between East Greenland and Denmark within the hippolytid shrimp, *Spirontocaris gaimardi*; consistent with the macro-ecological trend at the inter-specific level, POI was higher within the East Greenland population (Thorson 1936). Similar observations were subsequently made for numerous other species, for example within the barnacle *Semibalanus balanoides* (Crisp 1959, Barnes and Barnes 1965), provoking interest in variations in POI at the intra-specific level and across environmental gradients (e.g. Efford 1969, Lonsdale & Levinton 1985, Hadfield 1989). POI is now known to vary geographically across environmental gradients within many taxa; consistent with the macro-ecological trend in POI and development modes at the inter-specific level, higher POI is found in higher latitude populations within species (e.g. Lardies & Castilla 2001, Lardies & Wehrtmann 2001). POI is also known to vary temporally between seasons (e.g. Kerfoot 1974, Boddeke 1982), between years (e.g. Kattner et al. 1994, Urzúa et al. 2012), and perhaps over longer time scales (see Reed et al. 2012). Such POI variation at the intra-specific level can provide insights into the evolutionary processes that shaped the macro-ecology of development modes, which firstly drove local adaptation, speciation, and eventually, the diverse multitude of reproductive and development modes present in the aquatic environment.

Within this thesis, a greater understanding of the evolution of macro-ecological trends in POI and development modes is sought: through investigating the effects of POI, and the interaction between POI and environmental conditions, on larval development and developmental plasticity, a greater understanding of the role which POI plays in the evolution of larval development modes will be gained.

1.1 Maternal effects on Per Offspring Investment

Biotic and abiotic factors within an organism's environment are variable both spatially and temporally. An organism's ecological niche -the environment in which it may successfully survive and reproduce- is defined by its physiological tolerance of biotic and abiotic factors within its environment. Consequently, a species' spatial and temporal distribution is an expression of its ecological niche in space and time (Sexton et al. 2009). Accordingly, a species' distribution range may shift, expand, or contract over ecological as well as evolutionary periods of time (Pfenninger et al. 2007, Sexton et al. 2009). Importantly, an organism's ecological niche is not necessarily invariable; phenotypic plasticity –the ability of an organism's genotype to express multiple environmentally dependant phenotypes from a single genotype throughout the organism's ontogeny (Pigliucci 2005)- may provide physiological flexibility, permitting ecological niche plasticity. Phenotypic plasticity can involve changes to an organism's chemistry, physiology, development, morphology, or behaviour and thus ecology in response to the biotic and abiotic environment. For example, an organism's molecular, cellular, and systemic processes are optimised for a limited range of body temperatures, outside of which functional constraints arise (Pörtner & Farrell 2008). However, temperate species are able to shift their temperature range through adjustments in physiological performance such as changes in mitochondrial densities (Pörtner & Knust 2007). The above quote from Darwin's 'Origin of Species', preceeding this introduction, emphasises the role of genetic inheritance in determining an organisms genotype, but in doing so it diminishes the effects of environmental conditions on organism phenotype.

In spatially and temporally variable environments, phenotypic plasticity is thought to be important in enabling species to optimise and extend their distributions. A single phenotype is unlikely to provide high fitness across the spatially and temporally variable environments within a species' distribution and consequently, environmentally dependant changes in the phenotype (phenotypic plasticity) may provide increased environmental

tolerance (Via et al. 1995). Such environmentally dependant changes in the phenotype are thought to evolve in populations which experience predictable environmental change; e.g. the annual seasonal cycle in temperate regions (Stearns 1989, Via et al. 1995).

Changes in parental phenotype may affect offspring phenotype such that phenotypic plasticity spans generations. This 'trans-generational phenotypic plasticity' may increase ecological niche plasticity. The maternal environment is one of the most important factors influencing initial offspring phenotype, performance, and fitness (Wade 1998, Marshall et al. 2008). POI, a trait with fundamental ecological and evolutionary implications (Lack 1947, Vance 1973, Smith & Fretwell 1974), is influenced by the maternal environment: abiotic factors such as temperature (Blackenhorn 2000, Fischer et al. 2003 a, b, c) and biotic factors such as food availability (George et al. 1990, George 1996), density, competition, predator interactions have been associated with shifts in POI. The best studied is temperature: laboratory manipulations have documented greater POI with decreasing maternal temperature (Ernsting & Isaaks 1997, Blackenhorn 2000, Fischer et al. 2003 a, b, c, Fisher et al. 2009).

1.1.1 Problems with Per Offspring Investment proxies

Above, POI was briefly defined as 'the quantity and quality of resources allocated to individual offspring'. More specifically, POI is 'the [energy content] of a propagule once it has become independent of maternal nutritional investment. According to this definition, the [energy content] of a freely spawned egg is the appropriate measure of [POI] but the [energy content] of a direct developing snail egg before the embryo has ingested nurse eggs is not' (adapted from Marshall & Keough 2007 [text in square brackets]). Originally, Marshall and Keough (2007) defined 'offspring size' as 'the volume of a propagule once it has become independent of maternal nutritional investment. According to this definition, the size of a freely spawned egg is the appropriate measure of offspring size but the size of a direct developing snail egg before the embryo has ingested nurse eggs is not'. The difference between the definition for POI used here, and the definition for 'offspring size' used by Marshall and Keough (2007) highlights an important assumption commonly used in empirical studies of invertebrate reproductive ecology, which must be highlighted before discussing POI variations and plasticity at the intra-specific level. That is, studies of invertebrate reproductive ecology routinely use egg dimensions (often used to calculate egg volume) as a proxy for egg energy content (POI).

Few studies have assessed the relationship between egg dimensions and energy content, especially at the intra-specific level. At the inter-specific level, available data (predominantly for echinoderms) indicate a correlation between egg dimensions and egg energy content; i.e. larger eggs contain more energy than smaller eggs (Strathmann & Vedder 1977, McEdward & Chai 1991, Clarke 1993, Jaeckle 1995, McEdward & Morgan 2001). However, studies have found that similar sized eggs vary in composition, this may be as ecologically important as variations in dimensions (see Bernardo 1996, Fox & Czesak 2000). Further, the relationship between egg dimensions and energy content is not proportional (Strathmann & Vedder 1977, Turner & Lawrence 1979). Analyses of available data have found that whilst a positive correlation exists between egg dimensions and energy content, egg dimensions cannot always be used to accurately predict egg energy content as large eggs contain proportionally more energy than smaller eggs (McEdward & Morgan 2001, Moran et al. 2013).

The correlation between egg dimensions and energy content documented at the inter-specific level is often assumed to occur at the intra-specific level. However, studies which have tested this assumption have found a weak or no correlation between egg dimensions and energy content (Turner & Lawrence 1979, Jones & Simons 1983, McEdward & Coulter 1985, McEdward & Carson 1987, McEdward & Coulter 1987, McEdward & Chai 1991, Bridges 1993, Bernardo 1996 and references therein). These data indicate that within species, and often within the offspring of a single female, there is variation in the energy content of eggs with similar dimensions. Consequently, at the intra-specific level, egg dimensions are a poor proxy for egg energy content (McEdward & Carson 1987, McEdward & Coulter 1987, McEdward & Chai 1991, Jaeckle 1995, McEdward & Morgan 2001). It should be noted that in studies assessing the relationship between egg dimensions and energy content, echinoderms predominant.

Given that at the intra-specific level, the size of progeny is not an accurate or useful proxy for POI, here, it is defined as offspring energy content (see above). Note that the relationship between hatchling size and energy content are poorly reported. As a result of more complex morphology and anatomy, the relationship between size and energy content is probably weaker than that between egg size and energy content. Most literature concerning POI is size based; dry weight biomass data and elemental composition and fatty acid analyses are better proxies but relatively little used, though this is changing (e.g. Wehrtmann & Kattner 1998, Urzúa et al. 2012). Consequently, the following synopsis of

literature concerning variations in POI suffers from the assumption that egg volume is an accurate proxy for POI and the reader should consider these limitations.

1.1.2 Spatial variation in Per Offspring Investment

Variations in POI within species were first documented between populations from differing geographical locations; for example, across latitudinal gradients (Figure 1.1). Generally within marine invertebrates, but especially within crustaceans, a trend for increasing egg volume/biomass, coupled with decreasing fecundity, with increasing latitudes is now relatively well documented (e.g. Crisp 1959, Lönning & Wennerberg 1963, Hagström & Lönning 1967, Efford 1969, Van Dolah & Bird 1980, Lonsdale & Levinton 1985, Wägele 1987, Hadfield 1989, Clarke et al. 1991, Clarke & Gore 1992, Gallardo & Penchaszadeh 2001, Lardies & Castilla 2001, Lardies & Wehrtmann 2001, Timofeev & Sklyar 2002, Brante et al. 2003, Brante et al. 2004, Bas et al. 2007).

In a study assessing egg volume and egg dry weight for the commensal crab, *Pinnaxodes chilensis*, Lardies and Castilla (2001) documented increased egg volume and egg dry weight at higher latitudes along the Chilean coast. At 23°45'S, average egg volumes and egg dry weights for recently extruded (stage I) eggs were 0.048 mm^3 and 2.02 ± 0.19 μg, respectively. At higher latitudes (33°23'S and 39°24'S), for recently extruded (stage I) eggs, average volumes were 0.070 mm^3 and 0.072 mm^3, respectively, and average dry weights were 2.24 ± 0.21 μg and 2.29 ±0.26 μg, respectively (Lardies & Castilla 2001). Egg number per unit weight of female decreased at higher latitudes, indicating decreased fecundity coupled with increased egg volume and egg dry weight (Lardies & Castilla 2001). Similarly, in a study of the snapping shrimp, *Betaeus truncatus*, from three sites along the Chilean coast (30°07'S, 41°35'S, 42°25'S), Lardies and Wehrtmann (2001) found average volume (0.057, 0.090, 0.097 mm^3, respectively) and dry mass (17.60 ± 8.46 μg, 37.21 ± 5.21 μg, 44.08 ± 4.7 μg, respectively) of recently extruded (stage I) eggs increased with latitude. Coupled with this was a decrease in average clutch size from 399 eggs at 30°07'S latitude to 234 eggs at 42°25'S latitude. At these same three sites along the Chilean coast (30°07'S, 41°35'S, 42°25'S), Wehrtmann and Kattner (1998) assessed egg volume and egg fatty acid composition for the caridean shrimp, *Nauticaris magellanica*, and found no latitudinal cline in either volume or fatty acid composition of recently extruded (stage I) eggs. Similarly, Jones and Simons (1983) found no latitudinal cline in POI for the mud crab, *Helice crassa*, from New Zealand. Both studies did, however,

observed that the percentage change in egg volume during embryogenesis increased with increasing latitude (Jones & Simons 1983, Wehrtmann & Kattner 1998).

Shallow-water fauna are the subject of most studies documenting spatial (latitudinal) trends in POI. However, latitudinal trends in POI are also known from deep-sea species: Wägele (1987) documented greater egg volume at higher latitudes for the Antarctic isopod, *Ceratoserolis trilobitoides*. For the same species, Clarke and Gore (1992), documented increased egg volume and egg dry mass at higher latitudes. Similarly, Clarke et al. (1991) found a trend for greater egg volume and organic content towards high latitudes for the deep-water prawn, *Pandalus borealis*.

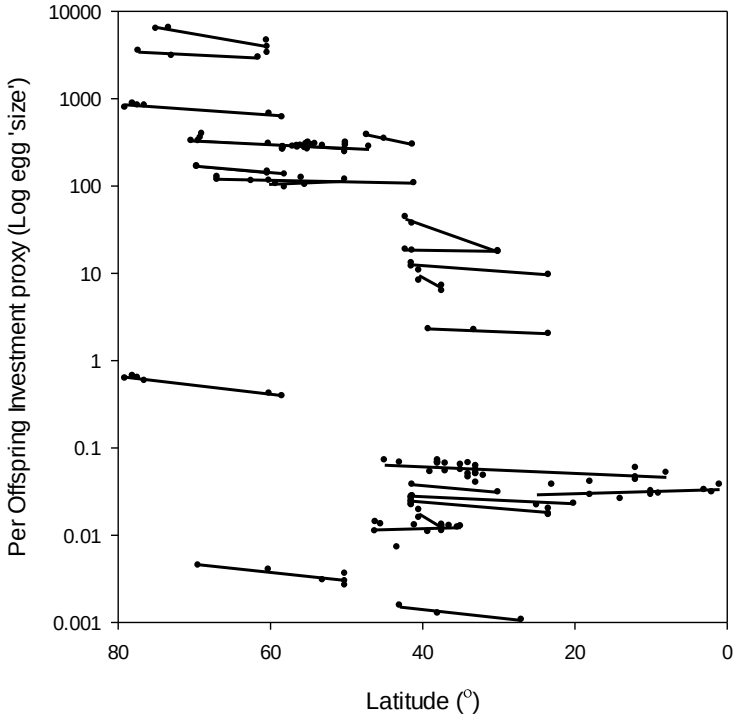

Figure 1.1. Per Offspring Investment variation across latitude for a range of taxa (crustaceans predominate). Units for egg 'size' (used as proxy for POI) differ between studies and between species and are not directly comparable. For data and references, please see Table 7.2 in Appendix 2

Latitude does not affect the biology of organisms, rather environmental factors which vary with latitude, such as temperature, affect biology directly (Jones & Simons 1983b). Non-latitudinal, spatial variation in POI is known. Within the North Atlantic, the barnacle, *Semibalanus balanoides*, shows latitudinal clines in POI on both the European and North American coasts (Barnes & Barnes 1965). POI on the North American coast is

greater than that at similar latitudes on the European coast: e.g. St John, Newfoundland, 47°34' latitude, egg length = 386 μm; Pornic, France, 47°07' latitude, egg length = 282 μm; Hammerfest, Norway, 70°40' latitude, egg length = 329 μm (Barnes & Barnes 1965). Consistent with the pattern that greater POI occurs at higher latitudes where cooler temperatures prevail, these data indicate a trend for larger eggs in areas with more severe winters and cooler summers, irrespective of latitude (Barnes & Barnes 1965). Spatial scale variation in POI between populations may result from selection acting on POI within populations resulting in local adaptation to the immediate environment. Alternatively, spatial scale POI variation may be a form of trans-generational plasticity, resulting from the effects of differing environments on reproduction. Temporal shifts in POI, usually occurring across the breeding season or between breeding seasons, are likely trans-generational plasticity, only.

1.1.3 Temporal plasticity in Per Offspring Investment

Temporal plasticity in POI is known for many species, again, particularly crustaceans (Boddeke 1982, Sheader 1983, Clarke et al. 1985, Sheader 1996, Sampedro et al. 1997, Oh & Hartnoll 2004, Bas et al. 2007, Urzúa et al. 2012, Urzúa & Anger 2013). Most studies document seasonal changes in POI within the same population of a species. For example, winter eggs of the common shrimp, *Crangon crangon*, from the North Sea are known to have greater volume, dry weight, and carbon content than summer eggs (Boddeke 1982, Oh & Hartnoll 2004, Urzúa et al. 2012, Urzúa & Anger 2013). These seasonal differences in POI are also evident in newly hatching larvae (Urzúa & Anger 2013). Interestingly, whilst Boddeke (1982) observed a decrease in fecundity coupled with increased egg volume during winter months, other studies have found no change in fecundity between winter and summer (Henderson & Holmes 1987, Oh & Hartnoll 2004).

For the anomuran crab, *Pisida longicornis*, in the Ría de Arousa (NW Spain), Sampedro et al. (1997) documented an increase in fecundity, coupled with a decrease in egg volume, from January to March-April. Similarly, Bas et al. (2007) collected ovigerous females of the grapsoid crab, *Neohelice granulatus*, from Mar Chiquita (37°45'S latitude) and San Antonio Bay (40°46'S latitude), Argentina, at the beginning (7th Oct 2002 and 26th Nov 2002, respectively) and end (5th Mar 2003 and 27th Jan 2003, respectively) of the reproductive season. Volume, dry mass, carbon, nitrogen, and energy content of eggs were higher at the beginning than at the end of the reproductive season at both sites (Bas et al.

2007). Seasonal variations in fecundity, egg size, and egg content are also known within copepod species (Guisande et al. 1996, Pond et al. 1996, Halsband-Lenk et al. 2001).

Seasonal changes in reproductive output have also been found for species with direct development. Sheader (1996) documented a seasonal effect on POI for the gammarid amphipod *Gammarus insensibilis*. In winter the maximum egg volume (~0.054 mm^3) was ~60% greater than the minimum summer value of 0.034 mm^3. Bell and Fish (1996) documented a marked seasonal variation in the reproductive output for a population of the amphipod, *Pectenogammarus planicrurus*, from Aberystwyth, Wales. During winter breeding, mean egg volume was 0.030 mm^3 and mean brood size was 1.4 eggs. During summer breeding, mean egg volume was 0.023 mm^3 and mean brood size was 5.9 eggs. Similar temporal changes in reproductive output have been documented for other amphipods (Sheader 1983, Clarke et al. 1985, Soto et al. 2006, Yu & Suh 2006).

Consistent with the relationship between POI variation and temperature at the spatial scale, temporal POI plasticity appears related to temperature, with higher POI occurring during cooler winter months. At both spatial and temporal scales, environmental factors other than temperature may be associated with differences in POI; however, temperature is a consistent factor between spatial and temporal scales and laboratory experiments have shown that temperature can drive POI plasticity.

1.1.4 Temperature-mediated Per Offspring Investment plasticity

Laboratory experiments have shown temperature mediated POI plasticity within many species, but mostly in aquatic and terrestrial arthropods (Kerfoot 1974, Perrin 1988, Blanckenhorn 2000, Fischer et al. 2003a, b, c, Liefting et al. 2010). Studies have transferred animals between temperature-controlled conditions months, weeks, and days before, or during oviposition. With few exceptions, greater POI has been observed under cooler conditions, consistent with field observations (Figure 1.2). For example, Patel and Crisp (1960) worked with several species of barnacle and demonstrated POI plasticity within all species: greater POI occurring at lower temperature; consistent with the trend in egg size over latitudinal gradient reported for *Semibalanus balanoides* (Crisp 1959, Barnes & Barnes 1965). Similarly, Perrin (1988) performed experiments on a cladoceran crustacean to separate the effects of maternal size on POI, which is affected by temperature, and the effects of temperature on POI; POI was not influenced by maternal size but was greater at cooler temperatures (Perrin 1988).

More recently, there have been a number of studies investigating temperature driven POI plasticity within terrestrial arthropods (Ernsting & Isaaks 1997, Blanckenhorn 2000, Ernsting & Isaaks 2000, Fischer et al. 2003a, b, c, Liefting et al. 2010). In all cases greater POI was documented under cooler conditions. For example, within the butterfly, *Bicyclus anynana*, mothers ovipositioning at 20 °C produced larger eggs than at 27 °C: 0.74 mm^2 and 0.66 mm^2, respectively (Fischer et al. 2003c), 0.737 ± 0.008 mm^2 and 0.627 ± 0.006 mm^2, respectively (Fischer et al. 2003a). Similarly, within the springtail, *Orchesella cincta*, POI was lower at 20 °C than at 16 °C in terms of egg volume (0.0054 ± 1.7 $*10^{-4}$ mm^3 vs. 0.0089 ± 2.7 $*10^{-4}$ mm^3, respectively), dry weight (2.23 ± 0.09 μg vs. 2.95 ± 0.10 μg, respectively), and percentage lipid content (13.99 % vs. 17.29 %, respectively) (Liefting et al. 2010). The consistent and pervasive nature of this plasticity has stimulated discussion over the fitness consequences, adaptive value, and potential physiological mechanisms of temperature-mediated shifts in POI.

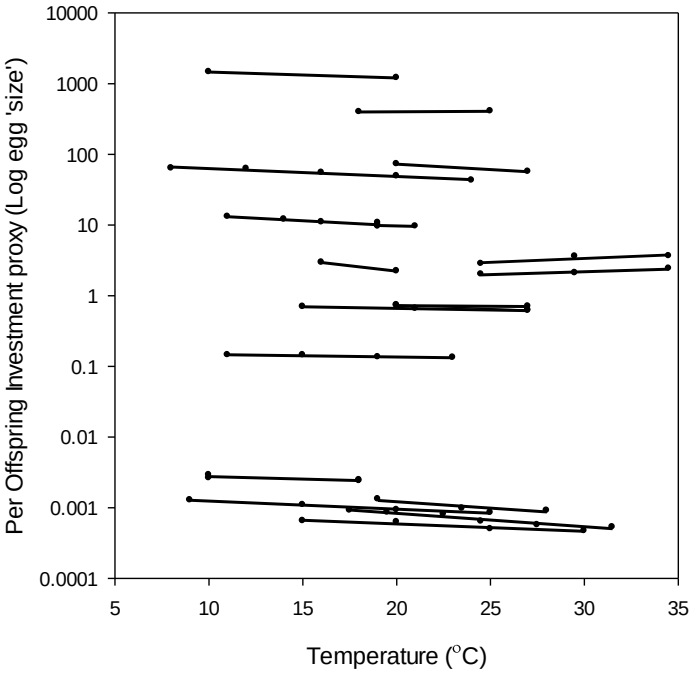

Figure 1.2. Per Offspring Investment plasticity across temperatures for a range of taxa (arthropods predominate). Units for egg 'size' (used as proxy for POI) differ between species and between studies and are not directly comparable. For data and references, please see Table 7.3 in Appendix 3

1.1.5 Fitness implications of Per Offspring Investment

Despite the demonstrating of temperature mediated variations in POI within species (Figure 1.2), the adaptive significance of this plasticity is not well studied. Few studies transfer eggs and offspring between temperatures to form a fully-crossed experimental design (e.g. Blanckenhorn 2000, Fischer et al. 2003a, b, Liefting et al. 2010, Burgess & Marshall 2011). These few studies have generally found that higher POI is more advantageous under all temperature conditions, yielding larger offspring size with higher hatching success, survivorship, and resistance to extreme conditions. Conversely, within a bryozoan species, smaller offspring produced under warmer temperatures had higher dispersal potential and greater metamorphosis success under all temperatures (Burgess & Marshall 2011). These studies do not support the adaptive hypothesis, but find that generally, higher POI is advantageous under all conditions. Importantly, the trade-off between fecundity and POI is not considered fully enough and it may be that under more benign conditions, the fecundity advantage associated with producing offspring of low POI outweighs the fitness advantage of producing offspring of high POI (Fischer et al. 2003b). Further, studies focus on the egg and larval stages, future studies must consider carry-over (latent) effects into adult stages and the full life cycle.

The ecological and evolutionary implications of changes in POI within a species have been investigated through artificial manipulations of POI, or comparisons of offspring with different POI. Within echinoderms, POI has been artificially reduced via centrifuging to remove lipids (Emlet & Høegh-Guldberg 1997), or by isolating cells from embryos at the two- and four-cell stage: so-called ‘twinning’ (Sinervo & McEdward 1988, Hart 1995, Allen et al. 2006, Allen 2012). These experiments indicate that changes in POI have differing implications within echinoderm species with feeding vs. non-feeding larvae. Reduced POI within feeding larvae is primarily associated with decreased development rate and increased development time, whilst for non-feeding larvae it is related to reduced juvenile size, only (Sinervo & McEdward 1988, Hart 1995, Emlet & Høegh-Guldberg 1997, Allen et al. 2006, Allen 2012). Within a species with feeding larvae, reduced POI causes initial larval morphology to converge with that of a congeneric species with relatively lower POI; indicating that species level differences in larval form and function may result from differences in POI (Sinervo & McEdward 1988). Reductions in POI appear to have stronger effects on larval development time in species with lesser POI, this effect is not linear but increases exponentially with lesser POI (Allen 2012).

Within crustaceans, specifically decapods, experiments have used between brood variations in POI to investigate the implications of differing POI for offspring fitness. Greater POI is correlated with greater survivorship, nutritional independence, and starvation resistance and has also been associated with reduced development time, higher growth rates and greater juvenile size (Giménez et al. 2004, Paschke et al. 2004, Anger et al. 2007, Anger & Hayd 2010).

Few studies have attempted to assess the implications of POI in the field. Field out-planting experiments within the intertidal gastropod, *Nucella ostrina*, found that larger hatchlings had greater growth, shorter time to maturity and higher survivorship, but these advantages were less under poorer conditions; i.e. conditions which resulted in higher overall mortality (Moran & Emlet 2001). Similarly, field experiments with the ascidian, *Microcosmus squamiger*, found that larger settled individuals had higher growth rates but not greater survivorship; between two life stages, larger offspring were beneficial (Rius et al. 2010).

Experiments assessing the effects of POI on egg and offspring fitness, and those focused on temperature driven shifts in POI, consistently find that high POI is advantageous under all conditions. Unfortunately, the trade-off between POI and fecundity and how this may change with environment has received too little attention at the intra-specific level and accurate conclusions on the adaptive value of POI plasticity are not possible. Before considering what advantages high POI under cooler conditions may be, the mechanism by which POI plasticity occurs must first be considered.

1.1.6 Physiological mechanisms by which Per Offspring Investment plasticity arises

The temperature-size rule is estimated to occur in >80 % of ectothermic organisms (including animals, plants, protozoans, and bacteria) and describes the tendency for ectotherms to grow to a larger size when reared under lower temperatures (Atkinson 1994). Van der Have and de Jong (1996) developed a biophysical model for describing the temperature-size rule, based on the Sharpe-Schoolfield equation, which links biological rates to enzyme kinetics (Sharpe & DeMichele 1977, Schoolfield et al. 1981). Development consists of differentiation and growth. Within van der Have and de Jong's model, differentiation and growth rates are each controlled by a single rate-limiting enzyme (as in the Sharpe-Schoolfield equation), further, differentiation and growth have

different temperature coefficients (Figure 1.3). Differences in the temperature coefficients of differentiation and growth determine the slope of the temperature-size reaction norm (Figure 1.3). Van der Have and de Jong (1996) consider that differentiation rates are primarily dependent upon DNA replication rates whilst growth rates are primarily dependent upon rates of protein synthesis. The temperature coefficients of DNA replication and protein synthesis differ as a result of the molecular sizes involved: ribosomal subunits involved in protein synthesis are much larger than DNA polymerase, the enzyme involved in DNA replication. Ribosomal subunits must diffuse within the cytoplasm, assemble into ribosomes and form complexes with mRNA. DNA polymerase must diffuse within the nucleus and assemble with DNA. As a result of the much larger size of ribosomal subunits, protein synthesis is limited by diffusion rates which are generally independent of temperature. Differentiation is limited not by diffusion rates but by enzymatic rates; consequently, the temperature coefficient of growth can be expected to be different and lower than that of differentiation.

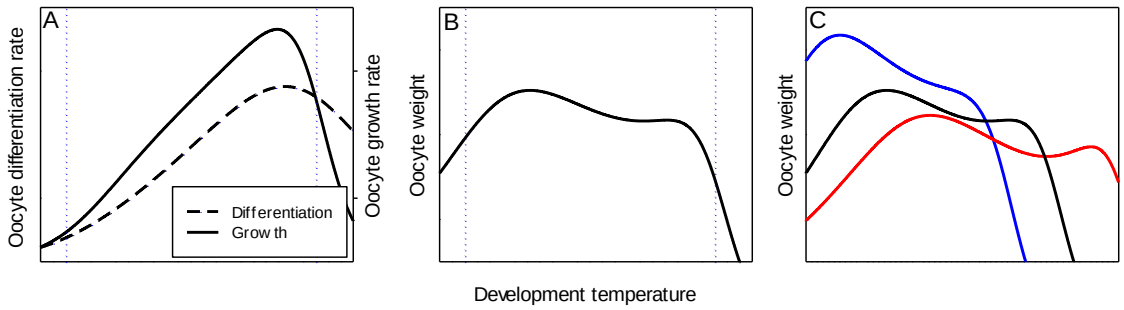

Figure 1.3. **A** oocyte differentiation and growth rates with development time. **B** oocyte weight against temperature. **C** different reaction norms resulting from differing reaction rate coefficients. **A** and **B** redrawn from Van der Have and de Jong (1996)

Van der Have and de Jong (1996) proposed that their biophysical model could be applied to temperature mediated propagule size. During oocyte production, oocyte size is determined by the relationship between oocyte production (differentiation) and vitellogenesis (growth). Studies of temperature driven POI plasticity within the carabid beetle *Notiophilus biguttatus* (Ernsting & Isaaks 1997, 2000), and of ovarian dynamics during temperature mediated POI plasticity in the yellow dung fly *Scathophaga stercoraria* (Blanckenhorn & Henseler 2005), and the tropical butterfly *Bicyclus anynana* (Steigenga & Fischer 2005) offer empirical support to van der Have and de Jong's (1996)

biophysical model and hypothesis. The biophysical model provides a description of how temperature may influence POI without inferring complex regulatory genes. Consequently, the temperature mediated POI plasticity (and the temperature-size rule) does not require a general adaptive explanation, which has, as yet, eluded researchers. Although this hypothesis implicates biophysical factors, natural selection may have manipulated such physiological processes resulting in adaptive POI plasticity (Steigenga & Fischer 2005). Adaptive evolutionary changes in the temperature coefficients of differentiation and growth may change the shape of the temperature-size reaction norm to become adaptive (Figure 1.3C).

Reduced somatic maintenance costs at lower temperatures have also been implicated in temperature mediated POI plasticity (Lonsdale & Levinton 1985, Avelar 1993, Fox & Czesak 2000); however, Ernsting and Isaaks (1997), Fischer et al. (2003b), and Steigenga and Fischer (2005) all provide evidence to the contrary. Ernsting and Isaaks (1997) reported that for the carabid beetle *Notiophilus bigattatus*, a limited food ration caused a reduction in total egg production (compared with an unlimited food ration) but POI was not influenced by food ration; indicating that the resources available for reproduction do not influence POI (Ernsting & Isaaks 1997, 2000). Fischer et al. (2003b) found that for the butterfly *Bicyclus anynana*, despite POI being lower at 27 °C compared to 20 °C, because fecundity was greater at the higher temperature (417.4 ± 89.5 eggs vs. 323.8 ± 92.7 eggs), the mean total egg mass was higher at 27 °C (175.2 ± 39.6 mg) than at 20 °C (140.9 ± 40.5 mg). Again, indicating that the resources available to reproduction do not affect POI. Further and for the same butterfly, Steigenga and Fischer (2005) demonstrated that reduced somatic maintenance costs at lower temperatures did not enable the allocation of greater resources to reproduction as reproductive investment was greater at higher temperatures.

Interestingly, Sheader (1983) highlighted that if the relative proportion of the intermoult period available for oocyte growth decreases with increasing temperature, then POI would decrease. Given that most temperature driven POI plasticity has been documented within arthropods, this hypothesis has received little attention.

1.1.7 Adaptive value of high POI under cooler temperatures

With increasing latitude there is an increasing mis-match between shorter periods of food availability, which result from increasing seasonality, and longer larval

development periods, which result from slower development rates at lower temperatures. This is thought to have selected for the macro-ecological trend in reproductive and development modes at the inter-specific level. Similarly, at the intra-specific level, POI plasticity may better provision higher latitude offspring for these conditions. Higher POI may provide greater independence from external food sources and enable greater development rates and more abbreviated development. However, POI plasticity and latitudinal clines are known for species with direct development (Sheader 1983, Clarke et al. 1985, Wägele 1987, Hadfield 1989, Clarke & Gore 1992, Bell & Fish 1996, Sheader 1996, Soto et al. 2006b, Yu & Suh 2006), indicating that a mis-match between short food availability and long larval development cannot be implicated for these species. Clarke (1992), suggested that longer embryonic and larval development at lower temperatures requires more energy for maintenance during this time; therefore greater POI is required to supply the energy for development at low temperatures.

1.2 Larval developmental plasticity

Phenotypic plasticity during development (developmental plasticity), enables organisms to cope better with environmental variations (Knowlton 1974, Boidron-Metairon 1988, Pfennig 1990, Hart & Strathmann 1994, Esperk et al. 2007). Changes in the morphology of feeding larvae of echinoderms and molluscs have been demonstrated in response to levels of food availability (Boidron-Metairon 1988, Strathmann et al. 1993, Hart & Strathmann 1994, Hadfield & Strathmann 1996). Feeding larvae within these groups are suspension feeders, collecting food particles on ciliated bands and then transporting these particles to the mouth. In response to low food availability, cilia lengths and ciliated band lengths, which collect food, and mouth and stomach sizes, which process food, may increase (Boidron-Metairon 1988, Strathmann et al. 1993, Hart & Strathmann 1994, Hadfield & Strathmann 1996). These morphological changes increase feeding and food processing efficiency, enabling the more rapid extraction and accumulation of energy from the environment.

Within crustaceans, development proceeds via a series of moults which are accompanied by changes and advances in morphology, physiology, and behaviour as larvae grow and develop into juveniles. Developmental plasticity is manifest as variations in the number of larval stages during development; no plasticity in feeding structure morphology has been observed within crustaceans, thus, no change in feeding capability

occurs in response to the environment. Additional larval stages, which may occur during development when conditions are unfavourable to growth and development, extend the larval phase: prolonging the period during which larvae may extract and accumulate energy from the environment.

Within echinoderms, there appears a latitudinal gradient in developmental plasticity in larval feeding structures: cold, temperate species show a greater degree of plasticity than warm, subtropical and tropical species (McAlister 2008 and references therein). Tropical echinoderm larvae have, relative to body size, large feeding structures (longer arms). Within temperate species and under high food conditions, larval feeding structures are not enlarged enabling early development of the juvenile rudiment. Under low food conditions, larval feeding structures are enlarged and development of the juvenile rudiment is delayed (Boidron-Metairon 1988, Strathmann et al. 1993, Hart & Strathmann 1994, Hadfield & Strathmann 1996). Early development of post-metamorphic features may be advantageous under conditions which slow development (e.g. low temperature) and when the season for reproduction may be short. Such developmental plasticity comes about through the selection away from the production of large 'superfluous' feeding structures and long larval phases when food is abundant, but the retaining of the 'option' to express them if needed, and may be permitted by increases in POI. Therefore, developmental plasticity maximises the fitness potential of higher POI.

Within crustaceans, few data are available on differences in developmental plasticity across environmental gradients. Thatje and Bacardit (2000) reported that within a high latitude population of the caridean shrimp, *Nauticaris magellanica*, larvae developed walking legs (juvenile features) earlier than lower latitude population. This would appear to be an analogous trend, the earlier development of juvenile features at higher latitudes to reduce larval period.

1.3 Interaction between Per Offspring Investment and larval developmental plasticity

Too few studies have assessed the interaction between POI and developmental plasticity within marine invertebrates; those which have are limited to echinoderms and crustaceans. The changes associated with the interaction between POI and developmental plasticity are often analogous to the changes induced by external food availability. Within echinoderm species with feeding larvae, reduced POI yields larvae with lower

developmental plasticity (McAlister 2007), suggesting that plasticity is enabled by higher POI and may maximise potential fitness advantages provided by higher POI. Similarly, facultative feeding larvae show less plasticity than feeding larvae (Podolsky & McAlister 2005). Within crustaceans, POI has been related to instar number: higher POI yields larvae which develop through fewer larval instars (Anger 2001, Giménez et al. 2004, Giménez 2006). These results suggest that high POI provides greater nutritional independence, and faster development through fewer instars.

The relationship between developmental plasticity and POI suggests that larvae which exhibit developmental plasticity are 'pre-adapted' to an evolutionary transition towards non-feeding development: i.e. in response to greater internal provisioning, larvae do not develop superfluous, large feeding structures or develop through superfluous, long pathways. Instead, larvae may develop juvenile features earlier, abbreviating development. Thus in the evolutionary transition towards non-feeding larvae, minimal genetic change is necessary, although once fully independent of external food supply, larvae may lose feeding (and even swimming) structures altogether and become obligatory non-feeding. This transition presumably requires additional genetic change. Also, developmental plasticity may increase selection for increasing POI with latitude as developmental plasticity maximises the potential fitness implications of POI. Under the same conditions, better provisioned larvae do not invest so heavily in superfluous feeding structures and long development pathway thus may out-compete more poorly provisioned larvae under unfavourable conditions.

1.4 Study species: *Palaemonetes varians* Leach, 1814 (Decapoda: Caridea: Palaemonidae)

Palaemonetes varians (Figure 1.4), commonly referred to as the variable shrimp or ditch shrimp, is a brackish-living species of the Palaemoninae Rafinesque, 1815, a subfamily of the caridean family Palaemonidae Rafinesque, 1815 (De Grave et al. 2009). The Palaemoninae (372 extant species) are numerically dominated by three genera: *Macrobrachium* Spence Bate, 1868 (243 species), *Palaemon* Weber, 1795 (41 species), and *Palaemonetes* Heller, 1869 (31 species) (De Grave et al. 2009). The synonymous genera *Palaemon* and *Palaemonetes* have marine, brackish, and freshwater representatives and are globally distributed in tropical and temperature regions (Ashelby et al. 2012). Several colonisations of freshwater habitats have likely occurred within the Palaemoninae

(Ashelby et al. 2012) and have been direct, via brackish waters (Vogt 2013). Fauna which inhabit brackish and freshwater environments show reproductive, developmental, and general life history adaptations to the low food and low salinity conditions in lotic systems (Vogt 2013). Given that there are representatives of the Palaemoninae in marine, brackish, and freshwaters, many reproductive, developmental, and life histories exist within this group. Consequently, representatives, particularly from *Macrobrachium*, have been used to study the evolutionary process of freshwater colonisation (see Ashelby et al. 2012, Anger 2013, Vogt 2013).

Figure 1.4 *Palaemonetes varians*, dorso-lateral view of adult female brooding late stage embryos; eyes in embryos visible as dark spots

Within the Palaemoninae, morphology is highly conserved making distinguishing taxa difficult and problematic (Ashelby et al. 2012 and references therein). This likely contributed to the grouping of several species of *Palaemonetes* as 'biological varieties' or sub-species within a *Palaemonetes varians* species complex, the type species of *Palaemonetes*. These biological varieties, *Palaemonetes varians microgenitor*, *P. varians mesogenitor*, and *P. varians macrogenitor*, are now classified as distinct species: *P. varians*, *P. mesogenitor*, and *P. antennarius*, respectively. In part, these biological varieties were defined by their differing reproductive and development traits, specifically egg size, larval morphology, and instar number. *P. mesogenitor* and *P. antennarius* inhabit freshwater river and lake systems and reproduce via larger eggs, larger larvae, and more abbreviated larval development than *P. varians*. In older literature, there is therefore some discussion about variation in egg size with habitat, and in larval development within the *Palaemonetes varians* species-complex (e.g. Gurney 1924, 1942, Thorson 1946).

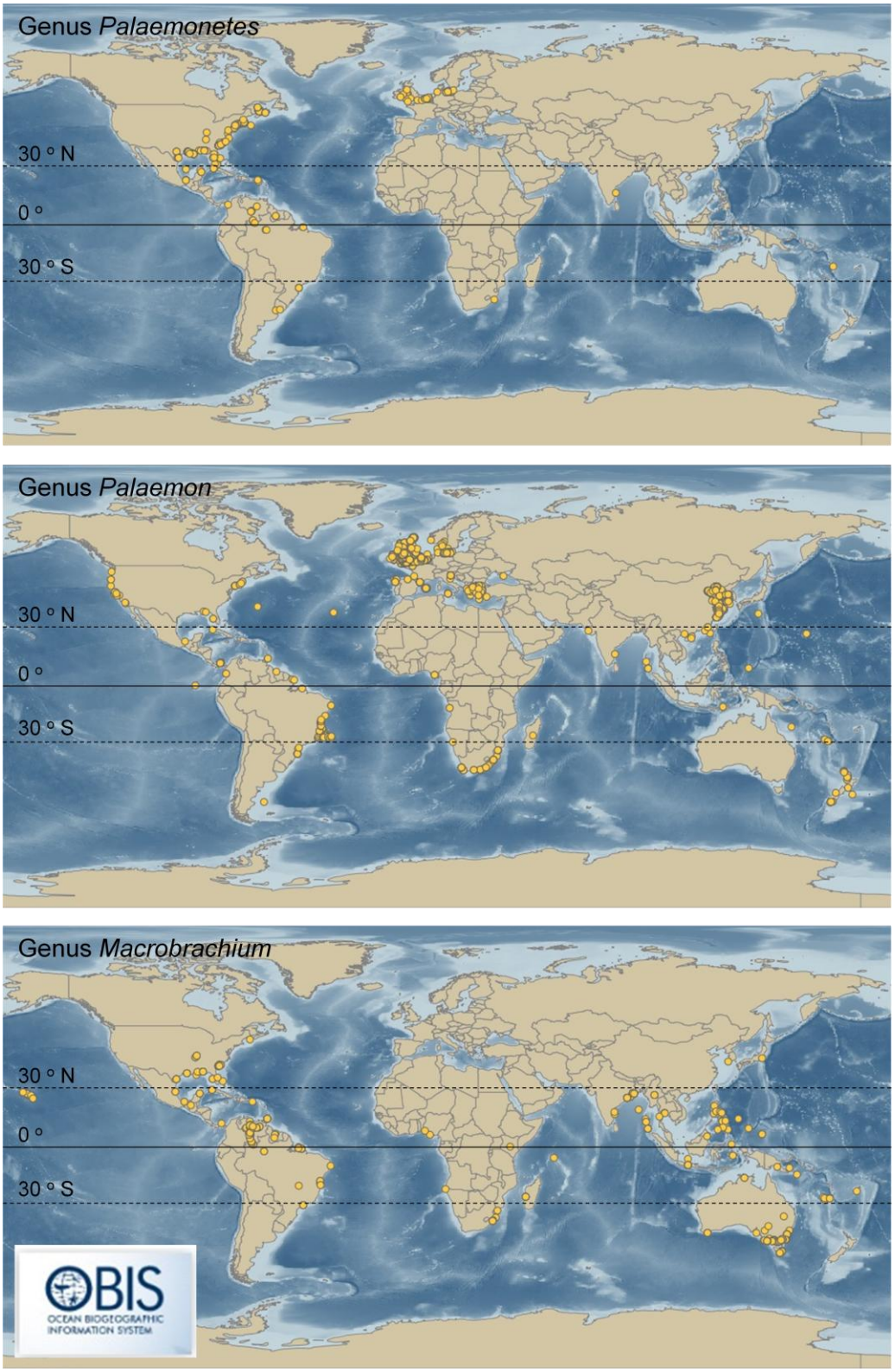

Figure 1.5. Global distributions of Palaemoninae shrimp of genus *Palaemonetes*, *Palaemon*, and *Macrobrachium*. Data from OBIS (accessed 10/09/13)

1.4.1 Recent evolution and ancestry

The Palaemonidae are generally thought to have arisen from an ancestral tropical marine clade (for review see Anger 2013). Within the Palaemoninae, the genera *Macrobrachium*, *Palaemon*, and *Palaemonetes* are absent from high latitude waters and generally have tropical, subtropical and temperature distributions (Figure 1.5). *Palaemon* and *Palaemonetes* are monophyletic: the '*Palaemon*' Clade (Ashelby et al. 2012). Phylogenetic analysis of this '*Palaemon*' Clade supports 6 clades which are largely associated with current biogeographic distributions of species: two Asian clades, an American clade comprising North and South American species, an Australian clade, an Atlanto-Pacific clade comprising European species, African species and an indo-pacific species, and an Indo-Pacific clade (Ashelby et al. 2012). The phylogeny of *Macrobrachium* is also associated with current biogeographic distribution (Murphy & Austin 2005). The global distribution of *Macrobrachium* coupled with the phylogeny of this group have been used to suggest that *Macrobrachium* radiated during the Mesozoic in the Tethys Sea (Anger 2013). The phylogeny of the '*Palaemon*' clade suggests similar phylobiogeography to that of *Macrobrachium* and, perhaps, similar evolutionary history. The '*Palaemon*' clade may too have originated, radiated and dispersed through the Tethrys Sea.

1.4.2 Distribution and ecology

Palaemonetes varians is abundant in shallow, brackish waters (2 - >45 salinity e.g. peripheral estuarine habitats, drainage channels, salt marshes, coastal ponds and lagoons) along the North-East Atlantic coast of Western Europe: from Southern Norway to Morocco and a few scattered locations within the Mediterranean Sea (Gurney 1924, Dolmen 1997, Falciai 2001, Hindley 2001, González-Ortegón & Cuesta 2006 and references therein). Within the UK, distribution is near ubiquitous as a result of the abundant estuarine and salt-marsh habitats and macro-tidal hydrodynamics, which aid larval dispersal between such habitats (Hindley 2001). These habitats may be highly turbid, prone to periods of hypoxia and commonly experience large fluctuations in temperature, salinity, and oxygen concentration on tidal, daily and seasonal cycles. *Palaemonetes varians* occurs at high population densities under such fluctuating conditions and, as such, has been the focus of physiological studies in relation to temperature (Cottin et al. 2010, Oliphant et al. 2011, Ravaux et al. 2012, Smith et al. 2013), salinity (Lofts 1956, Antonopoulou & Emson 1988, Nugegoda & Rainbow 1989), reproductive cycle (Bouchon 1991a, b), hypoxia and anoxia

(Nielsen & Hagerman 1998), metal uptake and detoxification (Nugegoda & Rainbow 1989, Rainbow et al. 2006, Gonzalez-Rey et al. 2007, Gonzalez-Rey et al. 2008, Rainbow & Smith 2013), the control of masculinisation (Frelon et al. 1993), and behavioural and ecological studies (Jefferies 1958, 1964, Roberts 1995, Hindley 2001, Dolmen et al. 2004, Aguzzi et al. 2005). Recently, *P. varians* has served as a shallow-water physiological comparison for studies of deep-sea hydrothermal vent shrimp (Gonzalez-Rey et al. 2007, Gonzalez-Rey et al. 2008, Cottin et al. 2010, Oliphant et al. 2011, Cottin et al. 2012, Smith et al. 2013) and has also received attention as an aquaculture crop and feed (Palma et al. 2008, 2009).

Within brackish water habitats, *P. varians* are benthic, associated with aquatic plants, algae, and over-hanging vegetation from the bank, rarely do shrimp swim in mid-water (Hindley 2001, personal observations). The diet of *Palaemonetes varians* is not well studied but probably varied, consisting of a wide variety of benthic and in-fauna, meio- and macro-fauna. A range of wading birds, sea birds, and fishes predate upon *P. varians*. Breed occurs in late spring/ early summer, but the number of broods per female per season and the age at which shrimp mature and may breed to are unreported.

1.4.3 Larval development

During animal development, individuals grow and develop from small, morphologically and physiologically simple forms into their larger and more complex juvenile and adult forms. The arthropod exoskeleton restricts growth and morphological development and is periodically shed and replaced with a larger carapace, which may also differ in morphology. This periodic moulting of the exoskeleton, which is necessary during the larval phase, gives rise to distinct stages of differing morphology. Within decapod crustaceans, these distinct morphological forms have been considered, described, and named as consistent stages of larval development within species.

Gurney (1942) distinguished four phases of decapod larval development: nauplius, protozoea, zoea, and post-larvae. Recently, Anger (2001) considered there to be only three larval phases: nauplius, zoea, and decapodid. These stages have differing modes of locomotion: antennal propulsion in nauplii, thoracic propulsion in zoea, and abdominal propulsion in decapodid. The number of larval instars or stages/moults during each larval phase varies inter-specifically and may also vary, although to a lesser extent, intra-specifically. Caridea and other ‘higher’ decapods pass through zoea and decapodid larval

phases only. Generally, the transitions between these phases may be morphologically, physiologically, and behaviourally well defined and have been considered as metamorphoses (Anger 2001).

Metamorphosis (Gr. *meta*- "change" + *morphe* "form") is widespread among animal taxa, occurring in amphibians, fish, tunicates, cnidarians, echinoderms, molluscs, and arthropods; consequently, interpretations of metamorphosis vary and a single definition may be inadequate (Bishop et al. 2006). Common themes in most definitions of metamorphosis include the transition to a sexual stage and changes in ecology between life stages. Among animal phyla, it may be acceptable to employ Michael Hadfield's definition: metamorphosis is the transition of an animal from a larva to a juvenile; larva being a post-hatching, free-living developmental stage which differs morphologically, physiologically, ecologically, and often in habitat from adults; juvenile being a non-reproductive stage but with most morphological, physiological, and ecological traits of adults; transition meaning all of the morphological, physiological, and behavioural modifications which occurring during the transformation from larva to juvenile (Bishop et al. 2006).

By this definition the transitions between larval phases, from nauplii to zoea and from zoea to decapodid, are not metamorphoses but are a continuation of a protracted metamorphosis. Transitions between larval phases may be considered a form of larval heteromorphosis, which in insect biology is a term used to describe rapid changes in morphology, physiology, and behaviour between larval stages (Nijhout 1994); this term has also been used within decapod biology to denote the growth of wrong appendages (Carmona-Suarez 1990). Consistent with Hadfield's definition, metamorphosis should be reserved for the transition from larva to juvenile. As such, metamorphosis within decapods can be considered to be protracted and analogous to the 'incomplete metamorphosis' within hemimetabolan insects (Nijhout 1994).

Within caridean shrimps, Anger (2001) considered the transition from zoea to decapodid and decapodid to juvenile to be gradual and non-metamorphic. Decapodid and megalopa stages are transitional between pelagic larval stages and benthic juvenile/adult stages and are often the stage at which larvae settle. Within brachyuran and anomuran crabs, the dorsoventrally flattened adult crab form with the abdomen tucked beneath the cephalothorax differs considerably from the more caridoid larval form. The brachyuran and

anomuran megalopae differ considerably from zoeal forms, being intermediate between zoeal and juvenile forms. Consequently, in moulting from the final zoea to the megalopa stage, considerable morphological changes occur (Figures 1.6 and 1.7). As such, the megalopa is easily distinguishable from zoeal stages. In contrast, the caridoid morphology is conserved between larval and adult body forms of non-crab decapods. Consequently, the requisite morphological transition between pelagic larval and benthic adult forms is not so significant and decapodid stages are morphologically similar to zoeal and juvenile stages; distinguishing between them, therefore, may be more problematic (Figures 1.8 and 1.9). Particularly within caridean shrimps, the morphological changes which occur during moulting from the final larval instar to the first juvenile instar may be no more extensive than changes during larval development.

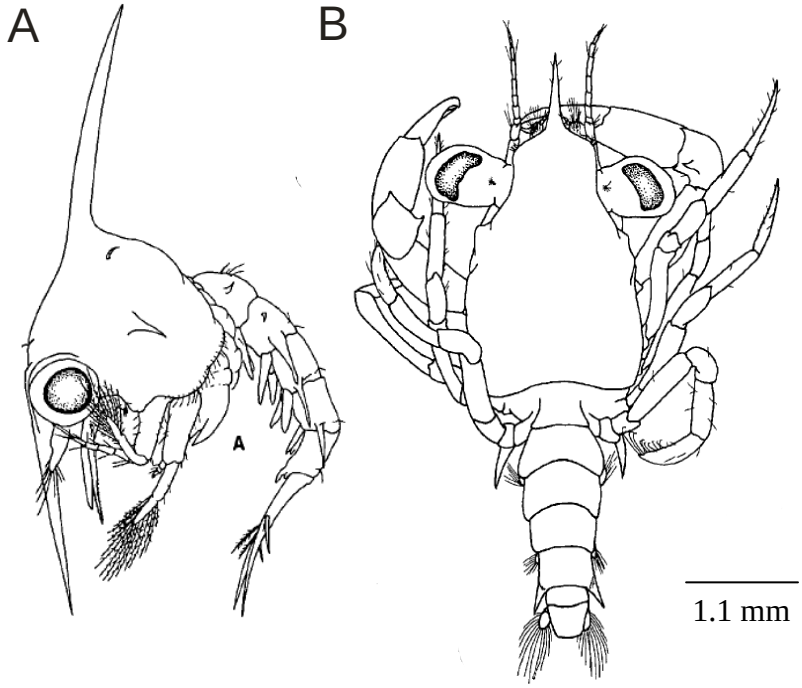

Figure 1.6. **A** lateral view of the final zoea (zoea VII) and **B** dorsal view of the megalopa of the speckled swimming crab, *Arenaeus cribrarius*; from Stuck and Truesdale (1988).

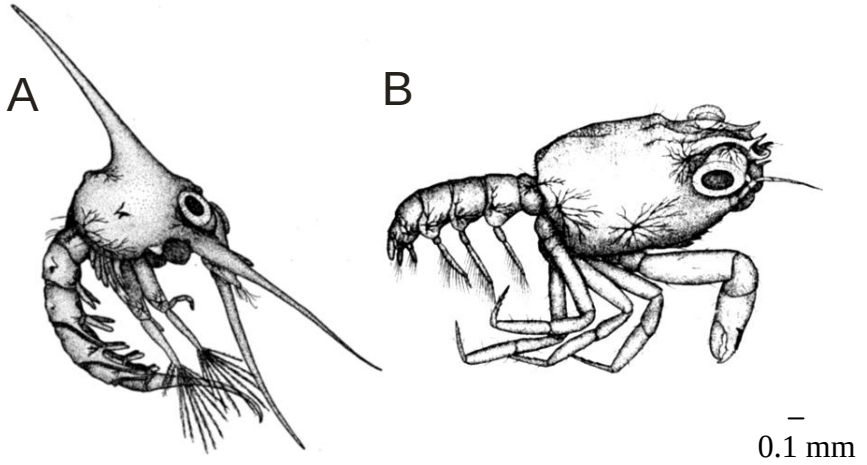

Figure 1.7. **A** lateral view of the final zoea (zoea 4) and **B** dorso-lateral view of the megalopa of the xanthid crab, *Hexapanopeus angustifrons*; from Costlow and Bookhout (1966)

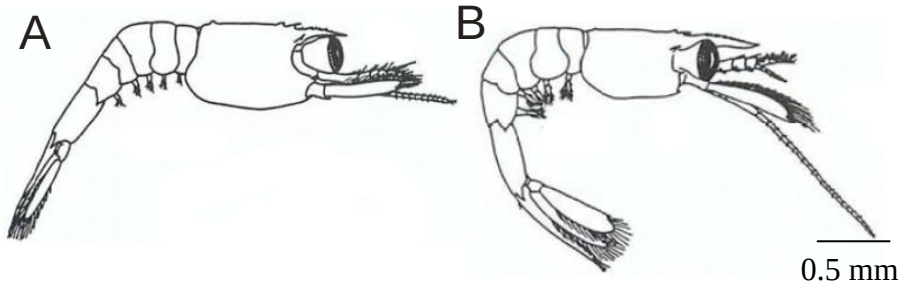

Figure 1.8. **A** lateral view of the final zoea (zoea IX) and **B** lateral view of the decapodid of the hippolytid shrimp, *Nautocaris magellanica* (thoracopods not shown); from Wehrtmann and Albornoz (1998)

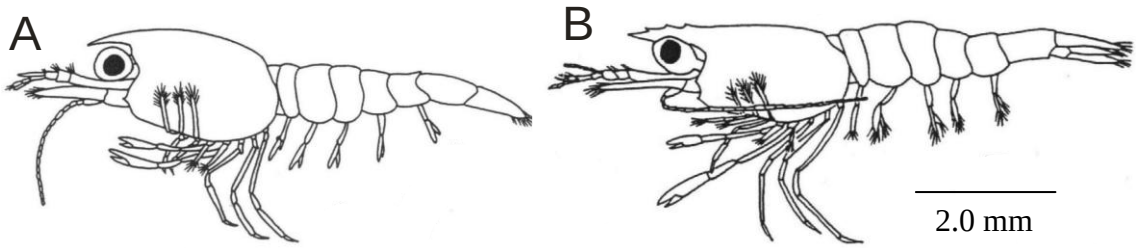

Figure 1.9. **A** lateral view of the final zoea (zoea 2) and **B** lateral view of the decapodid of the palaemonid shrimp, *Macrobrachium iheringi*; from Bueno and Rodrigues (1995)

Brachyuran and anomuran crabs generally develop through relatively fewer larval instars than other decapods and variations in the number of larval instars during development are uncommon. As such, brachyuran and anomuran larval development is relatively consistent (within species), morphological changes between instars are may be considerable, and megalopa are easily distinguishable from zoeal stages. In contrast,

caridean decapods (and other decapods generally) often develop through relatively more larval instars and the number during development may be highly variable. Consequently, morphological changes between instars are smaller than in brachyurans and anormurans and the magnitude of morphological changes may vary with larval instar number plasticity. The morphology of larval instars is a result of the interaction between the processes which control growth and development, and the processes which control the moult cycle (Knowlton 1974). As such, the magnitude of the difference in morphology between consecutive larval stages may differ between habitats with different environmental conditions such as temperature and food availability. Consequently, development is gradual and differentiating between late zoea and decapodid larvae is subjective. For example, late zoea of the palaemonid shrimp *Palaemonetes argentinus* have morphological and physiological traits which may be considered zoeal or decapodid and, as such, the classification of these larvae remains subjective (Anger 2001).

1.4.3.1 Palaemonetes varians larval development

Perhaps owing to the relative ease with which *Palaemonetes varians* larvae may be cultured under laboratory conditions, *P. varians* has been the subject of larval development studies for over 170 years (Fincham 1979). Fincham (1979) provided a detailed list of previous works describing the larval development of *Palaemonetes varians*, either in part or in full. As a result of the classification of *P. mesogenitor* and *P. antennarius* as 'biological variants' of *P. varians*, some of the works included in this list describe the larval development of *P. mesogenitor* or *P. antennarius*. Given the differing developmental traits of these 'biological variants', the larval development of *Palaemonetes varians* was considered polymorphic and even poecilogonic (Fincham 1979). In this context, Fincham (1979) provided a detailed description of the larval development of *P. varians*.

Previously, Gurney (1924) had given an accurate description of *Palaemonetes varians* larval development for shrimp sampled from Wells-Next-The-Sea, Blakeney, Cley-Next-The-Sea, and Salthouse (Norfolk, UK). Unfortunately, Gurney found that 'from stage 3 onwards the changes between moults become so inconsiderable that there cannot be said to be any true metamorphosis' and attributed 'the large proportion of "intermediate" stages to bad conditions provided'. Despite the limitations of the culturing techniques used, Gurney was able to identify five larval stages, the fifth of which had two sub-stages, 5A & 5B: the main differences being that in 5B the flagellum of the antenna is

longer and slender, the telson is longer and more slender, and the pleopods are longer and straight (Gurney 1924).

Fincham (1979) also described five zoea stages for the larval development of *P. varians* sampled from Burnham-on-Crouch estuary (Essex, UK) and reared at 22 ± 0.5 °C (2 °C below the water temperature within the ditches they were collected from). Unlike Gurney (1924), Fincham (1979) did not identify two variant forms of stage 5 nor any deviation from the five instar development; however, he noted considerable morphological variation in same stage instars throughout development. More recently, works based on *P. varians* sampled from Lymington (Hampshire, UK) have considered larval development to include several decapodid stages which correspond to the latter zoeal stages described by Fincham (1979) and Gurney (1924) (e.g. Kerr 2006, Mestre 2008, Smith 2008). Mestre (2008) considered development to proceed through three zoea and two decapodids (decapodids corresponded to zoea 4 and 5; Fincham 1979) and occasionally an additional one of two decapodid stages after the second decapodid. Similarly, Smith (2008) considered development to proceed through two zoeal stages and three decapodids (decapodids corresponded to zoea 3, 4, and 5; Fincham 1979) with an occasional additional decapodid stage after the third decapodid.

Variation in larval instar number and the morphology of same stage larvae is known for many decapods, especially caridean shrimp (Broad 1957, Knowlton 1974, Marakov 1974, Sandifer and Smith 1979). Such variation in morphology and instar number indicates desynchronisation of moulting and epigenesis resulting from differential control of these processes by external conditions (Knowlton 1974, Fincham 1979).

The five instar development within *Palaemonetes varians* is relatively abbreviated for a caridean shrimp and Fincham (1979) and Gurney (1924) noted that pereiopod development was more advanced in early zoeal stages compared with other Palaemoninae shrimp: at stage 2 only pereiopods 3 and 4 are rudimentary, pereiopod 3 is full formed by stage 3 and pereiopod 4 by stage 4. *Palaemonetes varians* is marine/brackish living and has a more abbreviated larval development (five instars) compared with other marine/brackish *Palaemoenetes* species: *P. vulgaris*, 7-11 instars (Broad 1957a); *P. pugio*, 7-11 instars (Broad 1957a); *P. intermedius*, 6-8 instars (Bray 1976). Gurney (1924) considered the habitat of adult *Palaemonetes varians* would favour a more abbreviated larval development ‘and it may only be the frequency of high tides flooding the marshes

that has prevented the acquisition of the type of development characteristic of the land-locked fresh-water form of the species'. Obviously, Gurney is referring to a separate species here, not a sub-species but the point remains valid.

Whilst the salinity tolerance of adult shrimp is relatively well studied, only one study on the salinity tolerance of larval *P. varians* was identified from the literature (Antonopoulou & Emson 1988). This study found that larval development within *P. varians* is feasible at salinities from 5 to 42, indicating that development can occur entirely under estuarine conditions (Antonopoulou & Emson 1988). *Palaemonetes varians* larvae have rarely been sampled from estuarine and marine waters; however, stage 1and 2 larvae and juvenile *P. varians* have been sampled from the lower Ria de Aveiro, Portugal (Pereira et al. 2000).

1.5 Thesis aims and objectives

1.5.1 Thesis rational

The effects of environmental conditions, such as temperature, salinity, and diet during larval development on larval survivorship, development, and growth, are relatively well studied in decapod larvae. Still, a greater physiological understanding of how the external environment affects larval development, particularly developmental plasticity, is requisite. Within decapod larvae, and within the larvae of other marine taxa, knowledge of how maternal effects influence larval development is poor. Further, how maternal effects interact with the larval environment and with larval developmental plasticity is lacking. Critically, understanding of the interaction between POI and environmental conditions during development and their joint influence on larval developmental plasticity is requisite for a better understanding of the evolutionary processes which shape the way in which larvae develop. Such understanding is valuable when considering ecological interactions in a thermally variable environment, evolutionary transitions between larval development modes, and the influence of past, present, and future climates, migrations, and range extensions on such transitions and evolutionary trajectories.

1.5.2 Thesis aims

The broad aims of this thesis were to investigate the interaction between POI and the larval environment on developmental plasticity, with an overall aim of providing a

better understanding of the interaction between these factors on offspring fitness within the context of evolutionary ecology.

Specifically, the thesis aims were:

A_1 to assess natural levels of plasticity in POI within a wild population of *Palaemonetes varians*, both within and between breeding seasons, and to correlate any variation to measured environmental conditions.

A_2 to investigate the effects of environmental conditions (specifically temperature) on the larval development of *Palaemonetes varians*, with special attention focused on developmental plasticity.

A_3 to investigate the interaction between between-brood-variation in POI and environmental conditions (specifically temperature) on the larval development of *Palaemonetes varians*, again with special attention focused on developmental plasticity.

1.5.3 Thesis objectives

To achieve the above aims, the following objectives were necessary (objective numbers correspond to aim numbers, above):

O_1 **i)** Measure the energetic content of newly extruded eggs and newly hatched larvae within a wild population of *Palaemonetes varians* several times during breeding seasons and over several breeding seasons; thus, providing an accurate measure of POI variation across time.

ii) Measure water temperature and salinity within the habitat of this wild population of *Palaemonetes varians* to provide environmental data to compliment POI data.

O_2 **i)** Culture larvae under controlled conditions within the laboratory at different temperatures to determine the thermal tolerance of *Palaemonetes varians* larval development and the effects of temperature on larval development and developmental plasticity. This includes measuring the effect of temperature and developmental plasticity on larval development time, juvenile mass after settlement, and calculating growth rates.

ii) Culture larvae under controlled conditions within the laboratory at different temperatures to investigate the effect of temperature on biochemical changes and

metabolism during larval development to better understand how temperature interacts with larval physiology and affects development. This includes taking respiration rate measurements, larval mass and biochemical composition (C, N) during development.

O_3 **i)** Culture larvae with differing POI under controlled conditions within the laboratory at different temperatures to determine how POI and its interaction with temperature effect larval development and developmental plasticity. This includes measuring the effect of temperature and developmental plasticity on larval development time, juvenile mass after settlement, and calculating growth rates.

1.5.4 Thesis hypothesis

Specific hypotheses are provided within individual chapters. The broad, overarching hypotheses investigated within this thesis were that larval instar number varies and is influenced by environmental factors. Further, per offspring investment influences this interaction. Finally, the interaction between per offspring investment and developmental plasticity has played a role in the evolution of larval developmental modes within decapods crustaceans.

1.5.5 Thesis structure

This thesis comprises a logical progression through the aims and objectives outlined above. In chapters which address the specific aims and objectives detailed above, hypotheses are highlighted within the introductions to these chapters in blue boxes. Firstly, the materials and methods used to collect data for this thesis are detailed in Chapter 2. Chapter 3 then deals with observations of variations in POI from field collected *P. varians* (**A_1**). Chapters 4 and 5 cover experiments which sought to assess the effects of temperature on larval development and developmental plasticity within *P. varians* (**A_2**). Chapter 6 details an experiment which investigated the effects of the interaction between POI and temperature on developmental plasticity (**A_3**). Finally, Chapter 7 synthesises these data, proposes a scenario for the evolution of the macro-ecological trend in POI and associated larval development modes, and finally outlines future work within this field.

2. Materials and Methods

Within this chapter, the general methodology used to collect data for chapters 3-6 is described. Methodological details specific to experiments performed for a chapter are described within the respective chapters. Similarly, statistical analyses of data are described in detail within the respective science chapters, only. The experiments comprising this thesis used the palaemoninae shrimp, *Palaemonetes varians* (Leach 1814) as a study species.

2.1 Collection site

Palaemonetes varians were collected from within an area of salt marsh, known as Oxey Marsh, south of Lymington town (Hampshire, UK; Figure 2.1). This area of salt marsh is bounded by seawalls and connected to the West Solent by a number of sluice gates within these seawalls. Throughout this salt-marsh are numerous inter-connected lagoons and drainage ditches; the collection site was a ~100 m section of drainage ditch (50.4423N, 1.3213W; Figure 2.1). This section of ditch is ~0.5 m deep and varies in width from ~1 to 3 m. Gauze, brambles, grasses, and other scrub vegetation over-hang the edges of the ditch and, during summer months, extensive weed grows within the ditch providing protection for invertebrate fauna (Figure 2.2). Predatory birds, such as white egrets and grey herons, and predatory fishes were noted in this ditch (personal observations). Shrimp were most often caught in high densities adjacent to the bank underneath over-hanging vegetation. During, or shortly after, periods of heavy rainfall and correspondingly low salinity within this section of ditch, shrimp were noticeably less common and had apparently migrated away from this area.

2.1.1 Site monitoring

Environmental conditions at the collection site were monitored to provide accurate information as to the variability in temperature and salinity within the ditch from which *Palaemonetes varians* were collected. Temperature and salinity were considered important abiotic factors which have a direct physiological effect on *Palaemonetes varians*. Temperature data loggers (nke instrumentation S2T600 Temperature data logger, Hennebont, France) were deployed at three sites along the ditch from April 2011 until August 2013, one at each end of the collection site and one in the middle (Figure 2.1). These loggers were cable tied to metal stakes which were pushed into the bottom sediment

adjacent to the bank underneath over hanging vegetation (Figure 2.2). Loggers were in contact with the ditch bottom and so were at a height that shrimp would frequent, indeed shrimp were observed on temperature loggers (personal observations). Temperature data were logged every 30 minutes; these data were periodically downloaded to a laptop taken into the field. The site was visited approximately monthly over three years (April 2011 to August 2013) and water samples were taken from adjacent to temperature data loggers during these visits. These water samples were then transported to the National Oceanography Centre Southampton (NOCS; Hampshire, UK) and their salinity measured using a Hach HQ30d portable conductivity meter.

2.2 *Palaemonetes varians* collection and processing

Adult *Palaemonetes varians* were collected for the purposes of: size frequency analysis throughout the breeding season; sampling recently extruded eggs and recently hatched larvae from ovigerous shrimp for measurements of POI; hatching larvae from ovgerous shrimp for use in experiments; and for breeding shrimp under controlled conditions within the laboratory.

Adult *Palaemonetes varians* were randomly collected by hand netting (using a fisherman's landing net with 6 mm mesh size). Shrimp were placed in 10 litre plastic buckets (fisherbrand, Fischer Scientific) containing water from the point of collection and which were sealed with water-tight lids. These buckets were then transferred (within one hour) to the research aquarium at the NOCS. Here, airstones, attached via airlines to air pumps (Hagen Elite products), were placed in the buckets to provide aeration whilst shrimp were processed.

2.2.1 Adult *Palaemonetes varians* for size frequency analysis

From February 2011 until July 2011, approximately 150 adult shrimp were sampled monthly for size frequency analysis of the population (24th Feb., 24th Mar., 27th Apr., 24th May, 24th Jun., 25th Jul.). During monthly samples in which ovigerous females were collected, those ovigerous females were treated as detailed below in section 2.2.2. Shrimp caught were, once at the NOCS, preserved in 4 % formalin. Later shrimp were sexed (following section 2.2.3; via the presence of the appendix masculine) and the post-orbit carapace length measured (following section 2.5.1).

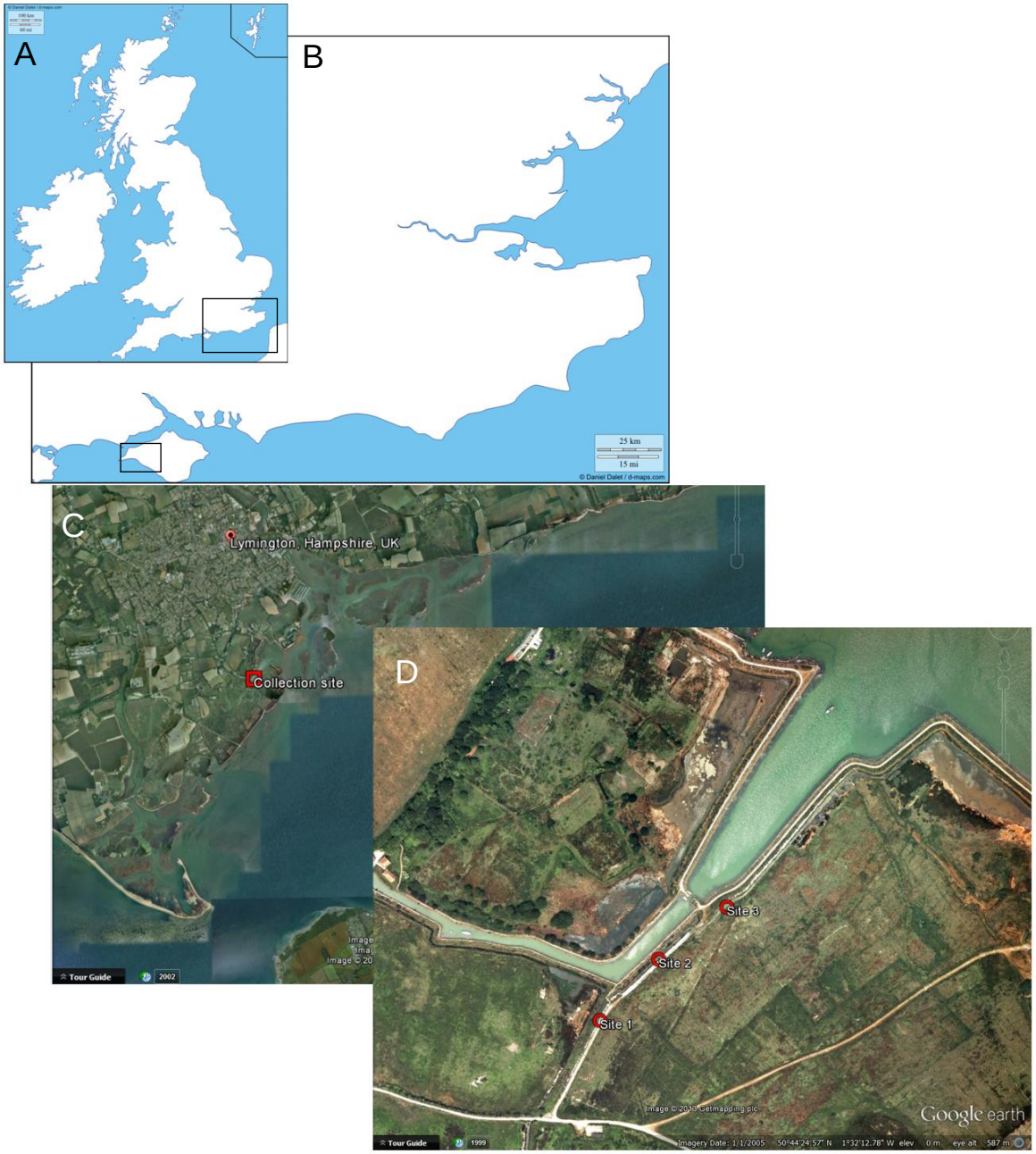

Figure 2.1 **A** map of the UK highlighting south-east England. **B** map of south-east England highlighting the location of the study site. **C** satellite image of the collection site relative to Lymington, Hampshire. **D** satellite image of sites 1-3 along the section of ditch from which collections of *Palaemonetes varians* were made (50.4423N, 1.3213W). A and B from Daniel Dalet/d-maps.com, C and D from Google Earth.

Figure 2.2 Photographs of the drainage ditch on Oxey Marsh from which *Palaemonetes varians* were collected. **A**, **B**, and **D** photographs of sites 1, 2, and 3 where temperature and salinity data were collected; arrows indicate precise locations. **C** photograph of an nke instrumentation S2T600 temperature data logger *in situ* at site 3.

2.2.2 Ovigerous *Palaemonetes varians*

Ovigerous *P. varians* were collected for sampling of newly extruded eggs and newly hatched larvae, and as a source of larvae to be used for experiments. These collections were done during the breeding season (Table 2.1).

Table 2.1. Collection dates of ovigerous *Palaemonetes varians*.

Year	April	May	June	July
2011	27th	24th	24th	27th
2012	25th	24th	26th	24th
2013	26th	28th	25th	23rd

Only ovigerous *P. varians*, identified by the presence of eggs or embryos attached to the pleopods under the abdomen, were retained and transported to NOCS; males and non-ovigerous females were immediately returned to the ditch. Of the ovigerous *P. varians* collected, only those individuals with broods of newly extruded eggs (stages I and II, see below) or embryos close to hatching (stages VII and VIII, see below) were required. The embryonic development of broods was, therefore, assessed and staged according to Müller et al. (2004).

Newly extruded eggs:

> *Stage I (spawning egg)*; no embryonic structures are visible on the surface of the egg. The chorion is transparent and attached to the vitelline membrane of the egg.
>
> *Stage II (cleavage)*; cleavage furrows evident and blastomeres are clearly visible.

Embryos close to hatching:

> Stage *VII (final post-nauplius)*; eyes are large and oval, defining the anterior region of the embryo. Post-nauplius appendages are well developed. Abdominal segments evident as is the telson; telson overlaps the optic lobes.
>
> *Stage VIII (pre-hatching embryo)*; eyes are large and round with ommatidia evident. Antennulae, antennae and mandibles are well developed. Abdomen is in five segments and the cephalothoracic carapace is formed.
>
> (after Müller et al. 2004)

Embryonic development of broods was assessed under a Leica S8AP0 dissection stereomicroscope with lighting provided by a Schott KL 1500 LCD halogen cold light with swan-neck attachment. Ovigerous *P. varians* with stage I and II eggs were selected for sampling of newly extruded eggs (see section 2.3.1). Ovigerous *P. varians* with stage VII and VIII embryos were selected for sampling of newly hatched larvae (see section 2.3.2) and for the hatching of larvae to be used in experiments (see section 2.4.3).

2.2.3 Non-ovigerous *Palaemonetes varians*

Non-ovigerous adult *P. varians* were collected for the inducing of breeding in the laboratory (see section 2.4.2). This collection was done outside of the breeding season, during autumn months (25th November, 2011). All adult *P. varians* hand-netted were

retained and transported to NOCS. For breeding, a sex ratio of ~2:1 females:males was desired within aquaria. *Palaemonetes varians* were sexed under a dissection stereomicroscope. Males were identified by the presence of the appendix masculine on the second pleopod pair, and females by the absence of this appendage.

2.3 Sampling of newly extruded eggs and newly hatched larvae

Newly extruded eggs (stage I and II) and newly hatched larvae (hatched from ovigerous females carrying stage VII and VIII embryos) were sampled for measurements of POI. Dry weight and carbon and nitrogen content of eggs decreases with embryonic development (e.g. Wehrtmann & Lopez 2003), therefore, it was important to sample newly extruded eggs only. Similarly, it was important to sample newly hatched larvae for the same reason.

2.3.1 Newly extruded eggs

Ovigerous *P. varians* with stage I and II eggs were blotted dry, placed within zip-lock plastic bags and frozen at -80 °C in a Kendro Laboratory Products model ULT1386-5-V39 -80 °C freezer. Later, shrimp were defrosted individually and 15 eggs were dissected away from their first right pleopod. Once dissected away from the pleopods, a second assessment of egg development was made under a Leica MZ16 dissection stereomicroscope, again in accordance with Müller et al. (2004); lighting was provided by two Leica CLS 100X lights, one with Leica Photonic Goose neck attachment. If eggs were more developed than previously thought, i.e. more advanced than stages I or II, they were discarded. Eggs were transferred individually to pre-weighed tin capsules (standard weight 6x4 mm, Elemental Microanalysis) and frozen at -80 °C. Later, samples were freeze-dried using a Thermo Scientific Heto PowerDry LL3000 freeze dryer, for 24 hours. These samples were stored in a Secador desiccator before being weighed for dry weight (see section 2.7).

For eggs sampled in all month during 2011, photographs were taken of eggs from five females. Eggs were photographed individually using a Leica DFCZ280 digital camera attached to a Leica MZ16 dissection stereomicroscope. Photographs were taken so that these images could later be used to take measurements of egg size and enable calculations of egg volume (see section 2.5.2).

2.3.2 Newly hatched larvae

Actively swimming larvae were separated from mothers (see section 2.4.1) using a plastic pipette. A sample of 15 larvae per female were individually blotted dry on tissue paper and transferred to pre-weighed tin capsules, frozen at -80 °C, and later freeze-dried for 24 hours. These samples were stored in a desiccator before being weighed for dry weight (see section 2.7).

2.4 *Palaemonetes varians* laboratory maintenance

Adult *Palaemonetes varians* were maintained in the laboratory for two purposes: to hatch larvae from ovigerous females for sampling and use in experiments and to breed adults under controlled and constant conditions. *Palaemonetes varians* were bred under constant conditions in the laboratory to attempt to reduce variability in POI between females.

2.4.1 Ovigerous *Palaemonetes varians*

Post-collection and -assessment of embryonic development (see section 2.2.1), ovigerous *P. varians* with stage VII and VIII embryos were isolated in 1 l plastic buckets (fisherbrand, Fischer Scientific) filled with ~850 ml of 32 salinity, 1 μm-filtered seawater acclimated to the field temperature at the time of collection (17 °C, 2011 or 15 °C, 2012). These buckets were then transferred to LMS Model 230 Series 2 Cooled Incubators (accuracy = ± 0.5 °C) set to the field temperature at the time of collection and 12:12 light:dark cycle. Although manufacturer specifications state ± 0.5 °C accuracy, temperature was found to fluctuate by ± ~1 °C over periods of 6-12 hours. During the period between collection and larval hatching, ovigerous shrimp were maintained under constant conditions at the field temperature at the time of collection (17 °C, 2011 or 15 °C, 2012), 32 salinity, 12:12 (light:dark) and fed Tetra Goldfish flakes three times per week to excess. Water changes (>70 %; 32 salinity, 1 μm-filtered seawater and 17 or 15 °C) were done every second day. Ovigerous shrimp were checked daily (ante meridiem, am) for hatched larvae.

2.4.2 Non-ovigerous *Palaemonetes varians*: breeding in the laboratory

Post-collection and -assessment of sex (see section 2.2.2), adult *P. varians* were transferred to two 14.5 l capacity transparent plastic aquaria containing 10 l of 32 salinity, 1 μm-filtered, seawater which had been acclimated to 11 °C: the field temperature at the time of collection. A sex ratio of 1.5:1 females:males (15 females and 10 males, per

aquarium) was established in the aquaria. Transparent lids for the aquaria were constructed from wooden frames and transparent acetate plastic, fixed to these frames using staples (Figure 2.3). Aeration was provided be airstones, attached via airlines to air pumps (Hagen Elite products), and inserted through holes in aquaria lids. The aquaria were placed within a Thermo Electron Corporation Haake W46 water bath. The temperature within these water baths was controlled using Thermo Electron Corporation Haake DC10 Heater and Circulators (accuracy = ± 0.02 K) and Haake EK20 Immersion Coolers. Lighting was provided by an Anglepoise Model 90PL lamp on a timer to control a light:dark cycle (Figure 2.3). The temperature within the water bath was increased by 1 °C per day until 15 °C was achieved, and the day length increased by 2 hours per day until a 18:6 light:dark cycle was achieved (day length was 8h 32m at time of collection). These conditions (15 °C and 18:6 light:dark) were chosen as warm temperatures and long day length have previously been found to induce breeding in *P. varians* (Bouchon 1991a, b). These conditions were then kept constant during breeding.

2.4.3 *Palaemonetes varians* larval culture

Larvae were cultured under controlled and constant laboratory conditions to assess the effects of temperature, starvation, POI, and their combination on larval development and particularly larval development plasticity. Only larvae hatching within ~10 days of the respective collection dates were used for experiments (mean = 5.2 ± 3.8 days). On hatching, actively swimming larvae were haphazardly separated from mothers (see section 2.4.1) using a 3 ml plastic pipette and transferred to Thermo Scientific Nunc 100 ml clear standard containers, containing ~ 80 ml of 32 salinity, 1 μm-filtered and UV-treated seawater. This seawater was acclimated to the temperature at which ovigerous females were maintained (17 °C, 2011 or 15 °C, 2012). These containers were placed 24 to a tray on plastic trays. Trays were stacked no more than three high and the top tray was covered by a piece of black plastic to minimise evaporation. Stacks of trays were placed in incubators set at the temperatures required in the respective experimental designs and to 12:12 light:dark cycle. Temperatures of 15, 17, 20, 25, 30 °C were used in experiments. For lower temperature (5 and 10 °C), stacks of trays were placed in temperature controlled rooms set to 5 and 10 °C and 12:12 light:dark cycle.

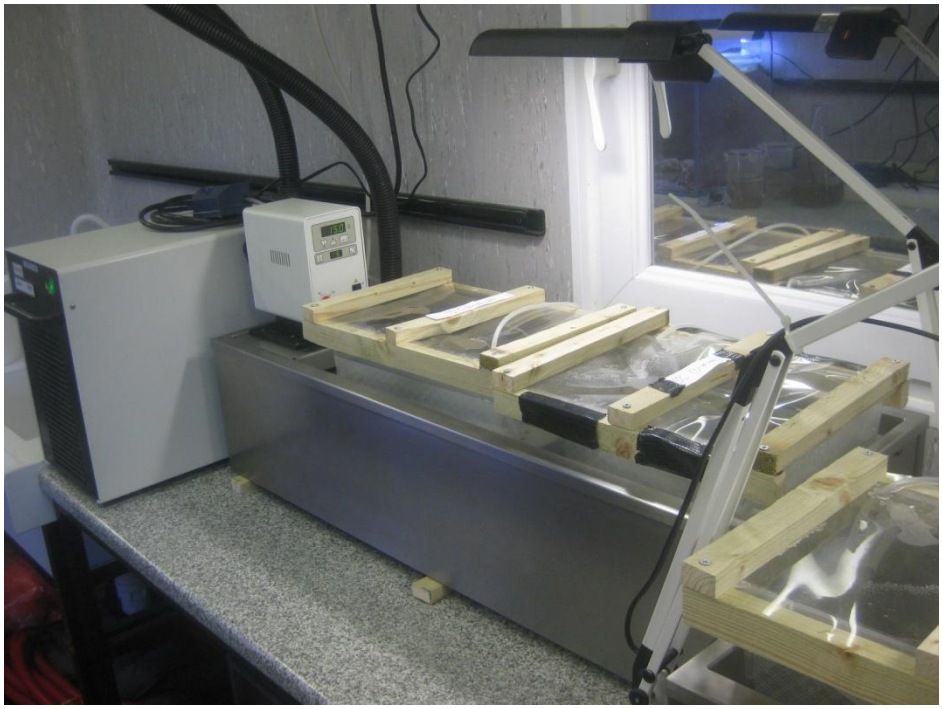

Figure 2.3 Photograph of the set-up used to breed *Palaemonetes varians* under laboratory conditions: 15 °C, 32 salinity, 18:6 light:dark. Thermo Electron Corporation Haake W46 water bath, temperature controlled by a Thermo Electron Corporation Haake DC10 Heater and Circulator (set to 15 °C) coupled with a Haake EK20 Immersion Coolers. Lighting was provided by an Anglepoise Model 90PL lamp on a timer.

Daily, larvae were individually transferred to a square watch glass (embryo glass) using a plastic pipette and inspected under a dissection stereomicroscope. Larvae were assessed for mortality and development. Mortality was assessed by the absence of a visible heartbeat and a milky whitish discolouration of tissues. Larval development was assessed by morphological changes (following Fincham 1979 see section 2.4.4) and by the presence of an exuvia, indicating moulting.

Whilst a larva was in the watch glass, 100 % of the water in its culture cup was changed. Approximately 80 ml of fresh 32 salinity, 1 μm-filtered and UV-treated seawater, acclimated to experimental temperatures, was poured into the container and the larva returned. This was repeated for all larvae. For larvae under high temperatures (25 and 30 °C), water changes were done daily. For larvae under cooler temperatures (5, 10, 15, 17, and 20 °C), water changes were done every second day.

In all experiments, larvae were starved during the first larval instar. On moulting to the second larval instar, and in experiments in which larvae were fed, freshly hatched

Artemia sp. nauplii were introduced into the cups in which larvae were cultured. *Artemia* sp. nauplii were hatched in *Artemia* columns (either 'Hobby Artemia' or 'ZM systems, decapsulated brineshrimp cysts'). *Artemia* sp. nauplii were never >2 days old (usually <1 day old) at the time of feeding. *Artemia* sp. nauplii were provided to excess and were refreshed with water changes (see above).

Upon reaching the juvenile stage, individuals were removed from their culture cups using a plastic pipette and blotted dry on tissue paper. Juveniles were then individually transferred to pre-weighed tine capsules and frozen at -80 °C. Later these samples were freeze dried for 24 hours so that dry weight measurements could be done (see section 2.7).

2.4.4 *Palaemonetes varians* larval nomenclature

Following Smith (2008) and Thatje (personal communications), in this thesis *Palaemonetes varians* is considered to develop through two zoea stages and three decapodid stages. Experiments within Chapters 4, 5, and 6 demonstrate that decapodids 1, 2, and 3 can all be the final larval instar before moulting to the juvenile stage, though moulting from decapodid 1 to the juvenile stage was only observed once.

The 'decapodid' stage is the final larval stage prior to the juvenile stage (Kaestner 1980, Anger 2001). Decapodids have functional pleopods and pereiopods (with or without natatory exopods), and the cephalic and anterior thoracopods (maxillipeds) function as mouthparts (Anger 2001). Also, pereiopod endopods are fully segemented and may be formed into chela (Anger 2001). As discussed earlier (see Chapter 1, section 1.4.3), differentiating between zoea and decapodid larval phases within caridean shrimp can be difficult and subjective. Often, larval instars may have features of both zoeal and decapodid stages. This is the case for the third, four, and fifth larval instars of *Palaemonetes varians*. Morphology varied between individual larvae within the same instar and generally appeared slightly more advanced than descriptions provided by Fincham (1979), though this description was used to differentiate between larval instars. Within stages 4 and 5, considered here as decapodid 2 and 3, pleopod movement was observed and larvae sometimes swam back and forth in a zig-zag pattern, alternating between thoracic and abdominal propulsion. This was less common in decapodid 2. Stage 3, considered here as decapodid 1, was the most zoeal like stage named as a decapodid. Within this stage, pleopods were not functional. This stage was considered a decapodid

given its large size and the fully developed periopods and chela formation (Thatje, personal communications).

Below are the main morphological and developmental changes between larval stages which were used to differentiate between instars (Figure 2.4) (following Fincham 1979 and pers. obs.). These changes are those which were obvious under a dissection stereomicroscope in living larvae and are, therefore, not extensive. The changes in italics are those which were not easily observed in living larvae. After decapodid 1, larvae occasionally moulted and the resulting morphology could not confidently be classified as decapodid 2 or 3 and as such was called 'decapodid'. There could be a series of 'decapodid' moults before the juvenile stage was reached.

Zoea 1- eyes are sessile. Rostrum is straight and the carapace is without spines. Telson is a simple broad fan shape and is continuous with somite 6. *Additionally, somites 1-5 have small pleopod buds. Pereiopods 1-5 are rudimentary and unsegmented.*

Zoea 2- eyes are stalked. Rostrum has a slight downward curve and the carapace has a dorso-medial spine and a pair of supra-orbital spines bent anteriorly. Telson is still a broad fan shape but two developing uropods are visible beneath the carapace. Somite 5 has a pair of spines on the posterior margin. Somites 1-5 have rudimentary pleopods. *Additionally, pereiopods 1, 2 are developed with exopodites and pereiopod 5 is developed but without exopodite. Pereiopods 3, 4 are rudimentary.*

Decapodid 1- carapace has two dorso-medial spines and a fronto-lateral spine beneath the eyes. Pleopods are larger but non-functioning. Telson is narrower but still fan shaped and has a pair of external uropods with another pair of uropods visible beneath the carapace. *Additionally, pereiopods and maxillipeds are developed (fully segmented).Pereiopods 1, 2 are developing into chela.*

Decapodid 2- carapace has three dorso-medial spines. Pleopods are more developed but non-functioning, though movement may be evident. Tail fan is almost fully developed, the telson is rectangular in shape and the two pairs of uropods are external and almost as long as the telson. Antennae are now prominent and obvious. *Additionally, exopods are reduced but still functional. Pereiopods 1, 2 are developing into chela.*

Decapodid 3- antennae are much longer. Pleopods are now fully developed and functional. *Additionally, exopods are further reduced. Pereiopods 1, 2 are developed into chela.*

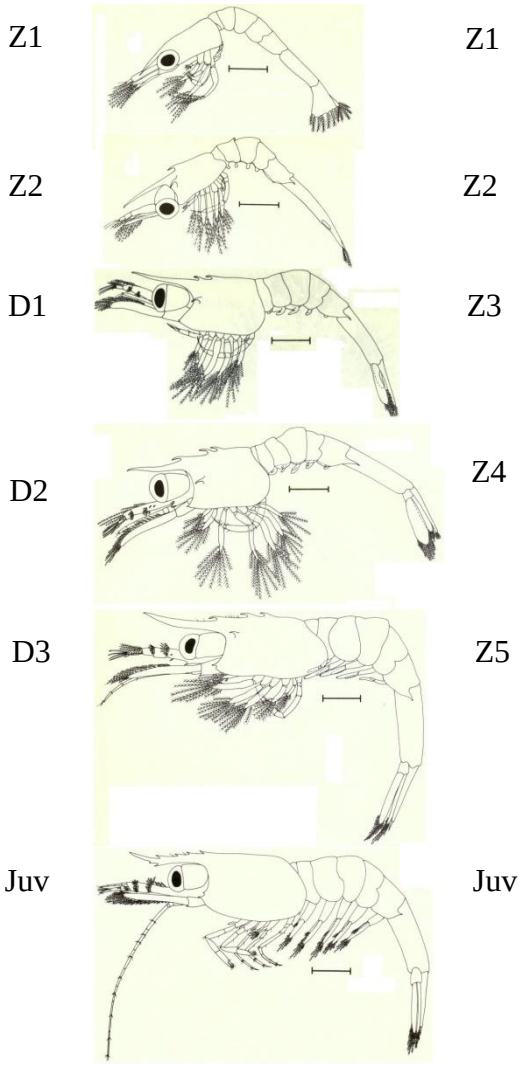

Figure 2.4. *Palaemonetes varians* larval development, consisting of five larval stages, as described by Fincham (1979). Larval instars are labelled; larval nomenclature used here is on the left and that used by Fincham (1979) is on the right. The first juvenile stage/post-larva is also shown. Scale bars = 0.5 mm. See Chapter 4, Figure 4.1 for development pathways observed during experiments here.

2.5 Morphological measurements

2.5.1 Maternal carapace length

Maternal carapace length (CL) was measured as a standard proxy for female size. Carapace length is related to total length and has been coupled with dry weight and energetic content, though the correlations between carapace length and these latter factors are weaker and more variable. Maternal carapace length was measured for females brooding newly extruded eggs (stage I and II) and females hatching larvae to assess the relationship between female size and POI.

Maternal carapace length (CL) was measured as post-orbit CL using digital vernier callipers (accuracy 0.01 mm) under a dissection stereomicroscope. Post-orbit CL was measured from the orbit behind the eye to the back of the carapace.

2.5.2 Egg volume measurements and calculations

Egg volume is often reported in literature as a proxy for egg energy content, but has received criticism as to its accuracy (Moran & McAlister 2009, Moran et al. 2013). Egg volumes were measured to compare with egg dry weight data to assess the accuracy of egg volume as a proxy for egg dry weight within *Palaemonetes varians*. Egg-length and -width were measured from digital photographs (see section 2.3.1) using the software SigmaScan V6. Within SigmaScan V6, the circumference of eggs was manually traced onto digital photographs via a computer mouse. The software then calculated the 'major axis' and 'minor axis' (length and width, respectively).

Egg volume was calculated using the equation for the volume of a prolate spheroid;

$$Volume = \frac{4}{3}\pi a^2 b$$

Where $a = \frac{1}{2}\ minor\ axis$ and $b = \frac{1}{2}\ major\ axis$.

2.6 Respiration rate measurements

The rate at which oxygen is consumed by an organism, or respiration rate, is a useful indicator of metabolic rate within the organism. This can provide insights into metabolic rate in response to temperature, stresses, or across developmental processes such as the moult cycle. This is important in understanding how environmental conditions influence organismal physiology.

Respiration rates were measured throughout larval development across a range of temperatures to understand better the effects of temperature on larval development and growth rates. Larval and juvenile respiration rates were measured by isolating individuals under constant conditions within water tight vials of set volume and for a set period of time. The depletion of oxygen within these vials over this set time, coupled with the measured dry weights of individuals, was used to calculate respiration rates, accounting for animal mass.

Larvae or juveniles were transferred individually into 2.8 ml plastic vials using a 3 ml plastic pipette. These vials were filled with 32 salinity, 1 μm-filtered and UV- treated seawater acclimated to experimental temperatures (15, 20, or 25 °C). Vials were sealed underwater to ensure no air bubbles were trapped inside. This was done within an aquarium containing 32 salinity, 1 μm-filtered and UV-treated seawater acclimated to experimental temperatures. A thermometer, placed in this aquarium, was used to monitor the water temperature. Given that all air was excluded from within the vials, the oxygen saturation of the water inside the vials, depleted by larvae, juveniles, or bacteria respiration, could not be replenished by oxygen diffusing into solution from air bubbles.

The accurate timing of isolation times was important. Setting-up treatment and control vials before the isolation period, and the measuring of oxygen saturation of water within vials after the isolation period, took time. Consequently, vials could not be set-up and isolated simultaneously but were staggered by two minutes. Respiration rates were expected to vary with temperature, being greater at higher temperatures. To ensure that oxygen saturations within vials did not decrease below 60 %, isolation times varied depending on temperature. At 15 °C vials were isolated for four hours, three and a half hours at 20 °C, and two hours at 25 °C. Blank vials served as controls. These were vials containing no larvae or juveniles but treated in the same way as treatment vials. One control vial was run per treatment vial. Treatment and control vials were run alternatively. Five replicate treatment vials (and therefore five control vials) were run per treatment. Once sealed, vials were transferred to incubators set to the treatment temperature. Lighting was on inside the incubators during the isolation times and as the vials were opaque, vial interiors were illuminated.

After the isolation period, vials were removed from the incubators and inverted several times to mix the content and disrupt any oxygen saturation gradient within the vial. Vials were then opened. The percentage oxygen saturation of water inside the vials was immediately measured using a Presens Microx TX 3 temperature-adjusted oxygen meter and microoptode (accuracy ± 0.4 % O_2 at 20.9 % O_2, ± 0.05 % O_2 at 0.2 % O_2). The microoptode, housed within a hypodermic needle and held by a clamp and stand, was inserted into the open vial. Daily, the microoptode was calibrated with fully aerated seawater which had been left to equilibrate for 30 minutes (100 % O_2 saturation) and seawater deoxygenated by over-saturation with sodium sulphite (0 % O_2 saturation). These calibration solutions were acclimated at the treatment temperatures before use. The value

taken for percentage oxygen saturation was that at which % O_2 measurements stabilised and within 2 minutes of vials being opened.

Values were calculated for the concentration of oxygen (μmol l^{-1}) for 100 % oxygen-saturated seawater under the temperature and salinity conditions used in experimental treatments according to Benson and Krause (1984). Following equation (1), these values coupled with measurements of percentage oxygen saturation of water inside the vials were used to calculate values for oxygen consumed during treatments (μmol l^{-1}). These values were then adjusted for vial volume, using equation (2), and isolation time, using equation (3), to yield values for oxygen consumption rate (μmol hr^{-1}).

(1)

$$O_2 consumed\ in\ \mu mol\ l^{-1} = \left(100\ \%\ O_2 in\ \mu mol\ l^{-1} - \left(\frac{vial\ \%\ O_2 concentration}{100}\right) * 100\ \%\ O_2 in\ \mu mol\ l^{-1}\right)$$

(2)

$$O_2\ consumed\ in\ \mu mol = \frac{(O_2 consumed\ in\ \mu mol\ l^{-1} * vial\ volume\ in\ ml)}{1000}$$

(3)

$$O_2\ consumption\ rate\ in\ \mu mol\ hr^{-1} = \left(\frac{O_2 consumed\ in\ \mu mol}{time\ in\ minutes}\right) * 60$$

Values for oxygen consumption rates of larvae and juveniles were then corrected for any bacterial consumption during isolation periods by subtracting values for experimental vials from those for control vials. These corrected values were then adjusted for larval/juvenile dry weight following equation (4).

(4)

$$O_2\ consumption\ rate\ in\ \mu mol\ mg^{-1}\ hr^{-1} = \frac{O_2\ consumption\ in\ \mu mol\ hr^{-1}}{dry\ weight\ in\ mg}$$

2.7 Dry weight (*W*) measurements

Dry weight (*W*) is a relatively useful proxy of POI (certainly more so than egg size/volume) (Moran & McAlister 2009). Dry weight was also measured to monitor growth during development and differences in juvenile dry weight at settlement in response to temperature and differing development pathways. Dry weight measurements

were taken after samples had been freeze-dried for 24 hours using a Thermo Scientific Heto PowerDry LL33000 freeze dryer and then stored in a Secador desiccator. Samples were weighed for dry weight (*W*; ug) using a Sartorius microbalance ME5 mass balance. This balance gave measurements of mass in mg to 3 decimal places, which were converted to µg.

2.8 Carbon:Nitrogen (C:N) analysis

Decapod larvae contain relatively little inorganic carbon and as such, carbon and nitrogen content can be used as accurate proxies for lipid and protein content, respectively (Anger & Harms 1990). The amount of lipid larvae are provisioned with is important in determining the level of food independence during development. Also, changes in lipid and protein content throughout development provide information on the relative uses of lipids and proteins for metabolism. Carbon and nitrogen content was measured to determine to amount of lipid reserves at hatching within *Palaemonetes varians* larvae, how these reserves were utilised throughout development, and whether temperature affected the rate of utilisation.

Elemental analysis was done using a Carlo ERBA instruments CHNS-O EA1108-elemental analyser. For calibration, a chitin standard was used comprising C = 44.71 %, N = 6.61 %, H = 6.79 %, O = 36.86 %. After calibration, one chitin standard was run per ten samples. The error permitted on the standard was ± 5 %. Elemental composition of samples was given as %. These values were converted to carbon mass and nitrogen mass (µg ind^{-1}) using values for sample dry weights.

2.9 Energetic calculations using oxygen consumption measurements

The consumption of oxygen can be converted into energy loss using equation (5), following Gnaiger (1983).

(5)

$$Energy\ loss\ in\ J\ ind^{-1}\ h^{-1} = O_2\ consumption\ in\ \mu mol\ hr^{-1} * 0.450\ J\ h^{-1}$$

Energy loss in J ind^{-1} h^{-1} were converted to energy loss J ind^{-1} d^{-1} by multiplying by 24 for measurements taken daily (first ten days) and by 48 for measurements taken every second day. These values were then added cumulatively to estimate total energy loss for development, or within stages of development.

2.10 Statistical analysis

Statistical analysis is described in detail within the methods sections of respective chapters. Statistical analysis was carried out in Minitab V16 and Ri386 3.0.1 (R Development Core Team 2013) and in accordance with Sokal and Rohlf (1995), Underwood (1997), and Zuur et al. (2009). Data were managed and simple calculation performed using Microsoft Excel. Figures were plotted using Sigmaplot V12 and diagrams were drawn using Microsoft Powerpoint.

3. Inter- and intra-annual variability in Per Offspring Investment (POI) within the caridean shrimp, *Palaemonetes varians*

3.1 Summary

The maternal environment may influence the quantity and quality of resources invested in offspring (per offspring provisioning; POI). This trait, in turn, affects larval fitness and may carry-over into early juvenile life. Here, temperature and salinity were monitored within a drainage ditch on Lymington salt marsh (Hampshire, UK) and POI measured across three breeding seasons in consecutive years to establish the influence of temperature on POI within the caridean shrimp, *Palaemonetes varians*. Results demonstrate that temperature and salinity are highly variable within Lymington salt marsh, both seasonally and diurnally. Diurnal variations were of greater magnitude than sub-tidal environments and may be comparable to the variations known for inter-tidal environments. Maternal size during the breeding season changed and was consistent across the three breeding seasons, suggesting that this change was related to the population structure. Size frequency analysis indicated that the population consisted of three female cohorts, the differing breeding times of these cohorts resulting in the change in maternal size across breeding seasons. POI varied throughout breeding seasons and between years, but was not correlated with environmental temperatures during gonad development. These data highlight the considerable variability, diurnal, seasonal, and inter-annual, experienced by salt marsh inhabiting fauna.

3.2 Introduction

The level of maternal resources invested into individual offspring is of fundamental importance within life histories biology, as this trait influences both maternal and offspring fitness (Marshall & Uller 2007). Environmental conditions within the maternal environment may determine the quantity and quality of maternal resources invested in individual offspring (George et al. 1990, George 1996, Urzúa et al. 2012, Urzúa & Anger 2013). Shifts in per offspring investment (POI) associated with changes in the maternal environment have been demonstrated for a range of taxa (although arthropods dominate) including: crustaceans (e.g. Kerfoot 1974, Perrin 1988, Sakwińska 1998, Fischer & Thatje 2008), bryozoans (Bownds et al. 2010), and insects (e.g. Ernsting & Isaaks 1997, Blanckenhorn 2000, Ernsting & Isaaks 2000, Fischer et al. 2003a, Fischer et al. 2003b, Fischer et al. 2003c, Berkebile et al. 2006, Geister et al. 2008, Liefting et al. 2010). The majority of such studies have focused on the effects of temperature on POI plasticity and have demonstrated higher POI under lower temperatures. For example, Kerfoot (1974) observed seasonal cycles in the cladoceran, *Bosmina longirostris*, which produce larger eggs (higher POI) in winter months. Kerfoot related this shift in POI to temperature changes between seasons, demonstrating the link between temperature and POI experimentally (Kerfoot 1974). Likewise, Perrin (1988) demonstrated temperature mediated POI plasticity in another cladoceran, *Simocephalus vetulus*; again, higher POI was found under lower temperatures.

Among crustaceans, seasonal shifts in POI have been documented for many taxa (e.g. Boddeke 1982, Sheader 1983, Clarke et al. 1985, Bell & Fish 1996, Guisande et al. 1996, Pond et al. 1996, Sheader 1996, Sampedro et al. 1997, Halsband-Lenk et al. 2001, Oh & Hartnoll 2004, Paschke et al. 2004, Soto et al. 2006, Yu & Suh 2006, Bas et al. 2007, Urzúa et al. 2012). A relatively well studied example of seasonal POI variation is that which occurs within the common brown shrimp, *Crangon crangon*. Common on sandy and muddy substrate in shallow, coastal areas of the Eastern Atlantic and particularly Northern Europe (Tiews 1970), this caridean shrimp reproduces from mid-winter throughout spring and into late summer and early autumn (Boddeke 1982, Oh & Hartnoll 2004, Urzúa et al. 2012). Across this breeding season, egg volume, dry weight, carbon and nitrogen mass, lipid and protein mass, and energy content decrease significantly from maximum values at the beginning of the season to minimum values throughout summer (Boddeke 1982, Henderson & Holmes 1987, Oh & Hartnoll 2004, Urzúa et al. 2012, Urzúa & Anger 2013).

Whilst Boddeke (1982) observed a decrease in fecundity coupled with increased egg volume during winter months, other studies have found no change in fecundity between winter and summer (Henderson & Holmes 1987, Oh & Hartnoll 2004). On shorter temporal scales, shifts to lower POI have been demonstrated over a few months for summer breeding crustaceans, and have been suggested to be 'reproductive exhaustion' (Sampedro et al. 1997, Bas et al. 2007). Seasonal temperature fluctuations are associated with shifts in POI (Kerfoot 1974, Perrin 1988). However, changes in POI over such a short time scale (a few months), and their relationship with temperature remains unclear.

Differences in POI between winter and summer eggs of *C. crangon* are associated with differences in the starvation resistance and point of saturation for secondary lecithotrophy within larvae, and have been associated with differing larval development pathways (Anger 2001, Paschke et al. 2004, Giménez 2006). Within *Palaemonetes varians*, differences in POI are associated with larval development plasticity and carry-over effects into early juvenile stages (see Chapter 6) and, as such, may have significant ecological implications. Interestingly, shifts in POI are known for crustaceans with no larval stage and direct development. For example, seasonal changes in POI and reproductive output have been found for peracarid crustacean species with direct development. Sheader (1996) documented a seasonal effect on offspring provisioning for the gammarid amphipod *Gammarus insensibilis*, whilst Bell and Fish (1996) documented a marked seasonal variation in the reproductive output for a population of the amphipod, *Pectenogammarus planicrurus*, from Aberystwyth (Wales, UK). Similar seasonal changes in reproductive output have been documented for other amphipods (Sheader 1983, Clarke et al. 1985, Soto et al. 2006, Yu & Suh 2006).

Here, POI in relation to environmental temperature and salinity is assessed for the salt marsh inhabiting palaemonid shrimp, *Palaemonetes varians*. These data will form an important and useful baseline to later laboratory studies detailed within this thesis. The following specific hypotheses were tested:

3.2.1 Hypotheses

H_1 POI (measured for both eggs and larvae) varies across breeding seasons and between breeding seasons

H_2 variations in POI are related to the temperatures during gonad development: POI is greater after gonad development during cooler temperatures

H_3 larval quality is affected both by POI of eggs and the conditions during embryonic development: larval quality being lower after embryonic development under warmer conditions

3.3 Materials and Methods

3.3.1 Collections and maintenance of *Palaemonetes varians*

Adult *Palaemonetes varians* were sampled monthly between February and July 2011 for size frequency analysis of the population following Chapter 2, section 2.2.1. Ovigerous *Palaemonetes varians* were collected as described in Chapter 2, section 2.2.2 and on dates detailed in Table 2.1. Only ovigerous shrimp, identified by the presences of eggs attached to the pleopods under the abdomen, were retained and were transported to the National Oceanography Centre Southampton research aquarium. Here, the development of broods was assessed under a stereo dissection microscope: see Chapter 2, section 2.2.2. Females with stage I and II eggs were immediately blotted on tissue paper, placed in plastic bags and frozen at -80 °C (see Chapter 2, section 2.3.1). Females with stage VII and VIII embryos were maintained following Chapter 2, section 2.4.1. Shrimp were checked every day (am) for hatched larvae.

3.3.2 Measuring egg and larval per offspring investment (POI)

Upon hatching, a sample of 15 newly hatched, actively swimming larvae per female were separated from mothers using a plastic pipette and preserved following Chapter 2, section 2.3.2. Larvae hatching in May and June, 2012 were analyses for carbon and nitrogen content analysis as proxies for lipid and protein content, respectively. Five larvae per female were analysed for elemental composition following Chapter 2, section 2.8. Ovigerous *P. varians* with recently extruded (stage I and II) eggs were defrosted, and a sample of 15 eggs dissected from the 1st right pleopod of each female and preserved following Chapter 2, section 2.3.1. The post-orbit carapace lengths of all females, those carrying stage I and II eggs, and those carrying stage VII and VIII embryos, were measured following Chapter 2, section 2.5.1.

3.3.3 Monitoring temperature and salinity within the maternal environment

Methods used to monitor temperature and salinity at the sampling site are detailed in Chapter 2, section 2.1.1. In addition, air temperature data and rainfall data were

downloaded from http://www.southamptonweather.co.uk/; accessed from October 2012 to August 2013.

3.3.4 Statistical analysis

Data were tested for normality of distribution and equality of variance using Kolmogorov-Smirnov Test and Levene's Test, respectively. Relationships between egg dry weight and egg volume were tested by linear regression analysis, whilst correlations between larval composition and larval dry weight were assessed by Spearman's rank order correlation. Analysis of variance of maternal carapace lengths, egg dry weight, and larval dry weight between months and between years were done by General Linear Model (GLM) ANOVA; *post hoc* testing (were appropriate) of factors (year) and (month) were done using the Sidak method. Larval dry weight data were normally distributed but were heteroscedastic and were weighted (1/*n*) for the analysis.

3.4 Results

3.4.1 Environmental variability

In all three years during which temperature was monitored (2011, 2012, 2013), seasonal fluctuations were observed, with minimum temperatures in late winter and maximum temperatures in summer (Figure 3.2). Minimum temperatures and the duration of low temperatures varied between years, as did maximum temperatures and the duration of high temperatures (Figure 3.2). For example, minimum temperatures varied between years being 0.98 °C on 2nd February 2012 and -0.14 °C on 12th March 2013. Maximum temperatures were 23.44 °C on 26th June 2011, 26.59 °C on 25th May 2012, and 25.38 on 19th July 2013; again, varying between years. In 2012, temperatures were less than 5 °C from early- to mid-February and were greater than 5 °C from 12th February 2012. In contrast, temperatures during 2013 decreased below 5 °C in mid-January and then fluctuated between ~2 and <10 °C and temperatures less than 5 °C were recorded as late as April (Figure 3.2). On top of the general seasonal temperature fluctuations, there were considerable short term temperature variations. In some cases temperature changes of ~6 °C within 24 hours were recorded (Figures 3.2 and 3.10A). Similarly, in March 2013 the temperature dropped by 11.9 °C in ~66 hours (Figure 3.2). The ditch monitored is connected to the West Solent via sluice gates; however, changes in tidal amplitude between neap and spring tides did not seem to affect daily temperature fluctuation (Figure 3.1). Water temperature closely matched air temperatures, though daily ranges were less, thus

temperature within the ditches appears more closely linked with weather and the terrestrial environment than tides and the marine environment.

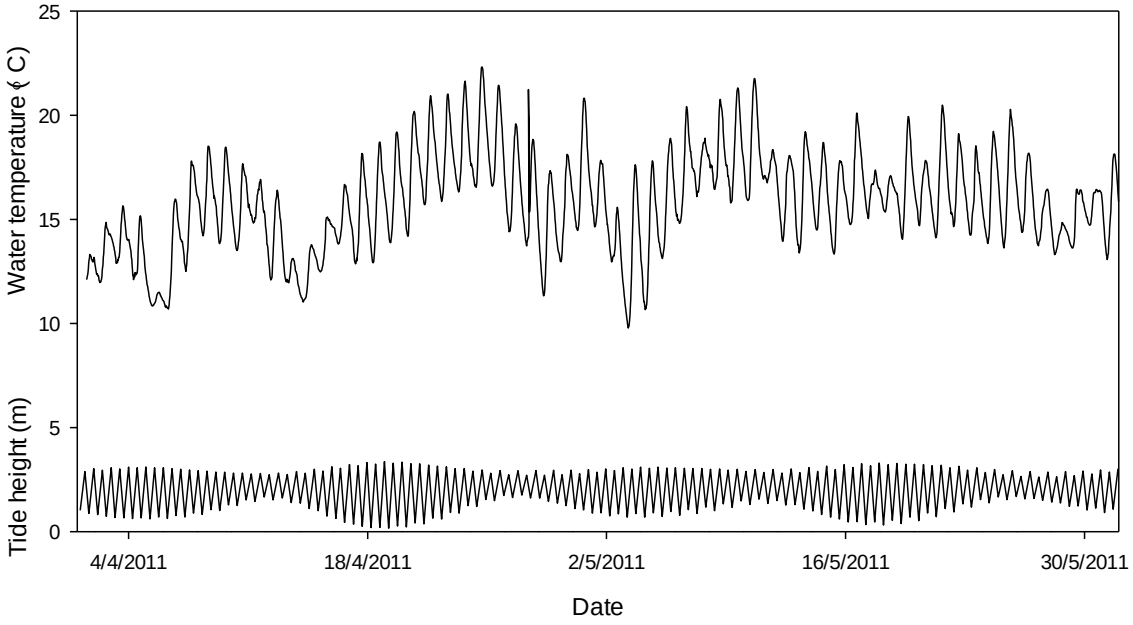

Figure 3.1 Water temperature data for Lymington salt marsh (Hampshire, UK) and tide height data for Lymington marina, adjacent to the salt marsh

Salinity was similarly variable, being generally lower during winter months and higher during summer months. Salinities of 0.46 ± 0.25 to 43.2 ± 0.5 were measured between 2011 and 2013 demonstrating the considerable seasonal variation in salinity. Salinity also varied between years. The autumn of 2011 and winter of 2012 were drier than the autumn of 2012 and winter of 2013 and, consequently, salinities were much higher in the autumn/winter of 2011/12 than that of 2012/13 (Figure 3.3). For example, salinity on 24th October 2011 was 42.2 ± 1.8 whilst on 24th October 2012 it was 4.4 ± 1.3. In contrast, the summer of 2012 was much wetter than 2011 and 2013 and, accordingly, salinities were much lower; for example, during the summer of 2012 recorded salinity ranged from 1.7 ± 0.3 to 17.8 ± 1.7 whilst for the summer of 2011 recorded salinities ranged from 36.9 ± 1.9 to 43.2 ± 0.5 and in the summer of 2013 recorded salinities ranged from 26.2 ± 1.0 to 31.8 ± 3.3 (Figure 3.3). Monthly salinity measurements presented here are too low resolution to identify short term fluctuations in salinity. However, the high variability in rainfall and salinity changes in response to rainfall suggests that salinity may be variable short term in response to rainfall and surface run-off, as well as seasonal.

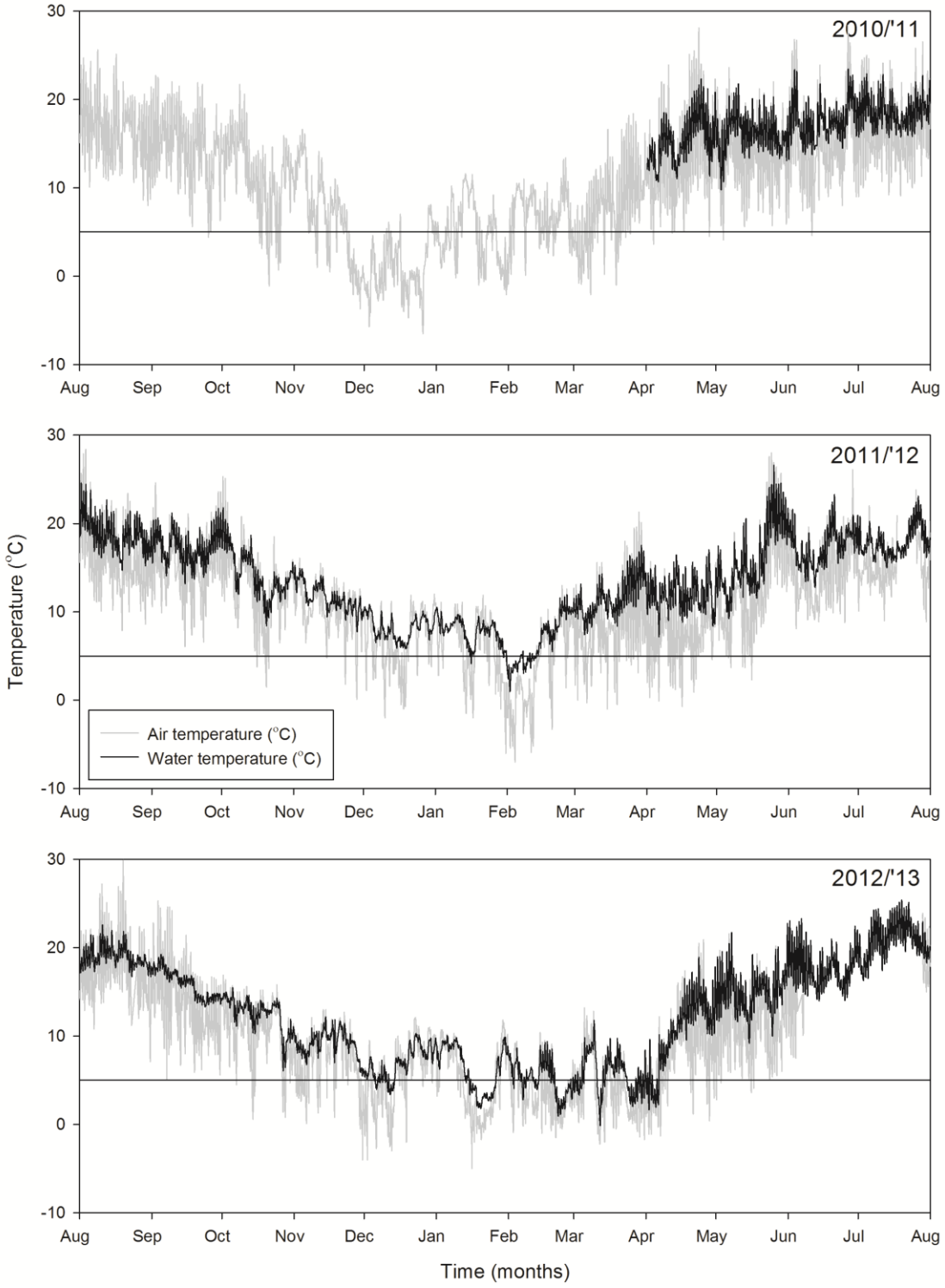

Figure 3.2 Average *in situ* water temperature for the ditch on Lymington salt marshes (Hampshire, UK) from which *Palaemonetes varians* were sampled; air temperature for Southampton (http://www.southamptonweather.co.uk/; accessed from October 2012 to August 2013) are also shown. Data are presented from August to August, which is estimated to be the annual cycle for breeding within *P. varians* here. Horizonal line indicates 5 °C, above which gonad maturation may occur in *P. varians* (Hindley 2001)

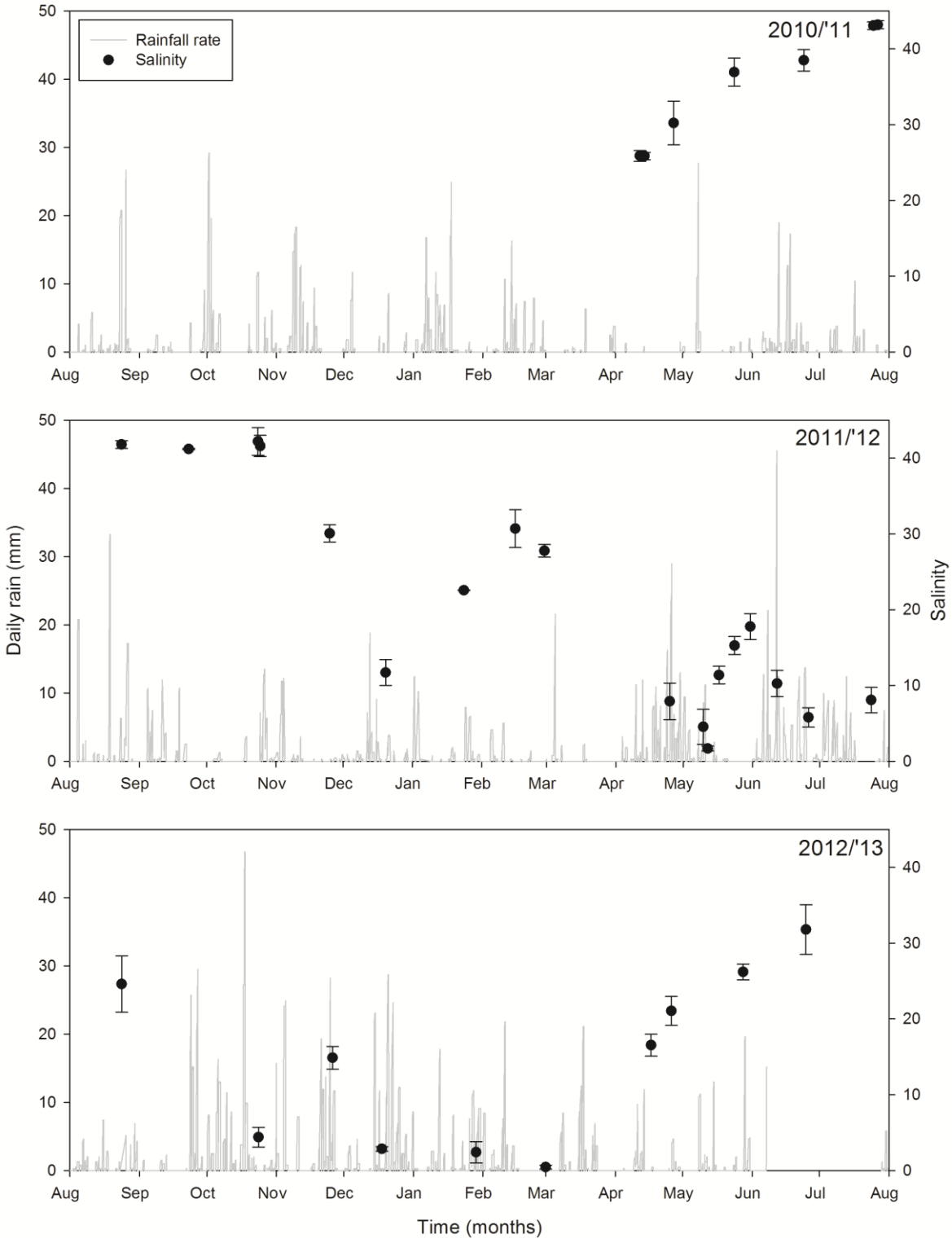

Figure 3.3 Average *in situ* salinity within the ditch on Lymington salt marsh (Hampshire, UK) from which *Palaemonetes varians* were sampled. Data are presented as means ± S.D. Rainfall data for Southampton (http://www.southamptonweather.co.uk/; accessed from October 2012 to August 2013) are also shown. Data are presented from August to August as this is considered as the annual breeding cycle for *P. varians* here

3.4.2 Size frequency

Throughout the period of sampling, the percentage of males in samples increased from 42.9 % in February to the highest value of 74.8 % in June (Table 3.1). Pre-orbital carapace lengths of *P. varians* sampled between February and June, 2001, ranged from 3.6 to 10.3 mm (Figure 3.4). Female carapace length ranged between 3.6 and 10.3 mm whilst males were generally limited to smaller size classes, with carapace length between 3.9 and 7.8 mm. Carapace length for females with stage I and II eggs (see Chapter 2, section 2.2.2) were between 4.4 and 8.9 mm, whilst for females with stage VII and VIII (see Chapter 2, section 2.2.2) they ranged from 6.6 to 9.8 mm. Size frequency data for males appeared to form two cohorts, whilst for females data formed three cohorts; the largest of which was lost through the breeding season (Figure 3.4). All three cohorts contained ovigerous females.

Table 3.1 Percentage of male *Palaemonetes varians* in samples for each month throughout the 2011 breeding season

	Feb.	Mar.	Apr.	May	Jun.	Jul.
% male	42.9	57.0	72.5	70.7	74.8	62.8

3.4.3 Sampling of POI

In all years, ovigerous *Palaemonetes varians* with stage I and II eggs were present in April collections, whilst ovigerous *P. varians* with stage VII and VIII embryos were absent. Similarly, shrimp with stage I and II eggs were rare in July, whilst shrimp with stage VII and VIII were present (Table 3.2, see also Figure 3.4 for 2011 data), suggesting that sampling encompassed the breeding season well. Further, ovigerous shrimp were absent in March and August.

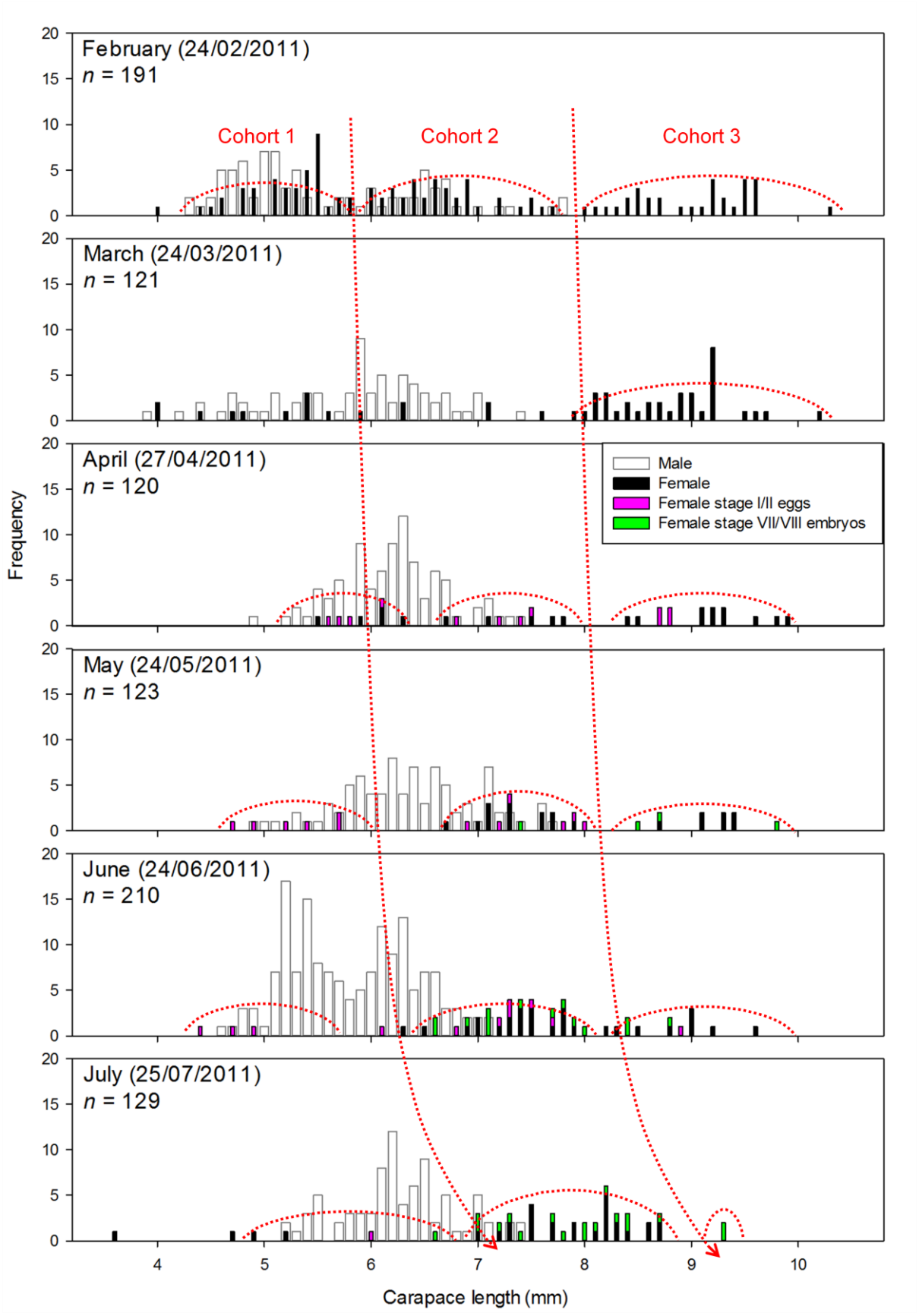

Figure 3.4 *Palaemonetes varians* size frequency data across the 2011 breeding season in Lymington salt marsh (Hampshire, UK). Plots are annotated to depict three female cohorts

Table 3.2 Numbers of *Palaemonetes varians* with A) stage I and II eggs and B) stage VII and VIII embryos sampled each month throughout the breeding season and over three consecutive years.

A)

Year	April	May	June	July
2011	12	12	11	1
2012	14	10	6	1
2013	4	-	4	1

B)

Year	April	May	June	July
2011	-	4	15	19
2012	-	13	12	12
2013	-	1	8	6

3.4.4 Relationship between egg volume and dry weight

Egg volume ranged from 0.178 to 0.334 mm^3 with a mean of 0.274 ± 0.024 mm^3 whilst egg dry weight ranged from 117 to 197 μg with a mean of 160.44 ± 14.06 μg. Egg volume was positively related to egg dry weight (Figure 3.5A; F = 233.55, $P < 0.001$), though the R^2 value was <0.5 ($R^2 = 0.41$; Table 3.3). In all months there was a positive relationship between egg volume and dry weight (April F = 23.31, $P < 0.001$; May F = 176.37, $P < 0.001$; June F = 59.36, $P < 0.001$); however, the R^2 values for April and June were much lower than that for May (Figure 3.5B, Table 3.3).

Table 3.3 Fitted parameters and correlation coefficients for the linear relationship ($y = a + bx$) between *Palaemonetes varians* egg volume and egg dry weight for the 2011 breeding season (see Figure 5 for data)

	a	*b*	R^2
All	0.09269	0.00113	0.41296
April	0.16924	0.00071	0.20040
May	0.01493	0.00156	0.59914
June	0.16257	0.00073	0.33657

3.4.5 Relationship between larval dry weight and elemental composition

Hatchling nitrogen composition (%*N*) ranged from 8.45 to 11.66 % with a mean of 9.91 ± 0.58 % whilst hatchling dry weight ranged from 101 to 172 μg with a mean of 133.31 ± 13.99 μg. At hatching, larvae of greater dry weight had slightly lower nitrogen

content (%*N*) than larvae of higher dry weight; this negative relationship was significant, but the r value was <0.5 (r= -0.318, $P < 0.001$) (Figure 3.6A). In contrast, hatchling larvae of greater dry weight had slightly greater carbon content than larvae of lower dry weight. This relationship was also significant (r = 0.341, $P < 0.001$) (Figure 3.6B). Hatchling carbon composition (%*C*) ranged from 36.16 to 54.39 % with a mean of 45.91 ± 2.28 %. As a consequence of the slightly negative relationship between nitrogen content and dry weight and the slightly positive relationship between carbon content and dry weight in hatchling larvae, the ratio of carbon to nitrogen (C:N) increased with increasing dry weight in newly hatched larvae (r= 0.451, $P < 0.001$) (Figure 3.6C). Hatchling C:N ratio ranged from 3.74 to 5.43 with a mean of 4.65 ± 0.34.

Hatchling nitrogen mass ranged from 9.71 to 16.94 μg ind^{-1} with a mean of 13.18 ± 1.35 μg ind^{-1}. Despite the negative relationship between nitrogen content (%*N*) and larval dry weight (Figure 3.7A), the mass of nitrogen was positively and strongly correlated with larval dry weight (r= 0.815, $P < 0.001$) and so, therefore, larvae of greater dry weight had considerably more nitrogen mass than larvae of lower dry weight (Figure 3.7A). Similarly, larvae of greater dry weight had considerably higher carbon mass, as there was a strong relationship between dry weight and carbon mass in newly hatched larvae (r = 0.923, $P < 0.001$) (Figure 3.7B). Hatchling carbon mass ranged from 41.58 to 85.94 μg ind^{-1} with a mean of 61.31 ± 8.01 μg ind^{-1}. Consequently, some larvae hatched with half the carbon mass (and lipid reserves) of other larvae (Figure 3.7B).

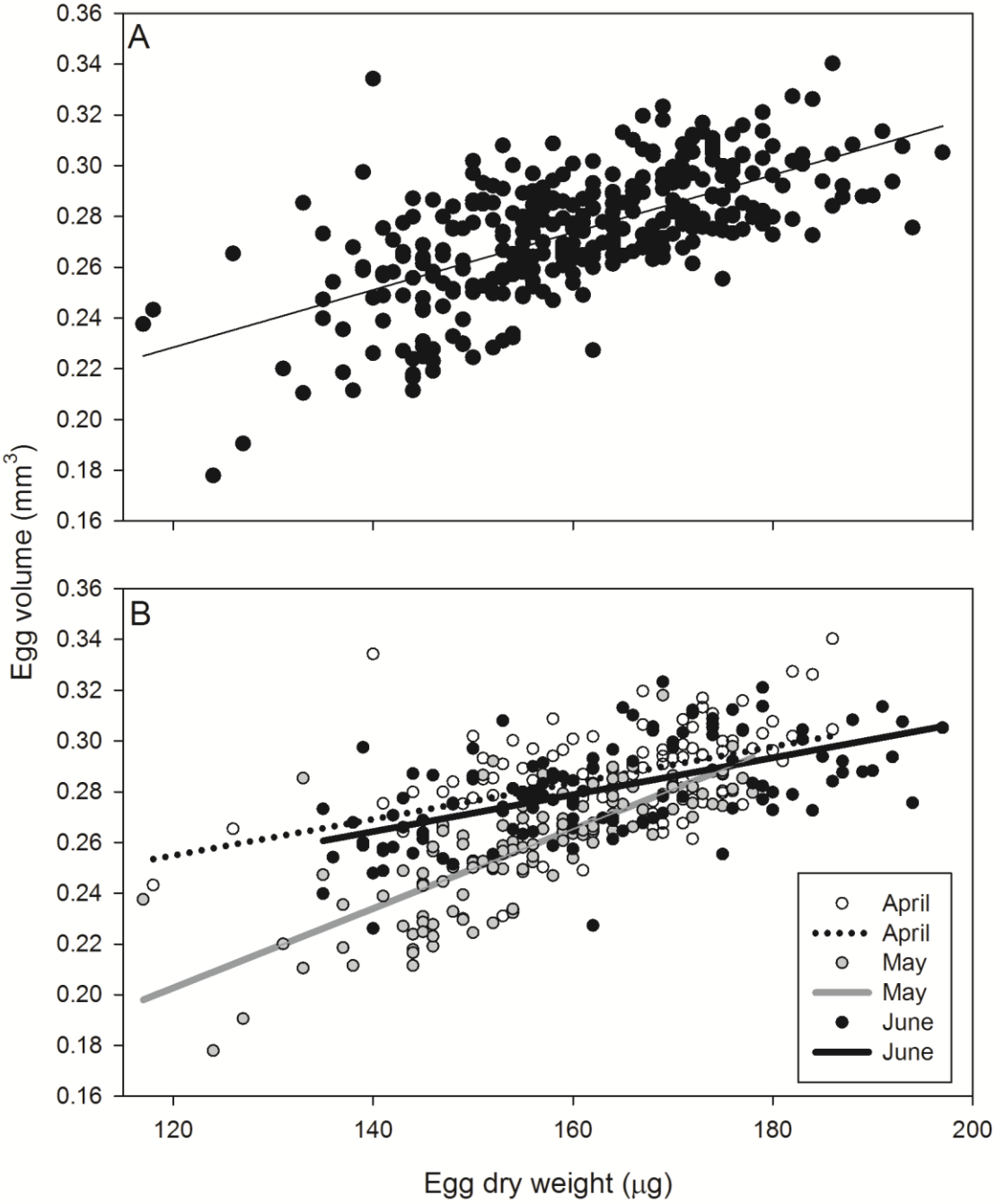

Figure 3.5 *Palaemonetes varians* relationship between egg volume and egg dry weight for **A** eggs sampled in 2011 and **B** eggs sampled from differing months within 2011 from Lymington salt marsh (Hampshire, UK) (see Table 3.3 for fitted parameters and correlation coefficients)

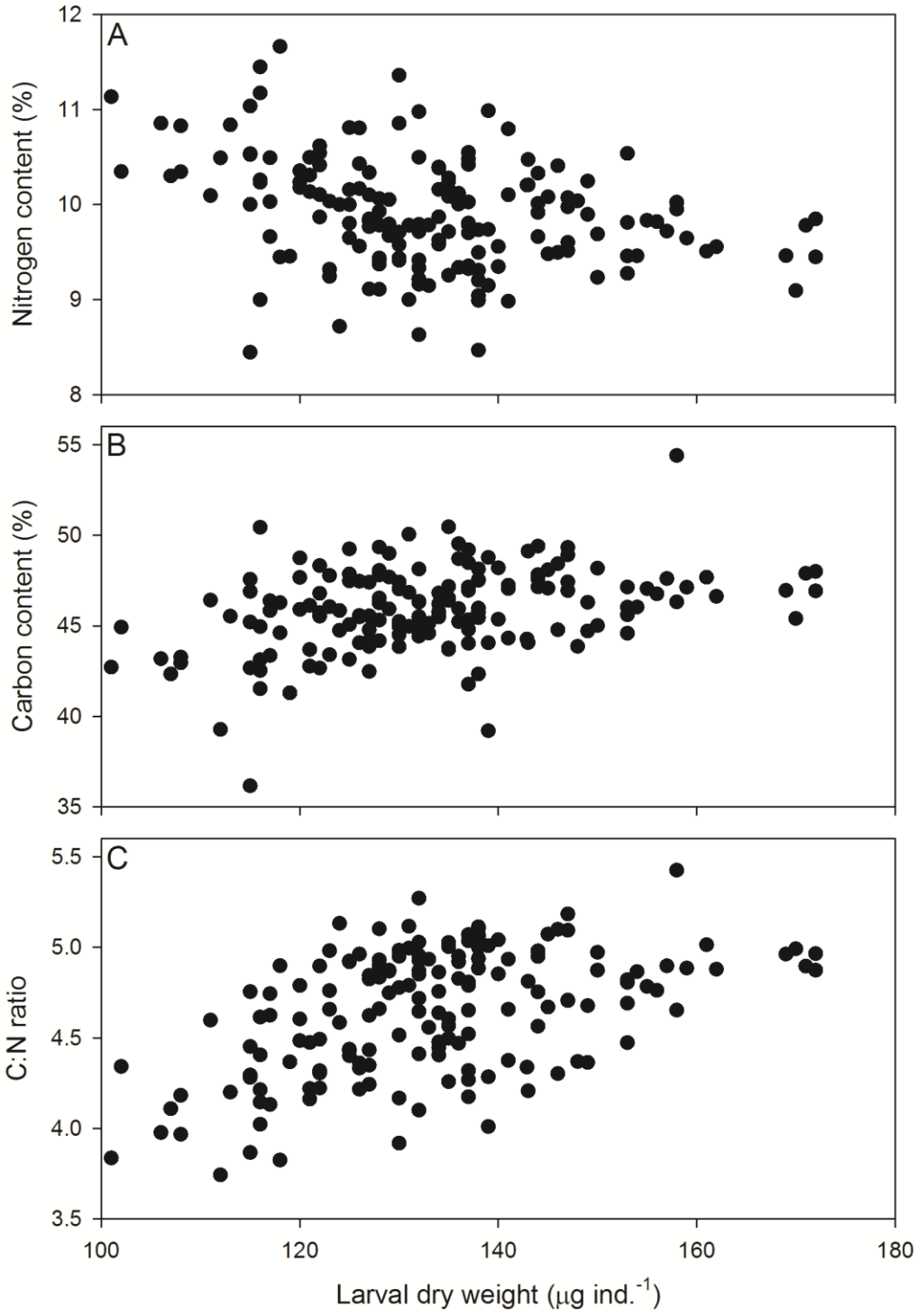

Figure 3.6 *Palaemonetes varians* larval dry weight and **A** nitrogen content, **B** carbon content, and **C** C:N ratio for larvae sampled in May and June of 2012 form Lymington salt marsh (Hampshire, UK)

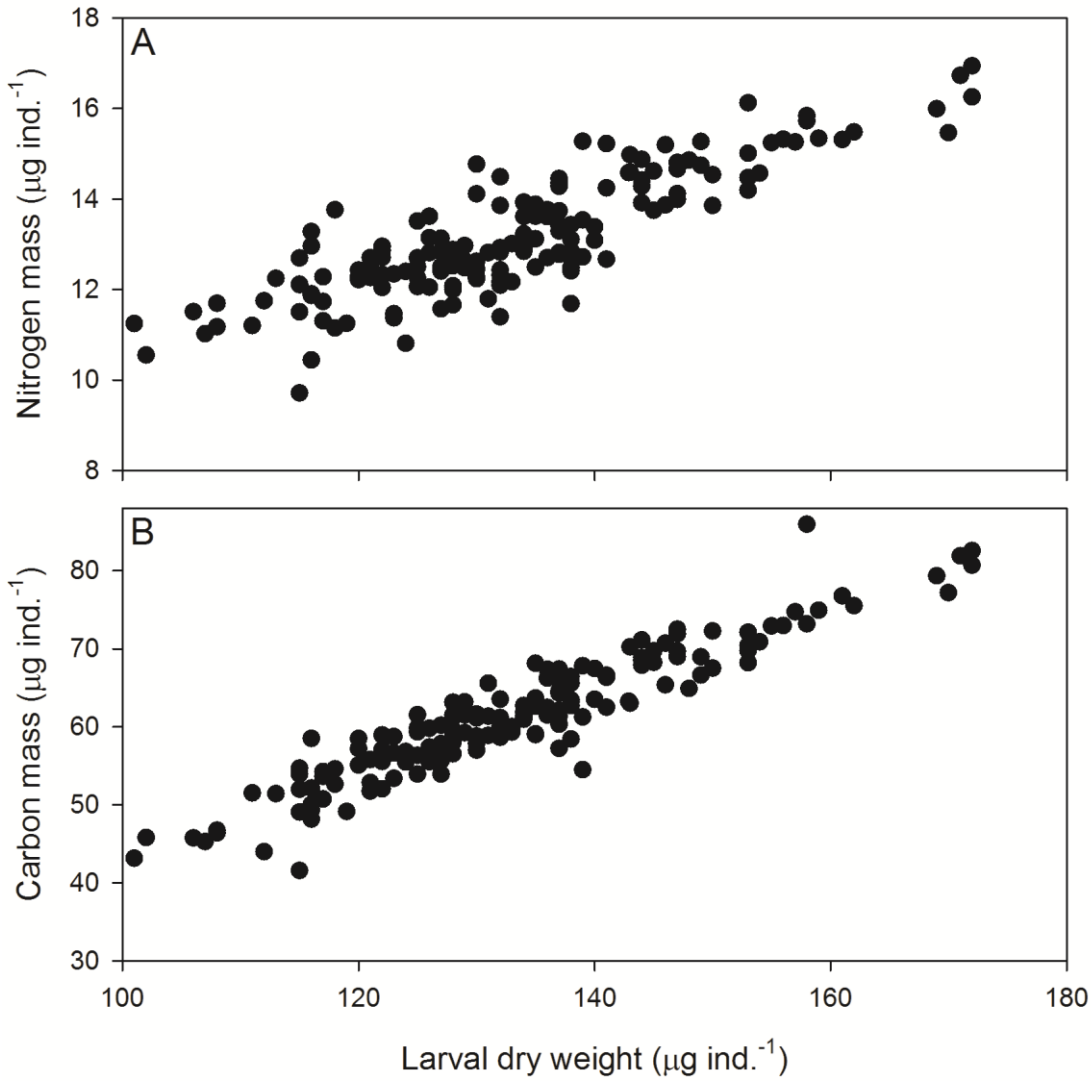

Figure 3.7 *Palaemonetes varians*. Correlations between hatchling dry weight and **A** nitrogen mass and **B** carbon mass for larvae hatched from a population in Lymington salt marsh (Hampshire, UK)

3.4.6 Maternal post-orbital carapace length

Maternal carapace length of ovigerous females carrying stage I and II eggs varied significantly between months (F = 8.31, *P* = 0.001) (Figure 3.8A). Maternal carapace length decreased from April to May (*P* = 0.0003) and then increased again from May to June (*P* = 0.0294). As there was no interaction between month and year (F = 1.85, *P* = 0.166), this trend was consistent between years (2011 and 2012). Maternal carapace length was different between years (F = 31.24, *P* < 0001), being greater in 2012 than in 2011 (*P* < 0.001).

Maternal carapace length of ovigerous females carrying stage VII and VIII embryos (and which hatched larvae) was significantly different between months (F = 8.81, $P < 0.001$), following the same pattern as above: decreasing from May to June ($P = 0.0094$) and increased again from June to July ($P = 0.0014$) (Figure 3.8B). There was no interaction between year and month, indicating that this trend was consistent between 2011, 2012, and 2013 (F = 1.35, $P = 0.260$). Maternal carapace length was different between years (F = 4.52, $P = 0.014$); those in 2011 were greater than those in 2013 ($P = 0.0203$), whilst those in 2012 were intermediate and were not different from either 2011 or 2013.

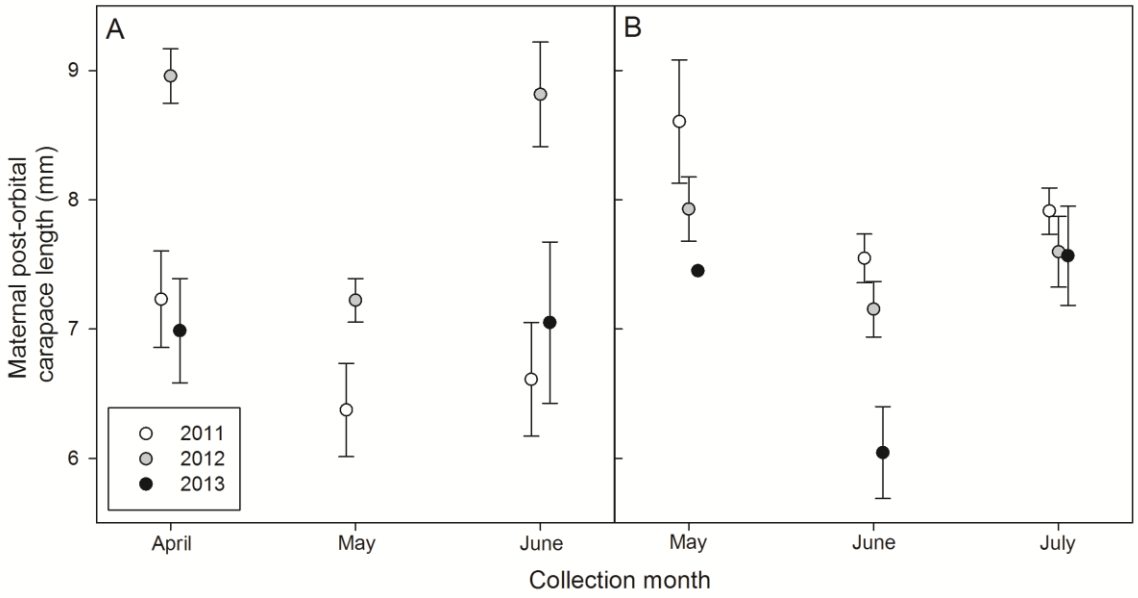

Figure 3.8 *Palaemonetes varians* maternal pre-orbital carapace length for **A** ovigerous shrimp carrying stage I and II eggs and **B** ovigerous shrimp carrying stage VII and VIII eggs (and which hatched larvae) across breeding seasons in three consecutive years (2011, 2012, 2013) form Lymington salt marsh (Hampshire, UK). Data are presented as means ± S.E. See Table 3.2 for female numbers sampled per month in each of the three years.

3.4.7 Egg and hatchling dry weight through the breeding season

The dry weight of newly extruded eggs varied significantly between 2011 and 2012 (F = 33.45, $P < 0.001$), between months within these years (F = 15.46, $P < 0.001$), and there was an interaction between month and year (F = 27.00, $P < 0.001$) indicating that variation in dry weight between months was different between years. Indeed, in 2011 the dry weight of newly extruded eggs did not vary between months (Figure 3.9A); however,

in 2012 dry weight decreased across the breeding season: from 162.78 ± 1.16 µg ind.$^{-1}$ in April to 157.06 ± 1.64 µg ind.$^{-1}$ in May ($P < 0.001$) and to 147.31 ± 1.06 µg ind.$^{-1}$ in June ($P < 0.001$) (Figure 3.9A).

The dry weight of newly hatched larvae varied significantly between years (F = 806.29, $P < 0.001$) and was generally highest in 2011 and lowest in 2013 (Figure 3.9B). Dry weight also varied between months (F = 11.96, $P < 0.001$). This variation between months was different for different years as there was a significant interaction between year and month (F = 168.66, $P < 0.001$). In 2011, larval dry weight decreased from 149.98 ± 2.29 µg ind.$^{-1}$ in May to 137.00 ± 0.86 µg ind.$^{-1}$ in June ($P < 0.001$) and increased again from June to July (142.06 ± 0.86 µg ind.$^{-1}$; $P = 0.0220$) (Figure 3.9B). In 2012, larval dry weight was consistent between May (127.97 ± 0.86 µg ind.$^{-1}$) and June (129.34 ± 1.13 µg ind.$^{-1}$) but then decreased significantly to 115.20 ± 0.94 µg ind.$^{-1}$ in July ($P < 0.001$). In 2013, larval dry weight increased across the breeding season from 91.67 ± 1.19 µg ind.$^{-1}$ in May to 110.23 ± 0.83 µg ind.$^{-1}$ in June ($P < 0.001$) and to 124.81 ± 1.16 µg ind.$^{-1}$ in July ($P < 0.001$).

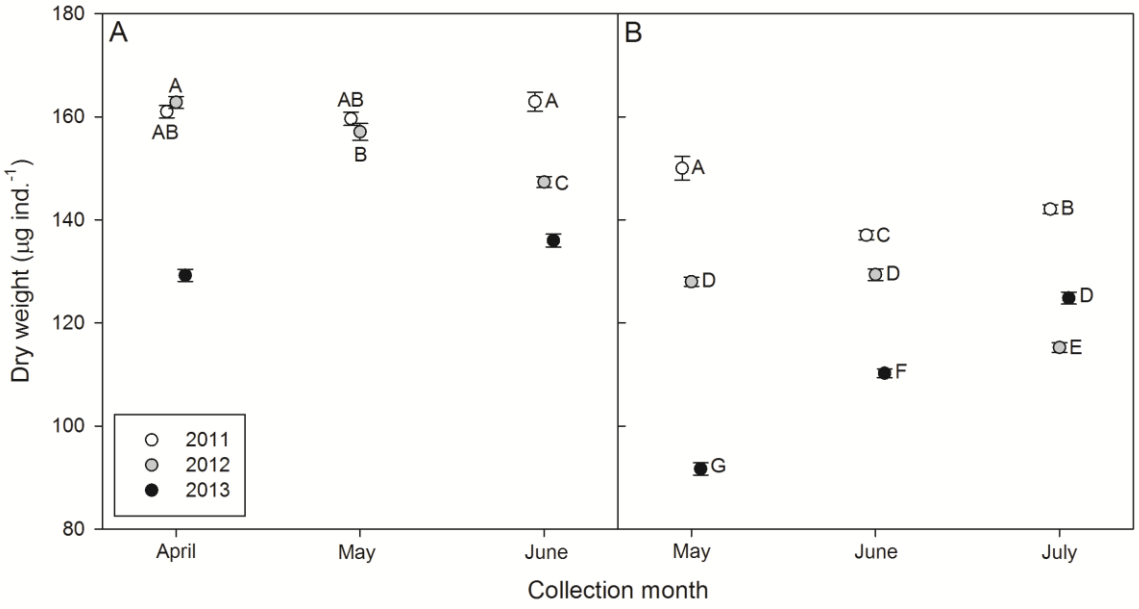

Figure 3.9 *Palaemonetes varians* dry weight of **A** stage I and II eggs and **B** newly hatched larvae across the breeding season in three consecutive years (2011, 2012, 2013) form Lymington salt marsh (Hampshire, UK). Data are presented as means ± S.E. Data points, which share letters, are statistically indistinct. See Table 3.2 for female numbers sampled per month in each of the three years; 15 eggs or larvae sampled per female.

3.5 Discussion

3.5.1 Environmental variation

Temperature fluctuated seasonally within the ditch from which *Palaemonetes varians* were sampled, between ~0 °C in winter and ~25 °C in summer (Figure 3.2). Such seasonal variations are of similar magnitude to those reported elsewhere for habitats in which *P. varians* have been found; for example, 0 - 33 °C within drainage ditches on salt marsh in the Cardiff area (Wales, UK) (Lofts 1956). During the period of monitoring for this study, temperature varied between a minimum of -0.14 °C (12th March 2013) to a maximum of 26.2 °C (25th May 2012) (Figure 3.2). The minimum temperatures recorded during winter appear to be close to the thermal limits of this population of *P. varians*, which were found to be ~0 °C; 100 % mortality occurred at -1 °C whilst at 0 °C mortality was low (Oliphant et al. 2011). The upper thermal limit of *P. varians* has been established as ~33 °C (Oliphant et al. 2011, Ravaux et al. 2012), although a preliminary experiment with a slower rate of temperature ramping found the upper thermal limit to be ~33-34 °C with 100 % mortality at 35 °C; shrimp were inactive between 32-34 °C unless stimulated mechanically (Oliphant, unpublished data). Further, Ravaux et al (2012) demonstrated thermal acclimation within *P. varians*; thermal limits were higher for shrimp acclimated at higher temperatures, indicating that this species can acclimate to seasonal temperature fluctuations. Such adjustments in the thermal tolerance window of *P. varians* likely better enable this species to cope with the short term fluctuations experienced in the salt marsh environment. Similarly, this thermal niche plasticity also enables shrimp within different populations to acclimate to local thermal conditions; optimising performance at local temperatures and enabling a wider spatial and temporal distribution.

The thermal tolerances of *Palaemonetes varians* closely match the minimum and maximum limits reported here and elsewhere (Lofts 1956, Jefferies 1964). Smith et al. (2012) reported a thermal preference for *P. varians* acclimated at 8 °C of ~ 18 °C, suggesting that, whilst extreme low temperatures of 0 °C could be tolerated, *P. varians* prefer higher temperatures. Certainly, moulting is thought to initiate above 9-10 °C only (Jefferies 1964), and maturation of the gonad above 5 °C (Hindley 2001). Such differences between temperature tolerance and preferred temperatures may be common among palaemonine shrimp; for example, Rodriguez and Ramirez (1997) demonstrated a thermal preference for *Macrobrachium tenellum* of between 21-23 °C and 31.5-33.6 °C (depending

on acclimation temperature) but reported temperatures at the site where animals were sampled had an average minimum of 10.5 ± 5.8 °C and an average maximum of 32.8 ± 5.08 °C over 10 year period, indicating that *M. tenellum* must tolerate much lower temperatures than its preferred thermal low.

Salinity also fluctuated considerably with a minimum of 0.46 ± 0.25 and a maximum of 43.2 ± 0.5 (Figure 3.3). The low salinity observed is comparable to that of 0.1 reported by Lofts (1956) but the high reported exceeds that of 36 reported by Lofts (1956). *Palaemonetes varians* are isotonic with their environment when salinity is ~25, when salinity is above or below this, respiration rates are elevated corresponding to greater energy usage on osmotic regulation (Lofts 1956). Although not tested explicitly, *Palaemonetes varians* appeared to migrate within ditches and in response to changing environmental conditions. On warm sunny days during spring and summer, shrimp (including ovigerous shrimp) were observed in sunny shallows, apparently 'sun bathing' on the dark muddy bottom (personal observations). During, or shortly after, periods of intense or prolonged rainfall, shrimp were scarce, having moved away from the sampling area (personal observations). In the very warm summer months of 2013 when both temperature and salinity were high, some shrimp appeared whitish in colour, especially across the tail region, and some ovigerous females had broods that appeared to be infected with a fungus/mould which caused eggs to appear yellowish in colour and easily rupture, suggesting that shrimp were not able to avoid unfavourable conditions (personal observations).

Daily temperature fluctuations within the ditch were considerable (Figures 1 and 10A). Within the sample period presented in Figure 3.10A, maximum temperature fluctuations during a daily cycle were ~6 °C. Similarly, within short periods of time, temperatures within the ditch could change considerably; for example in March 2013 temperatures dropped by 11.9 °C in ~66 hours (Figure 3.2). Jefferies (1964) also reported a temperature change of 9 °C within three days for a population of *P. varians* from Moreton (Cheshire, UK). The magnitude of variability recorded within the ditch on Lymington salt marsh was far greater than that measured for Bramble Bank; a sub-tidal mooring in the central Solent (Figure 3.9A). During the same period, whilst temperatures in the ditch on Lymington salt marsh fluctuated by ~4-6 °C across the daily cycle, temperatures on Bramble Bank in the central Solent fluctuated by <1 °C (Figure 3.10A). Temperature fluctuations recorded for Lymington salt marsh are intermediate in magnitude between the

sub-tidal observations and those measurements for inter-tidal habitats. For example, for inter-tidal pools within False Bay, San Juan Island (Washington, USA), Podolsky (2003) recorded temperature fluctuations of >22 °C during a single tidal cycle (Podolsky 2003) (Figure 3.10B).

The inter-tidal environment is thermally highly variable and can be considered an extreme environment (Helmuth 1998, 1999, Somero 2002). Similarly, salt marsh environments experience high thermal and salinity variability (Marsden 1976). Fauna inhabiting salt marsh environments must exhibit adaptations to this highly variable environment, as demonstrated for *P. varians* (e.g. Lofts 1956, Cottin et al. 2010, Oliphant et al. 2011, Smith et al. 2013) and other salt marsh fauna (e.g. Marsden 1976). Recruitment of larvae into tidal habitats and shallow-water salt marsh habitats is likely accompanied by a significant shift in thermal fluctuations and new recruits must tolerate such variations. Palaemonine shrimp, especially the genera *Palaemonetes* and *Palaemon*, inhabit shallow coastal, estuarine, brackish, salt marsh, and fresh water environments. Such habitats are thermally variable, especially relative to the sub-tidal environment, and these shrimp are likely adapted to high temperature variability and, as a group, show a tendency for thermally variable environments. Closely related bresilid hydrothermal vent shrimps (Tokuda et al. 2006, but see Li et al. 2011) inhabit the thermally dynamic environment of deep-sea hydrothermal vents, suggesting that a likely common ancestor of these extant shrimp, which are adapted to the diverse but thermally variable environments they inhabit, was tolerant of temperature variations. A number of studies have compared *Palaemonetes* and *Palaemon* species with bresilid vent shrimp (Gonzalez-Rey et al. 2007, Gonzalez-Rey et al. 2008, Cottin et al. 2010, Smith et al. 2013), finding that the shallow-water shrimp have high tolerance of thermal variations, but are less tolerant then their vent-living counter-parts. In comparison to the daily temperature fluctuations observed in Lymington salt marsh, temperature fluctuations observed within hydrothermal vent environments are of a similar, or slightly lesser, magnitude (Figure 3.10C). Over short time scales, temperature variations of 2-3 °C and occasionally 6 °C were observed, changes taking a few hours. Although not as large, on the scale of 2-3 °C, these changes take place on a more rapid scale. In a comparison between *P. varians* and the vent shrimp *Rimicaris exoculata*, the vent-living shrimp was found to better tolerate rapid fluctuations in temperature and extreme heat shock, *P. varians* demonstrated high tolerance of these temperature shocks (Cottin et al. 2010, Smith et al. 2013).

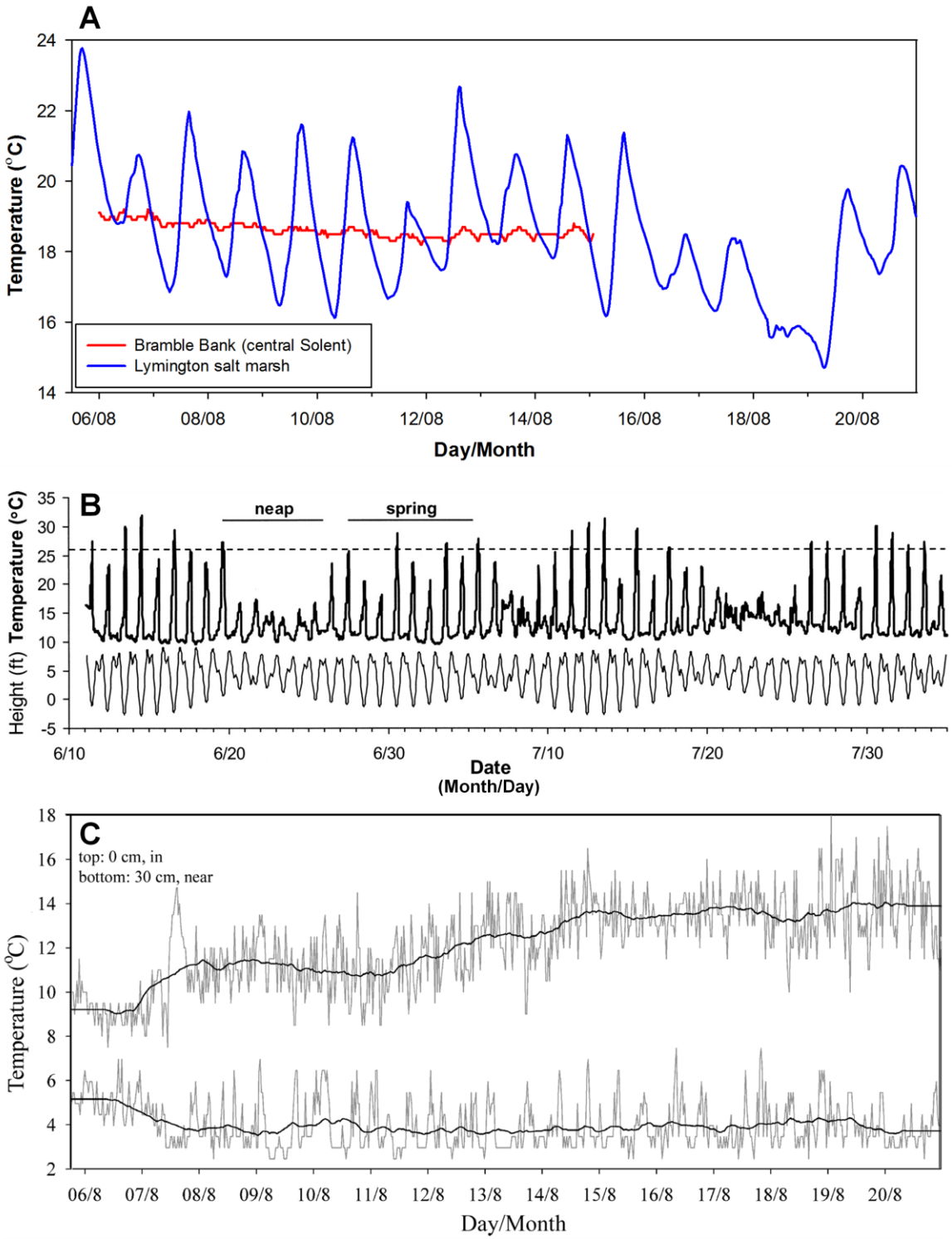

Figure 3.10 **A** Temperature time-series data for 6th to 20th August 2011 for Lymington salt marsh (data from nke instrument temperature data loggers) and for Bramble Bank central Solent sub-tidal (data from http://www.bramblemet.co.uk/ (accessed 08/12/13) Temp sensor Thermistor supplied by Campbell Scientific Limited. **B** inter-tidal tide pool water temperature time series data at False Bay, San Juan Island (Washington, USA) (data from Tidbit data loggers Onset Corp., MA) (From (Podolsky 2003)). **C** Temperature time series data for Endeavour Segment (depth 2220 m) of Juan de Fuca Ridge hydrothermal vents: temperature records are 0 cm (in-vent) and 30 cm (near vent)

from focused flow (from Bates et al. 2005) data from DS1921L-F50 Thermochron iButton temperature data loggers)

3.5.2 Inter- and intra-annual variation in maternal size

Maternal size varied across the breeding season for ovigerous *Palaemonetes varians* brooding newly extruded eggs and hatching larvae. This variation was consistent between years and between ovigerous *P. varians* brooding newly extruded eggs and hatching larvae (Figure 3.8), suggesting that the change in female size across the breeding season was influenced by population structure. Variations in the size of breeding females have been documented for *Palaemon macrodactylus*, female size within this species decreased across the breeding season (Vázquez et al. 2012). This decrease in female size was a result of large females breeding first, then all mature females breeding, lowering the average size. Large females then died off and a cohort of smaller females joined the breeding population, resulting in a further reduction in the average size of breeding females (Vázquez et al. 2012). Similarly, for two populations of *Palaemonetes pugio*, Alon and Stancyk (1982) found two female cohorts and noted variable breeding female size across the breeding season, with female size generally decreasing across the breeding season. Results presented here for *P. varians* suggest that there are three cohorts of females, all of which are mature and breed (Figure 3.4). It appears that early in the breeding season (April), females from cohort 3, cohort 2 and the largest females from cohort 1 breed. In mid-breeding season (May) females from cohort 2 and cohort 1 breed and by the end of the breeding season (June) most of the breeding females are from cohort 2 only, with a few females from cohorts 1 and 3. Across the breeding season, cohort 3 is lost as these old, large females die off. Also within cohort 1, there may be a decrease in size across the breeding season. This is likely associated with the small size of cohort 1 females and the high cost of producing eggs; thus, larger females in this cohort are able to breed first and smaller females breed later. As cohorts 2 and 3, and the largest females in cohort 1, breed first, followed by only cohorts 2 and 1, female size initially decreases, consistent with studies on *Palaemon macrodactylus* and *Palaemonetes pugio* (Alon & Stancyk 1982, Vázquez et al. 2012). Female size then remains constant or rises again as breeding females are mostly from cohort 2 only. Further, Hindley (2001) suggested that within a population of *P. varians* from the Ribble Estuary (Lancashire, UK), large females have a second round or breeding.

The population structure within Lymington salt marsh consisted of three female cohorts and two males cohorts. Differences in the timing of breeding between females of differing cohorts appears responsible for the changing size of breeding females over the summer. Unfortunately, concluding whether the three female cohorts represent three consecutive years of settlement or two years of settlement, the first year being cohort 3 and cohorts 1 and 2 being late and early settling shrimp of the second year, is not possible. Similarly, it is not possible to deduce whether males are limited to the smaller size categories as a result of earlier mortality, limited/cessation of growth, or a protandric hermaphroditic change of sex. A study across multiple years may help to elucidate such questions; alternatively, lab based study could assess this question with more certainty and could manipulate conditions to assess the influence of the environment on populations structure.

3.5.3 Egg volume as proxy for egg dry weight

Generally among marine invertebrates, but especially within echinoderms, a positive relationship between egg volume and egg energy content and organic dry weight is well documented and has repeatedly been demonstrated for numerous taxa (McEdward & Chai 1991, Clarke 1993, Jaeckle 1995, McEdward & Morgan 2001). This relationship is known at both the inter- and intra-specific level; larger eggs have more energy and higher organic dry weight (McEdward & Chai 1991, Clarke 1993, Jaeckle 1995, McEdward & Morgan 2001). The results presented here demonstrate that within *P. varians*, there is a positive relationship between egg volume and dry weight (Figure 3.5), consistent with previous literature (e.g. Clarke 1993).

The use of egg volume as a proxy for egg energy content has received criticism as, although a positive relationship exists, egg volume is not an accurate proxy for energy content (Moran & McAlister 2009, Moran et al. 2013). The results presented here for *Palaemonetes varians* are consistent with this. Although a positive relationship exists, this relationship was not strong ($R^2 = 0.41$) and considerable scatter was evident around the line of best-fit. Further, the correlation between egg volume and egg dry weight varied between months (Figure 3.5B). These data concur with previous studies (see Chapter 1, section 1.1.1), indicating that egg volume is a relatively poor proxy for mass and egg energy content.

3.5.4 Larval elemental composition

Interestingly, larval composition differed between larvae of differing dry weight (Figure 3.7). Larval protein content (%*N*) decreased with increasing larval dry weight whilst lipid content (%*C*) increased, as such, larger larvae had significantly greater lipid reserves than smaller larvae. At the inter-specific level for seven species of echinoderm, two polychaetes, and an oyster, Strathmann and Vedder (1977) found that smaller diameter eggs had proportionally greater organic matter reserves than larger diameter eggs. In contrast for *P. varians*, larger hatchlings have higher lipid content than smaller hatchlings (Figure 3.7). Owing to the greater mass of hatchlings with higher dry weight, both protein and lipid mass (nitrogen and carbon, respectively) were higher than for hatchlings with higher dry weights (Figure 3.7). Geister et al. (2009) demonstrated a similar trend for butterfly (*Bicyclus anynana*) eggs produced at different temperatures. Hatchlings from eggs produced at 20 °C had higher absolute amounts of water (+11.1 %), lipids (+20.4 %), and proteins (+35.7 %) than hatchlings from eggs produced at 27 °C (Geister et al. 2009); however, the relative composition of eggs produced at 20 and 27 °C was similar (Geister et al. 2009). In contrast, two populations of the echinoid, *Arbacia lixula*, were found to produce eggs which differed in size and lipid and protein content; larger eggs having relatively higher lipid and protein content (George et al. 1990). Similarly, echinoderms maintained under differing food rations produce differing size eggs with differing lipid and protein contents; those provided poor food ratios produced smaller eggs with relatively lower protein and lipid contents (see George 1996). Differences in hatchling dry weight and composition reported here for *P. varians* may indicate differing adult nutritional states. The possibility, however, that such differences arise through conditions during embryogenesis should not be ignored. For example, within the snapping shrimp, *Betaeus emarginatus*, temperature significantly affected larval size at hatching: larvae being smaller after developing at higher temperatures (Wehrtmann & Lopez 2003, see also Smith & Thatje 2013). Similarly, significant differences between embryos within broods may be associated with the position of embryos within the brood; i.e. on different pleopods (e.g. Pochelon et al. 2011).

3.5.5 Correlations between POI and environmental variables

Variations in POI are known within populations on temporal scales and have been demonstrated for decapod crustaceans inter-annually (e.g. Kattner et al., 1994), between seasons (e.g. Boddeke, 1982, Sheader 1983, Oh and Hartnoll, 2004, Urzua et al., 2012),

and within a single breeding season (e.g. Sampedro et al., 1997, Bas et al., 2007). Many of these examples have been associated with increasing temperatures gonad development (Kerfoot 1974, Perrin 1988). Results presented here demonstrate variations in POI within and between years; consequently, attempts were made to investigate the relationship between POI and environmental temperature during gonad development, and during embryonic development. Gonad maturation has been estimated to initiate once environmental temperatures rise above 5 °C (Hindley 2001). The period during winter, when environmental temperatures are below 5 °C, may be considered as winter dormancy; after which gonad maturation may take place (Hindley 2001). On this basis, total degree days for gonad maturation within *Palaemonetes varians* were calculated (Figure 3.11). Degree days were calculated using the formula:

$$Degree\ days = \left(\frac{T_{max} + T_{min}}{2}\right) - T_{base}$$

where T_{max} = maximum temperature of the day, T_{min} = minimum temperature of the day, and T_{base} being the temperature above which gonad maturation may occur (5 °C). If the degree days ≤0, no gonad maturation may occur. Degree days were calculated for each day from the end of winter dormancy and added cumulatively. It was found that from the winter dormancy in 2011 and 2012 until the first occurrence of ovigerous females during sampling in April, which was considered to be approximately the start of the breeding season, total degree days = ~450. This was not, however, consistent with 2013 when total degree days = ~220. Despite this, a value of 450 degree days up to the times of collections of ovigerous females brooding stage I and II eggs was used to estimate the period over which temperature may have influenced gonad maturation and thus egg POI (and egg POI adjusted for maternal size). No correlations between temperatures calculated from this method and egg POI (or egg POI adjusted for maternal size) were evident across all years. Temperature was then averaged from the estimated end point of the previous year and the times of collections of ovigerous females brooding stage I and II eggs. Again, no correlations were evident between temperatures calculated via this method and egg POI (or egg POI adjusted for maternal size).

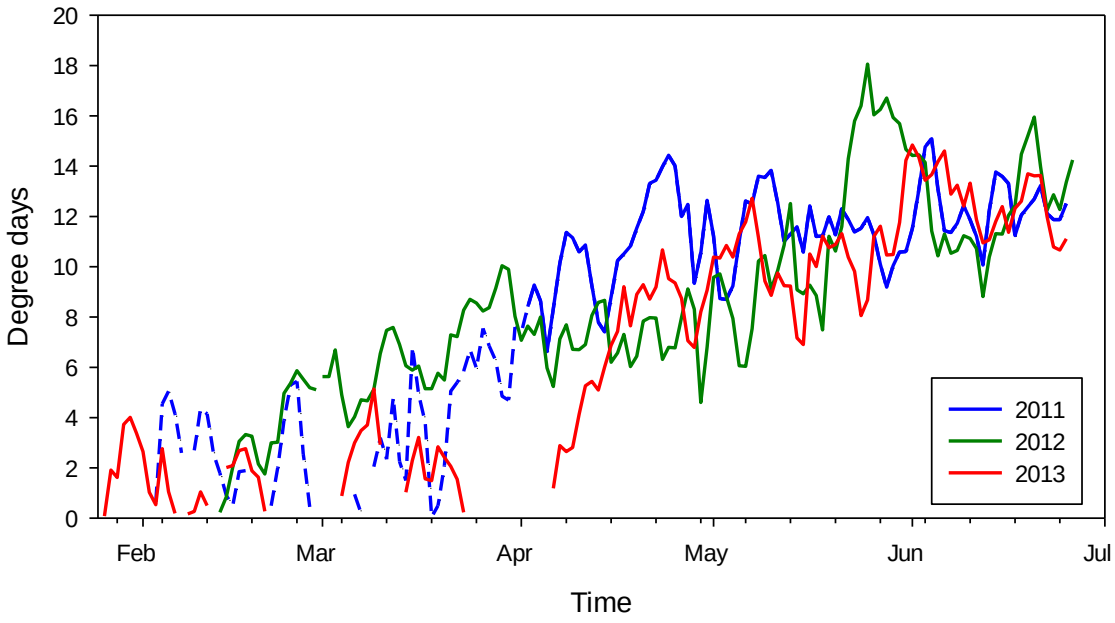

Figure 3.11 Degree days for three breeding seasons (2011, 2012, 2013) on Lymington salt marsh (Hampshire, UK). Dashed line for 2011 was calculated from air temperature, whilst all other data were calculated from water temperatures

Despite trends in POI, correlations with environmental temperatures have rarely been quantified. The lack of correlation between POI and temperature found here may indicate the poor method used to estimate the appropriate period over which to expect temperature to have influenced POI. Histology of shrimp gonads throughout the year would enable an accurate assessment of the onset of gametogenesis and the period over which gametogenesis takes place. From these data, the correct period over which to assess the influence of temperature on POI could be determined. However, the lack of correlation between temperature and POI, here, may be accurate, indicating that additional factors such as salinity, primary production, predation, influence POI. Alternatively, shifts in *P. varians* POI may be robust against the influence of temperature.

3.5.6 Maternal size and offspring POI

Among arthropods, female size is known to influence offspring provisioning. In general, larger females give rise to larger offspring, this relationship is often weak and is certainly not ubiquitous; indeed, negative relationships are documented and studies, which found no relationship, may be under reported (Fox & Czesak 2000, Marshall & Keough

2008). Within *Palaemonetes varians*, egg size is positively, though weakly, correlated to maternal size, similarly, larval size is positively correlated with maternal size (Figure 3.12A, B). Adaptive explanations have been proposed where correlations between maternal size and offspring size have been identified (Parker & Begon 1986, Fox & Czesak 2000, Sakai & Harada 2001, Marshall & Keough 2008). For example, larger mothers may provision offspring more efficiently than smaller mothers (Sakai & Harada 2001) or larger, more fecund, mothers may better provision offspring to compensate for higher levels of sibling competition (Parker and Begon 1986). More simply, this trend may arise from morphological and anatomical scaling, meaning that larger females are able to produce larger offspring (Fox & Czesak 2000). Too little data is presently available to evaluate the merits of the above hypotheses, especially within marine invertebrates (Marshall & Keough 2008). Intriguingly for *P. varians*, the relationship between maternal size and offspring size is greater for larvae than for eggs, suggesting that maternal size may have implications for embryonic development (Figure 3.12A, B). For example, larger females may be better able to ventilate the brood by pleopod flapping or may out-compete smaller females for the best environment in which to brood embryos.

Fox and Csezak (2000) highlighted a trend for larger females to invest a smaller proportion of their resources per offspring. *Palaemonetes varians* conforms to this trend; both egg and larval dry weight, adjusted for female size, decrease with increasing maternal size (Figure 3.12C, D). Assuming that the resource available to reproduction increases with maternal size, a decrease in egg and larval dry weight adjusted for female size suggests lower proportions of resource investment per offspring. Therefore, whilst larger females may produce larger offspring, the change is not proportional to the increase in maternal size, suggesting that the production of larger offspring is relatively less costly for larger females. Both the relationship between maternal size and offspring size, and the relationship between maternal size and the proportion of resources invested per offspring raise important questions for the timing of reproduction within semalparous animals such as *P. varians*. It would appear that reproducing as a large female produces better provisioned offspring; therefore, delaying reproduction until a larger size would appear advantageous. Investing energy into reproduction whilst at a smaller size appears disadvantageous. However, delaying reproduction may be costly too as the chances of suffering mortality before reproducing increase. There would, therefore, appear to be a trade-off between the timing of reproduction and the quality of offspring produced and this

trade-off would, presumably, differ between environments. More data is requisite to draw conclusions about such potential trade-offs.

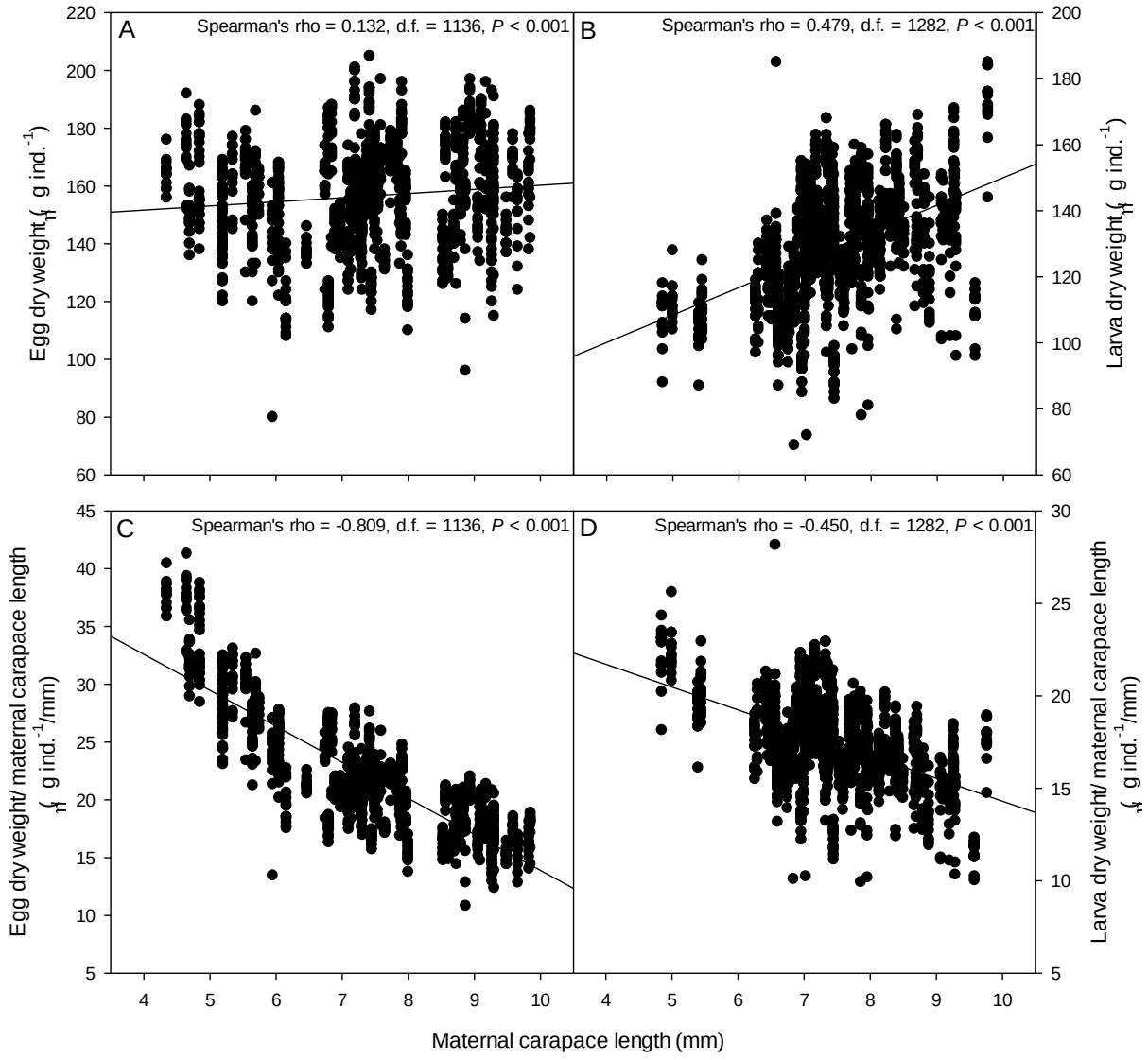

Figure 3.12 Correlations between maternal carapace length and **A** egg dry weight, **B** larva dry weight, **C** egg dry weight adjusted for maternal carapace length, and **D** larva dry weight adjusted for maternal carapace length. Spearman's correlation coefficients are shown.

Data presented here demonstrate highly variable temperatures and salinities on Lymington salt marsh, both with seasonal and diurnal cycles. Whilst POI varied across breeding seasons and between breeding seasons, no correlation was evident between POI and temperatures during gonad development. Correlations were found between egg volume and dry weight and between larval elemental content and dry weight.

3.5.7 Conclusions

- Both temperature and salinity are highly variable within the Lymington salt marsh environment. Conditions fluctuate on both a seasonal and diurnal scale.
- The *Palaemonetes varians* population structure comprises two male cohorts and three female cohorts. The relative ages of these cohorts were not determined here.
- Ovigerous female size varied across breeding seasons and was consistent between years. Differences in the timing of breeding between the three female cohorts were responsible for the consistent variation in the size of ovigerous females across the breeding season.
- Egg dry weight and egg volume were positively correlated; however, there was considerable scatter and a low R^2 value, thus egg volume is not an accurate proxy for egg dry weight (or energy content) within *Palaemonetes varians*.
- Larval composition varied with larval dry weight. Larval nitrogen content was negatively correlated with larval dry weight, whilst larval carbon content and the ratio between carbon and nitrogen were positively correlated with larval dry weight. Positive correlations with high R^2 values were found for the relationships between larval dry weight and nitrogen and carbon masses.
- Per offspring investment, measured for both newly extruded eggs and newly hatched larvae, varied across breeding seasons and between years. This variation was not consistent between years.
- Variations in POI were not correlated with temperature during gonad development.

4. The implications of temperature-mediated plasticity in larval instar number for development within a marine invertebrate, the shrimp *Palaemonetes varians*[*]

4.1 Summary

Variations in larval instar number are common among arthropods. Within this chapter the implications of temperature-mediated variations in larval instar number for larval development time, larval growth rates, and juvenile dry weight within *Palaemonetes varians* are assessed. In contrast with previous literature, which focuses on terrestrial arthropods, particularly model and pest species often of laboratory lines, wild shrimp were used, which differ in their life history from previous models. Newly-hatched *P. varians* larvae were first reared at 5, 10, 17, 25, and 30 °C to assess their thermal scope for development. Larvae developed at 17, 25, and 30 °C. At higher temperatures, larvae developed through fewer larval instars. Two dominant developmental pathways were observed; a short pathway of four instars and a long pathway of five instars. Longer developmental pathways of six to seven instars were rarely observed (mostly at lower temperatures) and consisted of additional instars as 'repeat' instars; i.e. little developmental advance over the preceding instar. To assess the implications of temperature-mediated variation in larval instar number, newly-hatched larvae were then reared at 15, 20, and 25 °C. Again, the proportion of larvae developing through four instars increased with temperature. At all temperatures, larval development time and juvenile dry weight were greater for larvae developing through five instars. Importantly, because of the increasing proportion of larvae developing through four instars with increasing temperature, larval traits associated with this pathway (reduced development time and juvenile dry weight) became more dominant. As a consequence of increasing growth rate with temperature, and the shift in the proportion of larvae developing through four instars, juvenile dry weight was greatest at the intermediate temperature (20 °C). At settlement *P. varians* juveniles do not follow the temperature-size rule; this is of importance for life-history ecology in response to environmental change, as well as for aquaculture applications.

[*] Published 'Oliphant A, Hauton C, Thatje S. (2013) The implications of temperature-mediated plasticity in larval instar number for development within a marine invertebrate, the shrimp *Palaemonetes varians*. PLoS ONE 8(9) e75785

4.2 Introduction

Variations in the number of instars during larval development, and the morphology of same stage larvae, within arthropod species is a relatively common plastic trait and is influenced by temperature, salinity, photoperiod, density, sex, food quantity, diet quality, humidity, and pollution (including antibiotics; for reviews see Knowlton 1974, Esperk et al. 2007). Within insects, variations in larval instar number are particularly common within orders Lepidoptera and Blattodea (roach spp.) whilst they are lacking within orders Diptera and Hymenoptera (excluding sawflies), suborder Heteroptera (true bugs) and superfamily Papilionoidea (butterflies; for review see Esperk et al. 2007). Within crustaceans, they are known for many planktotrophic decapod larvae and appear especially prevalent within euphausiacean, penaeidean, and caridean decapods but are less common within anomurans and brachyurans (Knowlton 1974).

Arthropods must periodically moult to grow and develop. Given this inherent biology, larval development is coupled with the moult cycle. Environmental conditions which differently affect the processes of moulting and of growth and development de-synchronise these processes and drive variations in larval instar number (Knowlton 1974, Fincham 1979). For some arthropod species, including the tobacco hornworm, *Manduca sexta*, and the forest tent caterpillar, *Malacosoma disstria*, species-specific genetically determined size thresholds (so-called 'critical weights') have been shown to initiate pupation and metamorphosis (Nijhout 1975, 1994, Kingsolver 2007, Etilé & Despland 2008). Environmental conditions, which slow growth and development, tend to extend larval development until this threshold is achieved and, consequently, result in the addition of larval instars (Nijhout 1975, 1994). In contrast, for those arthropod species with a fixed number of larval instars (e.g. the folivorous moth, *Epirrita autumnata*), adult size is strongly dependent on environmental conditions, indicating no size and development threshold for the initiation of metamorphosis (Tammaru 1998).

At the inter-specific level within the marine environment, there exists a macro-ecological gradient in larval instar number among marine crustaceans. With increasing latitude, often coupled with decreasing temperature and increasing seasonality and unpredictability of primary production, larval instar number is reduced (see Anger 2001, Thatje et al. 2003). This is termed abbreviated development and is accompanied by a greater degree of endotrophic development potential. At the intra-specific level,

temperature is known to influence larval instar number and has been associated both with additions in larval instar number, and the omitting of larval instars (Knowlton 1974, Criales & Anger 1986, Willott & Hassall 1998, Etilé & Despland 2008 for review see Esperk et al. 2007). In some cases, the influence of temperature on variations in larval instar number within marine crustacean species is consistent with the macro-ecological trend in larval instar number at the inter-specific level. For example, within the palaemonid shrimp, *Palaemonetes vulgaris*, larval development proceeds through more larval instars under warmer temperatures (Knowlton 1974).

Intra-specific variations in larval instar number are known to influence juvenile and adult body size within arthropods. Such variation in body size may have significant ecological implications. For example, within insects, which do not grow as adults, the accumulation of mass during larval stages affects adult size and several adult traits, including fecundity (Davidowitz et al. 2004). Temperature also influences body size among ectotherms. Estimated to occur in >80 % of ectotherms (including animals, plants, protozoans, and bacteria), temperature-dependant body size among ectotherms is described by the temperature-size rule (TSR); reared under lower temperatures, organisms grow to a larger body size (Atkinson 1994). However, the influence of temperature-mediated variations in larval instar number on body size and how this plasticity may affect the temperature-size rule are poorly reported.

In an experiment, which sought to identify the thermal limits of larval development within the shrimp, *Palaemonetes varians*, and the influence of temperature on larval development time and juvenile dry weight, we identified temperature-mediated variation in larval instar number. This variation in larval instar number affected both larval development time and juvenile dry weight. We were, therefore, provided the opportunity to assess the influence of temperature-mediated variations in larval instar number on larval development and on body size within *P. varians*.

Working with crustacean larvae is innately difficult because of their small size, the need for tedious individual rearing methods, and high maintenance requirements (Anger 2006). Coupled with the fact that the agricultural economic importance of insect pest species has driven research into the larval phase of insects, knowledge of insect larvae is far greater than that of crustaceans (Anger 2006). For example, studies assessing the influence of the environment, especially temperature, on development time, growth rate,

critical weight, larval instar number and subsequent adult mass have focused on insects, particularly pest and model species such as *Manduca sexta* (e.g. Davidowitz et al. 2004, Davidowitz & Nijhout 2004a, Nijhout et al. 2010). Laboratory lines of such species, raised under constant conditions and on standard diets for many hundreds of generations, have shown significantly different growth and development rates and less variable development compared with wild type organisms (e.g. Kingsolver 2007). The commercial exploitation of crustacean species within aquaculture now drives a need for understanding better the crustacean larval phase (e.g. Anger et al. 2009). Coupled with the fact that much of what we know about the arthropod larval phase is derived from laboratory lines of model and pest insects, here, we assess the implications of temperature-mediated variations in larval instars number within wild individuals of the caridean shrimp, *Palaemonetes varians*.

4.2.1 Hypotheses

The specific hypotheses tested were:

H_1 temperature influences larval instar number: larval development proceeds through fewer larval instars at higher temperatures

H_2 temperature influences juvenile size at settlement: juvenile size at settlement is smaller at higher temperatures

H_3 temperature influences larval development rate: larval development is more rapid at higher temperatures

H_4 larval development through fewer larval instars results in earlier juvenile settlement and juveniles of smaller size

4.3 Materials and Methods

Experiment 1 (Exp 1; thermal scope for larval development) assessed the temperature range across which successful larval development may take place. This experiment observed temperature-mediated variation in larval instar number, which influenced development time, growth rate and juvenile dry weight (*W*). Importantly, given the low fecundity of *Palaemonetes varians*, particularly among the individuals used, coupled with the high number of temperature treatments done, larvae from different females were not divided between all temperatures. Therefore, this experimental design did not account for maternal effects or heritable differences between larvae from different females. This prevented a detailed assessment of the influence of temperature-mediated

variation in larval instar number on larval development. Consequently, we carried out Experiment 2 (Exp 2; effects of developmental plasticity) to assess the effects of temperature-mediated variation in larval instar number on larval development time, larval growth rate, and juvenile DW.

For both experiments, larvae used were hatched from wild collected ovigerous *P. varians*. For Experiment 1, 15 ovigerous *P. varians* were collected between June and July, 2011. The field water temperature at the time of collection was 17 °C. For Experiment 2, 30 ovigerous *P. varians* were collected between May and June, 2012. The field water temperature was 15 °C at the time of collection. All collections and maintenance of shrimp followed the same protocol, detailed below.

4.3.1 Collections and maintenance

Ovigerous *Palaemonetes varians* were collected as described in Chapter 2, section 2.2.2. The embryonic development of broods was assessed and staged according to Müller et al. (2004); see section 2.2.1 .Ovigerous *P. varians* (with stage VII and VIII embryos) were maintained as described in Chapter 2, section 2.4.1.

4.3.2 Larval maintenance

4.3.2.1 Experiment 1 (thermal scope for larval development)

On hatching, actively swimming larvae were separated from 14 ovigerous *P. varians* and maintained as described in Chapter 2, section 2.4.3. Larvae were cultured at constant temperatures of 5, 10, 17, 25, and 30 °C and 12:12 (light:dark); n = 96 larvae from three to four females per treatment. This temperature range reflects temperatures naturally experienced by adult populations and the range of adult thermal tolerance (Jefferies 1958, Oliphant et al. 2011, Ravaux et al. 2012). Zoea 1 is facultative lecithotrophic (see Chapter 5); consequently in all treatments zoea 1 larvae were not fed. On moulting to zoea 2, larvae were fed freshly-hatched *Artemia* sp. nauplii (Hobby Artemia) to excess. Larval mortality and development, assessed by morphological changes and moulting (see Chapter 2, section 2.4.4) were monitored daily (am). On moulting to the juvenile stage, animals were preserved for measurements of dry weight (see Chapter 2, sections 2.4.3 and 2.7).

4.3.2.2 Experiment 2 (effects of developmental plasticity)

Larval maintenance followed the same protocol as above with the following exceptions. Larvae, hatched from 27 ovigerous *P. varians*, were reared at 15, 20, and 25 °C; this temperature range falls within that of viable larval development and, given the results of Experiment 1, was considered the most interesting in terms of temperature-mediated variation in larval instar number. Twelve larvae from each of the 27 ovigerous *P. varians* were placed at 15, 20, and 25 °C (a total of 324 larvae per temperature), thus this experimental design accounted for maternal effects and heritable differences between larvae from different females. Larvae were maintained as described in Chapter 2, section 2.4.3 and, on moulting to zoea 2, were fed freshly hatched *Artemia* sp. nauplii (ZM systems, decapsulated brineshrimp cysts) to excess.

4.3.3 Nomenclature of larval stages

Fincham described five zoeal stages for the larval development of *Palaemonetes varians* (Fincham 1979). Here, Fincham's (1979) morphological descriptions were followed to differentiate progressive larval stages; however, terminology for the naming of larval stages differed (see Chapter 2, section 2.4.4). The first two larval stages were assigned zoea 1 and zoea 2 (following Fincham 1979). The third, fourth, and fifth larval stages were assigned decapodid 1, decapodid 2, and decapodid 3 (following Kaestner 1980, Anger 2001). Fincham (1979) reported considerable morphological variation between same stage larvae. Here, morphological variation between same stage larvae was also observed. Rarely, moulting after decapodid 2 occurred with little advance in morphological development – so called 'repeat' moults/instars (Rochanaburanon & Williamson 1976, Fincham 1977, 1979). In Experiment 1, larvae undergoing 'repeat' instars developed through five to seven larval instars before metamorphosis. Although larval morphology advanced slightly with 'repeat' instars, morphological development was subtle between subsequent larval instars. Studies have identified and named intermediate larval instars of consistent morphology (Criales & Anger 1986, Hassall & Grayson 1987). Here, the number and morphology of 'repeat' instars was inconsistent and the naming of intermediate larval instars of consistent morphology was not possible. Instead 'repeat' instars are reported here ubiquitously as decapodid' (D' in figures). Within a single development pathway a decapodid' instar was considered to be more morphologically advanced than previous instars; however, decapodid' instars are not morphologically consistent between larvae with differing moult histories (i.e. in different development

pathways). In summary, decapodid 1, 2, and 3 reflect consistent morphological forms (with some morphological variability). Decapodid' does not reflect consistent morphological forms, but within the same developmental pathway can be considered to advance development.

4.3.4 Statistical analysis

The effects of development temperature, larval instar number, and the interaction between the two on larval development time, larval growth rate, and juvenile DW were analysed by general linear model (GLM) ANOVA; *post hoc*, multiple comparison of factors, development temperature (°C) and larval instar number (4 vs. 5 instars), were carried out using the Sidak method. Given the low numbers of larvae, which developed through 6 instars (8 of 972) analysis was done on 4 and 5 instars only. All statistical analysis was carried out using Minitab 16 statistical software and in accordance with Sokal and Rohlf (1995). Significance was accepted at $P < 0.05$, unless stated otherwise.

4.4 Results

4.4.1 Experiment 1 (thermal scope for larval development)

At 5 °C, all larvae failed to moult to zoea 2 and survived in zoea 1 for an average of 11.8 ± 6.1 days (max. 25 days, min. 3 days). At 10 °C, two larvae developed to juvenile (through five instars after 59 and 66 days; respectively); all other larvae died after an average of 55.8 ± 30.1 days (max. 107, min 1 day). Larval development at 10 °C proceeded normally until decapodid 2. After decapodid 2, very little to no development was evident between moults and so these were considered 'repeat' instars (decapodid'). The instar duration (and standard errors) of these 'repeat' instars (decapodid') was greater than for zoea 1, zoea 2, decapodid 1, and decapodid 2 (Table 4.1). Similarly, survivorship during 'repeat' moults (decapodid') was lower than during zoea 1, zoea 2, decapodid 1, and decapodid 2 moults (Table 4.1).

Successful larval development occurred at 17 °C (92.7 % metamorphosed to juvenile), 25 °C (86.5 %), and 30 °C (95.8 %). Larval development proceeded through varying development pathways, consisting of different numbers of larval instars (Figure 4.1A). Two development pathways were numerically dominant, a five instar pathway ("5" in Figure 4.1A; as described by Fincham (1979)) and a four instar pathway ("4" in Figure 4.1A); larvae metamorphosed to juveniles directly from decapodid 2, omitting decapodid

3. Alternative development pathways, consisting of five to seven larval instars predominantly occurred at 17 °C. The 5' development pathway proceeded via a single 'repeat' instar (decapodid') after decapodid 2; 6'[a] consisted of two repeat instars after decapodid 2; 6'[b] consisted of a single 'repeat' instar after decapodid 3; and 7' consisted of three 'repeat' moults after decapodid 2 (Figure 4.1A). Such development was rare, occurring in 11.2 % of larvae at 17 °C, 1.2 % at 25 °C, and was absent at 30 °C. Interestingly, at 30 °C a single larva metamorphosed to juvenile directly from decapodid 1, omitting decapodid 2 and 3. Temperature appeared to influence larval development pathway; larvae increasingly developed through four instars at higher temperatures. With increasing temperature, the proportion of larvae developing through five instars decreased from 0.84 at 17 °C to 0.37 at 30 °C. The number of larvae developing through four instars increased with temperature from 0.04 at 17 °C to 0.62 at 30 °C (Figure 4.1B).

Table 4.1 *Palaemonetes varians* instar duration (presented as means ± standard errors) and survival within instars and cumulative survival from hatching at 10 °C. Instars are indicated, initial n = 96. Dashed line indicates the point at which two individuals (2.1 %) metamorphosed to juvenile. Z indicate zoeal stages, D indicate decapodid stages.

		Z1	Z2	D1	D2	D'	D'	D'	D'
Instar	days	6.4	11.4	10.8	11.1	13.5	11.8	14.2	14.3
duration	(±S.E.)	(±0.2)	(±0.2)	(±0.1)	(±0.2)	(±0.7)	(±0.5)	(±1.6)	(±3.2)
Survival	%	90.6	88.5	93.5	93.1	79.1	62.3	39.4	23.1
	cum. %	**90.6**	**80.2**	**75.0**	**69.8**	**55.2**	**34.4**	**13.5**	**3.1**

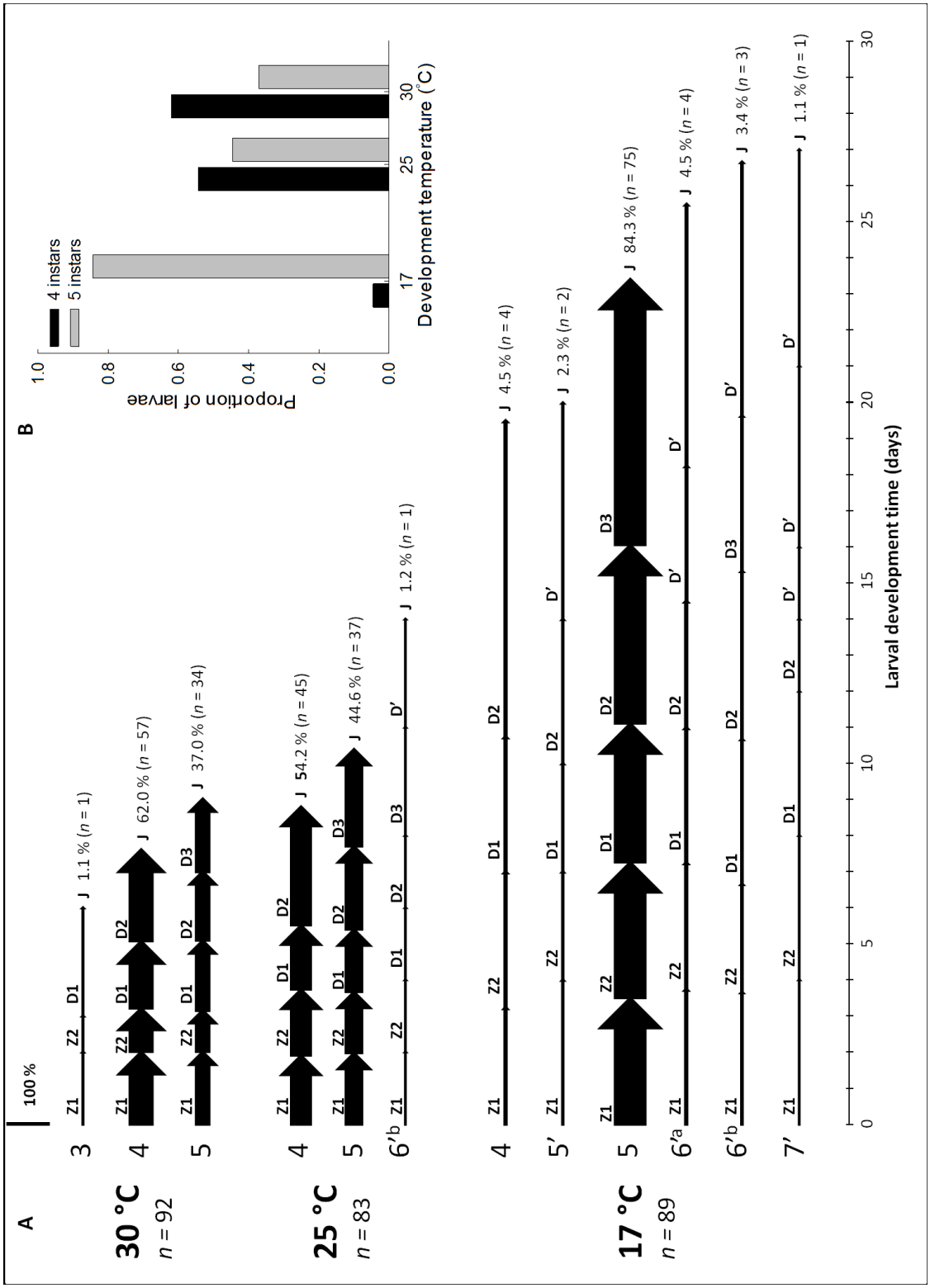

Figure 4.1 Temperature-mediated developmental plasticity within *Palaemonetes varians* larvae. **A** Diagram of *Palaemonetes varians* larval development pathways observed during Experiment 1 (thermal scope for larval development) reared at three temperatures (17, 25, 30 °C). Arrow height = per cent of larvae developing through a pathway (also indicated at the end of the development pathway), arrow length = instar duration (days). Development pathways and instars are indicated. **B** Proportions of larvae developing through four and five instars at 17, 25, and 30 °C.

4.4.2 Experiment 2 (effects of developmental plasticity)

4.4.2.1 Developmental plasticity

Larval instar number was influenced by temperature, consistent with the results of experiment 1; larvae increasingly developed through fewer instars with increasing temperature (Figure 4.2). The proportion of larvae developing through four instars increased from 0.09 at 15 °C to 0.59 at 25 °C, whilst the proportion developing through five instars decreased from 0.89 at 15 °C to 0.40 at 25 °C (Figure 4.2). The dominant pathway for larval development was, therefore, five instars at 15 °C, five instars at 20 °C, and four instars at 25 °C. Larval instar number was less variable than in experiment 1, larvae predominantly develop through four or five instars; <1 % of larvae developed through six instars.

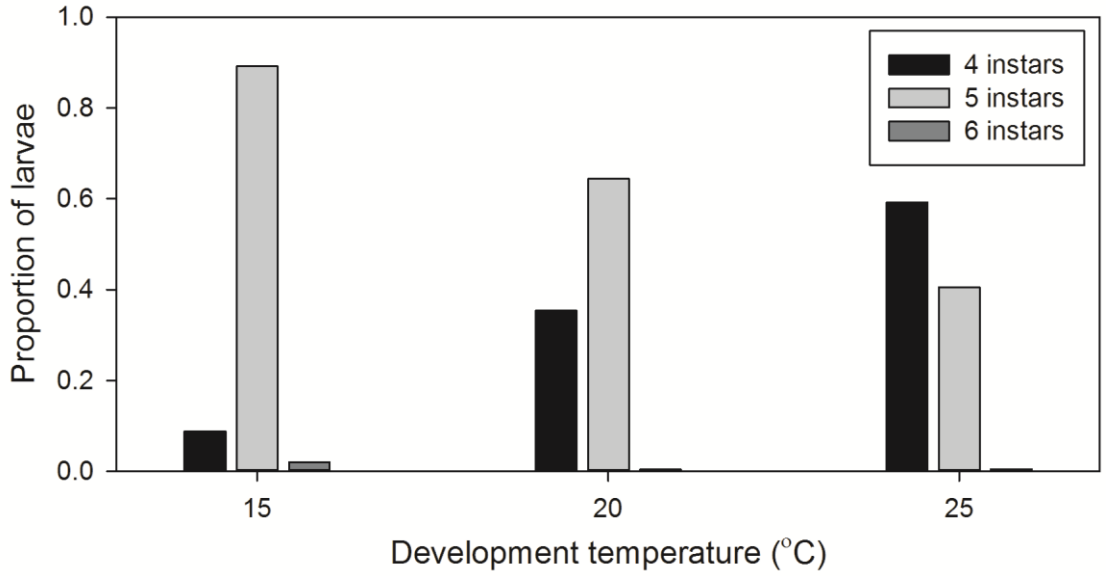

Figure 4.2 Proportions of *Palaemonetes varians* larvae developing through four, five, and six instars at 15, 20, and 25 °C.

4.4.2.2 Development time

The effects of development temperature, larval instar number, and the interaction between them, on development time were significant ($P < 0.001$ in all cases; Figure 4.3). Development time through both four and five instars decreased significantly with increasing temperature ($P < 0.001$ in all cases; Figure 4.3). Within development temperatures, development time was significantly longer for larvae developing through five instars ($P < 0.001$ in all cases; Figure 4.3). Development through five instars took 19.9

% longer at 15 °C, 18.9 % longer at 20 °C, and 23.1 % longer at 25 °C. The proportion of larvae developing through this longer development pathway (five instars) decreased with increasing temperature (Figure 4.3).

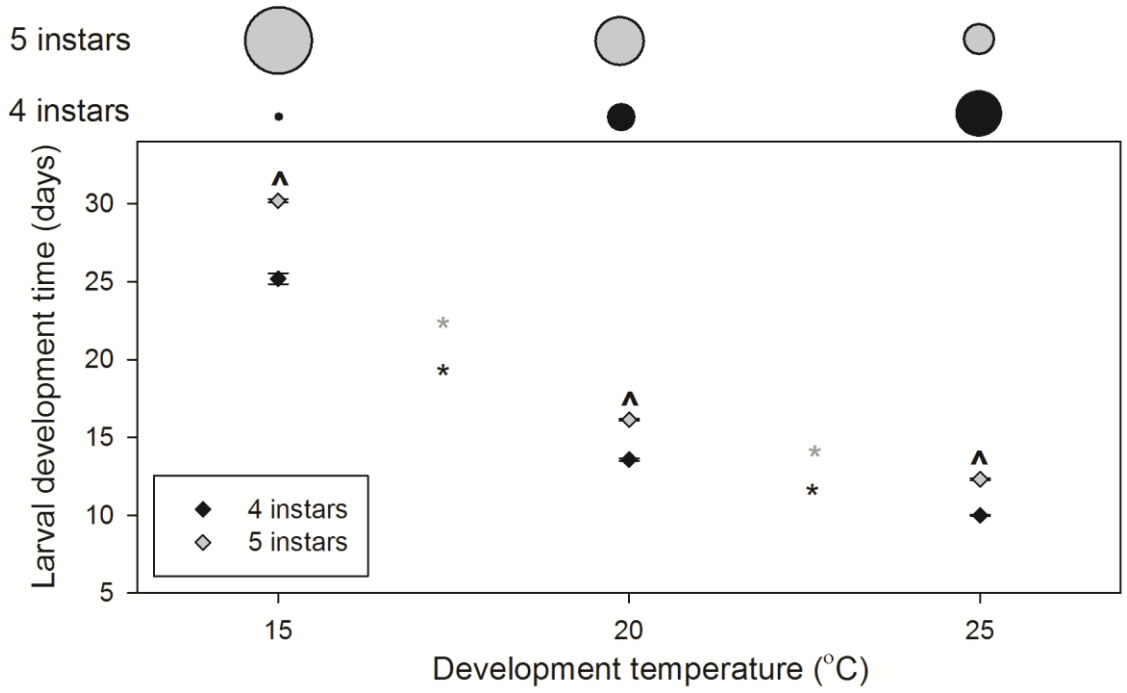

Figure 4.3 *Palaemonetes varians* larval development times through four and five instars at 15, 20, and 25 °C. Data are presented as means ± standard errors. Asterisks indicate significant differences between temperatures (*, five instars; *, four instars), ^ indicate differences (within temperatures) between larvae developing through four and five instars. Above the plot, circle diameters indicate the proportion of larvae developing through four (black) and five instars (grey) at each temperature.

4.4.2.3 Juvenile DW

Juvenile DW was significantly affected by development temperature ($P < 0.001$), larval instar number ($P < 0.001$) and the interaction between the two ($P = 0.043$; Figure 4.4). For both four and five instars, juvenile DW increased with increasing temperature between 15 and 20 °C, only ($P < 0.0001$; Figure 4.4). Within development temperatures, juvenile DW was greater for larvae developing through five instars ($P = 0.002$ at 15 °C, $P < 0.001$ at 20 and 25 °C; Figure 4.4). Juvenile DW after development through five instars was 13.3 % greater at 15 °C, 19.1 % greater at 20 °C, and 22.3 % greater at 25 °C. The proportion of larvae developing to a larger size through this longer development pathway (five instars) decreased with increasing temperature (Figure 4.4).

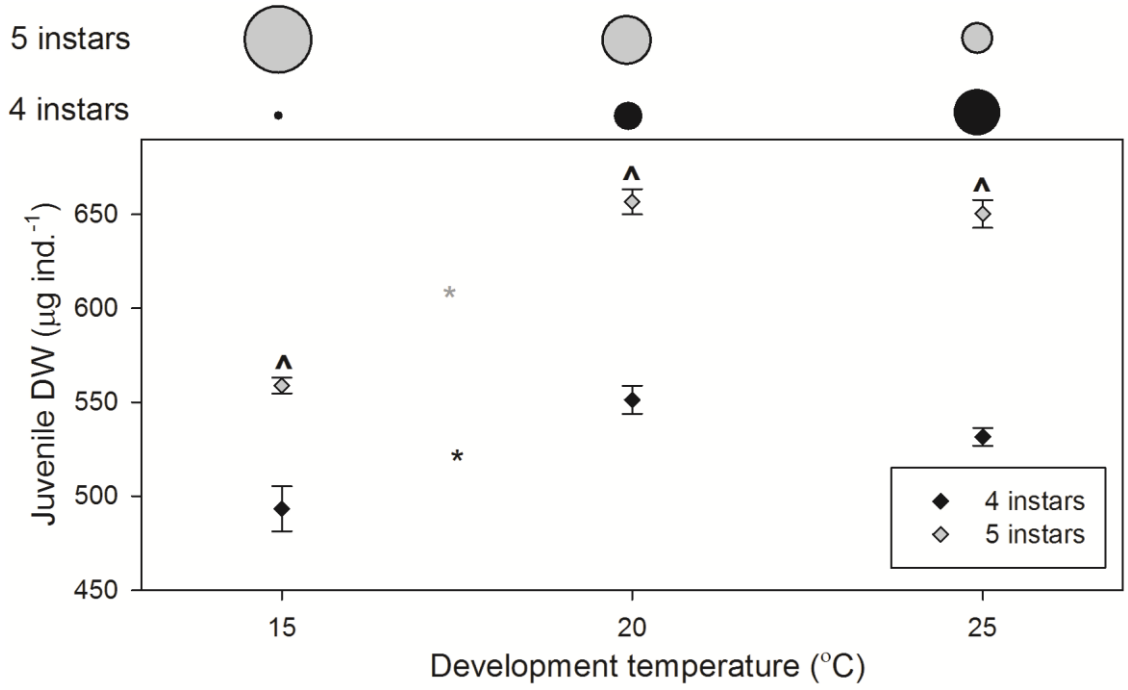

Figure 4.4 *Palaemonetes varians* juvenile DW after development through four or five larval instars at 15, 20, and 25 °C. Data are presented as means ± standard errors. Asterisks indicate significant differences between temperatures (*, five instars; *, four instars), ^ indicate differences (within temperatures) between larvae developing through four and five instars. Above the plot, circle diameters indicate the proportion of larvae developing through four (black) and five instars (grey) at each temperature.

4.4.2.4 Growth rate

Growth rates were affected by development temperature ($P < 0.001$), larval instar number ($P = 0.006$), but not the interaction between them (Figure 4.5). Growth rate for both four and five instars increased significantly with increasing temperature ($P < 0.001$ in all cases; Figure 4.5). Within development temperatures, differences in growth rates between larvae developing through four and five instars were significant at 25 °C, only ($P = 0.0013$).

4.5 Discussion

Temperature-mediated variation in larval instar number is known among arthropods; additional larval instars have been observed under both higher and lower temperatures (Knowlton 1974, Criales & Anger 1986, Willott & Hassall 1998, Etilé & Despland 2008) for review see (Esperk et al. 2007). Here, we demonstrate that under conditions of high growth rates (high temperature), *P. varians* larvae tend to omit larval instars whilst under conditions of low growth rates (low temperature) additional larval

instars may occur during development. With increasing temperature, greater growth and development rates likely provided the opportunity for earlier metamorphosis; i.e. larvae developed sufficiently by decapodid 2 to moult to juvenile directly, omitting decapodid 3. At lower temperatures, additional instars were observed as 'repeat' instars (decapodid') suggesting that morphogenesis and growth between larval instars was retarded. The predominance of additional larval instars under lower temperatures indicates that these temperatures are suboptimal for larval development within *P. varians*.

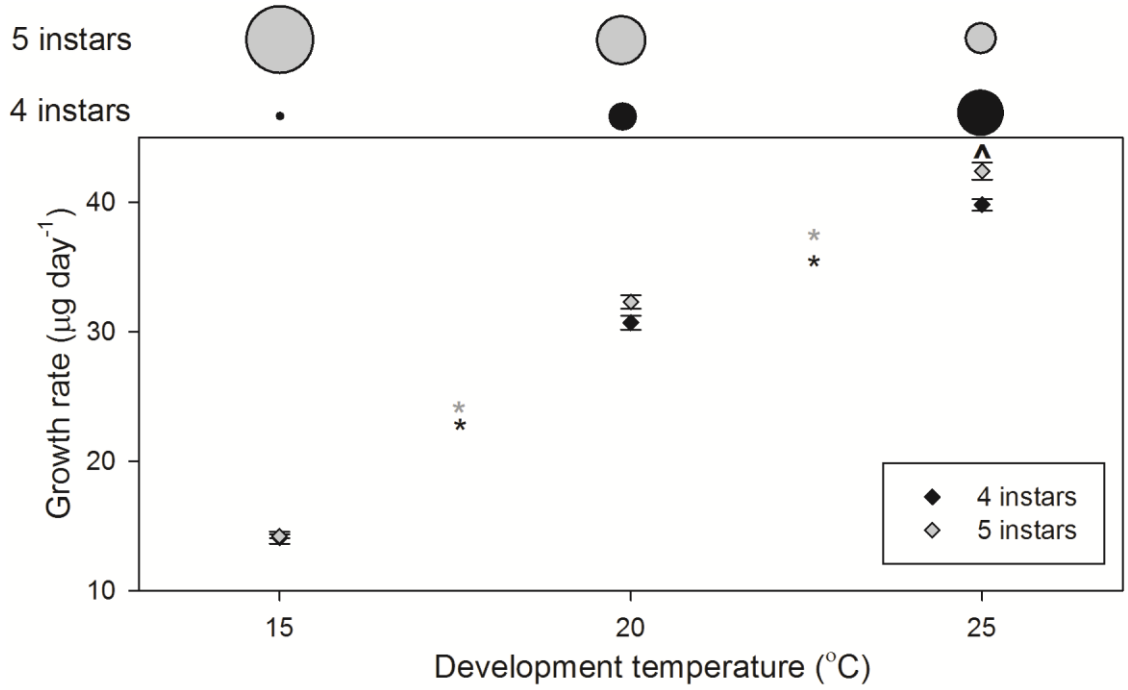

Figure 4.5 *Palaemonetes varians* growth rates for larvae developing through four and five instars at 15, 20, and 25 °C. Data are presented as means ± standard errors. Asterisks indicate significant differences between temperatures (*, five instars; *, four instars), ^ indicate differences (within temperatures) between larvae developing through four and five instars. Above the plot, circle diameters indicate the proportion of larvae developing through four (black) and five instars (grey) at each temperature.

Wild larvae from plankton trawls may be represented by fewer larval instars than laboratory reared larvae of the same species (Rochanaburanon & Williamson 1976, Fincham 1977, 1979). Morphological variation and differences in the extent of development and larval size are also known between wild and laboratory reared larvae (Thatje & Bacardit 2000, Wehrtmann & Albornoz 2003). Further, the extension of larval

development through a series of decapodid moults, each of which advances development little, has been considered a plastic response to a lack of settlement cue (Wehrtmann & Albornoz 2003). These observations indicate that laboratory experiments may not fully replicate the conditions under which wild larvae develop. Here, we document the effects of temperature on larval development and demonstrate temperature-mediated plasticity in larval instar number. The ability for decapod larvae to cope with sub-optimal conditions through plasticity in instar number is considered advantageous and may, potentially, occur in wild larvae where it may have ecological implications.

Among arthropods, variation in larval instar number is a common plastic trait. This trait results from the interaction between the inherent biology of arthropods, which must moult to grow and develop, and the differential effects of the environment on the moult cycle and on growth and development rates (Nijhout 1975, 1994, Kingsolver 2007). Unlike non-arthropods which grow incrementally, within arthropods variations in larval instar number may give rise to differences in juvenile and adult DW because, although growth and development thresholds may be reached during an instar, growth and development continues until the end of the moult cycle. For example, here we show that for *P. varians*, development through four instars is quicker and gives rise to juveniles of reduced DW than development through five instars, across all temperatures. Similarly, within the estuarine crab, *Neohelice (Chasmagnathus) granulata*, larvae may omit an instar during development; larvae, which do omit an instar metamorphose sooner but to juveniles of reduced DW (Pestana & Ostrensky 1995, Giménez et al. 2004). For the tobacco hornworm, *Manduca sexta*, and the forest tent caterpillar, *Malacosoma disstria*, larval instar number affects development time and mass; those larvae developing through more instars take longer to develop but do so to a larger size (Nijhout 1975, 1994, Kingsolver 2007). Considering the pervasive nature of variations in larval instar number coupled with the varied life histories among arthropods, moulting, which may give rise to considerable differences in larval development time and juvenile or adult DW, may have significant and diverse ecological implications. For example, within the holometabolous insects, reproductively active adults emerge after pupation. For *M. sexta*, pupal mass (which is influenced by larval instar number) is positively correlated with the number of mature eggs in the ovaries after eclosion (Davidowitz et al. 2004, Kingsolver 2007). For settling crustaceans, however, juveniles are not reproductively active but continue to grow into adulthood.

4.5.1 Insight into competence thresholds for settlement

Suboptimal environmental and ecological conditions tend to increase larval instar number and reduce morphogenesis between larval instars in crustaceans with high developmental plasticity (e.g. euphausids and carideans; Anger 2006). Diet is known to influence variations in larval instar number (Knowlton 1974, Esperk et al. 2007). Consequently, the use of different suppliers of *Artemia* sp. within Experiments 1 and 2 (Hobby vs. ZM systems, respectively) may explain differences in the frequency of additional instars, juvenile DW (Figure 4.6B) and larval growth rates (data not shown) between the experiments. Similarly, larvae were derived from different females and in different years. Maternal effects may therefore have influenced differences between experiments. Nevertheless, the effects of temperature on larval instar number were found to be consistent between experiments.

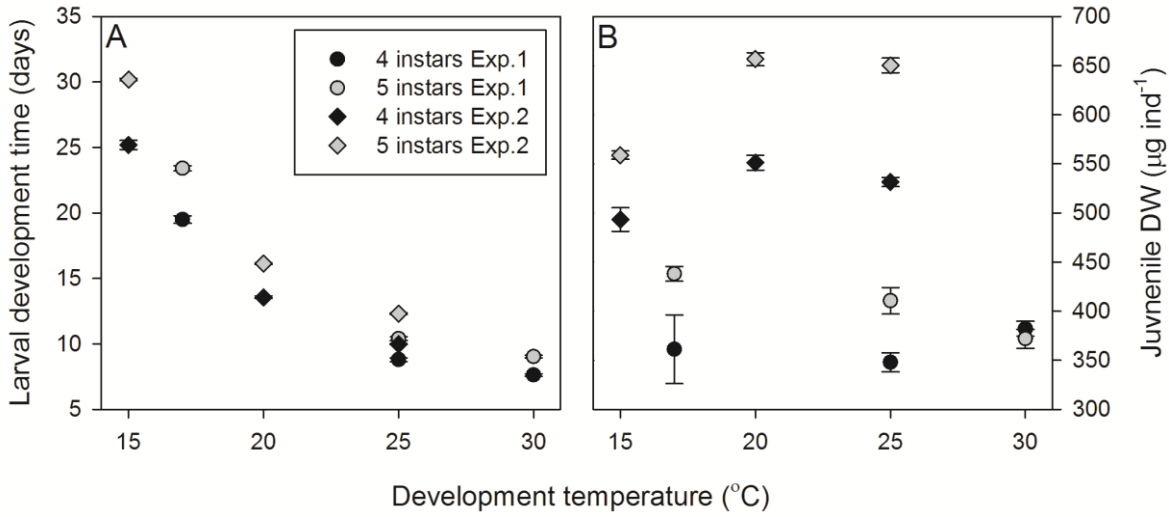

Figure 4.6 Comparison of larval development time and juvenile dry weight between Experiments 1 and 2. (A) *Palaemonetes varians* larval development times for larvae developing through four and five instars in Experiment 1 (at 17, 25, 30 °C) and in Experiment 2 (at 15, 20, 25 °C). Data are presented as means ± standard errors. (B) *P. varians* juvenile DW after development through four or five larval instars in Experiment 1 (at 17, 25, 30 °C) and in Experiment 2 (at 15, 20, 25 °C). Data are presented as means ± standard errors.

The potential effects of diet or uncontrolled maternal effects on the current study are reflected in a comparison of juvenile DWs between Experiments 1 and 2 (Figure 4.6A and 4.6B). Whilst larval development rates were consistent between both experiments the juvenile DWs were not. Coupled with temperature-mediated variations in larval instar numbers, these data suggest that *P. varians* do not develop to a threshold size but

continuously develop from larvae to juveniles. Environmental conditions which perturb the rate of development result in variations in larval instar number. We do not suggest that there is not a minimum size a larva may be, but that there is no size threshold for the end of larval development. This may be true of decapod larvae in general and requires further investigation.

4.5.2 Macro-ecological perspective

Temperature-mediated variations in larval instar number observed here for *Palaemonetes varians* are inconsistent with those made for *Palaemonetes vulgaris* and may be associated with the relative amounts of per offspring investment and associated endotrophic development potential within these species. *Palaemonetes vulgaris* is fully planktotrophic and unable to develop to its second larval stage (zoea 2) without feeding (Broad 1957b). *Palaemonetes varians* is facultative lecithotrophic during its first larval stage (zoea 1; see Chapter 5) and can develop to its third and sometimes fourth larval stages (decapodid 1 and 2, respectively) without feeding (see Chapters 6). The higher internal reserves present within *P. varians* larvae may allow for more rapid development at higher temperatures. For example, under starved conditions, *P. varians* larvae develop to more advanced larval stages under warmer conditions (see Chapters 6). Results were also inconsistent with the macro-ecological trend in larval instar number at the inter-specific level. At the inter-specific level, species with abbreviated development have high per offspring investment and high endotrophic development potential. Within *P. varians*, Chapter 7 showed that under the same conditions, larvae from broods of higher hatchling energy content developed through fewer larval instars. This suggests that the inconsistency between observations at the intra-specific level and those at the inter-specific level are linked to inter-specific differences in per offspring investment.

4.5.3 *Palaemonetes varians* and the temperature-size rule

Studies of larval development within insects have provided better understanding of the temperature-size rule. For example, within *Manduca sexta*, larval body size (which follows the temperature-size rule) was found to be a function of the "interval to cessation of growth (ICG)"- the period between attaining the critical weight for the initiation of pupation (which is thermally invariable) and the initiation of pupation. The ICG decreased with temperature, providing a shorter time for mass accumulation and resulting in smaller larvae at higher temperatures (Davidowitz et al. 2004, Davidowitz & Nijhout 2004). In contrast, Ghosh et al. (2013) identified genetically controlled shifts in the critical weight

for pupation with temperature within *Drosophila*. Importantly, such studies use laboratory lines of model species, which may differ significantly from wild types of the same species. For example, under the same constant conditions, wild type *Manduca sexta* exhibit temperature-mediated variation in larval instar number whilst laboratory types do not (Kingsolver 2007).

Our data suggest that *P. varians* do not develop to a size threshold but develop continuously to juveniles. Even so, temperature influences juvenile size. Variations in larval instar number, coupled with higher growth rates at higher temperature, gave rise to the greatest juvenile DWs at intermediate temperatures (20 °C). Consequently, temperature-mediated variation in larval instar number and its interaction with temperature-mediated growth rates is important in determining juvenile DW. Larval instar number is therefore of high importance in understanding the effects of temperature on decapods larval development. Interestingly, such temperature-mediated developmental plasticity is rarely considered in literature assessing the implications of temperature for larval growth, which is highly important to the aquaculture industry (e.g. Kumlu et al. 2000, Zacharia & Kakati 2004, Bermudes & Ritar 2008). For *Artemia franciscana*, Forster and Hirst (2012) found that for early ontogenetic stages, individuals were larger at higher temperatures and that the typical temperature-size rule became established only in later ontogenetic stages. This was thought to result from high temperature dependence in growth rate in early ontogenetic stages, which reduced through later stages. *Palaemonetes varians* may follow a similar pattern, with juvenile size being influenced by temperature-mediated variation in larval instar number and its interaction with temperature-mediated growth rates.

4.5.4 Ecological implications

Size at metamorphosis has important fitness consequences as larger individuals are considered fitter (Stearns 1992, Kingsolver & Pfennig 2004). For settling decapods, a large size may increase starvation resistance, increase predation success, and reduce cannibalism by fellow new-recruits (Giménez et al. 2004). Accordingly, *P. varians* juveniles which developed through five instars may be considered fitter at all temperatures. The juvenile environment may have important consequences for the fitness implications of size at metamorphosis. For example, under fed conditions, short pathway *N. granulata* juveniles grew faster than long pathway juveniles, indicating that any disadvantage of small size at metamorphosis may be reduced through subsequent instars (Giménez et al. 2004).

Similarly, Sandifer and Smith (1979) found no growth or development advantage for early (with fewer larval instars), compared to late (with more larval instars) metamorphosing juvenile *Macrobranchium rosenbergii*. Later metamorphosing juveniles were smaller (in terms of carapace length) than earlier metamorphosing juveniles; however, after three weeks, no difference was evident (Sandifer & Smith 1979). These findings suggest that the influence of developmental plasticity (and larval experience) on initial juvenile fitness (and possibly later life stages) is likely to depend on habitat characteristics; advantages associated with larger juveniles may be greater under suboptimal conditions (Giménez et al. 2004). For *P. varians*, conditions which drive high growth rates during the larval phase promote development through four instars and settlement at a reduced DW. An important factor influencing the effects of the larval experience on later life stages appears to be relationship between conditions during the larval phase and those after settlement. Poor conditions during development lead to development through more larval stages, giving rise to large juveniles. Good conditions during development lead to development through fewer larval instars, giving rise to smaller juveniles. The literature suggests that larger individuals are fitter if conditions after settlement are poor. If conditions after settlement are good, there is no advantage of large size. Consequently, if conditions during the larval period do not reflect those after settlement, variation in larval instar number may be disadvantageous.

For dispersive propagules such as the planktonic larvae of aquatic arthropods, additional larval instars and delayed recruitment (resulting from slower epigenesis under sub-optimal conditions) or the omission of larval instars and earlier recruitment (resulting from faster epigenesis under more favourable conditions) will affect larval transport. This indirect effect could enhance or reduce dispersion and affect the chances of finding suitable conditions for growth and settlement (Anger 2006). Strong dispersion promotes gene flow and is associated with colonisation/re-colonisation of habitats and low extinction rates; however, dispersal may be disadvantageous in the short-term (for review see Pechenik 1999). For example, when local conditions are optimum (either temporally or spatially) reduced development time to settlement may enhance local recruitment in the favourable habitat (Anger 2006). Further, a short planktonic phase may also reduce the risks of mortality via predation in the plankton. For *P. varians*, when conditions are favourable and larval development is rapid, local recruitment will be maximised. Conversely, if conditions are unfavourable, development will be slow, prolonging the larval period and increasing dispersal; possibly to more favourable environments.

4.5.5 Conclusions

- *Palaemonetes varians* larvae from Lymington salt marsh (Hampshire, UK) are able to develop fully within a temperature range between ≥10 °C and >30 °C (30 °C being the highest temperature tested here). This range is wider than that recorded *in situ* during summer months (see Chapter 3). This thermal window for larval development may reflect the tropical ancestry of palaemonid shrimp.
- Larval instar number during development is highly variable. Development through four and five instars was the predominant development pathways but more instars during development were observed.
- The occurrence of 'intermediate' stages, which were morphologically in-between stages, was documented. Variation in the number of larval instars during development coupled with the occurrence of 'intermediate' stages indicates the decoupling of development from moulting.
- Larval instar number during development is influenced by temperature: larvae increasingly develop through four instars with increasing temperature and the proportion developing through five instars decreases with increasing temperature.
- Within temperatures, larval development is more rapid through four instars
- Juvenile dry weight is greatest after development through five instars
- Larval development rate is greater at higher temperatures
- The interaction between the shift in dominant development pathway coupled with changes in growth rate result in the highest juvenile dry weight at intermediate temperatures

5. Energetic adaptations to larval export within the brackish living palaemonine shrimp *Palaemonetes varians*[*]

5.1 Summary

Decapod crustaceans have repeatedly colonised brackish, freshwater, and terrestrial environments. Many decapods which inhabit brackish and freshwater habitats export larvae into estuarine and coastal areas where conditions for larval development may be better. In this study, we assess the starvation resistance, biochemical composition and respiration rate during larval development, and the effects of temperature on these factors within the brackish living palaemonine shrimp, *Palaemonetes varians*. Our results demonstrate that *P. varians* is highly resistant to starvation and may be considered facultative lecithotrophic in its first and second larval instars and planktotrophic from its third instar. This high starvation resistance is associated with a relatively large size, high carbon content (~45%) and C:N ratio (~4.2), and visible yolk reserves at hatching. These energy reserves are interpreted as an adaptation to the exportation of larvae from peripheral adult environments into mid and lower estuarine waters. Respiration rates varied with the moult cycle and were similar between fed and unfed larvae, suggesting that starved larvae do not suppress their metabolism as an energy saving strategy. Despite higher respiration rates at higher temperatures, energy loss throughout development (estimated from respiration rates) increased with decreasing temperature, whilst larval growth and development rates decreased with increasing temperature. High energy reserves at hatching, as within *Palaemonetes varians*, is an important life history adaptation in the colonisation of brackish and freshwaters, initially enabling the exportation of larvae from adult environments and eventually enabling lecithotrophy and direct development.

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5.2 Introduction

The palaemonine shrimp, *Palaemonetes varians*, commonly known as the variable shrimp or ditch shrimp, is the most northerly and high latitude distributed species of the *Palaemonetes* genus, inhabiting peripheral estuarine and brackish water environments along western European coasts in the North-East Atlantic (Dolmen 1997, Falciai 2001, Hindley 2001, González-Ortegón & Cuesta 2006 and references therein). The Palaemoninae are dominated by the genera, *Macrobrachium*, *Palaemon*, and *Palaemonetes*, all of which have representatives in marine, brackish, and freshwater environments (De Grave et al. 2009, Vogt 2013). Among decapods, the evolutionary transition from marine, via brackish, to freshwater environments is associated with major evolutionary changes in reproduction and development (Anger 1995, 2001, Vogt 2013). Within estuarine and freshwater environments, decapods may exhibit behavioural and physiological traits which place them on a spectrum of life-cycle adaptation to the estuarine environment. At one extreme are decapods which display behavioural and physiological traits which enable larvae to be retained within the estuarine environment, usually by larvae accumulating in bottom water layers in which transport is generally upstream (Sandifer 1975, Strathmann 1982, Anger 2001). At the other extreme are decapods which exhibit traits promoting the exportation of larvae from freshwater and estuarine environments into coastal waters (Sandifer 1975, Strathmann 1982, Anger 2001). Most decapods show life-cycles which are somewhere between these extremes. Decapod estuarine life cycle adaptations are considered intermediates in the transition from marine to freshwater habitats and, as such, are of particular interest in studying the evolutionary processes of transition and adaptation to freshwater environments.

Marine decapods typically have long-lived feeding larval phases (though at high latitudes larval phases tend to be abbreviated and non-feeding; Thorson 1950, Anger 2001, Thatje et al. 2003), whilst freshwater decapods have abbreviated and non-feeding larval phases and even direct development (Anger 2001, Vogt 2013). Non-feeding larvae require maternally derived energy rich yolk to sustain development and, consequently, freshwater decapods produce fewer, more energy rich eggs and larvae than their marine counterparts (Vogt 2013, Urzúa et al. in press). Brackish and freshwater decapods which export larvae to lower estuarine and coastal waters produce eggs and larvae with intermediate energy reserves; relatively high lipid content at hatching enables high starvation resistance during early larval stages which are exported from adult habitats (Anger & Hayd 2009).

Within decapod larvae, carbon and nitrogen content are accurate proxies for lipid and protein, respectively (Anger & Harms 1990). Changes in the relative composition of these elements (C:N ratio) provide information about the relative utilisation of lipid and protein for energy metabolism (Anger & Harms 1990, Anger 2001). Inter-specific differences in the energy content of eggs along the gradient from marine to freshwater (and terrestrial) habitats have been highlighted in a comparison of carbon (lipid) content, C:N (lipid:protein) ratio and egg size within grapsoid crabs (see Anger 2001, pg 110). Egg size, lipid content, and lipid:protein ratio all increase along the gradient from marine to freshwater. Associated with this change in the energy content of eggs are a decrease in the number of larval instars during development and an increase in the extent to which larvae may develop in the absences of food (Hubschman & Broad 1974, Anger 2001). Studies have assessed changes in lipid content and lipid:protein ratio during larval development for both planktotrophic and lecithotrophic marine decapods (e.g. Dawirs 1986, Anger 1996, Anger & Ismael 1997, Anger 2001, Calcagno et al. 2003, Lovrich et al. 2003, Thatje et al. 2004b, Anger & Hayd 2009, Weiss et al. 2009, Urzúa et al. in press); however, few such studies investigated brackish and freshwater decapods (Torres et al. 2002, Anger et al. 2007, Anger & Hayd 2009, Anger et al. 2009, Anger & Hayd 2010, Urzúa et al. in press). Similarly, few studies of changes in lipid content and lipid:protein ratio during development have been made for differing temperatures (Dawirs et al. 1986, Anger 1987, Weiss et al. 2009) and none have been done in brackish and freshwater decapods.

Adult *Palaemonetes varians* inhabit peripheral estuarine and coastal habitats such as brackish-water drainage channels, salt marshes, and coastal ponds and lagoons, which are often under tidal influence and regularly flooded (Lofts 1956, Gurney 1924). The exporting of *P. varians* larvae from such habitats has been observed (Gurney 1924) and zoea 1 and 2 larvae and juvenile *P. varians* have been sampled from the lower Ria de Aveiro, Portugal (Pereira et al. 2000). Further, early descriptions of *P. varians* larval development used specimens obtained from plankton samples, indicating the presence of larvae in estuarine and coastal waters (see Gurney 1942 and Fincham 1979 for references). Larval development within *P. varians* is feasible at salinities from 5 to 42, indicating that development can occur entirely under estuarine conditions (Antonopoulou & Emson 1988) and the ubiquitous distribution of this species around the UK has been attributed to the abundance of suitable habitat and large macrotidal hydrodynamics which aid dispersal (Dolmen et al. 2004). These data indicate the exporting of larvae from peripheral adult

habitats into estuarine and coastal waters and dispersal through these environments; however, no study has yet followed the life cycle of *P. varians* larvae in wild populations.

Temperature affects all aspects of biology (Clarke 2003) and is one of the most important environmental factors governing growth and development rates in decapods, and ectotherm in general (Anger 2001). Within studies assessing the effects of temperature on growth, both alone and in combination with salinity and nutrition, measures of growth are often limited to changes in total length, carapace length, or dry weight during development (Rothlisberg 1979, Criales & Anger 1986). *Palaemonetes varians* is a strongly eurythermal species (Oliphant et al. 2011), and its larval development is successful at between ≥10 °C and 30 °C (see Chapter 4). For *P. varians* larvae, growth rate in terms of dry weight accumulation increased approximately linearly and development rate increased in an exponential fashion, between 15 and 25 °C (see Chapter 4). Further, larval instars number was also affected by temperature; larvae developed through four instars more often at higher temperatures and five instars more often at low temperatures (see Chapters 4 and 6). The effect of temperature on larval growth and development is significant and may have carry-over effects into early juvenile life (see Chapter 4). A greater understanding of the effects of temperature on the biochemical composition of larvae during development and post-settlement is requisite.

Here, the effects of temperature and starvation on changes in dry weight, lipid, protein contents, and respiration rate during development were assessed for *P. varians* larvae. These measurements were made to assess the physiological adaptation of *P. varians* to its brackish water distribution, and how temperature may affects the larval ecology of this species. As few data are available for brackish and freshwater decapods, and especially carideans, concerning changes in elemental composition during development, this study will contribute fundamentally to our understanding of the energetic changes necessary for development in the evolutionary transition to freshwater.

5.2.1 Hypotheses

H_1 *Palaemonetes varians* larvae are highly resistant to starvation within early development

H_2 growth rate, in terms of dry weight and elemental composition, will increase with increasing temperature

H_3 respiration rates will increase with increasing temperature and vary with the moult cycle

H_4 respiration rates will be lower for starved larvae at all temperatures

5.3 Materials and Methods

5.3.1 Adult *Palaemonetes varians* collection and maintenance

Larvae used in these experiments were bred in the laboratory under constant conditions using females collected from a wild population as described in Chapter 2, sections 2.2.3 and 2.4.2.

5.3.2 Larval maintenance

Two parallel experiments were run: Experiment 1, 'the effects of temperature on larval development', monitored larval development in terms of moulting frequency, the number of larval instars during development, overall development time, and juvenile dry weight at settlement for groups of 'fed' and 'unfed' larvae at three temperatures. This experiment repeated the work of earlier papers to demonstrate the repeatability of the results. Experiment 2, 'the effects of temperature on elemental composition during larval development', monitored larval development from a more physiological stand point; taking measurements of larval respiration rates, dry weight, and elemental composition throughout development, again for groups of 'fed' and 'unfed' larvae at three temperatures.

5.3.3 Experiment 1, 'the effects of temperature on larval development'

On hatching, actively swimming larvae were separated from 11 females and maintained as described in Chapter 2, section 2.4.3. Larvae were maintained under contant temperatures at 15, 20, and 25 °C and 12:12 (light:dark); 12 larvae from eight females were transferred to each temperature. The first instar (zoea 1) of *P. varians* is facultative lecithotrophic (Oliphant, unpublished data) and as such, the first instar was not fed. At each temperature six of the 12 larvae per female were not fed (unfed category). The remaining six larvae per female were fed from the start of the second instar (fed category). A total of 48 larvae were fed (i.e. six larvae from eight females) and 48 larvae were unfed (i.e. six larvae from eight females). Larvae were monitored daily (am) for mortality and development, assessed by morphological changes and moulting (following Fincham 1979)

as described in Chapter 2, section 2.4.4. On moulting to the juvenile stage, individuals preserved as described in section 2.4.3 and later weighed for dry weight, see section 2.7.

5.3.4 Experiment 2, 'the effects of temperature on elemental composition during development'

On hatching, actively swimming larvae were separated from 12 females, 11 of which were the same females as those used in Experiment 1; therefore, larvae used in both experiments were, for the most part, from the same broods. Larvae were maintained as described in Chapter 2, section 2.4.3. Larvae were divided between incubators set at 15, 20, and 25 °C and 12:12 (light:dark); larvae used for both Experiment 1 and 2 were maintained in the same incubators. At each temperature, a portion of the larvae from each female was not fed (unfed category) and a portion was fed (fed category); again, feeding was from the start of the second instar, as above. During development, larval respiration rate measurements were made as described in Chapter 2, section 2.6. Subsequently, larvae used for respiration rate measurements were blotted on tissue paper and transferred to pre-weighed tin capsules, frozen at -80 °C, and later freeze-dried for 24 hours and then weighed for W (μg). Carbon and nitrogen composition were measured as described in Chapter 2, section 2.8. Respiration rate, W, and elemental composition measurements were made daily during the initial ten days of larval development, then every second day thereafter for $n = 5$ unfed larvae and $n = 5$ fed larvae, at each temperature.

5.3.5 Statistical analysis

Data were tested for normality of distribution and equality of variance using Kolmogorov-Smirnov Test and Levene's Test, respectively. Box-Cox transformation was used to calculate the most likely successful power transformation for data. Where data were non-normally distributed and could not be successfully transformed to meet assumptions, non-parametric statistics were used.

5.3.5.1 Experiment 1

Larval development time data were analysed by non-parametric Kruskal-Wallis comparison and juvenile W data were analysed by General Linear Model (GLM) ANOVA with *post-hoc* testing using the Sidak method with temperature (°C) and instar (four vs. five instars) as factors.

5.3.5.2 Experiment 2

Data were tested for normality of distribution and equality of variance using Kolmogorov-Smirnov Test and Levene's Test, respectively. Box-Cox transformation was used to calculate the most likely successful power transformation for data. Where data were non-normally distributed and could not be successfully transformed to meet assumptions, non-parametric statistics were used.

At 15 °C, differences in larval *W* between fed and unfed larvae were analysed by non-parametric Kruskal-Wallis comparison and the relationships between larval *W* and larval age for both unfed and fed larvae were tested by Spearman's correlations. At 20 and 25 °C, differences in larval *W* between fed and unfed larvae were analysed by general linear model (GLM) ANOVA with *post-hoc* testing using the Sidak method with larval age and unfed vs. fed as factors. The relationships between larval *W* and larval age for fed and unfed larvae were assessed via linear regression analyses.

Larval *W* data for individual larval instars across at all temperatures were analysed by Kruskal-Wallis comparisons. Average daily growth per instar (both dry weight and carbon mass) data were analysed via GLM ANOVA; *post-hoc* testing (Sidak method) with temperature and larval instar number as factors. Average daily growth increment data were transformed by log(n + constant). Respiration rate data for fed larvae were analysed by one-way ANOVA and data for both fed and unfed larvae were analysed using GLM ANOVA; *post-hoc* testing (Sidak method) with larval age and fed vs. unfed as factors. Cumulative energy loss both within larval instars and throughout development were analysed using GLM ANOVA; *post-hoc* testing (Sidak method) with temperature and larval instar as factors. Carbon content data were analysed by non-parametric Kruskal-Wallis comparisons and the relationship between carbon content and larval age was assessed via Spearman's correlation. C:N data were analysed via one-way ANOVA with Tukey method *post-hoc* testing. All statistical analysis was done using Minitab v16 software and in accordance with Sokal and Rohlf (1995).

5.4 Results

5.4.1 Experiment 1

5.4.1.1 Effects of starvation on survival

Palaemonetes varians larvae showed extended periods of survival in the absence of food at all temperatures tested (Figure 5.1). Fed larvae moulted regularly whilst for unfed larvae, moulting continued regularly to a point, beyond which moulting became retarded and eventually ceased. At 15 °C the second instar intermoult period for unfed larvae appear considerably longer than that for fed larvae (Figure 5.1). Whilst fed larvae moulted to the third larval instar after 9.89 ± 0.1 days, unfed larvae moulted to the third instar after 12.3 ± 0.2 days. This corresponded to a considerable increase in mortality with survivorship decreasing from 48 larvae at 11 days to 33 larvae at 13 days, and subsequently 0 larvae by day 16 (Figure 5.1). At 20 and 25 °C the second instar intermoult periods for unfed larvae were similar to those for fed larvae (Figure 5.1). At both temperatures most larvae successfully moulted to the third larval instar and mortality rates increased a few days after this moult. For example, at 20 °C unfed larvae moulted to the third larval instar after 5.4 ± 0.1 days and mortality rates increased considerably from days 8 to 10. Similarly, at 25 °C unfed larvae moulted to the third larval instar after 4.2 ± 0.4 days and mortality rates increased considerably from days 7 to 9. Temperature influenced the extent of development with only 39.6 % of larvae moulting to the third larval instar at 15 °C whilst this figure was higher at 20 °C (95.8 %) and 25 °C (97.9 %).

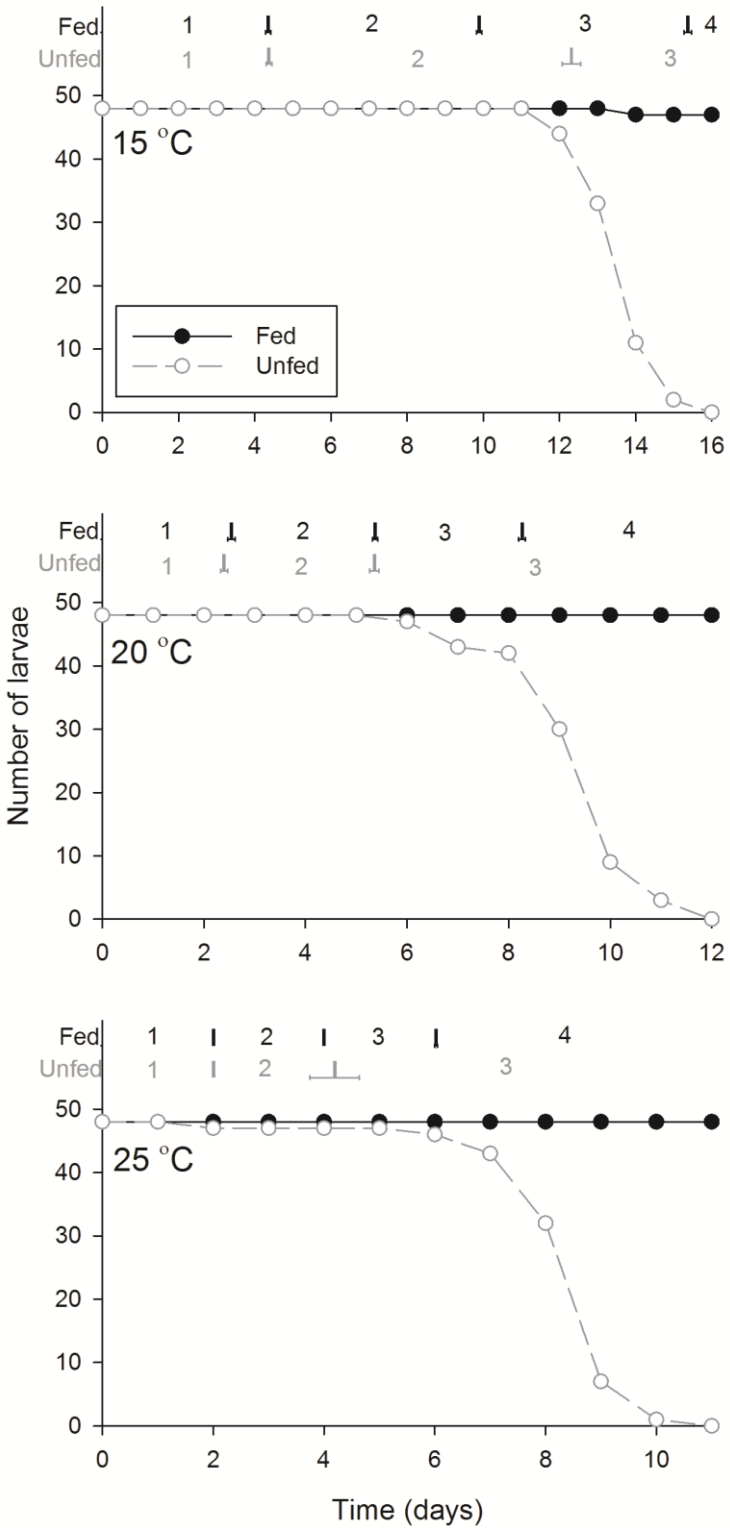

Figure 5.1 *Palaemonetes varians* survival (initial n = 48) of fed and unfed larvae at three temperatures (15, 20, 25 °C). Larval instar numbers and day of moulting (vertical black and grey lines; mean ± SD) for fed and unfed larvae are indicated. *NB.* All larvae, both fed and unfed groups, were not fed during the first larval instar

5.4.1.2 Effect of temperature on larval instar number

Temperature influenced the number of instars through which larvae developed (Figure 5.2A). With increasing temperature, the proportion of larvae developing through four instars increased from 0.33 at 15 °C to 0.63 at 25 °C, whilst the proportion developing through five instars decreased from 0.67 at 15 °C to 0.37 at 25 °C (Figure 5.2A). The dominant development pathway was, therefore, five instars at 15 °C and four instars at both 20 and 25 °C (Figure 5.2A).

Table 5.1 *Palaemonetes varians*. Fitted parameters (*a*, *b*) and correlation coefficients (*r*) for linear regressions ($y = a + bx$) describing the relationship between larval age and larval *W* for fed and unfed larvae at three temperatures (15, 20, 25 °C). At 15 °C, *r* value determined by Spearman's correlation and *a* and *b* calculated by pair-wise slopes. At 20 °C and for fed larvae, linear regression was done on transformed (*lambda* = 0.44) data

Unfed

Temp. (°C)	*a*	*b*	*r*
15	109.00	-0.11	-0.637
20	144.46	-8.19	-0.748
25	143.77	-9.82	-0.733

Fed

Temp. (°C)	*a*	*b*	*r*
15	38.20	17.40	0.973
20	10.93	0.75	0.914
25	-54.20	59.93	0.958

5.4.1.3 Effect of temperature and larval instar number on development time and juvenile W

Larval development time through both four and five instars decreased significantly with increasing temperature ($P \leq 0.001$ in all cases; Figure 5.2B). At all temperatures, mean larval development time was greater through five instars than that through four instars; however, these apparent differences were not supported by non-parametric Kruskal-Wallis comparisons ($P = 0.075$ at 15 °C, $P = 0.051$ at 20 °C, and $P = 0.058$ at 25 °C).

Juvenile *W* was highest for larvae developing through five instars at all temperature and for larvae developing through both four and five instars, juvenile *W* increased with increasing temperature (Figure 5.2C). Differences in juvenile *W* associated with temperature ($F = 24.93$, $P < 0.001$), larval instars number ($F = 79.19$, $P < 0.001$), and the

interaction between temperature and larval instars number (F = 3.53, P = 0.032) were statistically significant. At all temperatures, juvenile W for larvae developing through five instars was significant greater than that for larvae developing through four instars (T = 4.013, P = 0.0015 at 15 °C, T = 4.085, P = 0.0011 at 20 °C, and T = 7.362, P < 0.001 at 25 °C). For larvae developing through five instars, juvenile W was greater at 25 °C than at 20 °C (T = 4.662, P < 0.001).

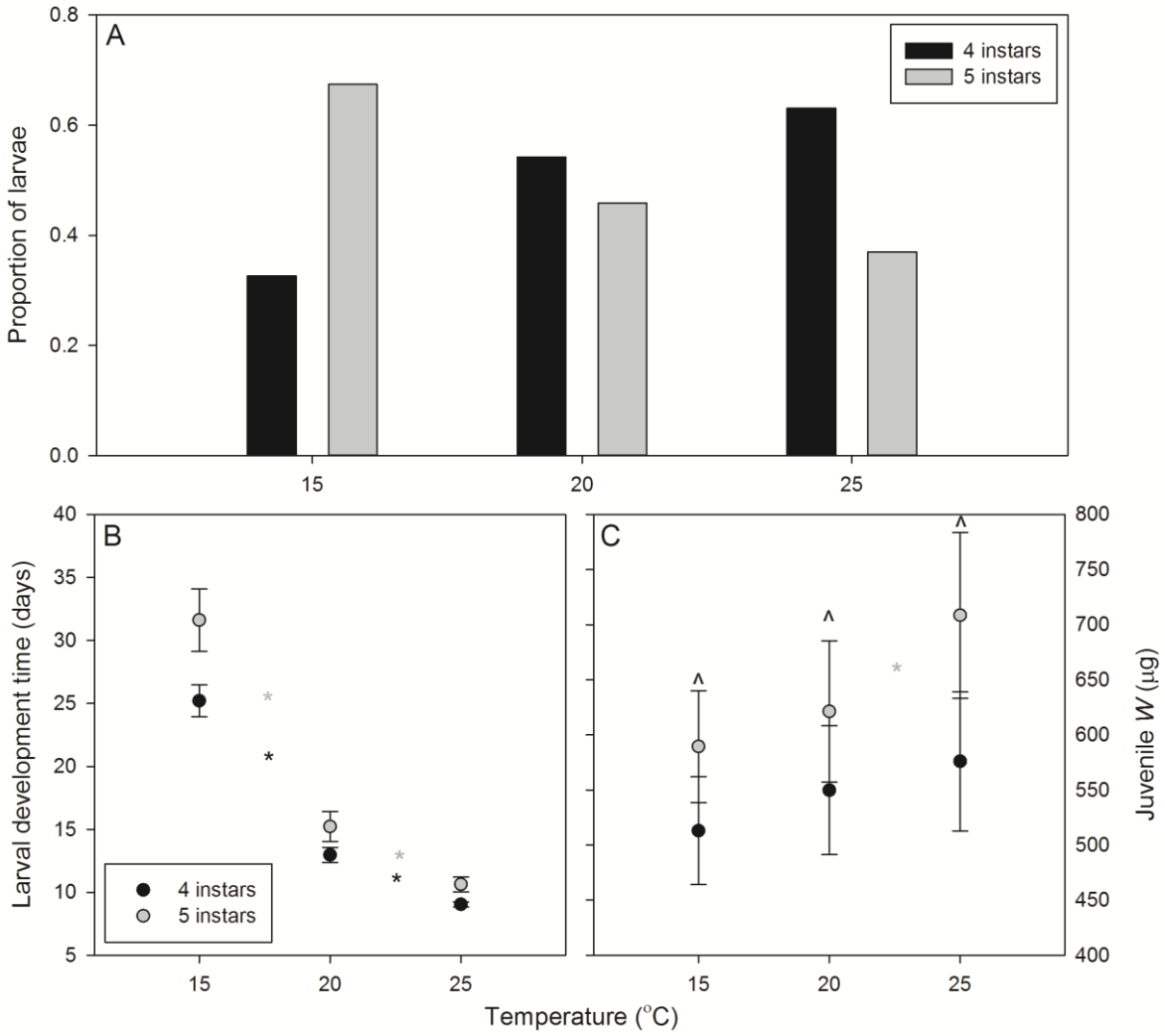

Figure 5.2 *Palaemonetes varians* **A** Proportions of larvae developing through four and five instars at each of three temperatures (15, 20, 25 °C). **B** Larval development time for larvae developing through four and five instars at each of three temperatures (15, 20, 25 °C). **C** Juvenile W for larvae developing through four and five instars at each of three temperatures (15, 20, 25 °C). Data are presented as means ± standard deviations. Asterisks indicate significant differences between temperatures (*, five instars; *, four instars), ^ indicate differences (within temperatures) between larvae developing through four and five instars

5.4.2 Experiment 2

5.4.2.1 Effect of starvation and of temperature on larval W during development

For fed larvae, larval *W* increased throughout development (i.e. with increasing larval age), whilst for unfed larvae, larval *W* decreased until mortality ensued, at all temperatures (Figure 5.3). These relationships appeared approximately linear and were analysed as such. For unfed larvae, negative linear relationships were evident between larval age and *W*, at all temperatures (at 15 °C, Spearman's correlation: $P < 0.001$ and at 20 and 25 °C, linear regressions: $F = 48.10$, $P < 0.001$ and $F = 38.22$, $P < 0.001$, respectively; see Table 5.1 for fitted parameters and correlation coefficients); indicating that larval *W* decreased significantly with larval age (Figure 3). For fed larvae, positive linear relationships were evident between larval age and *W*, at all temperatures (at 15 °C, Spearman's correlation: $P < 0.001$ and at 20 and 25 °C, linear regressions: $F = 347.09$, $P < 0.001$ and $F = 517.51$, $P < 0.001$, respectively; see Table 5.1 for fitted parameters and correlation coefficients); indicating that larval *W* increased significantly with larval age (Figure 5.3).

At 15 °C, differences in larval *W* was different between unfed and fed larvae from day 8 ($P = 0.027$; K-W) onwards; larval *W* being greater for fed larvae than unfed larvae. Larval *W* differed between unfed and fed larvae from day 5 ($P = 0.0023$; GLM ANOVA) onwards at 20 °C and day 4 ($P = 0.002$; GLM ANOVA) onwards at 25 °C; again fed larvae having greater *W*. At these temperatures, the onset of these differences corresponded to the moulting of both fed and unfed larvae to the third larval instar (Figure 5.3). The initial *W* of larvae of each instar were not different between temperatures; however, average daily growth per instar, in terms of dry weight, was affected by temperature ($F= 10.93$, $P < 0.001$), instar number ($F= 30.59$, $P < 0.001$) and the interaction between temperature and instar number ($F= 4.04$, $P = 0.001$) (Figure 2.A). Within the second larval instar, average daily growth rate per instar increased from 5.45 ± 2.58 μg *W* day^{-1} at 15 °C to 55.4 ± 15.66 μg *W* day^{-1} at 25 °C ($T= 4.52$, $P = 0.0032$). Similarly, within the fourth larval instar, average daily growth rate per instar increased from 18.80 ± 6.25 μg *W* day^{-1} at 15 °C to 79.75 ± 34.95 μg *W* day^{-1} at 25 °C ($T= 4.39$, $P = 0.0048$) (Figure 2.A).

Average daily growth per instar for carbon mass was similarly affected by temperature ($F= 17.53$, $P < 0.001$), larval instar number ($F= 37.99$. $P < 0.001$) and the interaction between these factors ($F= 5.01$, $P < 0.001$) (Figure 2.B). Again, average daily

growth per instar increased significantly within the second larval instar (T= 5.15, *P* = 0.0004) from 2.69 ± 1.03 μg *C* day^{-1} at 15 °C to 26.94 ± 5.91 μg *C* day^{-1} at 25 °C. Also, within the fourth larval instar, average daily growth per instar increased from 8.23 ± 2.62 μg *C* day^{-1} at 15 °C to 31.92 ± 9.04 μg *C* day^{-1} at 25 °C (T= 5.03, *P* = 0.0005).

5.4.2.2 Effect of temperature on respiration rate

At 15 °C, respiration rates varied with the moult cycle, generally being higher in post moult larvae and decreasing through the inter-moult period, especially for post-hatching first instar and post-moult second and third instar larvae (Figure 5.5). Although one-way ANOVA indicated a significant effect of larval age on respiration rates for fed larvae (F = 2.50, *P* = 0.001), variations in respiration rates associated with the moulting cycle were not statistically significant. Respiration rates of fed and unfed larvae were similar with high post-moult respiration rates, which decreased during the inter-moult period. No statistical differences were found between fed and unfed larvae (GLM ANOVA: F = 0.42, *P* = 0.517). Similarly, respiration rates of fed and unfed larvae were not different at 20 °C (GLM ANOVA: F = 0.859, *P* = 0.859) and 25 °C (GLM ANOVA: F = 0.04, *P* = 0.852). At these temperatures, respiration rates varied within larval instars, generally decreasing through the intermoult period; however, these changes were subtle (Figure 5.5). At 20 °C, there was no effect of larval age on respiration rate (one-way ANOVA: F = 0.98, *P* = 0.484), whilst at 25 °C there was (F = 2.94, *P* = 0.005). Again, there were no differences in respiration rates associated with the moult cycle. At all temperatures, the standard deviations of data for unfed larvae were greater than those for fed larvae.

Respiration rate data (MO_2; μmol O_2 hr^{-1}) were converted to energy loss data (J hr^{-1}) according to Gnaiger (1983) (1 μmol O_2 hr^{-1} = 0.450 J hr^{-1}). These values were then used to estimate energy loss per day and then cumulatively added to give an estimate of energy loss within individual larval instars and throughout development (Figures 5.6A, 5.6B). Cumulative energy loss within individual larval instar was affected by temperature (GLM ANOVA: F = 46.71, *P* < 0.001), generally being greater for larvae developing at 15 °C. Larval instar (F = 137.29, *P* < 0.001) and the interaction between temperature and instar (F = 12.73, *P* < 0.001) affected cumulative energy loss within individual larval instars.

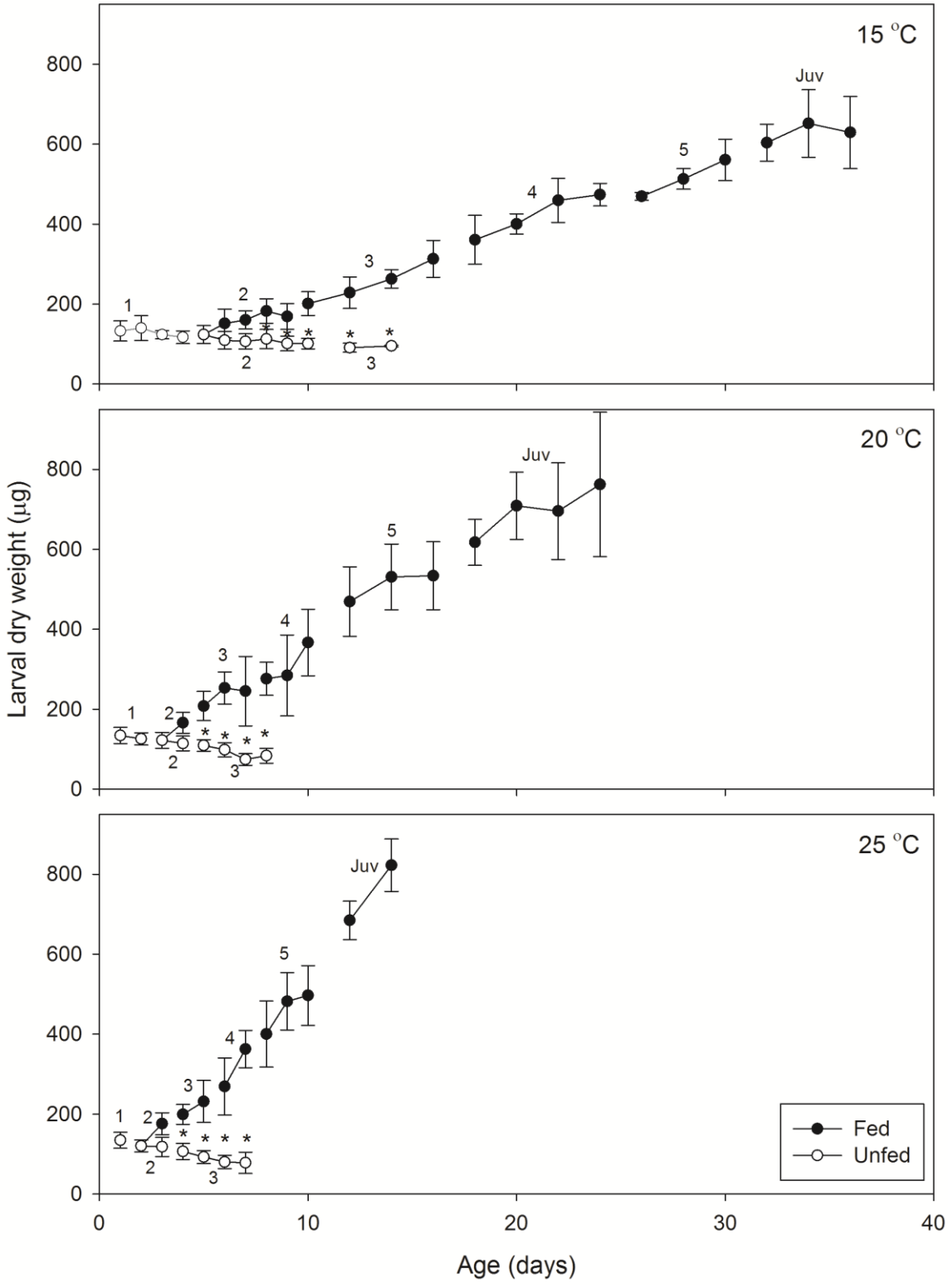

Figure 5.3. *Palaemonetes varians* larval dry weight throughout development for fed and unfed larvae at three temperatures (15, 20, 25 °C). Data points within the same instar are joined by lines, instar number is indicated, juv = juvenile. Data are presented as means ± standard deviations. Significant differences between fed and unfed larval dry weights are indicated by asterisks (*)

For example, during the second, third, and fourth instar, cumulative energy loss within these instars was significantly greater at 15 °C than at 20 and 25 °C, which were not distinct from one another (Figure 5.6A). For the fifth larval instar, cumulative energy loss during this instar was greatest at 20 °C and significantly higher than at 25 °C (Figure 5.6A). Cumulative energy loss throughout development was influenced by development temperature (GLM ANOVA: F = 44.18, $P < 0.001$), being greatest at 15 °C within all larval instars. Advancing larval instar also affected energy loss (F = 244.83, $P < 0.001$) but there was no interaction indicating that the trend was the same in all larval instars (Figure 5.6B).

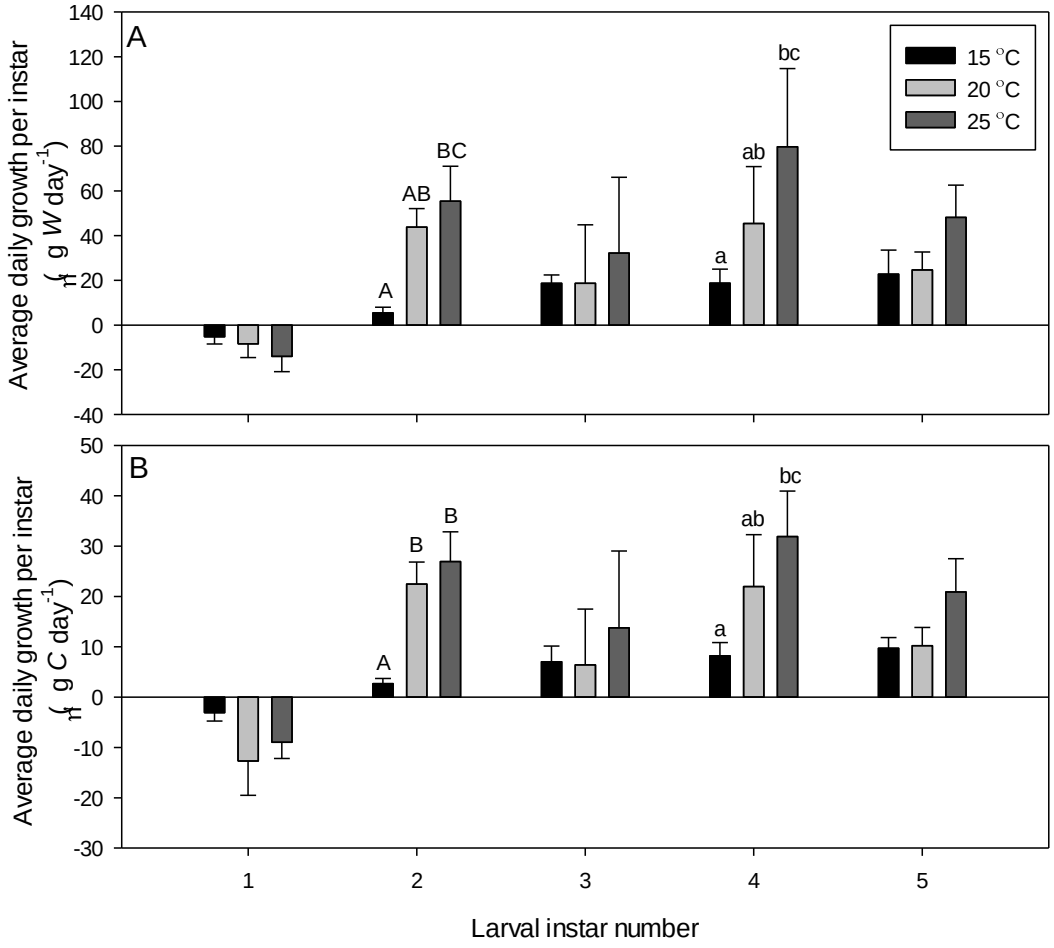

Figure 5.4 *Palaemonetes varians* average daily growth per instar for **A** dry weight and **B** carbon mass at three temperatures (15, 20, 25 °C). Data are presented at means ± standard deviations. Letters indicate differences between temperatures within instars

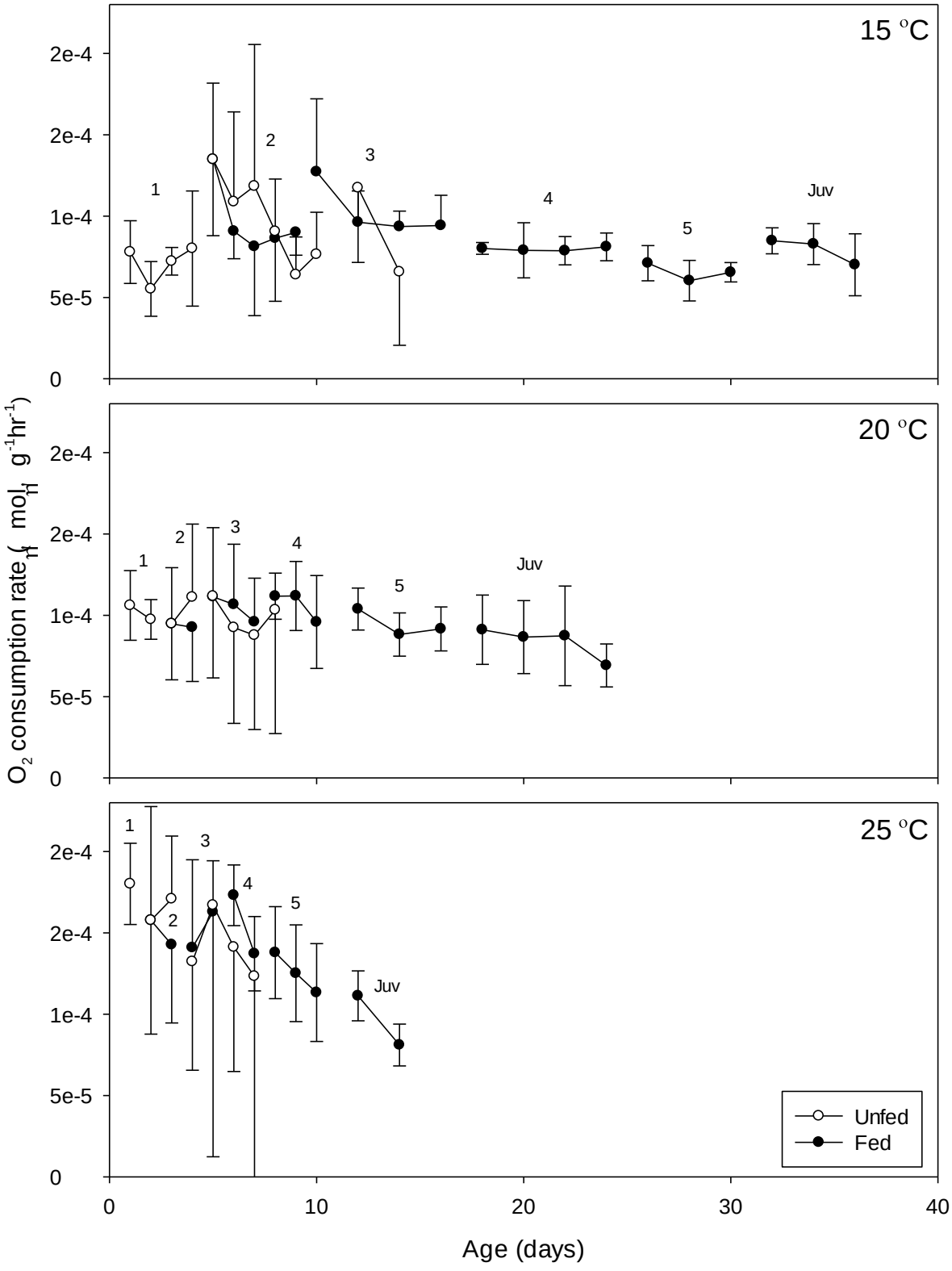

Figure 5.5 *Palaemonetes varians* respiration rates throughout development for fed and unfed larvae at three temperatures (15, 20, 25 °C). Data points within the same instar are joined by lines, instar number is indicated. Data are presented at means ± standard deviations

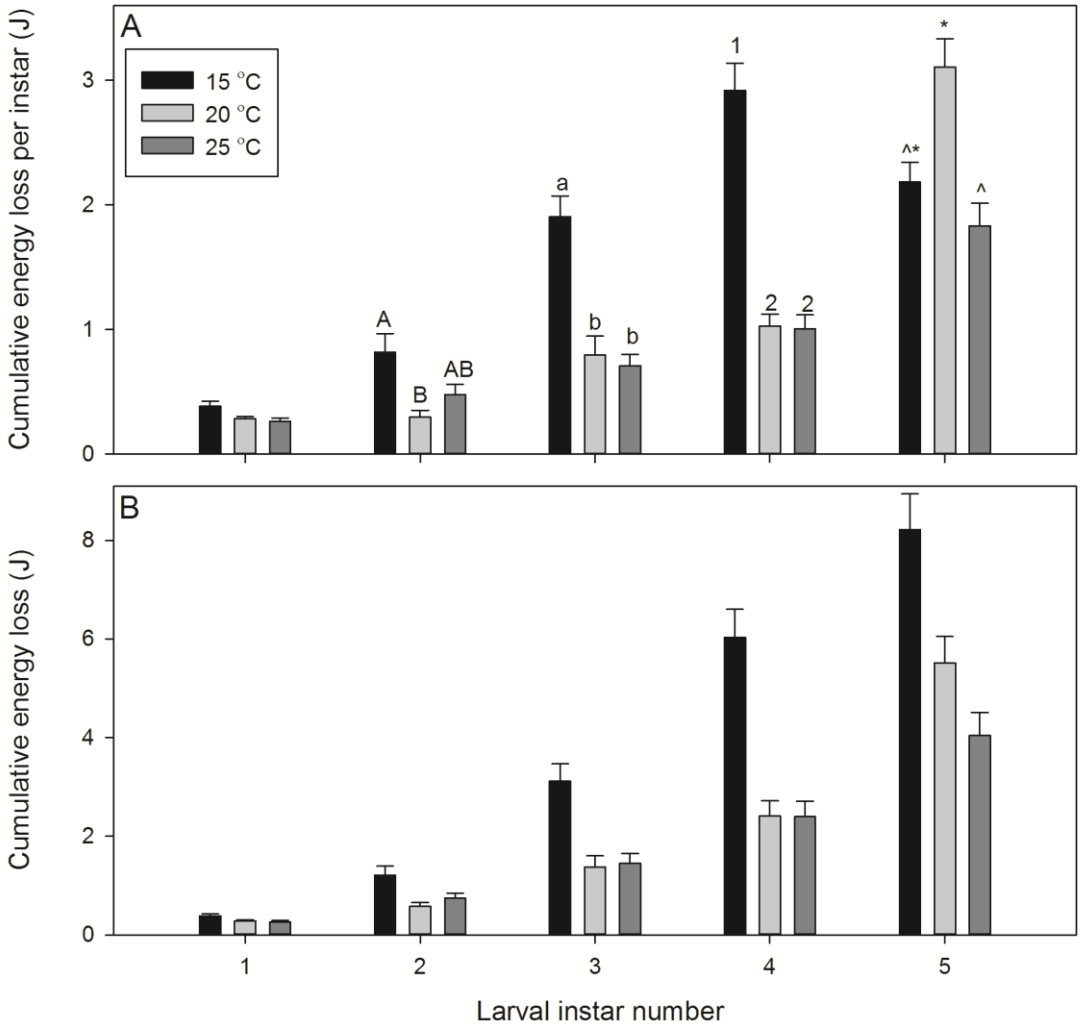

Figure 5.6 *Palaemonetes varians* **A** Cumulative energy loss within individual instars through development. **B** Cumulative energy loss throughout development. Data are means ± standard deviations. Differences between temperatures within instars are shown by letters, numbers, or symbols

5.4.2.3 The influence of temperature on elemental composition

Carbon content was approximately 45 % at hatching, whilst nitrogen content was approximately 11 %. The effects of temperature on larval dry weight (*W*), carbon mass (*C* µg), nitrogen mass (*N* µg), and carbon:nitrogen ratio (*C:N*) are shown in Table 5.2. Growth rates (final zoea *W* divided by initial hatchling *W*, * 100) were highest at 20 °C (409.84 ± 112.89 %) and lowest at 25 °C (369.71 ± 31.68 %), and was 394.85 ± 56.56 % at 15 °C. Growth factors F_G (carbon content of final zoea divided by freshly hatched zoea) were lowest at 20 °C (3.46 ± 1.06), highest at 15 °C (3.89 ± 0.49), and was 3.49 ± 0.42 at 25 °C.

Table 5.2 *Palaemonetes varians*. Changes in larval biomass and elemental composition throughout development at 15, 20, and 25 °C for larvae fed *Artemia* sp. nauplii. Dry mass (*W*), carbon and nitrogen mass (*C*, *N*), and C:N ratios; *n* = 5 replicate analyses

Temp. (°C)	Instar	Day	*W* (µg)		*C* (µg)		*N* (µg)		C:N	
			Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
15	1	1	132.800	25.332	58.045	11.019	13.678	2.105	4.225	0.171
	1	2	140.200	31.076	63.597	17.303	15.041	2.841	4.179	0.366
	1	3	123.600	10.164	53.079	6.394	13.608	1.196	3.892	0.157
	1	4	117.200	15.928	48.724	7.615	13.042	1.561	3.727	0.229
	2	5	147.200	26.310	62.359	13.731	15.028	2.786	4.126	0.225
	2	6	151.800	36.044	63.895	15.822	14.869	3.243	4.276	0.143
	2	7	160.400	22.876	70.156	14.566	16.057	2.910	4.354	0.138
	2	8	182.600	30.729	80.498	14.122	17.698	3.086	4.548	0.140
	2	9	169.000	32.031	73.100	13.053	16.639	3.141	4.401	0.080
	3	10	201.200	29.550	85.622	9.689	20.064	2.441	4.272	0.122
	3	12	228.600	39.336	100.805	19.676	18.848	11.166	4.271	0.100
	3	14	263.000	23.152	112.250	9.503	26.158	2.107	4.291	0.123
	3	16	313.200	46.370	127.827	26.565	29.141	6.465	4.403	0.156
	4	18	360.800	60.998	157.076	28.325	35.830	6.028	4.377	0.077
	4	20	400.400	25.314	174.544	11.684	39.966	3.096	4.370	0.066
	4	22	459.400	54.875	201.149	23.570	47.035	5.535	4.277	0.062
	4	24	473.600	28.343	206.458	12.836	49.006	3.342	4.215	0.093
	5	26	469.600	9.607	191.836	20.306	46.921	4.195	4.085	0.140
	5	28	513.000	25.797	221.719	16.192	55.000	2.816	4.029	0.122
	5	30	560.800	51.582	230.702	26.323	59.233	6.375	3.893	0.067
	J	32	603.800	46.165	251.487	21.190	64.589	4.473	3.891	0.075
	J	34	652.000	84.637	272.904	36.077	71.237	9.613	3.832	0.029
	J	36	629.400	90.544	256.827	41.308	66.799	10.269	3.841	0.052
20	1	1	134.000	20.211	66.351	9.133	14.960	1.572	4.424	0.265
	1	2	125.600	15.307	53.654	9.431	13.802	1.567	3.867	0.256
	2	3	122.000	20.075	48.350	6.859	14.112	1.882	3.955	0.275
	2	4	165.800	26.148	70.816	11.220	15.725	2.434	4.356	0.134
	3	5	207.800	36.396	89.394	14.314	20.517	3.415	4.362	0.083
	3	6	253.200	40.158	111.725	19.513	26.324	4.890	4.255	0.143
	3	7	245.200	86.952	102.228	34.944	23.673	7.194	4.261	0.251
	4	8	276.200	41.197	117.115	19.625	28.359	5.285	4.141	0.099
	4	9	284.400	101.434	121.440	45.374	29.705	11.370	4.096	0.153
	4	10	367.000	83.295	161.056	37.174	39.417	9.171	4.086	0.132
	5	12	469.200	86.975	201.003	41.102	49.311	10.174	4.077	0.120
	5	14	531.000	82.265	228.948	34.733	57.091	8.881	4.012	0.070
	5	16	534.000	85.194	222.578	32.354	57.582	7.908	3.863	0.081
	5	18	617.400	57.413	262.310	26.285	67.607	5.945	3.877	0.081
	J	20	708.800	84.200	278.910	30.792	74.231	8.149	3.757	0.060
	J	22	695.600	120.877	277.810	50.590	76.116	11.630	3.640	0.130
	J	24	762.400	180.542	280.437	53.319	73.210	16.961	3.860	0.178
25	1	1	134.600	19.844	61.357	10.458	14.696	1.872	4.158	0.196
	2	2	120.600	14.775	52.369	8.861	13.255	1.342	3.931	0.290
	2	3	176.000	27.331	61.334	35.750	17.588	2.335	4.352	0.172

3	4	199.400	24.966	86.082	10.402	20.226	2.547	4.262	0.195
3	5	231.600	52.524	99.864	23.000	23.569	5.889	4.258	0.177
4	6	269.250	71.309	90.921	57.175	27.528	7.282	4.129	0.185
4	7	362.600	46.645	152.558	29.123	36.792	7.329	4.152	0.062
5	8	400.400	82.773	170.498	36.202	41.703	8.994	4.093	0.111
5	9	482.200	71.709	206.257	26.166	51.466	7.717	4.018	0.112
5	10	496.800	74.764	212.325	28.183	52.537	6.723	4.040	0.084
J	12	684.600	48.169	282.935	19.720	73.855	5.507	3.833	0.055
J	14	822.600	65.401	330.550	26.062	87.784	8.258	3.770	0.056

Carbon content (% *W*) appeared to decrease with increasing development at all temperatures. Spearman's correlation indicated negative relationships between carbon content (% *W*) and larval age at 15 °C ($P = 0.009$), 20 °C ($P < 0.001$) and 25 °C ($P < 0.001$) (Figure 5.7; see Table 5.3 for fitted parameters and correlation coefficients). At all temperatures, carbon content appeared to decrease within the first instar, which was not fed, and then increase during the second instar, corresponding to the onset of larvae being fed (Figure 5.7). Non-parametric testing did not support these observations statistically. Similarly, C:N ratios appeared to decrease within the first instar and increase within the second instar at all temperatures (Figure 5.7); these changes were supported statistically. At 15 °C, C:N changed significantly through development ($F = 15.39$, $P < 0.001$). *Post-hoc* Tukey testing indicated that the C:N ratio decreased during the first instar from 4.23 ± 0.17 on day 1 to 3.73 ± 0.23 on day 4 was significant (Figure 5.7). The C:N ratio then increased from 4.13 ± 0.23 on day 5 to 4.55 ± 0.14 on day 8 during the second instar. C:N ratio decreased significantly between day 8 and day 10 (4.40 ± 0.08) and from day 16 until the end on the experiment (day 36).

At both 20 and 25 °C, larval C:N ratio changed significantly during development ($F = 8.66$, $P < 0.001$ and $F = 6.00$, $P < 0.001$, respectively), following similar patterns to those observed at 15 °C. *Post-hoc* testing indicated that the C:N ratio decreased during the first instar at 20 °C, from 4.42 ± 0.27 on day 1 to 3.87 ± 0.26 on day 2. As at 15 °C, C:N ratios increased during the second instar at 20 °C, from 3.96 ± 0.28 on day 3 to 4.36 ± 0.13 on day 4 (Figure 5.7). C:N ratio then decreased from day 12 onwards. At 25 °C, the decrease in C:N ratio through the first instar and the increase during the second instar were not significant. C:N ratio did, however, decrease significantly from day 10 onwards (Figure 5.7).

Table 5.3 *Palaemonetes varians*. Fitted parameters (a, b) and correlation coefficients (r) for linear regressions (y = a + bx) describing the relationship between larval age and carbon content (% *W*) at three temperatures (15, 20, 25 °C). At 15 °C, r value determined by Spearman's correlation and a and b calculated by pair-wise slopes.

Temp. (°C)	*a*	*b*	*r*
15	42.244	-0.040	-0.245
20	40.707	-0.210	-0.544
25	34.024	-0.301	-0.593

5.5 Discussion

The general decrease in both carbon content and C:N ratio during the larval development of *Palaemonetes varians* indicates the utilisation of stored lipid and the construction of muscle (Anger & Hayd 2009, Weiss et al. 2009). Changes in the relative elemental composition of *P. varians* larvae during development are not influenced by temperature; however, the timing and rate of changes are. We also demonstrate the impressive starvation resistance of *Palaemonetes varians* larvae which is associated with a high maternal energy investment in offspring and can be considered as an important evolutionary adaptation in the ecology of this species.

5.5.1 Biochemical composition, metabolism, and *Palaemonetes varians* larval ecology

Palaemonetes varians larvae are highly resistant to starvation, surviving for prolonged periods in the absence of food. Despite starvation, larval development may proceed to the third (results presented here) and even fourth larval instar (Oliphant & Thatje 2013); thus, *P. varians* can be considered facultative lecithotrophic in its first and second larval instars and planktotrophic in its third larval instar. The extent to which larvae developed was temperature dependant with a greater proportion of larvae developing to the third larval stage at higher temperatures (see also Chapter 6). This level of starvation resistance is greater than that observed for the North American *Palaemonetes* species, *P. vulgaris* and *P. pugio*: the former survives for ~5 days and is unable to moult whilst the latter survives for ~10 days and can moult to its second larval instar, only (Broad 1957a, b). *P. varians*' starvation resistance is more comparable with that of the palaemonine shrimp, *Macrobrachium amazonicum*, which is lecithotrophic in its first instar, facultative lecithotrophic in its second instar, planktotrophic in its third larval instar and can survive in the absence of food for ~12 days and moult to its third larval instar (Anger & Hayd 2009).

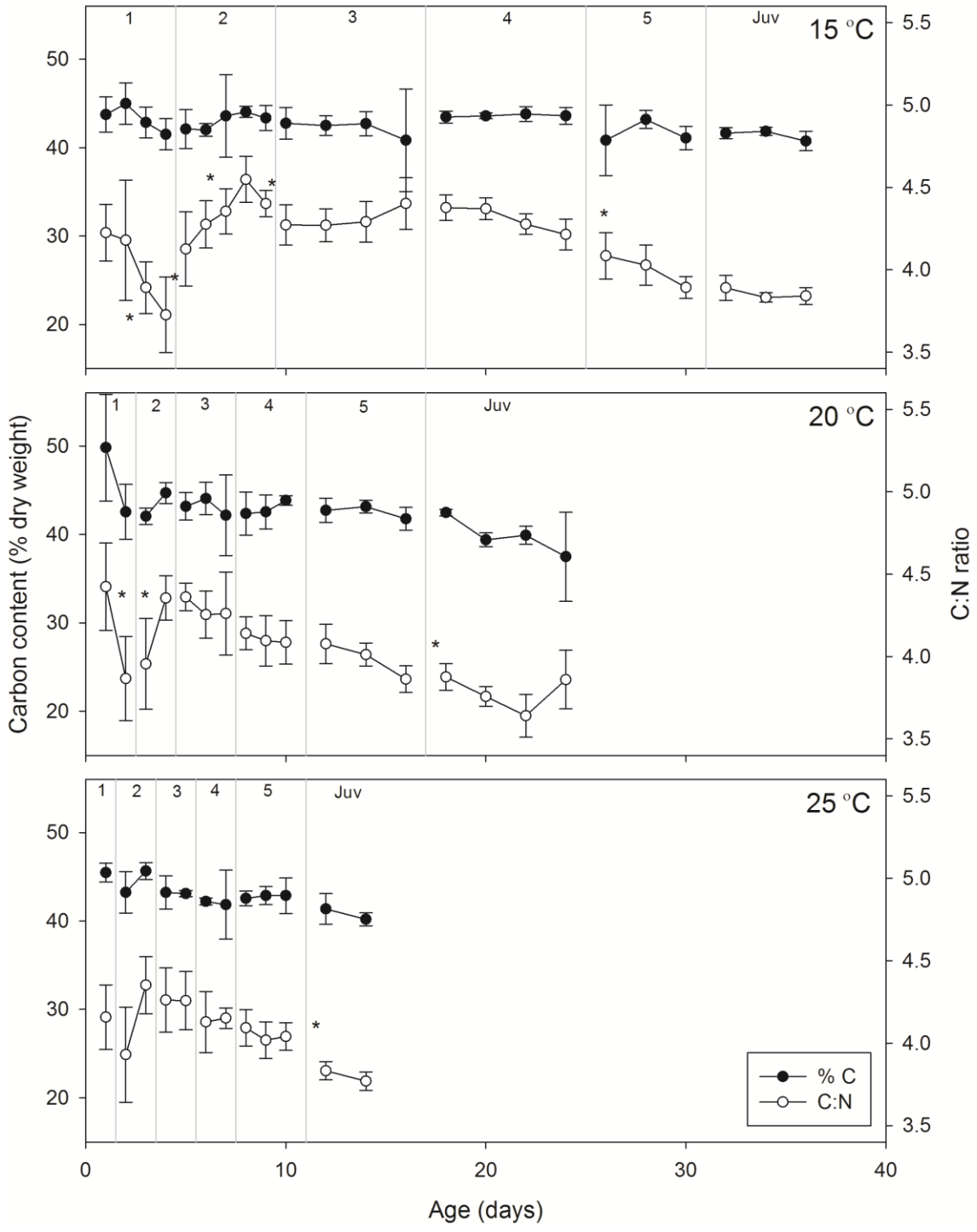

Figure 5.7 *Palaemonetes varians* carbon content (% *W*) and C:N ratio throughout development at three temperatures (15, 20, 25 °C). Instar numbers are indicated, juv = juvenile. Data are presented as means ± standard deviations. Significant changes in C:N ratios are indicated by asterisks (*)

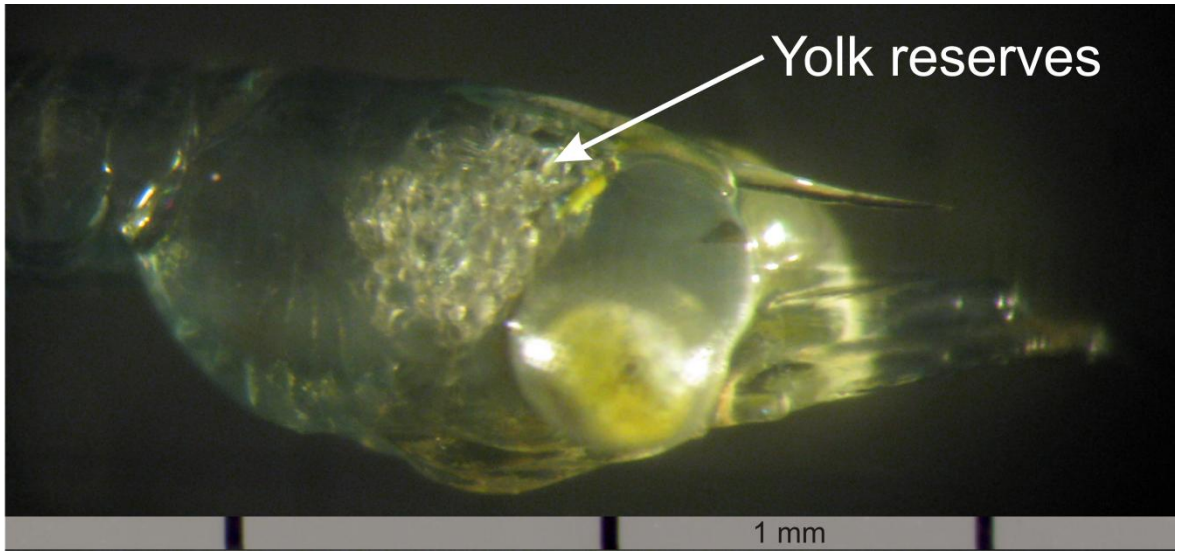

Figure 5.8 *Palaemonetes varians*. Photograph of a first instar (zoea 1) larva; lateral view of cephalothorax showing yolk reserves at hatching. Scale bar shows 1 mm increments

Inter-specific differences in starvation resistance among these palaemonine shrimp are likely a reflection of the extent to which these species are adapted to brackish and fresh water environments. *P. vulgaris* and *P. pugio* both inhabit estuarine environments but *P. vulgaris* occurs in deeper more saline waters whilst *P. pugio* occurs on mud flats and salt-marsh creeks (Jenner 1955). Adult salinity tolerances within these species reflect this distribution as *P. pugio* is more tolerant of lower salinities than *P. vulgaris*; larval salinity tolerances are similar, however (Knowlton & Kirby 1984, Knowlton & Schoen 1984). *Palaemonetes varians* inhabits more peripheral brackish-water habitats whilst *M. amazonicum* inhabits brackish and fresh waters as adults but the larvae of this species require salinities of 6 to 35 to develop (Moreira & McNamara 1986). Starvation resistance within early larval stages of brackish and fresh water palaemonine (and decapods in general) is considered an evolutionary adaptation to the export of larvae by river- and tidal-flow from adult environments into estuarine and coastal marine waters where conditions for larval development may be more favourable than those in the adult environment (Hovel & Morgan 1997, Anger 2001, Anger & Hayd 2009, Vogt 2013). The differing levels of starvation resistance among these palaemonine shrimp reflect the extent to which adults inhabit peripheral brackish and fresh water habitats and, consequently, the period of time taken for larvae to reach estuarine and coastal marine waters.

During the period of export from the adult environment, larvae may encounter low food availability and, consequently, high maternal energy investment per offspring is

selected for enabling starvation resistance in early stage larvae. Like *M. amazonicum* and the lecithotrophic palaemonid, *Palaemonetes zarqueiyi*, *P. varians* larvae hatch with visible maternally derived yolk reserves (Figure 5.8) (Anger & Hayd 2009, Urzúa et al. in press). Although *P. varians* larvae are similar in starvation resistance to *M. amazonicum*, the relative dry weight, carbon content and C:N ratio differ considerably between these species. Relative to *M. amazonicum*, *P. varians* has higher hatchling *W* (~62 μg < ~120μg, respectively), higher carbon mass (~33 μg < ~54 μg, respectively), but lower carbon content (as %*W*) (~54 % > ~45 %, respectively), and C:N ratio (~5.5 > ~4.2, respectively). *P. varians*, despite having lower carbon content and C:N ratio, has greater dry weight and carbon mass than *M. amazonicum*, which may be reflected in the more abbreviated development within *P. varians* (4 to 5 larval instars compared with 9 for *M. amazonicum*). Hubschman and Broad (1974) identified a continuum of *Palaemonetes* species occupying increasingly freshwater environments and which demonstrate increasingly abbreviated development. *P. varians* has moderately abbreviated development relative to both *P. pugio* and *P. vulgaris* (7-11 instars) which both hatch at a smaller size than *P. varians* (Broad 1957a, b, Hubschman & Broad 1974).

Limited data are available on elemental composition changes during larval development for caridean shrimp (Anger & Harms 1990, Thatje et al. 2004b, Anger & Hayd 2009, Anger et al. 2009, Anger & Hayd 2010, Urzúa et al. in press). Generally during larval development, the carbon content of planktotrophic larval decapods increases through successive larval stages, indicating the storage of lipids assimilated during larval feeding (Anger 2001, pg 194). Here, the experimental design meant that carbon content and C:N ratio changes during development were more complex than in previous studies. During the first instar, which was not fed, carbon content and C:N ratios decreased, indicating that maternally derived lipid reserves were preferentially utilised for energy metabolism (Anger 1998, 2001). At the onset of feeding, carbon content and C:N ratio initially increased during the second instar, evidencing an accumulation of lipid reserves. As for brachyuran crabs with feeding larvae, carbon content reported for larval development increases with advancing development, indicating the accumulation of lipid reserves: e.g. *Neohelice granulata*, *Hyas araneus*, *Carcinus maenas*, *Liocarcinus holsatus*, *Cancer setosus* and the anomuran crab, *Pagurus bernhardus* (Dawirs et al. 1986, Anger 1989, Anger et al. 1989, Harms 1990, Anger & Ismael 1997, Anger 2001, Weiss et al. 2009). After the second larval instar, development through subsequent larval instars

marked a gradual decrease in carbon content and C:N ratios. This utilisation of lipid reserves during development is consistent with results reported for the larvae development of *M. amazonicum* (Anger & Hayd 2009). This trend suggests the use of lipids and the formation of muscle structure (Anger & Hayd 2009, Weiss et al. 2009).

Typically, both carbon content and C:N ratio show short-term cyclical changes during development which correspond to the moult cycle (Dawirs et al. 1986, Anger 1989, Anger et al. 1989, Harms 1990, Anger & Ismael 1997, Anger 2001, Weiss et al. 2009). In post-moult larvae, carbon content is generally lower as a result of water and mineral uptake after moulting. Similarly, post-moult C:N ratios generally increase to a maximum in the inter-moult and then decrease through the pre-moult (Dawirs et al. 1986, Anger 1989, Anger et al. 1989, Harms 1990, Anger & Ismael 1997, Anger 2001, Weiss et al. 2009). Generally, these cyclical patterns in carbon content and C:N ratio are not evident in the results reported here for *P. varians*. This may point at the relatively low resolution within this study, although monitored daily, rapid development meant much of these cycles were missed.

5.5.2 Temperature effects on growth and elemental composition during *Palaemonetes varians* larval development

The results of Experiment 1 reported here indicate that the effects of temperature on the larval development of *P. varians*, in terms of development time, growth rate, juvenile dry weight, and developmental plasticity are consistent with results of Chapters 4 and 6. The implications of larval instar plasticity for post-settlement juvenile traits are poorly reported for decapods (Giménez et al. 2004), but may have significant ecological and evolutionary implications (Kingsolver 2007, Etilé & Despland 2008).

Few data are available on the effects of temperature on elemental composition and, thus, the utilisation of lipid and protein during development at different temperatures (e.g. Dawirs et al. 1986, Anger 1987, Weiss et al. 2009). For *P. varians*, the initial larval dry weights, carbon and nitrogen contents, and C:N ratios of post-moult larvae at each stage were not different between temperatures, suggesting that growth, development, and the moulting cycle were not de-coupled by temperature. However, the results of Experiment 1 and those reported in Chapter 4 and 6 demonstrate that temperature mediated developmental plasticity is driven by the decoupling of development from the moult cycle. Given the methods used in Experiment 2, it was not possible to separate larvae developing

through different larval instars and observe how growth rates and elemental composition may change with developmental plasticity. The results of Experiment 2 do, however, demonstrate a significant effect of temperature on growth rates: average daily growth per instar, both in terms of dry weight and carbon mass, increased with temperature. The second larval instar, which was the first to be fed, appeared particularly important in the assimilation of lipid resources which were subsequently utilised throughout development. Within the second instar, average daily growth rates for dry weight and carbon mass increased significantly with temperature (Figures 5.4). Increasing temperature has been found to increase the rate of carbon content growth, and thus lipid assimilation, within zoeal stages of the brachyuran crab *Carcinus maenas* (Dawirs et al. 1986) and early larval stages of the brachyuran crab *Cancer setosus* (Weiss et al. 2009). Interestingly, although growth rates were higher at 22 °C than 20 °C for *C. setosus*, larvae did not reach the juvenile stage at 22 °C (Weiss et al. 2009), indicating that higher growth rates do not necessarily imply better development conditions. This was consistent with the fact that carbon content remained generally stable whilst C:N ratio increased within *C. setosus*, indicating degradation of protein as an energy source, resulting from high metabolism, and indicative of suboptimal conditions (Weiss et al. 2009). In this present study, carbon content and C:N ratio decreased throughout development at all temperatures. For *C. setosus*, larvae developing at 16 °C showed a decreasing carbon content and C:N ratio and failed to develop fully to the juvenile stage; given the higher utilisation of lipid reserves, this temperature was interpreted as suboptimal for growth (Weiss et al. 2009). For *P. varians*, larval development is successful at all temperatures tested here and suggests that the decreasing carbon content and C:N ratio is not deleterious to development (Fincham 1979, see also Chapters 4 and 6). A similar decrease in carbon content within *M. amazonicum* was thought to indicate a 'programmed' degradation of maternal resources (Anger & Hayd 2009).

5.5.3 Temperature effects on respiration rate

The respiration rates of both fed and unfed larvae were not statistically different and varied cyclically with the moult cycle. Although respiration rate data of sufficiently high resolution to allow the observation of variation associated with the moult cycles is rare, available data indicate a consistent pattern: respiration rates (QO_2) are highest post-moult, decrease through the inter-moult period and may increase again pre-moult (Anger & Jacobi 1985, Jacobi & Anger 1985b, Anger et al. 1989, Anger et al. 1990, Carvalho &

Phan 1998, Anger 2001). High respiration rates at pre- and post-moult may be related to energy demanding 'reconstruction processes', whilst low inter-moult levels correspond to phase of little structural change and correspond to high mass accumulation (Anger 2001). Results presented here are consistent with this pattern. Unfed and fed larvae show the same pattern, indicating that respiration rate is not down-regulated in response to unfavourable conditions and that the moult cycle progresses as usual, being comparable to fed larvae. This is consistent with the results of Experiment 1 (Figure 5.1): larvae continue to develop and moult when starved. It appears, therefore, that *P. varians* larvae do not show energy saving traits, in terms of metabolic rate and development, in response to starvation.

Respiration rates generally increased with temperature, this has been demonstrated for adult *P. varians* (Oliphant et al. 2011) and other decapod larvae, generally (Moreira et al. 1980, Jacobi & Anger 1985a, Ismael et al. 1997). Importantly, cumulative energy losses were highest at the lower temperature. Despite relatively lower respiration rates, the longer development time resulted in overall higher energy loss. This indicates that development is more constrained and less efficient for *P. varians* at lower temperatures and could be a reason for lower growth rates at lower temperatures. The negative effect of low temperature on *P. varians* development is consistent with the ancestry of palaemonid shrimp which are thought to have evolved in tropical regions and are generally distributed in tropical and temperate regions; certainly *P. varians* is the most high latitude *Palaemonetes* sp. (De Grave et al. 2009, Ashelby et al. 2012, Anger 2013, OBIS database 2013). The lesser extent of development within unfed larvae at 15 °C, relative to development at higher temperatures, may be the influence of greater energy loss coupled with finite maternally derived energy resources. More energy may be utilised for basal metabolic costs leaving less available for development at this lower temperature; consequently, larval death from starvation occurs at a less advanced developmental stage relative to higher temperatures. Combined, greater energy loss and lower growth and development rates throughout development may yield more larval instars at lower temperatures.

Palaemonetes varians is highly resistant to starvation and may be considered facultative lecithotrophic in its first and second larval instars and planktotrophic from its third instar. This high starvation resistance is enabled by relatively high lipid content at hatching and the degradation of this energy resource throughout development. Such high maternal investment and high starvation resistance is an adaptation to the exporting of

larvae from peripheral adult environments into mid and lower estuarine environments were larval development takes place. Increasing maternal investment and the consequent decrease in fecundity are important life history adaptations in the colonisation of freshwater and terrestrial environments among decapods.

5.5.4 Conclusions

- *Palaemonetes varians* is facultative lecithotrophic in its first two larval instars (zoea 1 and 2) and planktotrophic from its third larval instar
- High starvation resistance is enabled by high carbon content (~45%) and high C:N ratio (~4.2) at hatching
- Lipid reserves are utilised throughout development as the major source of energy for metabolism
- Metabolic rate is not down regulated in response to starvation as an energy saving adaptation
- Temperature affects growth rates but not the composition of larvae
- Cumulative energy loss is greatest at lowest temperatures. Coupled with low growth rates, this may explain why development proceeds through more instars at lower temperatures

6. Per Offspring Investment implications for crustacean larval development: evolutionary insights into endotrophy and abbreviated development*

6.1 Summary

At the inter-specific level, Per Offspring Investment (POI) is associated with larval development mode and follows a macro-ecological trend within the marine environment; higher POI and its associated greater degree of endotrophy and abbreviated development is found in cooler, high latitude regions. Here, the implications of between-brood-variation in hatchling energy content (measured as carbon mass) on larval starvation resistance and developmental plasticity within the caridean shrimp, *Palaemonetes varians*, were assessed. Results demonstrate that greater POI provides increased endotrophy and the potential for abbreviated development. In the absence of food, *P. varians* larvae from broods of higher hatchling energy content developed to more advanced larval stages and survived for longer before succumbing to starvation. In the presence of food, *P. varians* larvae from broods of higher hatchling energy content developed through fewer larval instars, showed higher growth rates, and had shorter development times. Also, for larvae developing through the same number of larval instars, larvae from broods of higher hatchling energy content developed to greater juvenile dry weight. These data support the hypothesis that macro-ecological trends in development mode are driven by POI; environmentally mediated phenotypic plasticity in POI may therefore permit the evolution of the diverse and complex life cycles observed in the marine environment.

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6.2 Introduction

Per Offspring Investment (POI) -the quantity and quality of maternal resources allocated to a single offspring- is a trait with fundamental ecological and evolutionary implications (Vance 1973, Smith & Fretwell 1974, Christiansen & Fenchel 1979). POI may influence larval development, starvation resistance, larval ecology, planktonic longevity (for the dispersive propagules of marine invertebrates), and may have carry-over effects for juvenile and adult life (Fox 1994, Emlet & Høegh-Guldberg 1997, Ito 1997, Moran & Emlet 2001). Macro-ecological trends in POI, and its related larval development modes, at the inter-specific level, and geographic variation in POI at the intra-specific level, indicate past and continuing selection on this trait. The influence of changes in POI on larval development and their effect on later life stages is requisite if we are to understand how selective pressures act to change POI and development modes over evolutionary time scales.

At the inter-specific level, macro-ecological trends in POI (and its related development modes) are known for marine invertebrates (including marine arthropods); Danish ecologist, Gunnar Thorson first observed higher POI in species from higher/polar (cooler) latitudes relative to closely related species from lower/temperate (warmer) latitudes (Thorson 1936, 1950). Evidence also suggests an increase in POI with increasing depth within the oceans (Thorson 1961, 1964, King & Butler 1985, Pond et al. 1997). Based on observed trends in POI and knowledge of the relationship between POI and development mode, Thorson hypothesised that aplanktonic and planktonic non-feeding (lecithotrophic) larval development were more prevalent at high latitudes whilst planktonic feeding (planktotrophic) larval development was more prevalent at lower latitudes (Thorson 1936, 1950). A recent meta-analysis of life-history data supports Thorson's hypotheses on the macro-ecological trends in development mode. Marshall et al. (2012) demonstrated relationships between development mode and latitude and, within development modes, between offspring size and latitude. Importantly, and despite Mileikovsky (1971) proposing 'Thorson's Rule', the concept proposed by Thorson should be considered as a general macro-ecological concept, not an absolute rule (e.g. Thatje et al. 2005).

Inter-specific level variation in POI among marine invertebrates (and marine arthropods) is related to development mode; high POI is associated with high nutritional

independence (endotrophy/lecithotrophy) and abbreviated development whilst low POI is associated with planktotrophy (Thorson 1936, 1950). In arthropods, abbreviated development consists of larvae, which hatch in an advanced stage of development and proceed through relatively few larval instars before metamorphosing to juveniles; for example Antarctic shrimp (Thatje et al. 2003, Thatje et al. 2004a). High POI and abbreviated development are considered to be evolutionary adaptations to low or unpredictable food availability and/or the mis-match between short periods of primary production (resulting from extreme seasonality) and prolonged development (resulting from low temperatures) at high latitudes (Thorson 1950, Anger 2001, Thatje et al. 2003). High POI and abbreviated development are also known from brackish and freshwaters and are considered evolutionary adaptations to the limited food availability prevalent within these environments (Anger 2001).

Variation in POI is also known at the intra-specific level for marine invertebrates (including marine arthropods). Thorson observed that within a single species, populations from higher (cooler) latitudes exhibit greater POI than populations from lower (warmer) latitudes; reflecting the macro-ecological trend in POI at the inter-specific level (Thorson 1936). Mounting empirical evidence has demonstrated intra-specific variation in POI at both spatial and temporal scales; greater POI being observed under cooler conditions (often associated with higher latitude, greater water depth, or season; (e.g. Thorson 1950, Barnes & Barnes 1965, Urzúa et al. 2012). Intra-specific variation in POI at spatial scales may indicate genetic divergence and local adaptation between populations; a result of selective pressures acting on POI within those populations. Conversely, temporal variations in POI within a population demonstrate POI plasticity. Such plasticity is known at a range of scales, e.g. inter-annually, between seasons, and within a single breeding season (Boddeke 1982, Sheader 1983, Kattner et al. 1994, Sheader 1996, Oh & Hartnoll 2004, Urzúa et al. 2012, Urzúa & Anger 2013). POI plasticity is influenced by maternal size and age, food quantity and quality, population density, and temperature. Temperature-mediated shifts in POI may produce eggs which differ in size and dry weight (e.g. Blanckenhorn 2000, Fischer et al. 2003a, Fischer et al. 2003b, Liefting et al. 2010) and in relative yolk composition (e.g. water, carbohydrate, lipid, protein, and glycogen concentrations; Geister et al. 2008, Sloggett & Lorenz 2008, Liefting et al. 2010). Studies assessing the fitness implications of temperature-mediated POI have found that eggs with greater or intermediate POI are less prone to desiccation, more resistant to temperature shock, have

greater hatching success, and give rise to larger, fitter juveniles across a range of thermal conditions (Blanckenhorn 2000, Fischer et al. 2003a, b, Liefting et al. 2010).

In Chapter 4, temperature-mediated developmental plasticity was identified for *Palaemonetes varians* larvae from temperate European waters (Hampshire, UK). For *P. varians*, 'normal' larval development proceeds through five larval instars (Fincham 1979). Results in Chapter 4 demonstrate a four instar development pathway which omitted the final decapodid instar and which was temperature dependent, occurring more often under warmer conditions. Observations for the estuarine crab, *Neohelice* (*Chasmagnathus*) *granulata*, which also develops through either of two development pathways (Pestana & Ostrensky 1995), suggest that developmental plasticity is also influenced by hatchling dry weight (Giménez & Torres 2002). Larval instar number plasticity is an important trait among arthropod as it allows growth and development to a weight/development threshold under poor growth and development conditions (Nijhout 1994, Esperk et al. 2007, Kingsolver 2007, Etilé & Despland 2008). Variations in larval instar number are known to influence development time, growth rate, and juvenile/post-metamorphosis dry weight and, consequently, are of ecological and potentially evolutionary importance (Kingsolver 2007, Etilé & Despland 2008).

Here, we use between-brood-variation in hatchling (zoea 1) energy content to assess the implications of POI for larval starvation resistance, instar number, growth rate, and development time, and juvenile dry weight within the caridean shrimp, *Palaemonetes varians*. In accordance with Marshall and Keough (2007), we consider POI to be the energy content of a propagule once it has become independent of maternal nutritional investment. According to this definition, the energy content of a freely spawned egg is the appropriate measure of POI but the energy content of a direct developing snail egg before the embryo has ingested nurse eggs is not (adapted from Marshall & Keough 2008). We use the energy content of hatchling larvae as an approximation of POI as conditions during embryonic development can influence energy content at hatching (Wehrtmann & Lopez 2003). As proxy for energy content, propagule size/volume and propagule dry weight are routinely used in the literature. Given the inherent inaccuracies with the use of propagule volume as accurate proxy for energy content (Moran & McAlister 2009); here, we use propagule carbon mass ($\mu g\ ind^{-1}$) as accurate proxy for lipid content and thus energy content (following Anger & Nair 1979), a more accurate measure than has been used previously in like studies. The implications of POI for larval development and potential

carry-over effects for early juvenile life are essential if we are to understand the selection pressures which shape POI.

6.2.1 Hypotheses

The specific hypotheses tested were:

under fed conditions,

H_1 larvae hatching with higher POI are able to develop through fewer larval instars at all temperatures

H_2 larvae hatching with higher POI develop more quickly, to a larger size at settling, and have higher growth rates

under starved conditions,

H_3 larvae hatching with higher POI are able to develop to more advanced larval stages before death from starvation occurs

H_4 larvae hatching with higher POI will survive for longer before death from starvation.

H_5 the influence of POI on larval development will be greatest at lower temperature

6.3 Materials and Methods

6.3.1 Collection and maintenance of ovigerous *Palaemonetes varians*

Ovigerous *Palaemonetes varians* were collected on 24th May, 12th and 26th June, 2012 as described in Chapter 2, section 2.2.2. Ovigerous shrimp were maintained, following section 2.4.1. Ovigerous females were checked daily (at ~09:00) for hatched larvae. Between collection and larval hatching, ovigerous females were maintained at 15 °C as described in Chapter 2, section 2.4.1.

6.3.2 Larval maintenance

On hatching, actively swimming larvae were separated from 37 ovigerous *P. varians* and maintained as described in Chapter 2, section 2.4.3. To calculate a brood average dry weight (DW, μg ind^{-1}) and carbon mass (μg $ind.^{-1}$), a sample of 15 newly hatched larvae per brood (i.e. 15 larvae from each of the 37 ovigerous *P. varians*) were blotted on tissue paper, transferred individually to pre-weighed tin capsules and frozen at -

80 °C. They were later freeze-dried (for 24 hours) and weighed for DW (see section 2.7). Carbon and nitrogen (C and N) composition of n = 5 samples (from each of the 37 broods) was measured (see section 2.8).

For each brood, n = 24 larvae were transferred to temperature-controlled incubators set at 15, 20, and 25 °C, 12:12 (light:dark) (i.e. a total of n = 72 larvae per brood separated across three temperatures) and maintained as described in Chapter 2, section 2.4.3. This temperature range is within the tolerable range for viable *P. varians* larval development (see Chapter 4). Of n = 24 larvae for each brood at each temperature, n = 12 larvae were fed and n = 12 were not fed (non-fed category). Zoea 1 of *P. varians* is facultative lecithotrophic (see Chapter 5); consequently, larvae from both categories (fed and non-fed) were not fed during zoea 1 (15 °C = 4.0 ± 0.5 days, 20 °C = 2.3 ± 0.5 days, 25 °C = 2.1 ± 0.3 days). On moulting to zoea 2, larvae within the fed group only were fed freshly hatched *Artemia* sp. nauplii to excess. Therefore, fed vs non-fed treatments were done only after larvae attained zoea 2. Larval mortality and development, assessed by morphological changes and moulting were monitored daily (am, between ~09:00 – 11:00) as described in section 2.4.4. On moulting to the juvenile stage, animals were preserved as described in Chapter 2, section 2.4.3 and later weighed for dry weight (see section 2.7).

6.3.3 Statistical analysis.

For non-fed larvae, logistic regression was used to analyse the relationship between brood average carbon mass (μg ind.$^{-1}$) and the proportion of larvae developing to decapodid 1 or decapodid 2 before starvation. Similarly, for fed larvae, logistic regression was used to analyse the relationship between brood average carbon mass and the proportion of larvae developing through four instars. To analyse the effects of brood average carbon mass on starvation resistance (days), Analysis of Covariance (ANCOVA) was used, with brood average carbon mass as a continuous predictor and temperature as a factor. The model was fitted using the Generalised Least Squares method and was weighted by a variance structure, which accounted for heterogeneity within data. ANCOVA was also used to assess the effect of brood average carbon mass on larval development time (days), juvenile DW (μg ind^{-1}), and larval growth rate (μg ind^{-1} days^{-1}). Brood average carbon mass was a continuous predictor and temperature and instar number (four and five instars) were factors. Again, models were fitted using the GLS method and were weighted by variance structures, which accounted for heterogeneity within data. Post hoc testing was done using the least squares mean method for factor combinations

(temperature and instar number) and adjusted (Tukey method) for multiple comparisons. Statistical analysis was carried out using Minitab 16 (for logistic regression) and Ri386 3.0.1 (for ANCOVA and post hoc testing) software and in accordance with Sokal & Rohlf (1995) and Zuur et al. (2009).

6.4 Results

6.4.1 Influence of brood average carbon mass on starvation resistance

The stage to which larvae developed before succumbing to starvation was affected by brood average carbon mass. At 15 °C, the proportion of larvae developing to decapodid 1 before succumbing to starvation significantly increased with increasing carbon mass ($P < 0.001$, G = 87.70; Figure 6.1). At 25 °C, the proportion of larvae developing to decapodid 1 before succumbing to starvation significantly decreased with increasing carbon mass ($P = 0.001$, G = 13.90) whilst the proportion of larvae developing to decapodid 2 before succumbing to starvation significantly increased with increasing carbon mass ($P = 0.002$, G = 18.21; Figure 6.1). At 20 °C, neither the proportion of larvae developing to decapodid 1 ($P = 0.801$, G = 0.06) nor decapodid 2 ($P = 0.998$, G = 5.63) before succumbing to starvation was significantly related to carbon mass.

Brood average carbon mass significantly affected starvation resistance; larvae from broods of greater brood average carbon mass were more resistant to starvation (Figure 6.2, Table 6.1). Starvation resistance increased with decreasing temperature (Figure 6.2, Table 6.1). There was, however, no significant interaction between brood average carbon mass and temperature (Table 6.1). As the interaction between carbon mass and temperature was non-significant, no post hoc testing was done.

6.4.2 Influence of brood average carbon mass on developmental plasticity

The number of larval instars through which larvae developed was significantly affected by brood average carbon mass. At all temperatures, the proportion of larvae developing to juvenile through four instars significantly increased with increasing brood average carbon mass ($P < 0.001$ in all cases; 15 °C, G =18.54; 20 °C, G = 30.13; 25 °C, G = 24.52; Fig. 6.3).

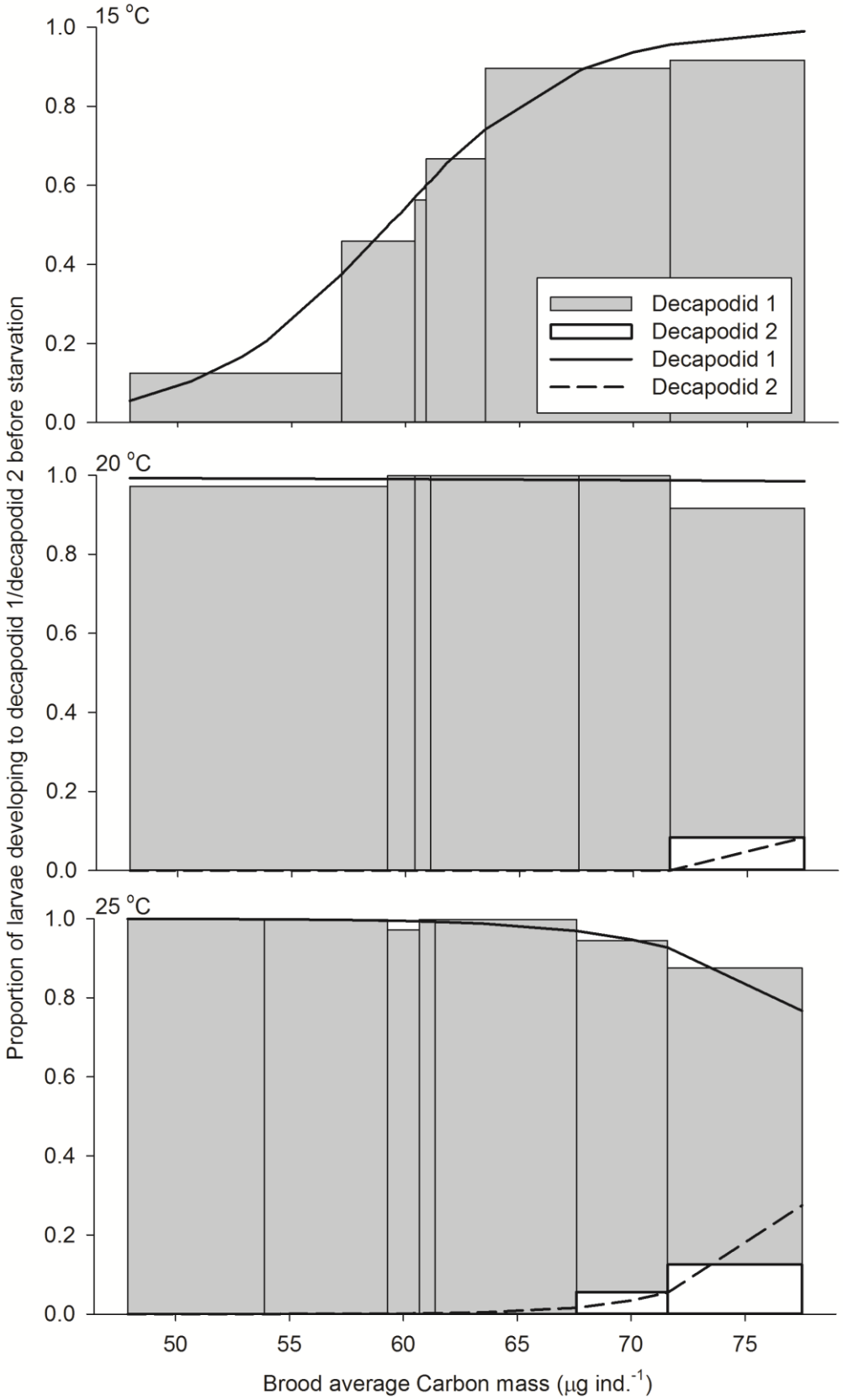

Figure 6.1 *Palaemonetes varians*. Logistic regressions of the proportion of larvae developing to decapodid 1 and decapodid 2 before succumbing to starvation against brood average carbon mass (µg ind.$^{-1}$) at 15, 20, and 25 °C. Bars indicate proportions data, which were divided into intervals for graphical purposes only. Bar widths may vary between temperatures as the intervals were chosen so that each bar represented a similar n. Best-fit-lines for logistic regressions are shown

Table 6.1 The effects of brood average carbon mass (C. mass) and temperature (Temp.), and their interaction on starvation resistance within *Palaemonetes varians*

	DF	F-value	*P*-value
Starvation resistance			
C. mass	1	56.67	<0.0001
Temp.	2	1121.40	<0.0001
C. mass*Temp.	2	1.11	0.3299
Error	690		

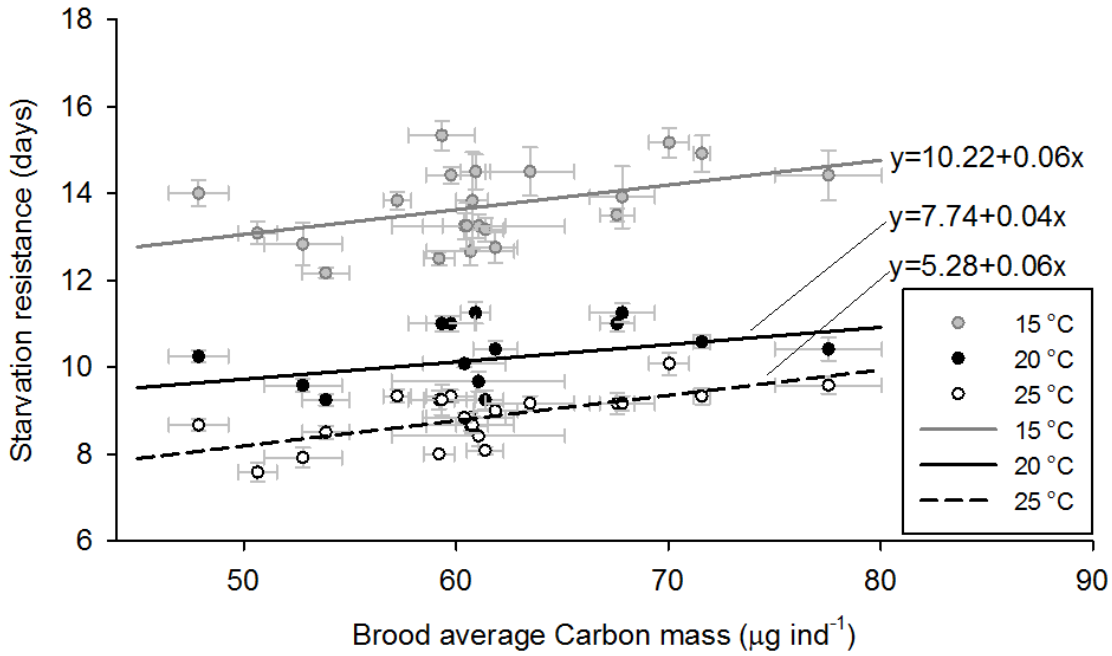

Figure 6.2 *Palaemonetes varians*. Starvation resistance against brood average Carbon mass at 15, 20, and 25 °C. For clarity, data are presented as means ± standard error, although statistical analysis was done on raw data. Best-fit-lines (fitted by General Least Squares (GLS) method) and their equations are shown

6.4.3 Influence of brood average carbon mass on larval development time, juvenile DW, and larval growth rate

Larval development time was affected by brood average carbon mass, temperature, and instar number during development (Figures 6.4A and 6.5A, Table 6.2). There were significant interactions between carbon mass and temperature, between temperature and instar number, and between carbon mass, temperature and instar number (Figures 6.4A and 6.5A, Table 6.2). Post hoc testing indicated that the trend between carbon mass and larval development time for larvae developing through four instars at 15 °C was significantly different from that for larvae developing through four and five instars at both 20 and 25 °C

(P <0.05 in all cases). The trend was not significantly different from that at 15 °C for larvae developing through five instars (P = 0.1062). All other trends were also statistically in-distinct.

Juvenile DW was also affected by brood average carbon mass, temperature, and instar number during development (Figures 6.4B and 6.5B, Table 6.2). There were significant interactions between carbon mass and instar number, temperature and instar number, and between carbon mass, temperature, instar number (Figures 6.4B and 6.5B, Table 6.2). Post hoc testing indicated that the trend between carbon mass and juvenile DW for larvae developing through four instars at 20 °C was significantly different from that for larvae developing through four instars at 15 and 25 °C and five instars at 20 °C (P <0.005 in all cases), and five instars at 25 °C (P <0.05). All other trends were statistically in-distinct.

Larval growth rate was also affected by brood average carbon mass, temperature, and instar number during development (Figures 6.4C and 6.5C, Table 6.2). There were significant interactions between carbon mass and instar number, and carbon mass, temperature and instar number (Figures 6.4C and 6.5C, Table 6.2). As the interaction between carbon mass, temperature and instar number was non-significant for larval growth rate (Table 6.2), no post hoc testing was done.

6.5 Discussion

At the inter-specific level among marine arthropods, high POI is related to endotrophy and abbreviated development and is associated with environments in which food production is limited or unpredictable (Thorson 1950, Anger 2001). Results presented here demonstrate the advantage of greater POI under a range of temperatures and the implications of POI for larval development and starvation resistance at the intra-specific level.

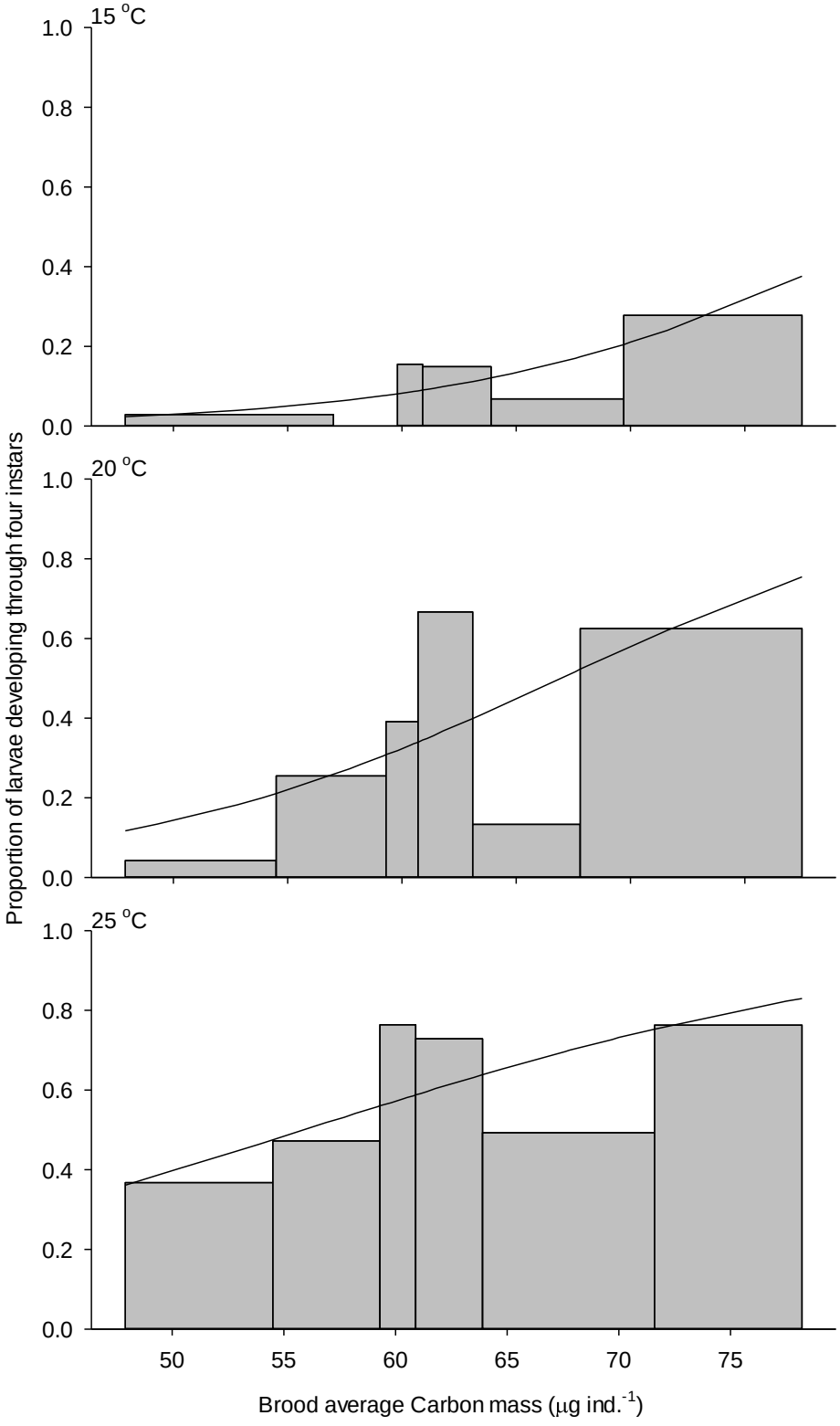

Figure 6.3 *Palaemonetes varians*. Logistic regressions of the proportion of larvae developing through four instars against brood average carbon mass (μg ind.$^{-1}$) at 15, 20, and 25 °C. Bars indicate proportions data, which were divided into intervals for graphical purposes only. Bar widths may vary between temperatures as the intervals were chosen so that each bar represented a similar n. Best-fit-lines for logistic regressions are shown

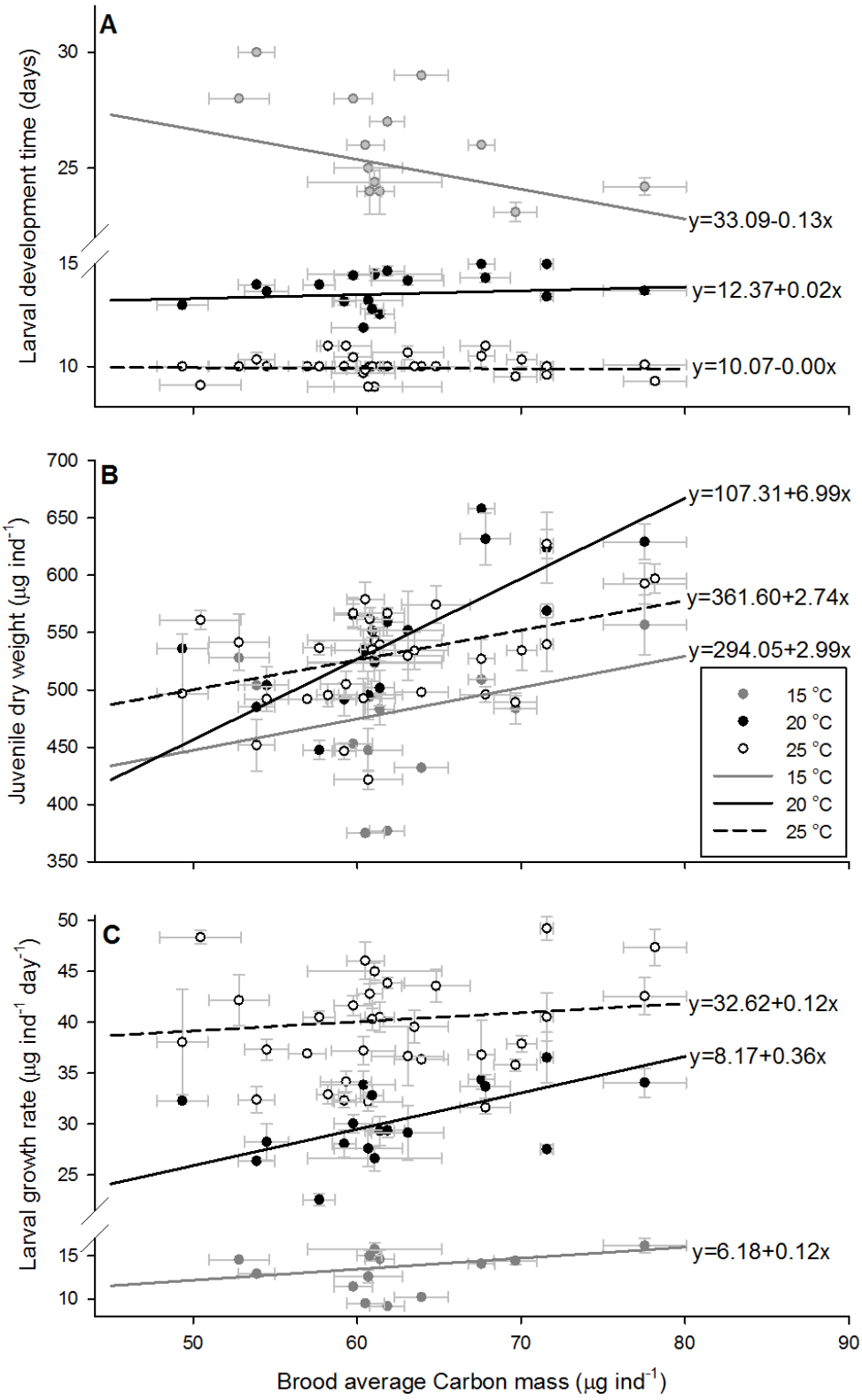

Figure 6.4 *Palaemonetes varians*. **A** larval development time, **B** juvenile dry weight, and **C** larval growth rate against brood average carbon mass (µg ind.$^{-1}$) for larvae developing through four instars at 15, 20, and 25 °C. For clarity, data are presented as means ± standard error, although statistical analyses were done on raw data. Best-fit-lines (fitted by General Least Squares (GLS) method) and their equations are shown.

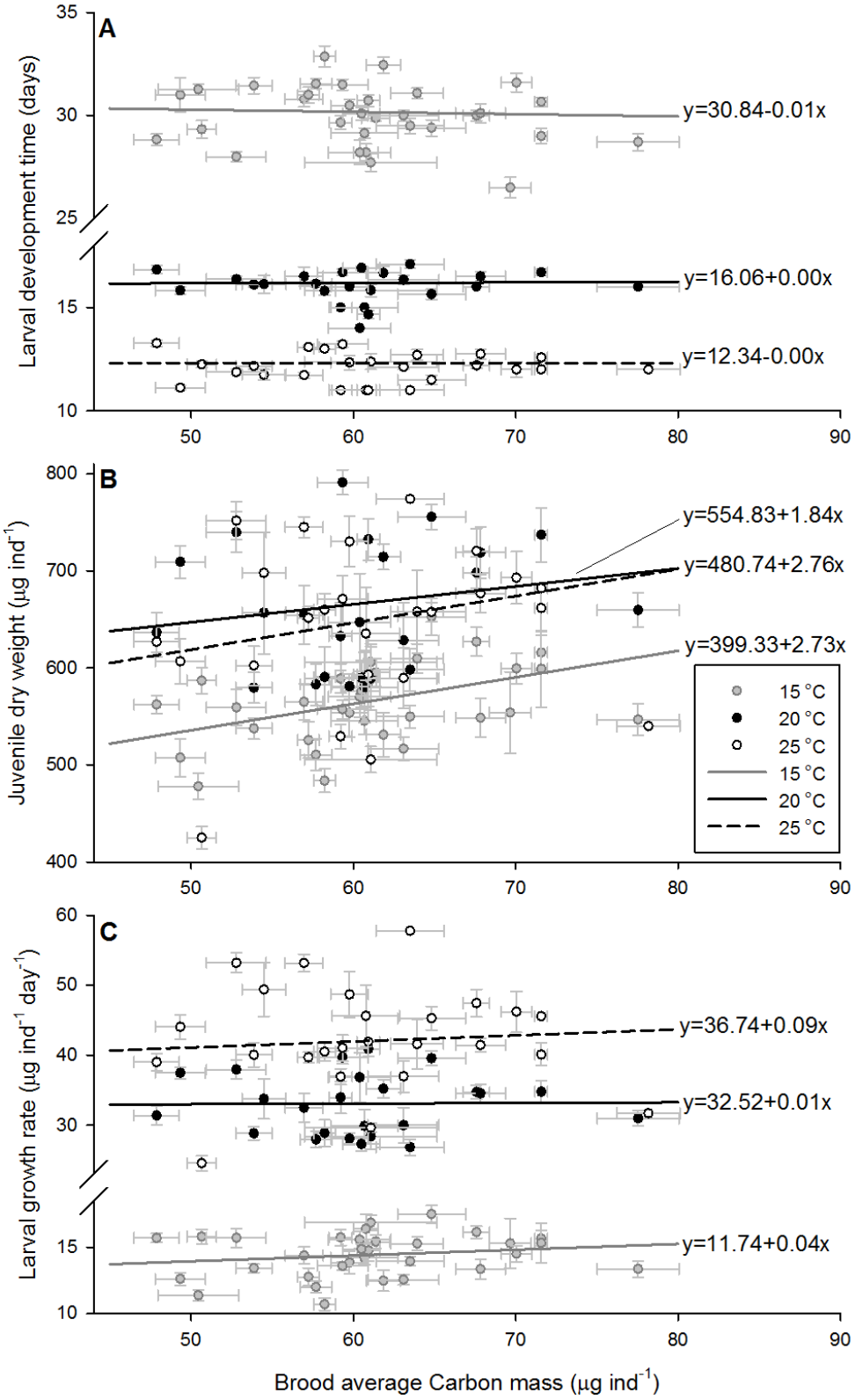

Figure 6.5 *Palaemonetes varians.* **A** larval development time, **B** juvenile dry weight, and **C** larval growth rate against brood average carbon mass (µg ind^{-1}) for larvae developing through five instars at 15, 20, and 25 °C. For clarity, data are presented as means ± standard error, although statistical analyses were done on raw data. Best-fit-lines (fitted by General Least Squares (GLS) method) and their equations are shown.

Table 6.2 The effects of brood average carbon mass (C. mass), temperature (Temp.), and instar number (Instar), and their interactions on larval development time, juvenile dry weight, and larval growth rate within *Palaemonetes varians*. ANCOVA analysis, with brood average carbon mass as a continuous predictor and temperature and instar number (four and five instars) as factors. Models fitted using the GLS method and weighted by variance structures which accounted for heterogeneity within data

	DF	**F-value**	***P*-value**
Larval development time			
C. mass	1	794.70	<0.0001
Temp.	2	20215.85	<0.0001
Instar	1	1758.55	<0.0001
C. mass*Temp.	2	7.52	0.0006
C. mass*Instar	1	1.49	0.2225
Temp.*Instar	2	45.16	<0.0001
C. mass*Temp.*Instar	2	3.78	0.0231
Error	1036		
Juvenile dry weight			
C. mass	1	23.77	<0.0001
Temp.	2	41.31	<0.0001
Instar	1	444.71	<0.0001
C. mass*Temp.	2	1.79	0.1669
C. mass*Instar	1	7.38	0.0067
Temp.*Instar	2	4.02	0.0182
C. mass*Temp.*Instar	2	4.38	0.0128
Error	1036		
Larval growth rate			
C. mass	1	34.564	<0.0001
Temp.	2	3370.120	<0.0001
Instar	1	13.955	0.0002
C. mass*Temp.	2	0.713	0.4905
C. mass*Instar	1	11.020	0.0009
Temp.*Instar	2	3.379	0.0345
C. mass*Temp.*Instar	2	2.945	0.0530
Error	1036		

Most studies assessing the implications of POI for larval development and carry-over effects have manipulated POI either directly, by removing yolk (Emlet & Høegh-Guldberg 1997) or by 'twinning' two- and four-cell embryos (Sinervo & McEdward 1988, Hart 1995), or indirectly, by maternal effects on POI; e.g. temperature-mediated POI plasticity (e.g. Blanckenhorn 2000, Fischer et al. 2003a, Bownds et al. 2010, Liefting et al. 2010). Such studies have found that POI influences size at metamorphosis, post-metamorphosis growth, and juvenile survivorship (Sinervo & McEdward 1988, Hart 1995,

Emlet & Høegh-Guldberg 1997). Knowledge on the influence of POI on larval developmental plasticity is poor. Poorer still is our knowledge of the interaction between POI and environmental factors and their combined effect on larval developmental plasticity. Here, we show that between-brood-variations in hatchling energy content (measured as carbon mass) significantly affects larval starvation resistance, instars number, growth rate, and development time, and has potential implications for juvenile and adult performance through differences in initial juvenile DW.

In the absence of food, *P. varians* larvae from broods of higher hatchling energy content developed to more advanced larval stages and survived for longer before succumbing to starvation, indicating a higher degree of nutritional independence (endotrophy). Within the caridean shrimp *Crangon crangon*, greater POI is known to increase starvation resistance and provide an earlier point of saturation for secondary lecithotrophy (Paschke et al. 2004). Similarly, for the butterfly *Bicyclus anynana*, greater POI increases starvation resistance, and general survivorship (Fischer et al. 2003a).

In the presence of food, *P. varians* larvae from broods of higher hatchling energy content developed through fewer larval instars, showed higher growth rates, and had shorter development times. Further, for those larvae developing through the same number of larval instars, larvae from broods of higher hatchling energy content developed to greater juvenile DW. The significant interactions between hatchling energy content and instar number for both juvenile DW and larval growth rate, coupled with the greater slope of the best-fit-lines for larvae developing through four instars, indicates stronger trends between hatchling energy content and larval traits for those larvae developing through four instars. Similarly, the relationship between hatchling energy content and larval development time was significantly stronger for larvae developing through four instars at 15 °C. With increasing hatchling energy content, larvae are better able to develop through fewer instars, though the effects of hatchling energy content on larval traits may be greater than if larvae were to develop through five instars. Development through five instars appears to buffer larval traits against initial energy reserves as larval development time, juvenile DW, and larval growth rate are less strongly coupled with hatchling energy content.

Evidence suggests that greater POI allows development of *C. crangon* and *Neohelice* (*Chasmagnathus*) *granulata* larvae through fewer instars (see Anger 2001,

Giménez & Torres 2002, Giménez 2006). Importantly, at all temperatures, juveniles that developed through four instars had significantly lower DW than those that developed through five instars. Thus, larger hatchlings (with greater energy content) generally metamorphosed at a smaller size after a shorter development time which consisted of fewer larval instars. This trend is similar to that at the inter-specific level for North American shrimp species in the genus *Palaemonetes* (Table 6.3; after Hubschman & Broad 1974); those *Palaemonetes* sp. producing larger hatchlings, metamorphose at a smaller juvenile size, have a shorter pelagic period, and develop through fewer larval instars. Although this trend in life histories occurs across a salinity gradient, the ecological basis for this trend is analogous with that of latitudinal trends in life histories; planktonic food availability becomes more limited with increasing latitude and with decreasing marine connectivity (Anger 2001, Anger & Hayd 2009).

At the intra-specific level, variation in larval instar number is a common plastic trait among arthropods and is usually associated with the addition of larval instars, which are thought to extend larval development, enabling larvae to achieve a development and size threshold under poor growth and development conditions (Nijhout 1994, Kingsolver 2007, Etilé & Despland 2008) (for reviews see Knowlton 1974, Esperk et al. 2007). Additional larval instars may enable development to, or even to surpass, a threshold; however, this comes at the disadvantage of longer development period and the fitness implications associated with such (Kingsolver 2007). Omitting larval instars is more poorly reported but is associated with more favourable growth conditions and has been shown to give rise to juveniles of lesser DW after a shorter development time (see Chapter 4). The potential to develop more rapidly (by omitting larval instars) and to tolerate unfavourable growth and development conditions without extending larval development (through additional instars) would appear advantageous. Development through relatively fewer larval instars under more unfavourable conditions is associated with greater POI and may be selected for in environments in which unfavourable growth and development conditions prevail, i.e. in cooler high latitude or deep-sea environments where food availability for plankotrophic larvae is limited or unpredictable.

The omitting of larval instars under favourable conditions and the addition of instars under unfavourable conditions may appear to contradict observations made at the macro-ecological scale; where arthropods show increasingly abbreviated development (fewer larval instars) with increasing cold (e.g. latitude: Thatje & Bacardit 2000, Thatje et

al. 2003). Importantly, observations of omitting and adding instars under favourable and unfavourable conditions are at the intra-specific level among individuals with similar POI. The macro-ecological trend in abbreviated development is at the inter-specific level between species with different levels of POI. The results of this study, and those of other works, demonstrate the importance of POI in enabling development through fewer larval instars. Thus, macro-ecological patterns in larval development are likely primarily influenced by POI and secondarily influenced by developmental plasticity and larval experience.

Table 6.3 Size at hatching and metamorphosis, and larval period and larval instar number for species of *Palaemonetes* from differing environments. *P. kadiakensis* is highlighted (grey); this species does not fit the general trend and is considered a recent colonist of the freshwater environment (Hubschman & Broad 1974).

Species	Reference(s)	Env[t]	Trophy	Hatchling size (mm)	Juvenile size (mm)	larval period	No. instars
P.vulgaris	(Broad 1957)	marine/ brackish	fully planktotrophic	2.3	6.3	15->30	7-11
P. pugio	(Broad 1957)	marine/ brackish	planktotrophic (zoea 1 facultative lecithotrophic)	2.6	6.2	15-> 40	7-11
P. atrinubes	(Bray 1976)	marine/ brackish	planktotrophic (facultative lecithotrophy unknown)	2.9-3.1	7.0-8.1	20-44	7-10
P. intermedius	(Hubschman & Broad 1974)	marine/ brackish	planktotrophic (facultative lecithotrophy unknown)	3.5	7	≥13	6-8
P. varians	(Fincham 1979)	marine/ brackish	planktotrophic (zoea 1 facultative lecithotrophic)	3.8 (3.5-4.1)	6.4 (6.0-7.0)	-	5
	Oliphant et al. (under review)			-	-	7-66	3-7
	This study			-	-	9-36	4-6
P. kadiakensis	(Broad & Hubschman 1963)	freshwater	planktotrophic (facultative lecithotrophy unknown)	4.4	7.5	16-30	5-8

P. paludosus	(Dobkin 1963)	freshwater	lecithotrophic (facultative lecithotrophy unknown)	3.8	4.5	5-10	3
P. cummingi	(Dobkin 1971)	freshwater	lecithotrophic (facultative lecithotrophy unknown)	4.8	5.5	9	3
P. australis	(Bray 1976)	freshwater	lecithotrophic (facultative lecithotrophy unknown)	5.0 (4.4-5.6)	5.2 (4.5-5.8)	4-17	3
P. zariquieyi	(Guerao 1993)	freshwater	fully lecithotrophic	3.9 - 4.03	4.2-4.6	4.5-6	3
P. ivonicus	(Magalhães 1986)	freshwater	fully lecithotrophic	4.55±0.14	5.27±0.32	4-6	3
P. mercedaes	(Magalhães 1988)	freshwater	fully lecithotrophic	4.68±0.03	4.92±0.24	5	1

Our results at the intra-specific level emulate observations at the inter-specific level and demonstrate that greater POI provides increased endotrophy and the potential for abbreviated development. A greater degree of endotrophy can be assumed to be advantageous in environments of limited or unpredictable food supply, and low temperatures when larval development is prolonged relative to food availability. POI plasticity, a widespread trait among taxa, therefore has significant ecological implications and can confidently be assumed to drive evolutionary trajectories.

Our data support the hypothesis that the evolutionary transition towards endotrophy and abbreviated development is driven by increasing POI, which provides greater energy reserves and therefore, increasing nutritional independence (endotrophy). Such energy reserves may allow and/or promote more rapid development, especially when food is scarce or patchy, or when feeding is difficult (i.e. during periods of dark for visual predators). For some species, high POI allows development within a larval stage or a sequence of larval stages without feeding; such larvae often retain functioning feeding structures and feed in the presence of food, so called facultative lecithotrophy (Anger 2001). The varying degrees of facultative lecithotrophy are considered intermediates between planktotrophy and full lecithotrophy. The evolution of full lecithotrophy leads to the redundancy of feeding structures. Fully lecithotrophic development is often obligatory lecithotrophic because larvae are unable to process external food; a result of under-

developed feeding structures and low activity of several key digestive enzymes (Saborowski et al. 2006). The evolutionary transition towards complete endotrophy (full lecithotrophy) is likely a result of increasing POI and its influence on endotrophy and abbreviated development; however, the conclusion of increasing POI (and its influence on endotrophy and abbreviated development) is direct development, evident in peracarids, and freshwater and terrestrial decapods (Anger 1995).

Temperature-mediated POI plasticity is hypothesised to be an emergent property of the physiochemical nature of organisms; resulting from the differing effect of temperature on the processes of differentiation (egg production) and growth (yolk production) (Van der Have & de Jong 1996). High POI is advantageous, especially under conditions of limited/unpredictable food availability and low growth potential. Macro-ecological trends in development mode are governed by POI; environmentally mediated phenotypic plasticity in POI may therefore permit the evolution of the diverse and complex life cycles observed in the marine environment.

6.5.1 Conclusion

- Larvae from broods of greater POI develop through fewer larval instars at all temperatures
- Larvae from broods of greater POI develop to more advanced larval stages under starved conditions
- The effect of POI on larval instar number was consistent across temperatures
- Larval development rate, larval growth rate, and juvenile DW are affected by POI
- Variation in larval instar number may maximise fitness potential of higher POI

7. Synopsis

In this thesis the palaemonine shrimp, *Palaemonetes varians*, was used as a study species to assess the effects of the interaction between maternal provisioning (per offspring investment; POI) and the environment (specifically temperature effects, but also see Appendix 1 for starvation effects) on larval developmental plasticity. The rational for this work was to understand better the role of the interaction between POI and developmental plasticity in the evolution of developmental modes across environmental gradients. Whilst the effects of POI, environmental conditions, and their combination have been studied for marine invertebrates, including crustaceans, the implications of their interaction for developmental plasticity is rarely considered. Only within echinoderms have the implications of the interaction between POI and environmental conditions been considered for developmental plasticity (McAlister 2007, 2008).

Within echinoderms, feeding structure size is a plastic trait in response to food availability (e.g. Boidron-Metairon 1988, Hart & Strathmann 1994). Importantly, the interaction between POI and the environment appears to affect feeding-arm plasticity: higher POI enables a greater magnitude of plasticity under food limited conditions (McAlister 2007, 2008). The magnitude of developmental plasticity may vary at the inter-specific level across a latitudinal gradient, generally being greater in cold temperate species (with higher POI) than tropical species (McAlister 2007, 2008). Within arthropods, developmental plasticity is manifest as variations in the number and form of larval instars during development (Knowlton 1974, Esperk et al. 2007). The number of larval instars during development varies between species and is generally less in cold regions and with low/short food availability (Wehrtmann & Albornoz 1998, Anger 2001, Thatje et al. 2003, Thatje et al. 2004b). Therefore, the role of the interaction between POI and environmental conditions on variations in larval instar number during larval development may provide insights into the role developmental plasticity plays in the evolution of larval development modes, especially within decapod crustaceans. This thesis investigates this question and in doing so has three major themes which are synthesised here: (1) POI plasticity in relation to environmental conditions (Chapter 3); (2) developmental plasticity, mediated by environmental conditions during development (Chapters 4 and 5); (3) the interaction between POI and environmental conditions on developmental plasticity (Chapter 6, also

see Appendix 1). Before these major themes are addressed, data presented in this thesis contributed a better understanding of the physiology and ecology of *Palaemonetes varians*, and the physical environment which it inhabits, and are discussed in the following section (below).

7.1 Physiology and ecology of *Palaemonetes varians*

A significant outcome of this thesis, though not a primary aim, are data presented here which contribute to a greater understanding of the physiological tolerances of *Palaemonetes varians* larvae, the ecology of larvae (and adults), as well as the physical environment of salt marshes. Data presented in Chapter 3 demonstrate the high magnitude of temperature and salinity variability within Lymington salt marsh; a maximum temperature of 26.59 °C and a minimum temperature of -0.14 °C were measured between 2011 and 2013. The range of temperatures between winter and summer were generally ~25 °C, diurnal temperature fluctuations of ~6 °C, and large changes within short time frames, for example 11.9 °C within 66 hours, were recorded. Similarly, salinity was highly variable and ranged between 0.5 and 43 over the period monitored. These data demonstrate the high variability within salt marsh environments, which has been reported previously (Lofts 1956, Jefferies 1958, 1964, Marsden 1976).

Organisms living in highly variable environments exhibit adaptations to cope with rapid and high magnitude changes in, for example, temperature and salinity. Animals inhabiting inter-tidal rocky shores, many of which are sessile, are highly adapted to temperature and salinity fluctuations rarely experienced in other marine environments (for review see Somero 2002). Salt marsh inhabiting fauna are also adapted to a variable environment and may demonstrate avoidance behaviour (e.g. Marsden 1976); anecdotal evidence from personal observations suggests that adult *Palaemonetes varians* (including ovigerous females) move within ditches. Previous studies have demonstrated high physiological tolerance of temperature and salinity within *P. varians*. For example, Lofts (1956) found that *P. varians* were tolerant of salinities between 1.7 and 66 for several days whilst the thermal tolerance of *P. varians* has been demonstrated to range from ~0 °C to ~33 °C (Oliphant et al. 2011, Ravaux et al. 2012). Further, Cottin et al (2010) subjected *P. varians* to a heat shock of 18 °C (from 10 to 28 °C) within 90 minutes (a rate of 0.2 °C min^{-1}) whilst Smith et al (2013) demonstrated that *P. varians* are tolerant of acute thermal shocks of 17 °C, brought about by transferring shrimp directly into water of much higher

temperatures. *Palaemonetes varians* has also shown high tolerance of high hydrostatic pressure and has become a study species for assessing the effects of hydrostatic pressure on shallow-water fauna (Oliphant et al. 2011, Cottin et al. 2012, New et al. in press). This species, therefore, appears highly tolerant of environmental perturbations.

Data presented in Chapter 4 demonstrate the wide thermal tolerance of *Palaemonetes varians* larvae which survived for several days at 5 °C, several weeks at 10 °C and developed fully at temperatures between 15 and 30 °C. During the summer period, when larvae were likely to be free swimming in the ditches on Lymington salt marsh (from mid-May to the end of July), temperatures here ranged from 9.2 to 26.6 °C and were mostly in the region of 15 to 20 °C; mean temperatures being 18.0 ± 2.5 °C throughout this summer period across the three years (2011, 2012, 2013). The thermal tolerance of *P. varians* larvae, therefore, fits well with the temperature range likely experienced during development for this population. Similarly, Antononopoulou and Emson (1988) demonstrated that the salinity range over which *P. varians* larval development is feasible is from 5 to 42, matching well the salinity range of between 5.8 and 43.2 which was recorded during the summer period from mid-May onward (from 2011 to 2013). The wide temperature and salinity tolerances in both adult and larvae of *Palaemonetes varians* are likely key to this species success in salt marsh environments; *P. varians* also exhibits life history adaptations to its adult habitat.

7.1.1 Energetic adaptations to larval export

Many species of estuarine, mangrove, salt marsh and freshwater decapods exhibit life cycle adaptations to their adult habitat; exporting larvae from adult habitats into lower estuarine and coastal marine waters where conditions for development may be more favourable than within the adult environment (for examples see Anger 2001). For example, the larvae of such species are often highly resistant to starvation within early development, allowing the export of larvae, during which process larvae may experience prolonged periods of low food availability. Data presented in Chapter 5 demonstrates the high carbon to nitrogen ratio (~4.2) and carbon content (~45 %), indicating that larvae hatch with high amounts of lipid. Further, yolk reserves are visible through the carapace at hatching, which is known for other palaemonine shrimp with facultative and obligatory lecithotrophy (Anger & Hayd 2009, Urzúa et al. in press). The high starvation resistance within early development of *P. varians* was also demonstrated in Chapter 5; *P. varians* larvae can be considered as facultative lecithotrophic in the first and second larval instars and

planktotrophic from the third larval instar. The relatively high starvation resistance within *P. varians* is likely an evolutionary adaptation to the adult habitat and the exporting of larvae from salt marsh environments into more favourable lower estuarine and coastal marine areas, where conditions for larval growth, development, and survivorship are better.

7.1.2 Temperature and larval development

Chapters 4 and 5 demonstrated higher larval growth and development rates at higher temperatures. Further, Chapter 5 demonstrated higher larval average daily growth per instar, both in terms of dry weight and carbon mass, at higher temperatures. Coupled with this was a decrease in energy loss through respiration during development at higher temperatures, indicating that larval development was more constrained at lower temperatures. These data may reflect the tropical ancestry of the palaemonine shrimp (see Chapter 1, section 1.4.1 and Anger 2013 for review of *Macrobrachium*). Constrained growth under low temperature conditions may explain why development proceeds through more larval instars than under warmer conditions. Chapters 4, 5, and 6 demonstrate temperature-mediated variation in larval instar number; larvae developing through fewer larval instars under warmer conditions. Development through differing numbers of instars affected juvenile dry weight and development time. Interestingly, the interaction between temperature-mediated changes in the proportion of larvae developing through differing numbers of instars coupled with increasing growth rates with temperature resulted in juveniles with the greatest dry weight settling at 20 °C, which closely reflects average summer temperatures for this population (~18 °C).

7.2 POI plasticity

Considerable POI plasticity has been reported for many crustaceans and especially decapods (e.g. Sheader 1983, Urzúa et al. 2012, Urzúa & Anger 2013). POI plasticity is associated with seasonal temperature differences (Kerfoot 1974, Perrin 1988) and temperature during gonad development (Ernsting & Isaaks 1997, Blanckenhorn 2000, Ernsting & Isaaks 2000, Fischer et al. 2003a, b, c, Liefting et al. 2010). In Chapter 3, considerable intra- and inter-annual variations in environmental temperature and salinity were reported for the ditch on Lymington salt marsh from which *Palaemonetes varians* were sampled. Consequently, gameteogenesis, embryonic development, and larval development took place under highly variable conditions, which likely differed between years. Given previous literature, these differing conditions during gametogenesis may be

expected to have yielded differing POI and correlations between the conditions during gonad development and POI might be found. Indeed, POI differed intra-annually across breeding seasons and inter-annually between breeding seasons. However, no correlation was found between POI and temperature during the period over which gonad development was estimated to occur, using the methods detailed in Chapter 3.

An attempt was made to breed *Palaemonetes varians* in the laboratory under constant conditions at three temperatures (10, 15, and 20 °C). Adult shrimp were sampled in November 2011 and acclimated to conditions of 10, 15, and 20 °C, and 18:6 light:dark. This long light period was intended to simulate a summertime light regime as long day lengths have previously been found to stimulate breeding in *Palaemonetes varians* (Bouchon 1991a, b). Breeding was highly successful at 15 °C, but less so at 20 °C as females rapidly dropped their eggs. At 10 °C, females also dropped their eggs almost immediately after extrusion. As such, many larvae were obtained at 15 °C, very few at 20 °C, and none at 10 °C. Eggs, sampled from the bottom of aquaria at both 10 and 20 °C, were fertilised as development was evident.

The dry weights of larvae bred at 15 and 20 °C were compared and no difference was found. Also, larval dry weights within 15 and 20 °C were highly variable. It is likely that residual gonad was present upon collection and that gametogenesis was already under way. Consequently, despite differing temperature conditions during gonad development, POI was not significantly different. A second attempt at breeding *Palaemonetes varians* was made. The intention was to breed shrimp at 13, 15, and 18 °C. The first brood from each female at each temperature would be disregarded, so that all breeding was known to have taken place under the experimental temperature. Unfortunately, as a result of time limitation, this experiment was not completed.

Data presented in Chapter 3 demonstrate variation in POI (for both eggs and larvae) between females, intra-annually across breeding seasons, and inter-annually between breeding seasons. POI has repeatedly been demonstrated to influence offspring fitness both in the larval phase and in the early juvenile stages. Further, Chapter 6 and Appendix 1 demonstrate the effect of the interaction between larval POI and the environment on developmental plasticity and larval development. The POI variations reported in Chapter 3 likely have significant ecological implications for larvae (see Chapters 4 and 6 for discussion). Evolutionary changes in POI, selected for through the influence of POI on

larval development and early juvenile stages, proceed through the selection for higher or lower POI and may be considered to be buffered by temperature-mediated plasticity in POI. Genetic accommodation may be a mechanism by which selection may occur, incorporating plasticity in POI.

7.2.1 Genetic accommodation of temperature-mediated plasticity in POI

The evolution of inter-population differences and inter-specific differences in POI may well proceed via genetic accommodation of temperature-mediated plasticity in POI. Genetic accommodation is adaptive genetic change through selection on the regulation of environmentally induced phentoypes (Suzuki & Nijhout 2006, Ledón-Rettig et al. 2008, Moczek et al. 2011). Genetic accommodation relies on the existence of heritable, genetic variation (among individuals within a population) in the propensity to express an advantageous phenotype in response to environmental conditions; the genes, gene combinations, or alleles responsible for a greater tendency to express an advantageous trait would then be favoured, selected for, and their frequency within the population increased (Suzuki & Nijhout 2006, Ledón-Rettig et al. 2008, Moczek et al. 2011). For example, Waddington (1959) demonstrated plasticity in anal papillae area, which are involved in osmotic regulation, for *Drosophila melanogaster* larvae grown under differing salt exposures. After breeding populations under increasing salt concentrations, and thus stringent selection, selected strains had higher anal papillae area than unselected strains when grown under the same conditions (Figure 7.1A). Further, selected strains were more plastic as changes in anal papillae area in response to salt concentrations during growth were greater in magnitude than unselected strains (Figure 7.1A). These data indicate that in the original stock, there was heritable variation in the area of anal papillae and the tendency for a plastic response to changes in the environment (Figure 7.1B). Under selection, genes, gene combinations, or alleles responsible for greater anal papillae area and greater magnitude of plasticity in this trait in response to salt concentrations were favoured and selected for.

In terms of plasticity in POI and the evolution of differing levels of POI between populations and, eventually, between species, we must consider that POI and plasticity in POI are genetically determined and heritable traits. Figure 7.1C shows the reaction norms which may be expected from an ‘unselected’ population bred at a range of temperatures. If this population is cold selected, i.e. bred under cooler conditions under which higher POI is favoured, we might expect to see a differing reaction norm with higher overall POI and a

greater magnitude of plasticity in POI in response to cooler temperatures. Conversely, if the population is warm selected, i.e. bred under warmer conditions under which lower POI is favoured, we might expect to see a reaction norm with lower POI and a greater magnitude of plasticity in POI in response to warmer temperatures (Figure 7.1.C).

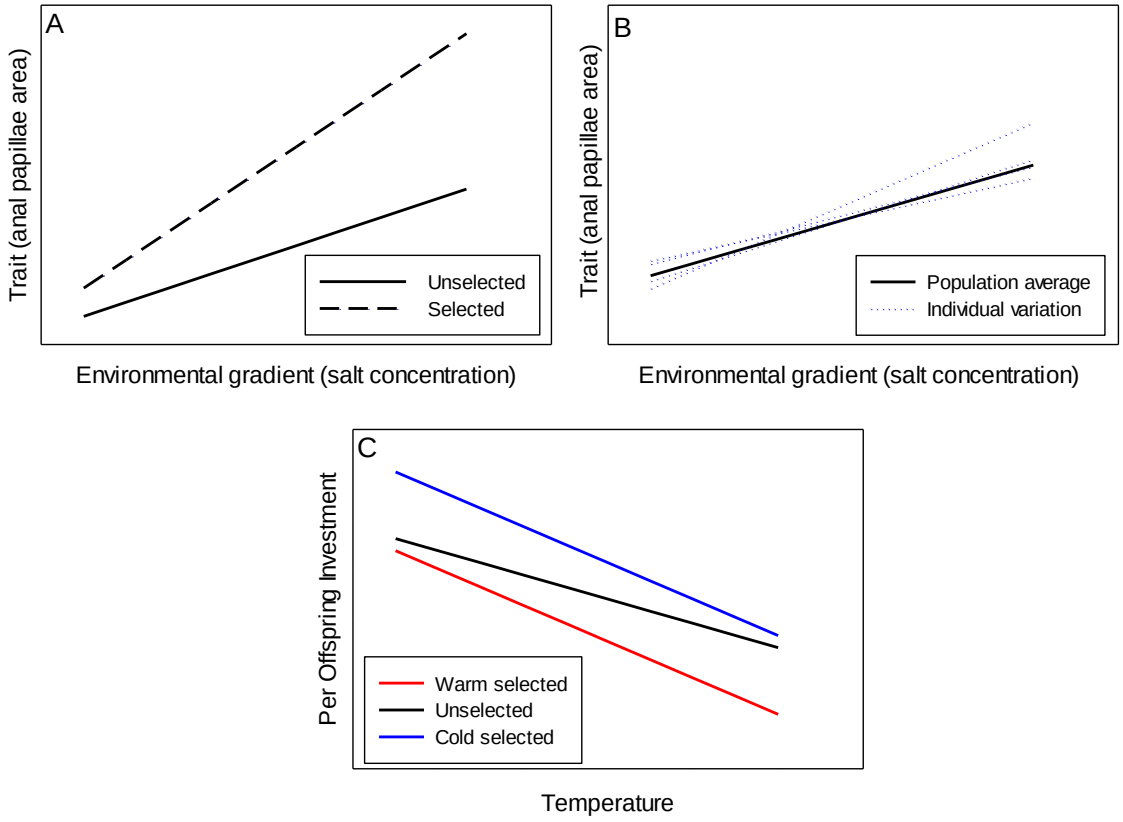

Figure 7.1 Genetic accommodation **A** anal papillae area for larvae grown under a range of salt concentrations for selected (for salt tolerance) and unselected strains of *Drosophila melanogaster*; conceptualised from Waddington (1959) **B** reaction norms for anal papillae area for larvae grown under a range of salt concentrations for the unselected strain, showing population average and individual level variation. **C** potential POI under a range of temperatures for unselected, cold selected, and warm selected strains of a species.

Like many arthropods, POI within the tropical butterfly, *Bicyclus anynana*, is plastic and temperature mediated, with greater POI occurring under cooler conditions (Fischer et al. 2003a, Fischer et al. 2003b, Fischer et al. 2003c). Fischer et al (2004) found that between butterfly families POI was variable, heritable, and the plastic response of POI to temperature varied between families; though this was non-significant (Figure 7.2A). Selection experiments have been conducted on this butterfly species, selecting for both

large and small eggs (Fischer et al. 2006a, b) (Figure 7.2B). Selection was successful, supporting Fischer et al (2004) findings that it is a heritable trait. Strains were selected for large or small eggs under constant conditions of 27 °C (Fischer et al. 2006b). Despite selection favouring extreme egg sizes (large or small), plasticity in response to temperature was maintained (Figure 7.2B); results being similar to the hypothesised change in reaction norms depicted in Figure 7.1C. Selection was, however, not based on the relationship between temperature, POI, and the fitness implications of POI, but on selection for extreme egg size (large or small). As such, this example does not constitute 'cold selected' and 'warm selected' strains (Figure 7.1C).

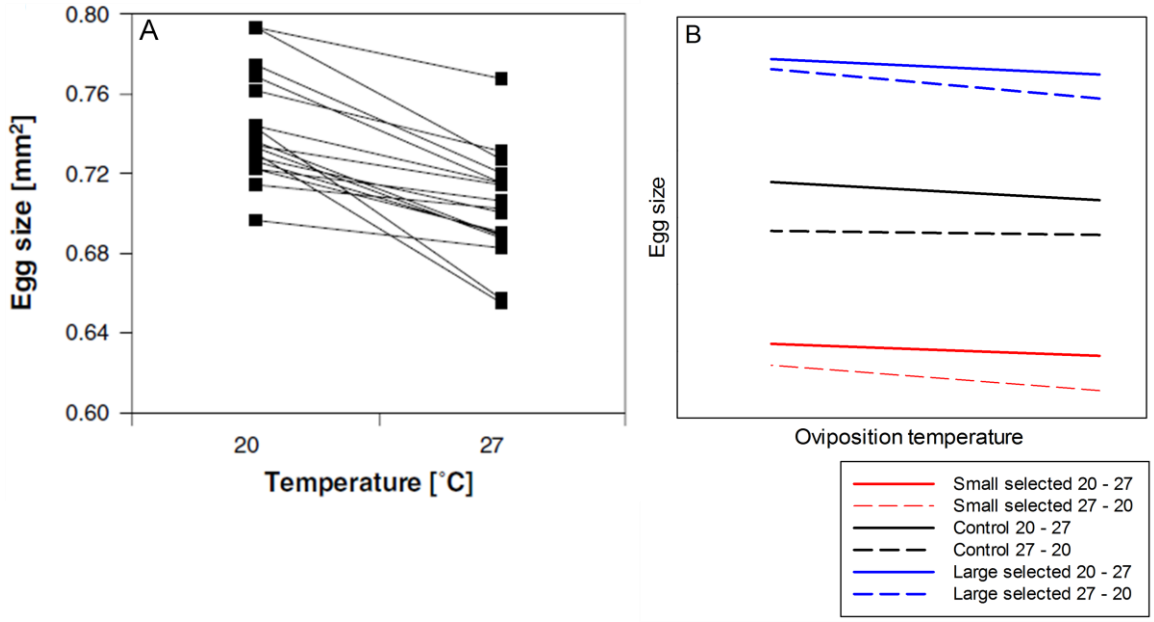

Figure 7.2 **A** *Bicyclus anynana* egg size (proxy for POI) for sisters breeding at 20 and 27 °C. Lines between temperatures join sisters. **B** conceptualisation of *Bicyclus anynana* egg size (proxy for POI) for small egg size selected, unselected (control), and large egg size selected strains raised and bred at 20 °C and then transferred to 27 °C, and strains raised and bred at 27 °C and then transferred to 20 °C

Fischer et al (2006a) suggested that their findings were not compatible with the biophysical model proposed by Van der Have and de Jong (1996) to explain temperature-mediated shifts in body size and POI (see Chapter 1, section 1.1.6). However, Van der Have and de Jong proposed that natural selection could act upon the physiology purported to give rise to temperature-mediated size differences. Van der Have and de Jong proposed that differences in the temperature coefficients of differentiation and growth could lead to

differences in body size and POI at different temperatures, thus an adaptive explanation for temperature-mediated shifts in body size were not necessary (Van der Have & de Jong 1996) (see Chapter 1, section 1.1.6). However, this scenario does not preclude the evolution of POI plasticity as genetic variation in the temperature coefficients of differentiation and growth could lead to the evolution of adaptive temperature-mediated shifts in body size or POI (Van der Have & de Jong 1996). Therefore, if heritable differences in the temperature coefficients of differentiation and growth exist, and the plastic response to temperature varies between families, then selection for greater plasticity in response to temperature could favour genes, gene combinations, or alleles responsible for the temperature coefficients of differentiation and growth and give rise to a population of differing POI and plasticity in POI in response to temperature; as in Figure 7.1C.

7.3 Developmental plasticity: variation in larval instar number

Developmental plasticity within decapod larval development, i.e. variations in the number of larval instars during the larval phase, was considered a laboratory artefact and efforts were made to determine the various stages of larval development, ignoring this variation (e.g. Gurney 1924). However, the demonstrating of variations in the number of larval instars during development in response to environmental conditions during this time raises important ecological considerations which were only later, and only partially, explored. Data presented within this thesis demonstrate novel aspects of plasticity in larval instar number and their ecological consequences within *Palaemonetes varians*.

7.3.1 Environmental influences

Within the larval development of *Palaemonetes varians*, of other decapods, and of insects, variations in the number of larval instars and in the morphology of same stage larval instars are known (Gurney 1924, Knowlton 1974, Fincham 1979, Esperk et al. 2007). Experiments comprising this thesis repeatedly demonstrate the influence of temperature on variations in the number of larval instars during the larval development of *Palaemonetes varians*; larvae increasingly develop through four rather than five instars with increasing temperature (within the tolerable range for larval development) (Chapters 4, 5, and 6).

Variations in larval instar number arise from the inherent biology of arthropods, which periodically shed their exoskeleton. Variations in the number of moults and the morphology of same stage moults arise because the process of moulting is not coupled

with the processes of growth and development (Knowlton 1974). It is my opinion that plasticity in the number of larval instars is probably not an evolved trait but the continuation of the normal moulting process; arising through a lack of 'feedback' between the control of moulting and the rate and extent of growth and development. This appears most evident in the occurrence of repeat moults, in which morphology changed little between subsequent instars, indicating the continuing of the moult cycle despite the retarding of development (Chapter 4).

Generally, variations in the number of larval instars during development are less common in anomurans and brachyurans than in other decapods (Knowlton 1974, Anger 2001, 2006). Variations in larval instar number are also less common in high latitude decapods, including caridean shrimp, than in lower latitude decapods (Wehrtmann & Albornoz 1998, Thatje et al. 2004b). This difference in plasticity may be associated with more abbreviated development in high latitude decapods and in anomurans and brachyurans relative to carideans and other decapods. Similarly, anomuran and brachyuran larvae generally have higher C:N ratios than carideans; this higher lipid to protein ratio may fuel more abbreviated and consistent larval development within these groups.

Temperature significantly influences variations in larval instar number with fewer larval instars observed during development at higher temperatures. The influence of temperature on larval development within *P. varians* contrasts with that for *P. vulgaris* in which fewer moults were observed at lower temperatures (Knowlton 1974). Larval growth and moulting rates presented in Chapter 4 indicate faster rates of both at higher temperatures. A more in depth experiment detailed in Chapter 5 showed that larval growth rates both in terms of dry weight increase and carbon mass increase were greater at higher temperatures. Further, the amount of energy loss through metabolism during development was greater at lower temperatures. Together, these data indicate that larval growth and development is constrained at lower temperatures. This may reflect the tropical distribution of ancestral palaemonine shrimp and could explain temperature mediated variations in larval instar number. Under cooler conditions, developmental and growth processes are retarded relative to the moulting cycle, despite a decrease in moult frequency. Consequently, the amount of development and growth per moult is less at lower temperatures and more moults must occur before juvenile morphology is reached (see Chapter 4).

For *Palaemonetes varians*, Gurney (1924) noted that development through the first three larval stages (i.e. two moults) were consistent among larvae. Only after the third larval stage were variations in moulting and morphology observed (Gurney 1924). The results presented in Chapters 4, 5, and 6 are consistent with Gurney's findings. Only after the third larval stage were variations in morphology between moults observed. This may be associated with the high starvation resistance demonstrated for *P. varians* within Chapter 5 and associated with high lipid reserves upon hatching. Consequently, larvae hatch with sufficient yolk reserves to develop to the third larval instar and can be considered facultative lecithotrophic in the first and second larval instars and planktotrophic from the third larval instar (Chapter 5). These high lipid reserves may reduce variability in development and hence variations in morphology within early larval development enabling the consistent development within the first three larval instars observed here and by Gurney (1924).

Within *Palaemonetes* spp., the quantity and quality of food provided to larvae affects larval instar number; additional larval instars during development are more frequent when food is limited or of poor quality (e.g. Broad 1957b, Knowlton 1974). Inadvertently, the effects of food quality on larval development were assessed for *P. varians* (Chapter 4). Differing *Artemia* sp. suppliers were used in two comparable experiments. In 'Experiment 1 (thermal scope for larval development)' (see Chapter 4), juveniles settled at a smaller size and additional moults in the larval phase were more frequently observed than in 'Experiment 2 (effects of developmental plasticity)'. These data suggest that the quality of food provided to larvae in 'Experiment 1' was of lower quality than that provided to larvae in 'Experiment 2', resulting in slower development, lower growth rates and so, consequently, more moults during development and smaller size at settlement; consistent with Broad (1957b) and Knowlton (1974). Data presented within Appendix 1 indicate that higher POI enables larvae to escape the effects (or buffers the effects) of limited or poor quality food on larval instar number and are discussed in section 7.3.

7.3.2 Evolutionary perspective

The life cycle of decapod crustaceans is fundamentally different from that of holometabolous insects, which are the subject of much of the research on larval growth, development, and larval instar number within arthropods (e.g. Nijhout 1975, Nijhout 1994, Davidowitz et al. 2004, Davidowitz & Nijhout 2004, Kingsolver 2007, Ghosh et al. 2013). Generally within decapods the larval phase is short-lived, undergoes rapid development,

and experiences little growth relative to the adult phase, and is the primary dispersive life phase. Larvae settle as immature juveniles which grow into mature adults. In contrast, the larval phase within holometabolous insects, is long-lived, undergoes relatively little development and experiences rapid growth, relative to the adult stage which is the primary dispersal life stage. At eclosion, holometabolous insects are mature adults and do not grow in this life phase.

Results presented in Chapter 5, 'Temperature-mediated Developmental Plasticity', indicate that within *Palaemonetes varians*, larval instar number variation results from the decoupling of development and the moulting cycle; no 'critical weight' threshold appeared evident in initiating the end of the larval phase. Instead, larvae developed continuously into juveniles. This is in contrast to holometabolous insects in which variation in larval instar number is caused by the decoupling of growth from the moulting cycle (Nijhout & Williams 1974a, b, Nijhout 1975). A threshold species-specific 'critical weight' must be achieved for the initiation of pupation and metamorphosis (Nijhout & Williams 1974a, b, Nijhout 1975). If this threshold is not reached then larvae undergo additional instars (Nijhout & Williams 1974a, b, Nijhout 1975). Given that adult holometabolous insects do not grow, the attaining of the 'critical weight' is critical to adult traits e.g. fecundity (Davidowitz et al. 2004).

The causes of variation in larval instar number appear inextricably linked to the 'functions' of the larval phase in decapods and holometabolous insects: decapod larvae must develop to the juvenile morphology but weight at settlement is less important, whilst holometabolous insects grow to a weight threshold but develop little in the larval phase. This difference in life cycles and its consequences for larval development provides important insight into the evolution of facultative lecithotrophy and obligatory lecithotrophy within decapod crustaceans. The combination of the decoupling of development from moulting and the lack of a growth threshold for the transition between larval and juvenile forms has enabled selection for non-feeding abbreviated larval development despite the cost of juveniles settling at a relatively small size.

Variations in larval instar number have implications for larval development time and juvenile dry weight at settlement, both of these traits may have significant ecological implications (see Chapter 4 for discussion). Larval development time affects the dispersal potential of larvae and may enhance local settlement when conditions are good, through

fast development and short larval phase, and increase dispersal away from poor conditions through slow development and prolonged larval phase (Anger 2006). Juvenile size at settlement is considered an important factor influencing fitness, with larger juveniles less likely to suffer predation, better able to compete for resources, and more resistant to starvation (Giménez et al. 2004). Critically, factors which influence developmental plasticity are important in affecting these traits and their ecological implications. Consequently, selection for larger juveniles at settling or faster development will select for factors influencing developmental plasticity.

7.4 POI interaction with developmental plasticity

The relationship between POI and several larval and juvenile traits have been assessed for a range of marine invertebrate taxa. Despite this, relatively little research has assessed the interaction between POI and developmental plasticity (see Chapter 1, section1.3). The study detailed in Chapter 6, 'Evolutionary insights into endotrophy and abbreviated development', demonstrates that for *Palaemonetes varians*, larvae hatching with higher energy content develop through fewer larval instars before reaching the juvenile stage; this was consistent under all temperature conditions. Further, larvae with higher energy content develop through fewer larval instars under food limited conditions (see Appendix 1). Both temperature and diet are known to influence variations in larval instar number (Broad 1957b, Knowlton 1974) (Chapter 4). Results presented in Chapter 6 and Appendix 1 indicate that high POI buffers larvae against the effects of poor environmental conditions during larval development (Figure 7.3).

Results presented in Chapter 5, 'Energetic adaptations to larval export', demonstrated the high energy content of hatchling larvae and the considerable starvation resistance within *Palaemonetes varians*, which is likely an evolutionary adaptation to the ecology of this species. Higher POI is associated with the development of larvae to more advanced stages in the absence of food and greater tolerance of starvation. The association of higher POI with development through fewer larval instars may be related to higher energy reserves enabling more rapid development and development independent of external resources.

Under poor growth conditions, higher POI and the associated quicker development time through fewer larval instars may be advantageous and so selection would favour high POI (Figure 7.3A). In high latitude regions, where high seasonality results in short-lived

primary productivity and low temperatures result in slow development rates, more rapid larval development can be considered advantageous (Figure 7.3A). Consequently, developmental plasticity in terms of variations in larval instar number, which is not an evolved trait, may maximise the effects of high POI and contribute to the selection for higher POI under poor conditions. Those larvae with lower POI, which extend the larval period through developmental plasticity, may be outcompeted during the larval phase or during the juvenile phase, and not favoured (Figure 7.3A). At lower latitudes, this trend may be similar, however, the more benign conditions may make lower POI and the associated higher fecundity more favourable (Figure 7.3C). Across a latitudinal gradient, selection may therefore favour higher POI at high latitudes and low POI (but high fecundity) at lower latitudes (Figure 7.3B).

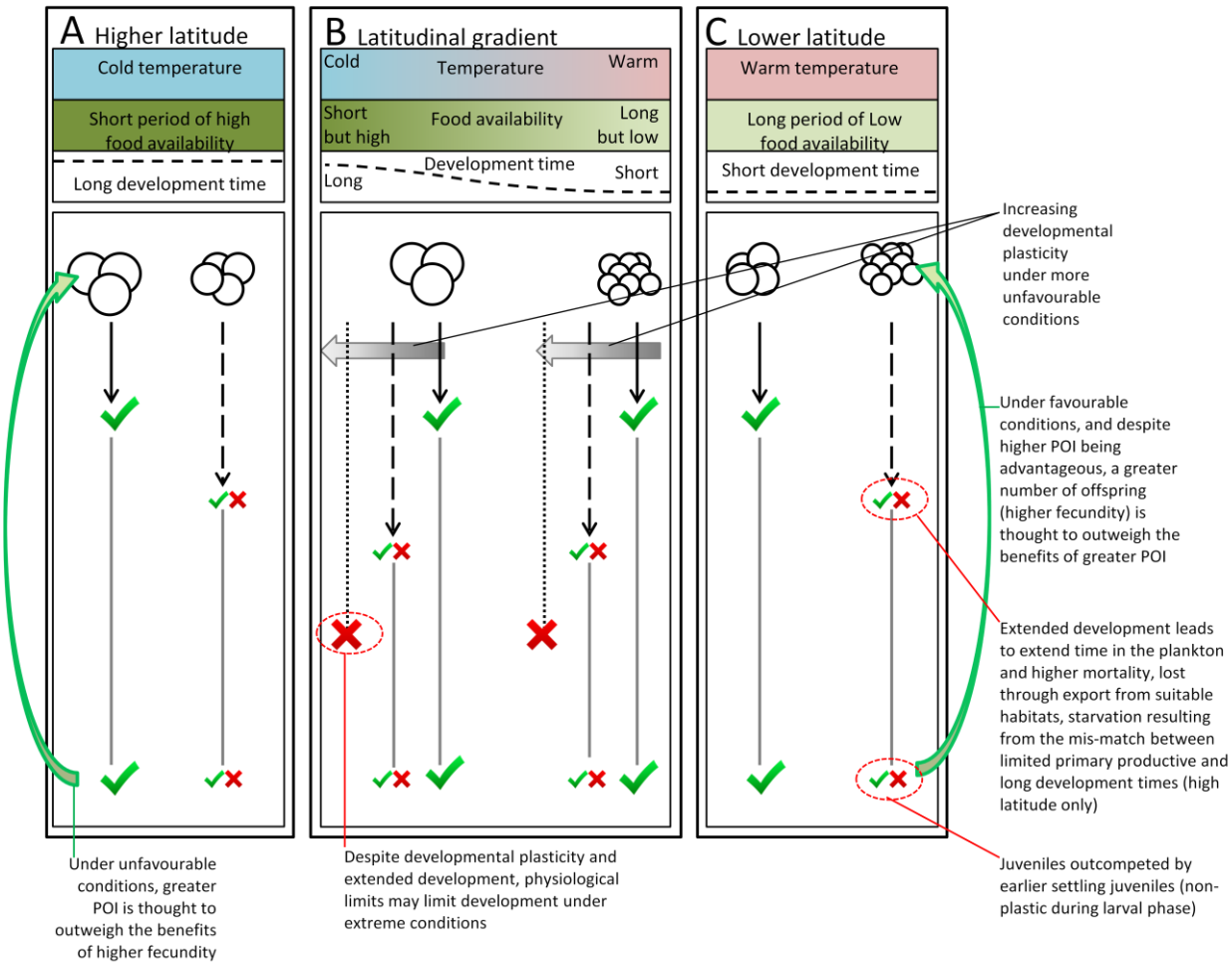

Figure 7.3 Conceptual diagram of the interaction between POI and environmental conditions and the effects of this interaction on larval development and early juvenile stages. **A** High latitude regions **B** across a latitudinal gradient **C** low latitude regions

7.4.1 Abbreviation of development

For both planktotrophic and lecithotrophic decapods larvae, greater POI at hatching is associated with greater juvenile size at settling (Figure 7.4). For lecithotrophs, which do not grow during development but lose mass as a result of metabolism of lipid reserves (Anger 1996, Calcagno et al. 2003, Lovrich et al. 2003, Urzúa et al. in press), juvenile size at settlement is significantly less than that of planktotrophs with comparable POI at hatching (Figure 7.4). Lecithotrophic development appears, therefore, to come at the cost of a small settling juvenile size. Further, the relationship between hatchling size and juvenile size for the subantarctic shrimp, *Campylonotus vagans*, falls below the line of best fit for other planktotrophs (Figure 7.4). This species hatches with high POI, has high starvation resistance and develops through an abbreviated larval phase comprising two zoeal stages and one decpodid (Thatje et al. 2004a, b). These larval traits are adaptations to the high latitude environment which *C. vagans* inhabits (Thatje et al. 2004a, b). Interestingly, *C. vagans* juveniles are smaller than expected for a planktotroph with its level of POI. This is likely a result of the abbreviated development within this species, which may limit the assimilation and accumulation of resources from the environment.

Palaemonetes varians falls slightly below the line of best fit. Importantly, data for the relationship between hatchling dry weight and juvenile dry weight from 'Experiment 2 (effects of developmental plasticity)' (Chapter 4) highlight an important difference between larvae developing through four instars (relatively abbreviated development) and larvae developing through five instars (Figure 7.5). *Palaemonetes varians* larvae, which develop through five instars, generally have lower dry weight at hatching but grow into juveniles of higher dry weight than larvae developing through four instars, which hatch with greater dry weight but develop to juveniles of small size (Figure 7.5). The abbreviated development enabled by higher POI results in lower juvenile dry weight, presumably because there is less time available to the larvae for the assimilation of mass from the environment. This is consistent with the trend in the relationship between hatchling dry weight and juvenile dry weight at the inter-specific level between planktotrophs with extended development and planktotrophs with abbreviated development and lecithotrophs (Figure 7.4).

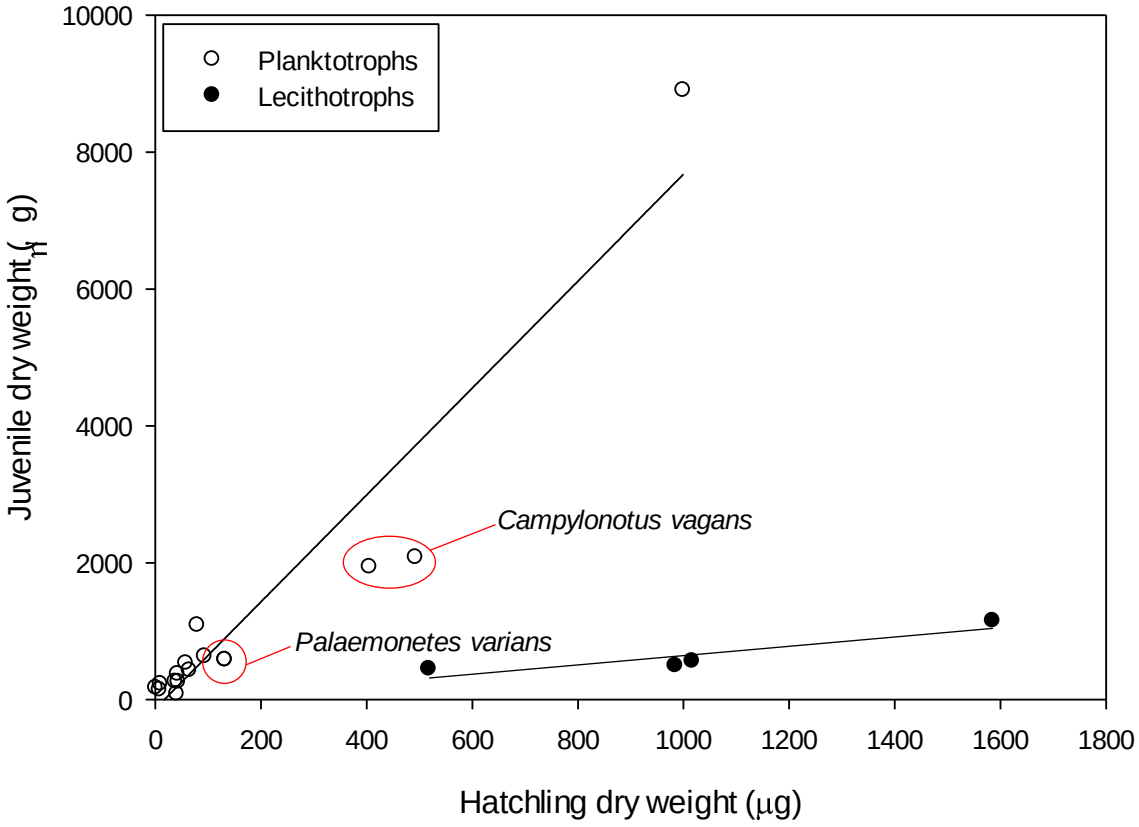

Figure 7.4 The relationship between hatchling and juvenile dry weight for planktotrophic and lecithotrophic decapod crustaceans. Values for *Palaemonetes varians* shown are averages from 'Experiment 2 (effects of developmental plasticity)', Chapter 4.

In environments in which abbreviated development is favoured, selection may drive increases in POI which enables abbreviated development. The influence of developmental plasticity (variations in larval instar number), which maximises the potential fitness benefits of greater POI by enabling larvae to settle as juveniles more quickly, is potentially important in the evolutionary transition towards abbreviated development. The abbreviation of development within decapods is accompanied by a decrease in the tendency for variations in larval instar number. Similarly, within some groups (brachyuran and anoruman crabs) variations in larval instar number are rare.

In section 7.3.1 I stated that plasticity in larval instar number was not an evolved trait but the continuation of the normal moulting cycle; arising through the a lack of feedback between the moulting cycle and the processes of growth and development (Knowlton 1974, Fincham 1979). However, if no feedback between the moulting cycle and

the processes of growth and development is present within decapods, how might variations in larval instar number become depressed, despite variable environmental conditions during development? A definitive answer is lacking. It may be that a mechanism of feedback between the moulting cycle and development is present but depressed in decapods with high larval instar number variation. On the other hand, the influence of the environment on the moulting cycle and on development may be more similar in decapods with low variation in larval instar number. As such, these processes are not differently affected by the environment but remain coupled despite environmental variation. Excessive moulting during the larval development would appear wasteful, especially under poor conditions. Within lecithotrophic crabs, exuvia are thin, minimising energy loss. As such, evolutionary transitions towards abbreviated development (which minimises exuvia loss) would also be accompanied by decreasing variation brought about by greater coupling of moulting and development.

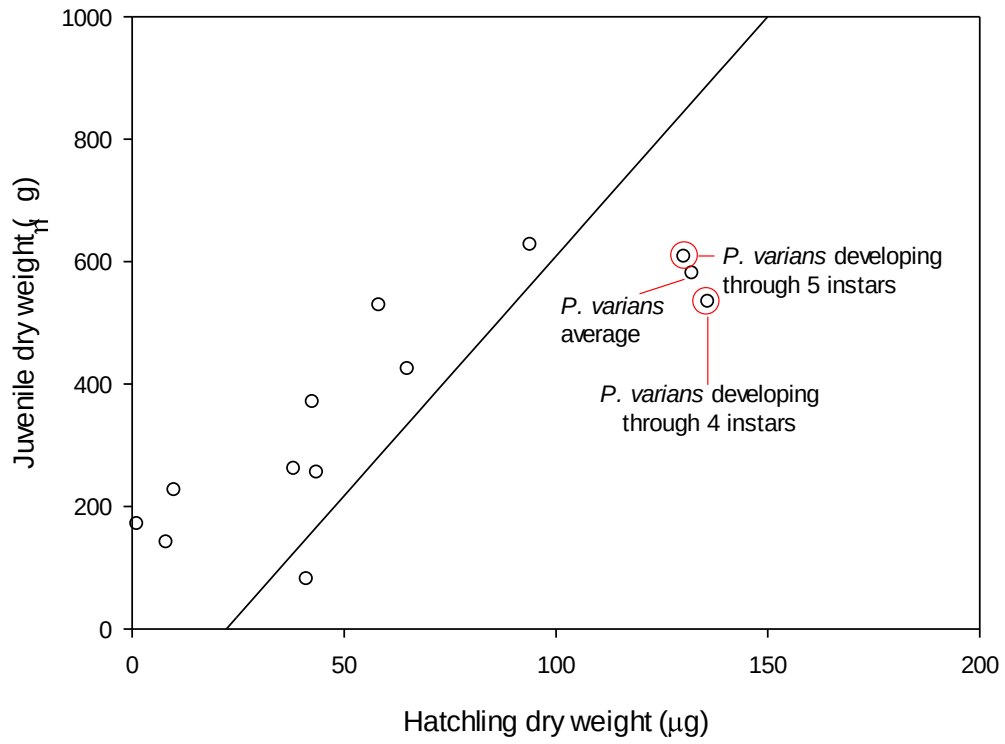

Figure 7.5 The relationship between hatchling and juvenile dry weight for planktotrophic and lecithotrophic decapod crustaceans. Graph is a magnification of the bottom left of Figure 7.4. Average values of *Palaemonetes varians* are shown, plus values for *P. varians* larvae developing through four and five instars; data from 'Experiment 2 (effects of developmental plasticity)', Chapter 4.

7.4.2 Lecithotrophic development

The influence of POI on starvation resistance has been studied for a number of marine invertebrate taxa (and terrestrial invertebrates) including echinoderms, and crustaceans (Paschke et al. 2004). Data presented here in Chapter 6 are consistent with previous studies and demonstrate that larvae hatching with higher POI are able to survive in the absence of food for longer. Further, data presented in Chapter 6 demonstrates that larvae hatching with greater POI are able to develop to more advanced larval stages before succumbing to starvation. These results indicate that higher POI enables development to continue in the absence of food and may be the mechanism through which lecithotrophy evolves. Further, with increasing POI promoting abbreviated development, decapod larvae are 'pre-adapted' to shorten development. As such, increasing POI likely leads to both development in the absence of exogenous food and the abbreviation of development.

7.5 Perspective: evolution of the macro-ecological trend in POI and development mode within marine invertebrates

Data presented in this thesis, and those of other studies suggest the following scenario for the evolution of the macro-ecological trend in POI and development modes within the marine environment at the inter-specific level:

Clades originate in the tropics with 'ancestral larval forms': swimming and feeding larval phases (Jablonski & Lutz 1983). Seasonal variations are low; consequently, food availability is consistently low. Larval development is constrained by low food availability, but not the timing of this food availability. Selection favours larval forms with large feeding structures relative to their body size; as conditions are consistent, developmental plasticity is redundant (McAlister 2008). From the tropics, clades are progressively 'exported' pole-ward (Jablonski & Lutz 1983). At higher latitudes, lower temperatures, which reduce larval development rate, and higher seasonal variation, which reduces the period of food availability, predominate. Larval development is constrained by shorter periods of food availability relative to the longer periods required for larval development under lower temperatures. Selection, therefore, favours higher development rates and thus higher POI, which enables earlier investment in juvenile features. Selection also favours greater developmental plasticity, which maximise the potential advantages of higher POI. Under high food conditions, developmental plasticity enables echinoderm and bivalve larvae to preferentially invest energy reserves into the development of post-metamorphic

juvenile organs rather than superfluous large feeding structures, thus reducing development time. For decapod larvae, developmental plasticity enables larvae to develop through fewer larval instars, thus developing more quickly. Under low food conditions, echinoderm and bivalve larvae preferentially develop large feeding structures, rather than investing in the development of post-metamorphic organs, thus survivorship is higher. Decapod larvae are able to extend the larval period through additional instars, enabling development to the juvenile stage. Developmental plasticity therefore maximises the potential fitness benefits of higher POI. Under good conditions, yolk reserves are invested in juvenile features and development proceeds through either a larval stage with reduced larval features (e.g. feeding structures) or a shorter larval stage. Under poor conditions, yolk reserves are invested into larval feeding structures to improve the efficiency of energy assimilation from the environment, or the larval stage is extended to allow more time for the assimilation of energy. Developmental plasticity – environmentally-dependent shifts in the trade-off in energy-reserve investment between the development of feeding structures and of post-metamorphic organs- increases with latitude.

As clades continue their pole-ward 'migration', temperature further decreases and seasonality further intensifies. Consequently, larval food availability becomes increasingly limited to shorter time periods. Selection, therefore, continues to favour increases in POI, which increase development rate and provide greater independence from external food sources. Given the capability for developmental plasticity in response to external food availability, larvae are 'pre-adapted' to exhibit energy saving traits in response to increases in internal food supply. Thus, a greater internal food resource has a similar effect on morphology and development as high external food source. Larvae preferentially invest resources into development of juvenile features rather than larval features, i.e. larvae with relatively smaller feeding structures, early investment into post-metamorphic structures, shorter development times, and for decapods, fewer larval instars. Thus, the evolutionary transition from feeding to facultative feeding and non-feeding larvae requires minimal genetic change. The evolution of obligate, non-feeding larvae through the complete loss of feeding function and the reduction in swimming ability via changes in morphology requires genetic change.

POI plasticity appears advantageous for species with large latitudinal distributions: at the higher latitude limits of a species' distribution greater POI would supply energy reserves for earlier investment in post-metamorphic juvenile features. The evolution of

developmental plasticity and POI plasticity enables greater reproductive success under sub-optimal conditions, allowing the extension of species distributions, spatially. There is, as yet, no evidence for variations in POI plasticity across latitude.

Importantly, the above scenario is a general concept and not all-encompassing. In reality, there are other, additional constraints and selective pressures acting on POI and development mode, in varying life stages. Within some clades, there may be no transition towards non-feeding larvae at higher latitudes. Also, clades may migrate equator-ward from high latitudes, submerge into the deep oceans and re-emerge again; 'mixing' the general macro-ecological trend in POI and development mode.

7.6 Future work on the evolution of larval development modes

The results of this thesis demonstrate, based on a single species, the potential influence of the interactions between POI, environmental conditions, and developmental plasticity on the evolution of decapod larval development modes. Future work must seek to investigate this process in action and expand the work to a wide range of taxa.

Previous literature in this field has focused on inter-specific comparisons. Those studies, which have made intra-specific comparisons on the influence of POI on larval development, have done so through artificial methods of POI reduction. Further, this work is predominantly focused on echinoderms. Both field and lab based observations of intra-specific divergences in POI and associated changes in larval development are lacking. Therefore, to elucidate the merits of the above scenario (see section 7.2.2, above) and to understand better the evolution of the macro-ecological trend in POI and development modes among marine invertebrates, studies comparing the relative magnitudes of phenotypic plasticity during the larval phase and maternal effects on POI between populations across latitudinal and environmental gradients are requisite at the intra-specific level. Such studies will identify hypothesised differences in plasticity selected for in the field. Intra-specific comparisons of taxa from tropical, temperate and polar regions, and inter-specific comparisons between species from differing climate zones will evidence any differences in plasticity across a latitudinal gradient. The use of population genetics to assess the connectedness of populations with differing levels of POI and magnitudes of POI plasticity could help to establish divergences in development mode. Useful comparisons between species from eurythermal and stenotherm environments and between semelparous and iteroparous species would further assessing the importance of

developmental plasticity between environments and differing life histories. In the long term, selective breeding experiments, which are presently lacking within this field, could observe the process of development mode divergence and demonstrate the role of developmental plasticity in this divergence.

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Appendix 1

Under review: Marine Biology (submitted 28/10/2013)

The influence of Per Offspring Investment (POI) and starvation on larval developmental plasticity within the palaemonid shrimp, *Palaemonetes varians*

Andrew Oliphant, Matteo C. Ichino and Sven Thatje

Abstract At the inter-specific level, per offspring investment (POI), degree of abbreviated development, and lecithotrophic potential all increase with increasing latitude and freshwater penetration among crustaceans. These traits are considered adaptations to conditions of decreasing growth potential. We hypothesis that the relationship between POI and abbreviated development also occurs at the intra-specific level. We studied the caridean shrimp, *Palaemonetes varians*, to investigate the hypothesis that under food-limited conditions, higher POI enables development through fewer larval instars. Larvae from broods of greater POI (measured as hatchling dry weight, *W*, and categorised as 'small', 'medium', or 'large') generally developed through fewer larval instars. With increasing starvation period, larval development time increased and larval growth rate, juvenile *W*, juvenile carbon mass, and juvenile C:N ratio all decreased. Larval development time decreased with increasing larval *W* category. In contrast larval growth rate, juvenile *W*, juvenile carbon mass, and juvenile C:N ratio all increased with increasing larval *W* categorys. There were no interactions between starvation treatment and larval *W* category for any larval or juvenile trait, except larval development time. These results are consistent with the relationship between POI and larval instar number (abbreviation of development) at the inter-specific level and support the concept that macro-ecological trends in development modes at the inter-specific level may be driven by selection occurring on POI at the intra-specific level.

Keywords: larval instar number, POI, reproduction, endotrophy, *Palaemonetes varians*

Introduction

At the inter-specific level among marine invertebrates, per offspring investment (POI) – the quantity and quality of resources allocated to individual offspring – is related to larval development mode. On the continuum of POI, larvae with low POI are planktotrophic (feeding larvae, which consume planktonic flora and fauna) whilst those

with high POI are lecithotrophic (non-feeding larvae, which utilise internal yolk reserves); in between are varying degrees of planktotrophic and lecithotrophic development with facultative feeding ability. Within the marine environment, greater POI, associated with lecithotrophic and abbreviated larval phases, is increasingly prevalent at high latitudes whilst lower POI and planktotrophic development is increasingly rare (Thorson 1950; Clarke 1992; Thatje et al. 2003; Marshall et al. 2012). Increasing POI, lecithotrophic, and abbreviated larval phases are considered evolutionary adaptations to low or unpredictable food availability and/or the mismatch between short periods of primary production (resulting from high seasonality) and prolonged development (resulting from low temperatures) at high latitudes (Thorson 1950; Anger 2001; Thatje et al. 2003). This macro-ecological trend in the reproductive and developmental ecology of marine invertebrates, often called 'Thorson's Rule' (Mileikovsky 1971), is one of the most important within our oceans as it determines reproductive success, gene flow and, consequently, influences species distributions, species diversity, and rates of evolution.

To understand how the evolution of inter-specific differences in reproductive and development modes arise, we must understand the implications of variations in reproductive and development traits at the intra-specific level. Selection on marine invertebrate POI and the evolution of developmental modes is reliant on intra-specific differences in POI and the effects of this trait on maternal fitness and on larval development and 'carry-over' or 'latent' effects into later life stages (Arthur 2000, Pechenik 2006, Marshall and Keough 2008). 'Carry-over' or 'latent' effects, originate from embryonic and larval experiences and are expressed in juvenile and adult life stages; for example, the effects of delayed metamorphosis on post-metamorphic growth, development, and survival (Pechenik 1990, 2006). The effects of POI on larval development have been assessed via the artificial manipulation and reduction of POI within echinoderms; for example, echinoderm embryos have been twinned at the two- or four-cell stage to reduce POI, or lipid has been removed via centrifuging (Sinervo and McEdward 1988; Hart 1995; Emlet and Høegh-Guldberg 1997; Allen 2012). Such experiments have demonstrated that reduced POI is associated with lower development rates, greater development time, and reduced juvenile size. Reduced POI can also cause initial larval morphology to be consistent with that of species with lower POI (Sinervo and McEdward 1988).

Within crustaceans, the effects of POI on larval development have been assessed through comparisons of larvae from broods of differing POI. Greater POI is related to

reduced development time, higher growth rates, greater juvenile size, higher starvation resistance, and greater survivorship, especially under unfavourable conditions (Giménez et al. 2004; Paschke et al. 2004; Oliphant and Thatje 2013). Critically, POI can interact with larval developmental plasticity (e.g. McAlister 2007; Oliphant and Thatje 2013); for example, within crustaceans POI can affect the number of larval instars during development (Giménez et al. 2004; Oliphant and Thatje 2013), which may have significant ecological and evolutionary implications (Davidowitz et al. 2004; Kingsolver 2007; Oliphant et al. 2013). Higher POI is associated with development through fewer larval instars (Giménez et al. 2004; Oliphant and Thatje 2013). Development through differing numbers of larval instars may affect juvenile size at metamorphosis, which in turn influences juvenile competitive interactions, starvation resistance, and survivorship (Giménez et al. 2004; Oliphant and Thatje 2013). This variation in larval instar number is consistent with inter-specific level trends in abbreviated development; which is manifest as larvae that hatch in advanced stages of development and develop through fewer larval instars, and is associated with increases in POI (Anger 2001; Thatje et al. 2003).

Despite macro-ecological trends within crustaceans in POI and abbreviated development and the assumed association of changes in these traits with food availability, no study has assessed the implications of POI in combination with starvation stress for larval development plasticity (variation in larval instar number). Such studies are requisite if we are to understand better the evolution of invertebrate life histories and their macro-ecological trends. Consequently, in this study the assumption that higher POI enables development through fewer larval instars is tested in relation to periods of starvation. We hypothesise that under conditions of limited food availability crustacean larvae with higher POI are better able to develop through fewer larval instars than their lower POI counterparts, and so are less likely to prolong larval development through additional larval instars.

Palaemonetes varians is a palaemonine shrimp, which inhabits brackish waters and is considered an intermediate species between marine and freshwater habitats (Dolmen 1997; Falciai 2001; Hindley 2001; González-Ortegón and Cuesta 2006; Oliphant and Thatje 2014 and references therein). *Palaemonetes varians* larvae exhibit physiological and ecological adaptations to their life cycle: larvae hatch with relatively high POI which can sustain early development and is considered an adaptation to the exporting of larvae from the adult habitat into lower estuarine and coastal marine waters (Strathmann 1982;

Anger 2001; Oliphant and Thatje 2014). Recently, larval instar number was found to be temperature dependant within *P. varians* (Oliphant et al. 2013), but also influenced by POI (Oliphant and Thatje 2013). Here, we use *P. varians* as a study species to test the above hypothesis.

Materials and methods

Palaemonetes varians larvae used for this experiment were obtained from wild caught ovigerous females and from wild caught shrimp which were brought into the aquarium before becoming ovigerous (aquarium population); all shrimp were from Lymington, UK (50.4423N, 1.3213W). The aquarium population was established in November, 2011 and larvae hatched from March - May, 2012; it was assumed that no acclimation to aquarium conditions would be evident within these larvae.

Collection and maintenance

Wild ovigerous *Palaemonetes varians* were collected via hand-netting from Lymington salt-marshes in May 2012; shrimp were then transported in a sealed 10 litre bucket containing water from the point of collection to the research aquarium at the National Oceanography Centre Southampton. Here, the embryonic development of broods was assessed and staged following Müller et al. (2004). Ovigerous *P. varians* with stage VII (final post-nauplius) and VIII (pre-hatching embryo) embryos were isolated individually in 1 litre plastic beakers containing ~850 ml of 15 °C, 32 salinity, 1μm-filtered seawater and transferred to temperature-controlled incubators set to 15 °C and 12:12 (L:D). Similarly, ovigerous *P. varians* from the aquarium population were isolated following the same method. Between isolation and larval hatching, all isolated ovigerous shrimp were fed Tetra goldfish flakes three times per week to excess. Water changes (>70 %; 15 °C, 32 salinity, 1μm-filtered seawater) were done every second day and shrimp were checked daily (am) for hatching larvae.

Larval culture

Upon hatching, actively swimming larvae were separated from 18 females (12 aquarium, 6 wild) and isolated individually in plastic beakers containing ~80 ml of 15 °C, 32 salinity, 1 μm-filtered and UV-treated seawater. To assess brood average larval dry weight (*W*; μg ind.$^{-1}$), five to fifteen larvae per female were immediately, randomly selected and blotted on tissue paper, transferred individually to pre-weighed tin capsules, frozen at -80 °C and later freeze dried (for 24 hours) and weighed (μg). Remaining larvae

were transferred to temperature-controlled incubators set to 20 °C and 12:12 (L:D). The first and second larval instars of *Palaemonetes varians* development are facultative lecithotrophic (Oliphant and Thatje 2014); an adaptation to the export of larvae from the adult habitat into lower estuarine and coastal marine waters (Strathmann 1982; Anger 2001). As such, the first larval instar was starved in all treatments (following Oliphant et al. 2013; Oliphant and Thatje 2013), thus simulating food limitation during export from the adult habitat. On moulting to the second larval instar, larvae were divided among three groups (n = 100 per group); those fed immediately (0 days starvation, 0S), those fed after two days of starvation (2S), and those fed after four days of starvation (4S). Larvae were fed freshly hatched *Artemia* sp. nauplii to excess and water changes were done every second day (100 %; 20 °C, 32 salinity, 1 μm-filtered and UV-treated seawater). Larval mortality and development, assessed by morphological changes and moulting (following Fincham 1979) were monitored daily (am). The number of larval instars, i.e. the number of moults passed through during the larval phase, during larval development was monitored. On moulting to the juvenile stage, shrimp were blotted on tissue paper, transferred individually to pre-weighed tin capsules, frozen at -80 °C and later freeze-dried (for 24 hours), and weighed (μg) and analysed for carbon and nitrogen composition using a Carlo ERBA instruments CHNS-O EA1108-elemental analyser.

Dry weight as accurate proxy for POI

Egg/offspring size (often converted to volume) and egg/offspring W are almost exclusively used as proxies for POI. Given the relatively poor correlation between propagule size and POI, size has received criticism in its use as proxy for POI (Moran and McAlister 2009); consequently, here we use hatchling W. Further, a strong relationship between W (μg ind.$^{-1}$) and carbon mass (μg C ind.$^{-1}$) has been recorded in hatchling *P. varians* larvae (Oliphant 2014).

Statistical analysis

Data were assessed for normality of distribution and homogeneity of variance using Kolmogorov-Smirnov Test and Levene's Test, respectively. Box-Cox transformation was used to calculate the most likely successful power transformation for data. Where data were non-normally distributed and could not be successfully transformed to meet assumptions, non-parametric statistics were used.

Between-brood-variation in brood average larval *W* (μg ind.$^{-1}$) was analysed by Kruskal-Wallis comparison. Differences in the proportion of larvae developing through differing numbers of larval instars were assessed via logistic regression. Larval development time (days) data were analysed by Kruskal-Wallis comparison. Larval growth rate (μg ind.$^{-1}$ day^{-1}), juvenile *W* (μg ind.$^{-1}$), juvenile carbon mass (μg C ind.$^{-1}$), and juvenile carbon:nitrogen ratio were analysed by General Linear Model ANOVA; factors were starvation treatment (0S, 2S, 4S) and larval *W* category (small, medium, large). Statistical analyses were done using Minitab v16 software and in accordance with Sokal and Rohlf (1995).

Results

Brood average larval *W* (μg ind.$^{-1}$) differed between broods (H = 143.28, $P < 0.001$; K-W) (Figure 7.6A). To assess the effect of larval hatchling size (POI) on traits during larval development and juvenile traits after settlement, broods were grouped into three larval *W* categories according to statistical differences in brood average larval *W* (Figure 7.6): broods grouped into the 'large' category were significantly heavier than broods grouped into the 'small' category, 'medium' broods overlapped in weight with 'large' and 'small' broods (Figure 7.6A). Differences in average larval *W* between larval *W* categories were significant ($P < 0.001$ in all cases; Figure 7.6B).

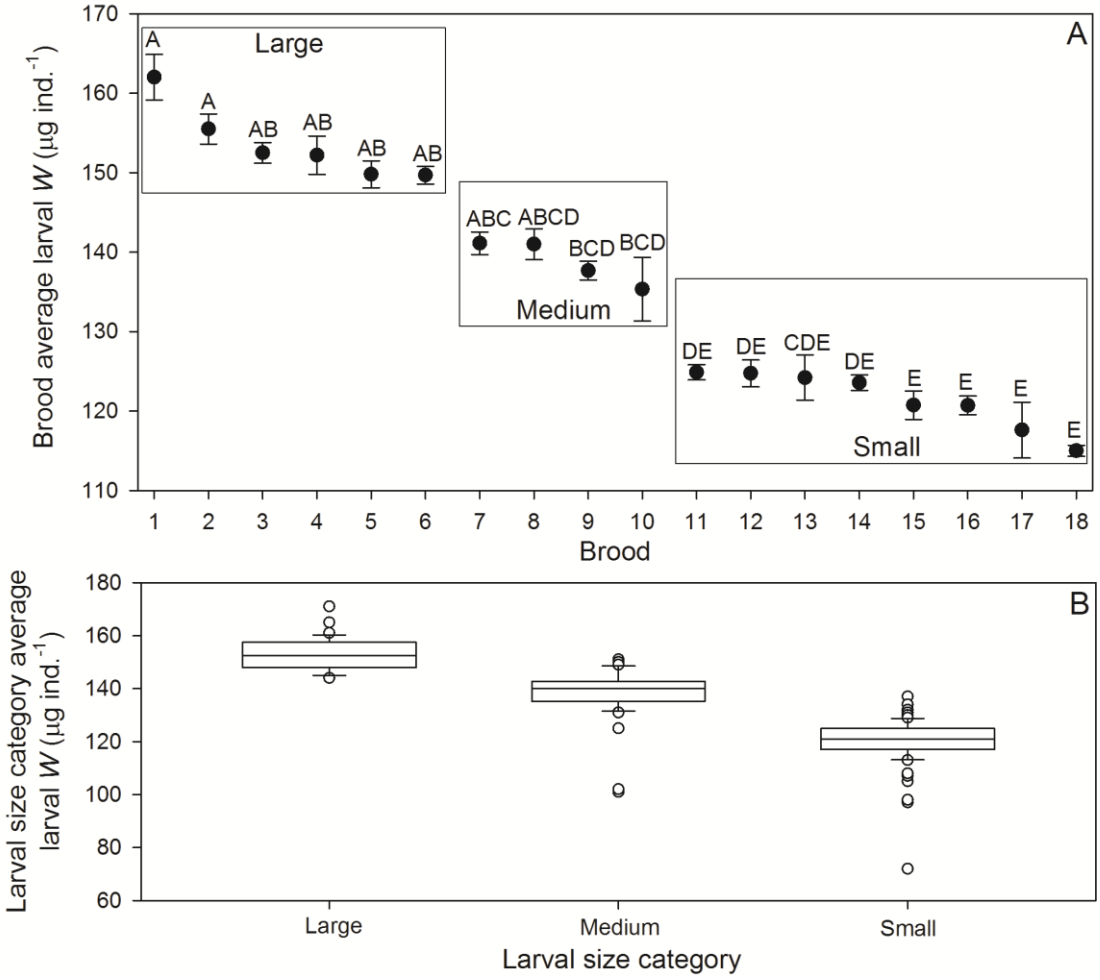

Figure 7.6. *Palaemonetes varians* **A** variation in brood average larval W (µg ind.$^{-1}$) between broods of larvae used in this experiment. Data are presented as means ± standard errors. Letters indicate significant differences between broods at the $P \leq 0.01$ level. Larvae from broods were grouped into three categories (large, medium, small) as indicated based on differences in brood average larval *W*. **B** boxplots of larval size categories (large, medium, small) used in this experiment.

Developmental plasticity

The proportion of larvae from all weight categories pooled developing through four instars was highest under the 0S treatment (0.53). Under the 2S treatment, the proportion of larvae developing through four instars was lower (0.08) and was lowest for 4S treated larvae (0.03). Relative to development under the 0S treatment, larvae were 0.08 times as likely to develop through four instars under the 2S treatment ($P < 0.001$) and 0.03 times as likely under the 4S treatment ($P < 0.001$). The proportion of larvae from all weight categories pooled developing through five instars was lowest under the 0S treatment (0.46), highest under the 2S treatment (0.80), and was 0.49 under the 4S treatment. The

likelihood of larvae developing through five instars was 4.64 times as likely under the 2S treatment as under the 0S treatment ($P < 0.001$), but under the 4S treatment larvae were no more or less likely to develop through five instars than under the 0S treatment. The proportion of larvae from all weight categories pooled developing through six instars was higher under greater starvation treatments: 0.01 under the 0S treatment, 0.12 under the 2S treatment, and 0.49 under the 4S treatment. Indeed, relative to development under the 0S treatment, larvae were 12.19 times as likely under the 2S treatment ($P < 0.018$) and 86.13 times as likely under the 4S treatment ($P < 0.001$) to develop through six instars.

For 'large' larvae, there was no change in the likelihood that larvae would develop through either four, five, or six instars across the starvation treatment (0S, 2S, 4S) (Figure 7.7). For 'medium' larvae, the proportion of larvae developing through four instars was 0.82 under 0S treatment, 0.10 under the 2S treatment, and no 'medium' larvae developed through four instars under the 4S treatment (Figure 7.7). Relative to the 0S treatment, 'medium' larvae were 0.02 times as likely to develop through four instars under the 2S treatment ($P = 0.002$) (Figure 7.7). The proportion of 'medium' larvae developing through five instars was greatest under the 2S treatment (0.80), being 0.18 and 0.14 under the 0S and 4S treatments, respectively (Figure 7.7). 'Medium' larvae were 18.67 times as likely to develop through five instars under the 2S treatment as under the 0S treatment ($P = 0.004$), there being no difference in likelihood between 0S and 4S treatments. For 'medium' larvae, development through six instars was absent under the 0S treatment, 0.10 under the 2S treatment, and was 0.86 under the 4S treatment; larvae were 54.00 times as likely to develop through six instars under the 4S treatment as under the 0S treatment ($P = 0.008$) (Figure 7.7).

For 'small' larvae, the proportion of larvae developing through four instars was 0.58 under the 0S treatment, 0.02 under the 2S treatment, and larvae did not develop through four instars under the 4S treatment. Development through four instars was 0.02 times as likely under the 2S treatment as under the 0S treatment ($P < 0.001$) (Figure 7.7). Development through five instars was 0.40 under the 0S treatment, 0.80 under the 2S treatment and 0.26 under the 4S treatment; 'Small' larvae were 6.27 times as likely to develop through five instars under the 2S treatment as under the 0S treatment ($P < 0.001$). Development through six instars was 0.02 under the 0S treatment, 0.17 under the 2S treatment, and 0.74 under the 4S treatment. Relative to development under the 0S

treatment, larvae were 9.89 times as likely to develop through six instars under the 2S treatment ($P = 0.034$) and 135.12 times as likely under the 4S treatment ($P < 0.001$).

Under the 0S treatment, 'large' larvae were 4.83 times as likely to develop through five instars ($P = 0.004$) and 0.23 times as likely to develop through 4 instars ($P = 0.007$) as 'small' larvae, whilst there was no odds ratio difference between 'medium' and 'small' larvae. Relative to 'small' larvae, 'large' larvae were 10.23 times as likely to develop through four instars ($P = 0.039$) under the 2S treatment and under the 4S treatment, 'large' larvae were 19.17 times as likely to develop through five instars ($P < 0.001$) and 0.02 times as likely to develop through 6 instars ($P < 0.001$) as 'small' larvae. Again, there were no odds ratio differences between 'small' and 'medium' larvae.

Development

Larval development time differed between larval weight categories and starvation treatments ($P < 0.001$; K-W, Figure 7.8A). With increasing starvation period, larval development time became longer for all starvation treatments. For larvae in the 0S and 2S starvation treatments, larval development time was not different between larval weight categories. For larvae in the 4S starvation category, development time was slower in 'small' larvae (21.4 ± 0.3 days) compared to 'large' larvae (19.0 ± 0.5 days); 'medium' larvae developed in 21.6 ± 0.8 days which was not different from either 'small' or 'large' larvae.

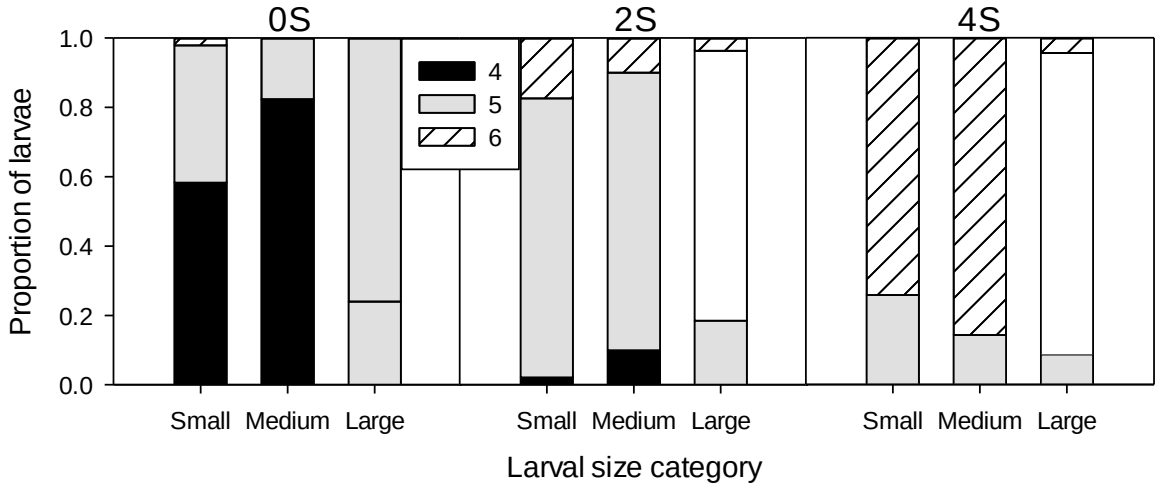

Figure 7.7 Proportions of *Palaemonetes varians* larvae developing through four, five, or six larval instars for small, medium, and large *W* category larvae under 0S, 2S, and 4S starvation treatments

Larval growth rate differed between larval weight categories and starvation treatments, but there was no interaction between these factors (Table 7.1). Larval growth rate was higher for larvae in higher weight categories, whilst it was lower for larvae under higher starvation stress (Figure 7.8B). Similarly, juvenile weight differed between larval weight categories and between starvation treatments, but there was no interaction between these factors (Table 7.1) (Figure 7.9A). Juvenile weight was higher for larvae in higher weight categories, whilst it was lower for larvae under higher starvation stress (Figure 7.9A).

Both juvenile carbon mass and juvenile C:N ratio differed between larval weight categories and between starvation treatments (Table 7.1, Figures 7.9B, C). Again, there was no interaction between these factors (Table 1). Juvenile carbon mass was higher for larvae in higher weight categories, whilst it was lower for larvae under higher starvation stress (Figure 7.9B). Similarly, juvenile C:N ratio was generally was higher for larvae in higher weight categories, whilst it was lower for larvae under higher starvation stress (Figure 7.9C).

Discussion

In the present study, we have demonstrated that under starvation stress greater POI enables larval development through fewer larval instars. Further, POI influences larval traits such as development time and growth rate, and post-settlement juvenile traits such as dry weight, carbon mass and C:N ratio. The effects of POI on larval and juvenile traits appear independent of the level of starvation stress.

Larval instar number is plastic at the intra-specific level within many arthropod species, and is thought to extend larval development under poor growth and development conditions, enabling larvae to achieve a development and size threshold (Nijhout 1975; Nijhout 1994; Esperk et al. 2007). Larval instar number is known to vary with environmental conditions including food quantity and diet (Broad 1957; Knowlton 1974; Esperk et al. 2007). In this study, increasing periods of starvation resulted in increased numbers of larval instars during development. This agrees with studies, which demonstrate that developmental plasticity permits growth to a size threshold (Nijhout 1975; Nijhout 1994). However, within *P. varians* empirical evidence suggests that there is no size threshold for the end of the larval period but that larvae develop continuously to the juvenile stage (Oliphant et al. 2013). Similarly, data presented in this study support this

conclusion (Figure 7.10). Although development through five instars generally leads to larger juveniles than development through four instars (within starvation treatments), juvenile size decreases with increasing starvation periods despite the shift in the proportion of larvae developing through five and six instars (Figure 7.10). No size threshold for the end of the larval period is evident for *P. varians* and plasticity in larval instar number, therefore, results from retarded development and the decoupling of moulting from development (Knowlton 1974).

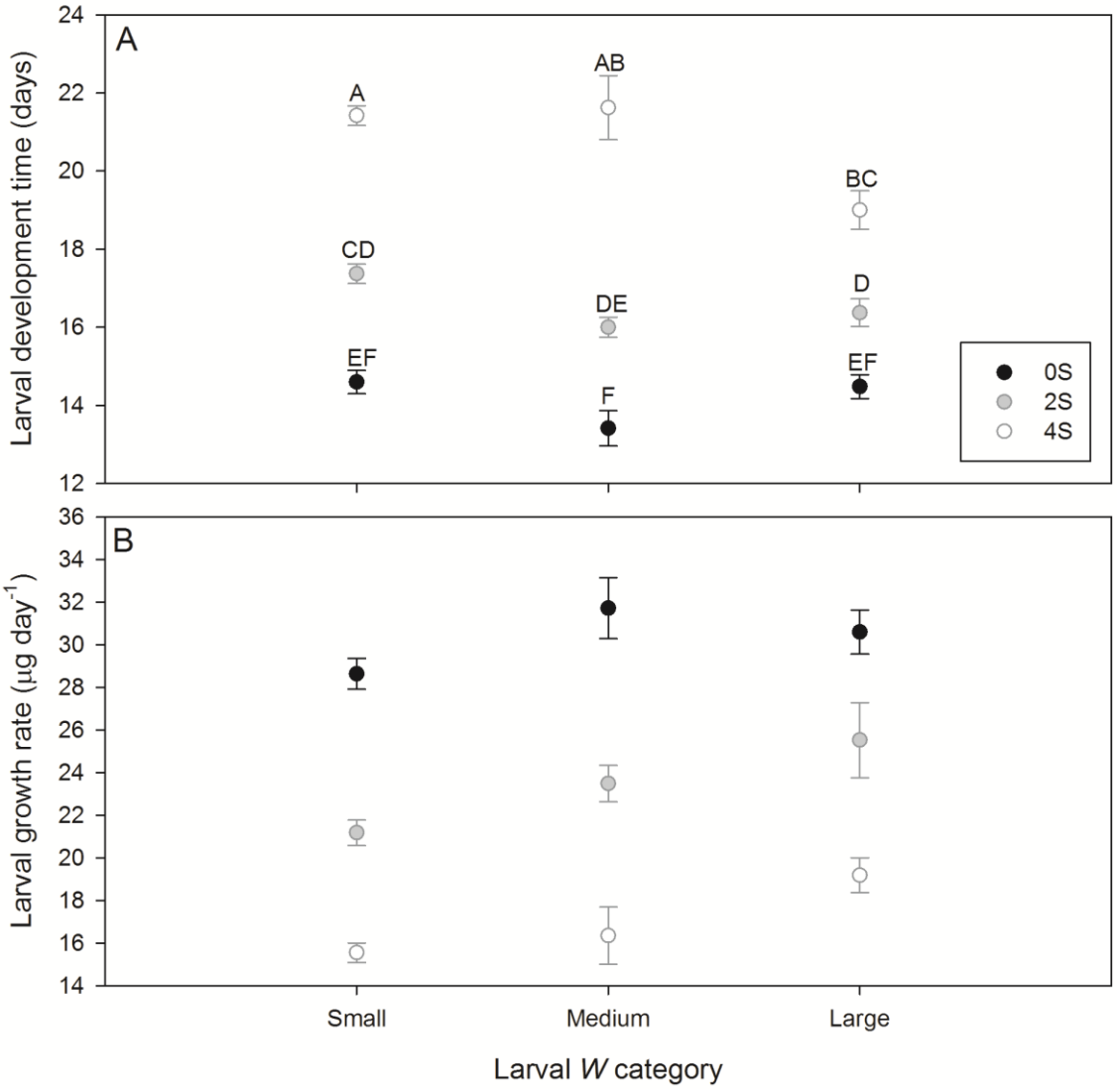

Figure 7.8 *Palaemonetes varians* **A** larval development time (days) and **B** larval growth rate (µg ind.$^{-1}$ day^{-1}) for small, medium, and large *W* category larvae under 0S, 2S, and 4S starvation treatments. Data are presented as means ± standard errors. Letters indicate significant differences between data points at the $P \leq 0.01$ level.

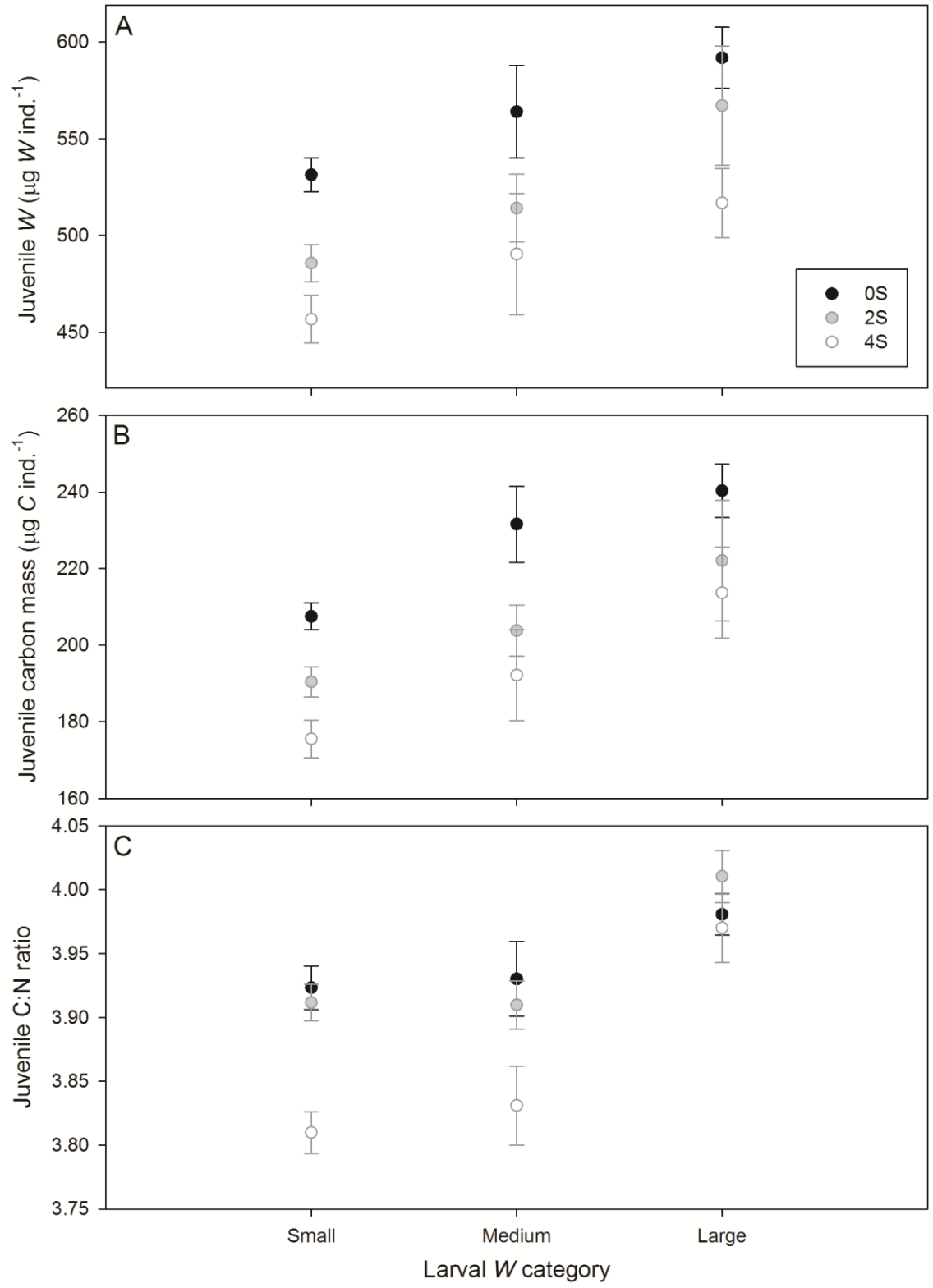

Figure 7.9 *Palaemonetes varians* **A** juvenile *W* (µg *W* ind.$^{-1}$), **B** juvenile carbon mass (µg *C* ind.$^{-1}$), and **C** juvenile C:N ratio for small, medium, and large *W* category larvae under 0S, 2S, and 4S starvation treatments. Data are presented as means ± standard errors.

Table 7.1 *Palaemonetes varians*. The effects of starvation, larval *W*, and their interaction on larval growth rate, juvenile *W*, juvenile carbon mass, and juvenile C:N ratio. General Linear Model ANalysis Of VAriance (GLM ANOVA) with starvation treatment (0S, 2S, 4S) and larval *W* category (small, medium, large) as factors.

Larval growth rate

Source	d.f.	Adjusted Mean square	*F*-ratio	*P*-value
Starvation treatment	2	4.8127	119.88	< 0.001
Larval *W* category	2	0.4780	11.91	< 0.001
Interaction	4	0.0524	1.30	0.269
Error	221	0.0401		

Juvenile *W*

Source	d.f.	Adjusted Mean square	*F*-ratio	*P*-value
Starvation treatment	2	1.52E-04	13.65	< 0.001
Larval *W* category	2	1.51E-04	13.57	< 0.001
Interaction	4	8.00E-07	0.08	0.990
Error	222	1.11E-05		

Juvenile *C* mass

Source	d.f.	Adjusted Mean square	*F*-ratio	*P*-value
Starvation treatment	2	8.40E-06	15.18	< 0.001
Larval *W* category	2	9.70E-06	17.54	< 0.001
Interaction	4	3.00E-07	0.48	0.753
Error	189	6.00E-07		

Juvenile C:N ratio

Source	d.f.	Adjusted Mean square	*F*-ratio	*P*-value
Starvation treatment	2	0.0787	8.74	< 0.001
Larval *W* category	2	0.2127	23.62	< 0.001
Interaction	4	0.0158	1.75	0.140
Error	193	0.0090		

Generally, the addition of larval instars is coupled with longer development times and gives rise to larger individuals at metamorphosis/settlement (Giménez et al. 2004; Kingsolver 2007; Etilé and Despland 2008; Oliphant et al. 2013). Consequently, this form of developmental plasticity may have ecological and evolutionary implications (Kingsolver 2007; Etilé and Despland 2008; Oliphant et al. 2013). The ability to tolerate unfavourable growth and development conditions, evident as larval development without the addition of extra larval instars and development through fewer larval instars would appear advantageous, especially under unfavourable growth conditions. Here, *P. varians* larvae with greater POI developed through fewer larval instars under starvation stress. This likely resulted from greater internal reserves, which enabled development to continue via endotrophy in the absence of external food. Evidence from studies of echinoids indicates

that mass-specific metabolic rate may not change with egg volume; consequently, changes in egg size do not affect larval starvation resistance as egg material is primarily used for construction of the larval body (Moran and Allen 2007). *Palaemonetes varians* hatch with considerable energy reserves and are highly resistant to starvation, which reflects the larval ecology of this species (Oliphant and Thatje 2014). The effects of larval size on larval development and post-juvenile traits are likely related to differences in energy reserves at hatching (Oliphant and Thatje 2013).

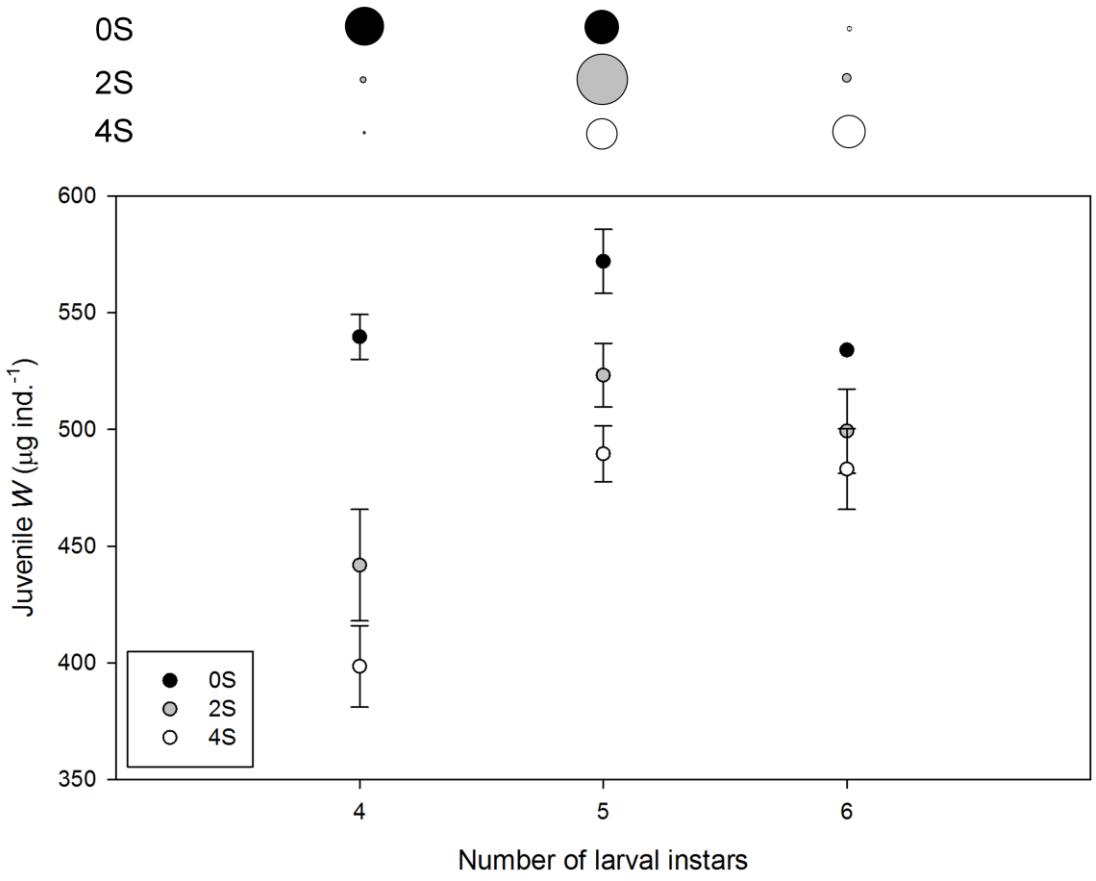

Figure 7.10 *Palaemonetes varians* juvenile *W* (µg ind.$^{-1}$) after development through four, five, and six larval instars and under three starvation treatments (0S, 2S, 4S). Data are presented as means ± standard errors. Above the plots, circle diameters indicate the proportion of larvae developing through four, five, and six instars from 0S (black), 2S (grey), and 4S (white) starvation treatments.

The relationship between POI and larval instar number, i.e. larger larvae develop more often through fewer larval instars, was not evident for larvae in the 0S starvation treatment. The lack of this relationship for 0S treated larvae is inconsistent with the results of Oliphant and Thatje (2013) who demonstrated that *P. varians* larvae hatching with higher POI developed through fewer larval instars than larvae hatching with lower POI, at

three temperatures, including 20 °C. Here, larvae within the 'medium' larval weight category appeared more plastic in the number of larval instars during development relative to both 'large' and 'small' larvae; under the 0S treatment the highest proportion of 'medium' larvae developed through four instars, whilst the highest proportion developed through six instars under the 4S starvation treatment. The effects of this greater plasticity appear evident in larval development time and larval growth rate data (Figure 7.8A, B): data points for 'medium' larvae have the greater range between starvation treatments. Why 'medium' larvae should be more plastic than either 'large' larvae or, especially, 'small' larvae remains unclear.

Under food limited conditions, high POI is important in tolerating starvation and enabling development through the 'normal' number of larval instars; i.e. development without additional instars. This would appear advantageous in environments where periods of food limitation are common, such as high latitude regions and freshwater. Along the environmental gradient from marine to freshwater, crustaceans (one of the few marine taxa to have colonised freshwater) display a trend in their reproductive and developmental ecology, which can be considered similar to 'Thorson's Rule'. With increasing penetration into freshwater habitats, species increasingly reproduce by lecithotrophic and abbreviated larval phases (Anger 2001; Vogt 2013). Increasing POI, lecithotrophic, and abbreviated larval phases are considered evolutionary adaptations to low food availability within freshwater environments (Anger 2001; Vogt 2013).

Interestingly, the effects of POI on larval development time and growth rate, and juvenile dry weight, carbon mass, and C:N ratio were consistent across starvation treatments, indicating that high POI is no more advantageous under high starvation stress than it is under relatively benign food limitation. Similarly, Hartmann et al. (2013) found that offspring size did not mediate latent effects of harsh environments within two species of coral: *Agaricia humilis* and *Montastraea faveolata*. For the gastropod, *Nucella ostrina*, Moran and Emlet (2001) found that the advantage of large hatchling size was decreased under more severe environmental conditions. The effects of the larval experience on latent effects within post-metamorphic individuals may be affected by larval energetic reserves so that offspring with higher POI are better able to tolerate adverse conditions (Marshall and Keough 2007). In this study and unlike previous studies, the adverse environments experienced by larvae were high levels of nutritional stress, which directly affect larval energetic reserves. Within *Palaemonetes varians*, POI did not mediate the direct effects of

the larval environment on development, nor on latent effects; larvae with higher POI were better under all conditions tested. Post-settlement juvenile traits (latent effects), which were influenced by both POI and starvation treatment (though not the interaction of these factors), will likely influence early juvenile survivorship and growth rate (Jarrett and Pechenik 1997; Giménez et al. 2004; Giménez 2010).

The relationship between POI and larval instar number is consistent with the relationship between these traits at the inter-specific level among species adapted to differing environments. This may indicate that evolutionary changes to more abbreviated larval development are relatively straight-forward as crustacean larvae are 'pre-adapted', through plasticity in larval instar number, to abbreviate development with increased POI, as demonstrated here and by Oliphant and Thatje (2013). Similarly, and within an echinoid species, Sinervo and McEdward (1988) demonstrated that artificial reductions in POI caused initial larval morphology to converge with that of a congeneric species with relatively lower POI; indicating that species level differences in larval form and function may result from differences in POI (Sinervo and McEdward 1988).

At the intra-specific level, our results indicate that POI is important in determining larval instar number, especially under conditions of low growth and development potential. Results presented here support the hypothesis that POI influences larval instar number and is consistent with the trend at the inter-specific level. Consequently, we highlight a development trait, which may be important in driving selection for POI. The influence of this trait (POI) is not constrained to the larval period but is carried over into early juvenile life.

Appendix 2

Table 7.2 Latitudinal clines in egg 'size' among marine invertebrates

Species	Latitude	Egg 'size'	±	Unit	Reference
Semibalanus balanoides	70.40	329.0		µm	Barnes & Barnes (1965)
(Balanus balanoides)	69.20	356.0		µm	
	69.00	396.0		µm	
	58.35	274.0		µm	
	58.35	273.0		µm	
	58.35	263.0		µm	
	58.35	269.0		µm	
	58.37	263.0		µm	
	58.37	264.0		µm	
	58.33	275.0		µm	
	58.33	278.0		µm	
	58.33	279.0		µm	
	56.51	288.0		µm	
	56.43	276.0		µm	
	55.46	280.0		µm	
	55.46	275.0		µm	
	55.46	273.0		µm	
	55.02	314.0		µm	
	55.12	263.0		µm	
	50.12	293.0		µm	
	50.12	314.0		µm	
	54.13	303.0		µm	
	57.06	283.0		µm	
	55.31	302.0		µm	
	54.51	293.0		µm	
	56.03	290.0		µm	
	47.07	282.0		µm	
Bold from NW Atlantic	**47.34**	**386.0**		**µm**	
	45.05	**348.0**		**µm**	
	41.32	**299.0**		**µm**	
Semibalanus balanoides	69.50	327.0		µm	Crisp (1959)
(Balanus balanoides)	60.24	304.0		µm	
	53.13	289.0		µm	
	50.23	260.0		µm	
	50.23	263.0		µm	
	50.22	245.0		µm	
Strongylocentrotus droebachiensis	58.14	136.0		µm	Vasseur (1949)
	60.38	146.0		µm	Hagstrom and Lonning (1967)
	69.66	168.0		µm	
Strongylocentrotus pallidus	60.38	139.0		µm	Hagstrom and Lonning (1967)
	69.66	166.0		µm	
Psammechinus miliaris	60.19	115.0		µm	Lonning and Wennerberg (1963)
	58.14	96.6		µm	
	55.46	103.3		µm	
	50.22	118.4		µm	
Pseudocalanus sp.	67.00	127.0		µm	Mclaren (1965)
	67.00	118.2		µm	
	62.52	114.2		µm	
	59.30	105.3		µm	
	55.94	124.0		µm	
	41.10	108.0		µm	
Ceretolis sp.	61.61	2970.0	180.0	µm	Wägele (1987)
	72.97	3090.0	190.0	µm	
	77.37	3550.0	240.0	µm	

Neohelice granulatus	37.45	0.013		mm^3	Bas et al. (2007)
(Chasmagnathus granulatus)	37.45	0.011		mm^3	
	40.46	0.020		mm^3	
	40.46	0.016		mm^3	
Cancer setosus	20.14	0.023		mm^3	Brante et al. (2003)
	41.32	0.028		mm^3	
Cancer setosus	23.45	0.017		mm^3	Fischer et al. (2009)
	23.45	0.020		mm^3	
	23.45	0.018		mm^3	
	41.44	0.022		mm^3	
	41.44	0.025		mm^3	
	41.44	0.023		mm^3	
	41.44	0.024		mm^3	
	41.44	0.028		mm^3	
	41.44	0.026		mm^3	
	41.44	0.022		mm^3	
	41.44	0.027		mm^3	
Pandalus borealis	79.10	0.624	0.073	mm^3	Clarke et al. (1991)
	78.10	0.668	0.067	mm^3	
	77.45	0.637	0.117	mm^3	
	76.55	0.585	0.033	mm^3	
	60.16	0.419	0.056	mm^3	
	58.47	0.392	0.033	mm^3	
Emerita analoga	45.00	0.072		mm^3	Efford (1969)
	43.00	0.068		mm^3	
	39.00	0.053		mm^3	
	38.00	0.068		mm^3	
	38.00	0.072		mm^3	
	38.00	0.068		mm^3	
	38.00	0.066		mm^3	
	37.00	0.066		mm^3	
	35.00	0.056		mm^3	
	35.00	0.064		mm^3	
	34.00	0.046		mm^3	
	34.00	0.067		mm^3	
	34.00	0.050		mm^3	
	33.00	0.040		mm^3	
	33.00	0.050		mm^3	
	33.00	0.051		mm^3	
	32.00	0.048		mm^3	
	8.00	0.052		mm^3	
	12.00	0.043		mm^3	
	12.00	0.046		mm^3	
	12.00	0.059		mm^3	
	33.00	0.062		mm^3	
	33.00	0.056		mm^3	
	37.00	0.054		mm^3	
Emerita rathbunae	25.00	0.022		mm^3	Efford (1969)
	23.00	0.038		mm^3	
	18.00	0.029		mm^3	
	18.00	0.041		mm^3	
	14.00	0.026		mm^3	
	10.00	0.032		mm^3	
	10.00	0.029		mm^3	
	9.00	0.030		mm^3	
	1.00	0.038		mm^3	
	2.00	0.031		mm^3	
	3.00	0.033		mm^3	
Helice crassa	35.00	0.01265	0.00141	mm^3	Jones and Simon (1983)
	35.40	0.01227	0.00185	mm^3	
	36.50	0.01277	0.00222	mm^3	

	37.41	0.01265	0.00240	mm^3	
	39.30	0.01094	0.00196	mm^3	
	41.05	0.01297	0.00221	mm^3	
	43.33	0.00726	0.00077	mm^3	
	45.50	0.01337	0.00223	mm^3	
	46.27	0.01111	0.00140	mm^3	
	46.22	0.01420	0.00203	mm^3	
Scottolana canadensis	43.00	0.00156		mm^3	Lonsdale and Levinton (1985)
	38.00	0.00127		mm^3	
	27.00	0.00107		mm^3	
Nauticaris magellanica	30.08	0.031		mm^3	Wehrtmann and Kattner (1998)
	41.35	0.038		mm^3	
Semibalanus balanoides	69.50	0.00449		mm^3	Crisp (1959)
(Balanus balanoides)	60.24	0.00402		mm^3	
	53.13	0.00305		mm^3	
	50.23	0.00298		mm^3	
	50.23	0.00266		mm^3	
	50.22	0.00362		mm^3	
Neohelice granulatus	37.45	7.2		µg	Bas et al. (2007)
(Chasmagnathus granulatus)	37.45	6.3		µg	
	40.46	10.8		µg	
	40.46	8.3		µg	
Cancer setosus	23.45	9.6	0.70	µg	Fischer et al. (2009)
	41.44	12.0	0.80	µg	
	41.44	13.1	1.10	µg	
Ceratoserolis trilobitoides	60.41	3350.0	0.33	µg	Clarke and Gorny (1992)
	60.41	3940.0	0.14	µg	
	60.50	4650.0	0.37	µg	
	75.03	6330.0	0.07	µg	
	73.35	6460.0	0.00	µg	
Pandalus borealis	79.10	792.0	0.042	µg	Clarke et al. (1991)
	78.10	882.0	0.078	µg	
	77.45	841.0	0.135	µg	
	76.55	841.0	0.118	µg	
	60.16	673.0	0.096	µg	
	58.47	614.0	0.036	µg	
Pinnaxodes chilensis	23.45	2.0	0.19	µg	Lardies and Castilla (2001)
	33.23	2.2	0.21	µg	
	39.24	2.3	0.26	µg	
Betaeus truncatus	30.07	17.6	8.46	µg	Lardies and Wehrtmann (2001)
	41.35	37.2	5.21	µg	
	42.25	44.1	4.70	µg	
Nauticaris magellanica	30.08	17.9	2.31	µg	Wehrtmann and Kattner (1998)
	41.35	18.2	2.57	µg	
	42.25	18.7	1.88	µg	

Appendix 3

Table 7.3 Temperature plasticity in egg 'size' among invertebrates

Species	Temperature (°C)	Egg 'size'	Unit	Reference
Bicyclus anynana	20.0	0.723	mm^2	Fischer et al. (2004)
	27.0	0.703	mm^2	
Bicyclus anynana	20.0	0.737	mm^2	Fischer et al. (2003a)
	27.0	0.627	mm^2	
Chthamalus stellatus	15.0	0.00065	mm^3	Patel and Crisp (1960)
	20.0	0.00063	mm^3	
	25.0	0.00050	mm^3	
	30.0	0.00047	mm^3	
Balanus perforatus	19.0	0.00132	mm^3	Patel and Crisp (1960)
	23.5	0.00098	mm^3	
	28.0	0.00091	mm^3	
Elminius modestus	9.0	0.00128	mm^3	Patel and Crisp (1960)
	15.0	0.00110	mm^3	
	20.0	0.00093	mm^3	
	25.0	0.00085	mm^3	
Balanus amphirite	17.5	0.00092	mm^3	Patel and Crisp (1960)
	19.5	0.00086	mm^3	
	22.5	0.00081	mm^3	
	24.5	0.00064	mm^3	
	27.5	0.00057	mm^3	
	31.5	0.00053	mm^3	
Scatophaga stercoraria	11.0	0.145	mm^3	Blanckenhorn (2000)
	15.0	0.144	mm^3	
	19.0	0.135	mm^3	
	23.0	0.133	mm^3	
Daphnia pulex	10.0	0.00263	mm^3	Brambilla (1982)
	18.0	0.00246	mm^3	
	10.0	0.00291	mm^3	
	18.0	0.00239	mm^3	
Notiophilus biguttatus	8.0	63.30	ug	Ernsting & Isaaks (2000)
	12.0	62.40	ug	
	16.0	54.90	ug	
	20.0	49.00	ug	
	24.0	42.70	ug	
Bicyclus anynana	20.0	72.814	ug	Geister et al. (2009)
	27.0	56.589	ug	
Orchesella cincta	16.0	2.95	ug	Liefting et al. (2010)
	20.0	2.23	ug	
Cancer setosus	19.0	9.60	ug	Fischer et al.(2009)
	19.0	10.10	ug	
	21.0	9.60	ug	
	11.0	13.10	ug	
	16.0	11.00	ug	
	19.0	10.70	ug	
	19.0	9.60	ug	
	14.0	12.00	ug	
Cochliomyia hominivorax	24.5	2.01	mg	Berkebile et al. (2006)
	29.5	2.09	mg	
	34.5	2.42	mg	
	24.5	2.82	mg	
	29.5	3.59	mg	
	34.5	3.64	mg	