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Physiological capacities of Gammarid amphipods to survive environmental change

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Summary

The aim of this thesis was to investigate the abilities of confamilial gammarid amphipods with differing latitudinal distribution patterns to compensate for environmental change and to examine the consequences. Interesting variations were observed in the strategies employed by gammarid amphipods to cope with environmental change. The boreal/temperate species, *Gammarus duebeni*, exhibits a high upper thermal tolerance and acclimatory ability, but at the cost of reduced growth and reproductive output. The warmer-water *G. locusta* appears to have a narrower tolerance and reduced ability to adjust to rapid temperature change, but at stable conditions within its thermal window and at higher salinities it is able to out-perform *G. duebeni*. *G. duebeni* may therefore be more likely to survive environmental change, but *G. locusta* populations may be better able to recover from high mortality events. A similar upper thermal limit and temperature independence of metabolic was observed in the subarctic/boreal species *G. oceanicus* compared with *G. duebeni*, however G. *oceanicus* also demonstrated r-selected life history traits. *G. oceanicus* and *G. locusta* occupy overlapping geographical ranges and the same low shore niche, but *G. oceanicus* appears to have a broader tolerance at a lower optimal temperature than *G. locusta*. Intraspecific variation was observed between two populations of *G. duebeni* from Wales (53 °N) and Norway (70 °N). Following acclimation to common temperatures, the northern population was characterised by reduced upper thermal limits and rates of oxygen uptake, a greater thermal sensitivity of oxygen uptake, and evidence of the upregulation of protein synthesis and growth rates in the cold. The influence of the combined factors of salinity and temperature on physiological rate processes in *G. duebeni* was complex. *G. duebeni* was less able to adjust to rapid temperature change when acclimated to salinities equivalent to full strength seawater than when held in brackish conditions.

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Abbreviations

ANCOVA	Analysis of co-variance
ANOVA	Analysis of variance
A_s	Absolute rates of protein synthesis
CS	Citrate synthase
K_{RNA}	RNA activity
k_s	Fractional rates of protein synthesis
LSD	Least significant difference
MCA	Metabolic cold adaptation
MO_2	Rate of oxygen uptake
N	Sample number
Na^+	Sodium ion
Na^+/K^+-ATPase	Sodium-potassium adenosine triphosphatase
PO_2	Oxygen partial pressure
Q_{10}	Temperature coefficient
RNA	Ribonucleic acid
PSRE	Protein synthesis retention efficiency
SEM	Standard error of the mean
STPD	Standard temperature and pressure
UTL	Upper thermal limit
ω^2	Measure of association

Chapter 1

General introduction

Temperature is considered one of the most significant environmental factors, exerting a strong influence over physiological rate processes (Peck et al., 2009) from a whole-animal to molecular level, and acting as a selection agent in determining the distribution of species (Stevens, 1989). Recent warming events (Parry et al., 2007) have been associated with range shifts in aquatic organisms (Barry et al., 1995; Calosi et al., 2008; Parmesan, 2006) and have lent importance to the study of thermal tolerance and its underlying physiology. Climate change is likely to negatively impact species or populations that inhabit areas close to their thermal limits and lack the ability to acclimate or adapt to temperature change, while some species may incur an increase in fitness if current temperatures are below their thermal optima or if warming allows range expansion.

To understand the relative vulnerability of a population or species to temperature change, there is a need to understand the thermal responses of individuals. Natural variations in thermal responses of ectothermic animals have a long history of measurement, often using latitude as a thermal gradient (Parmesan, 2006), however many theories on trends over the thermal gradient remain controversial (Pörtner et al., 2007). Differences in thermal responses have been attributed to variations in thermal tolerance (Kuo & Sanford, 2009), the thermal sensitivity of physiological processes during acute temperature change (Sokolova & Portner, 2003), the mechanisms underlying the short term survival of individuals during heat shock (Tomanek & Somero, 1999), and the ability to acclimate and adapt to long term exposure (Stillman, 2003). In the laboratory, acclimation studies can be used to examine the effect of temperature alone on inter and intraspecific variation in thermal tolerance and physiological rate processes, to control for the confounding effect of natural variations in photoperiod and resource availability, and to explore phenotypic plasticity. The present study examines thermal tolerance in gammarid amphipods, which are widely and abundantly distributed along the coastal fringes of the Atlantic and Arctic Oceans, and investigates their physiological capacity to compensate for environmental change by utilising a series of acclimation experiments. The current thesis will focus on the effects of acclimation temperature and salinity on two important biological rate processes: rates of metabolism and protein synthesis, and relate any observed differences to associated changes in growth rate and life-history traits.

1.1. Thermal tolerance and thermal sensitivity

Studies of the inverse relationship between upper critical temperature and latitude date back nearly a century (Mayer, 1914). Species occupying the polar regions are thought to be more susceptible to temperature variation, due to the relatively narrow window of thermal tolerance they possess compared to temperate counterparts (Peck et al., 2009). Many Antarctic species have been shown to have upper critical limits only a few degrees above their current habitat temperatures (Somero, 2010), and in the face of climate change will therefore need to adapt to survive.

A decline in upper critical limits with latitude has been well documented in ectotherms such as the killifish *Fundulus heteroclitus* (Fangue et al., 2006) and intertidal snail *Nucella canaliculata* (Kuo & Sanford, 2009). Although latitudinal trends in critical thermal limits have not been studied in the gammarids, comparisons have been made between stenothermal and eurythermal populations. Timofeyev et al. (2009) studied thermal tolerances in two freshwater gammarid species occupying environments with differing levels of temperature variation. The species endemic to Lake Baikal, *Eulimnogammarus cyaneus*, demonstrated a lower tolerance to thermal stress than populations of *Gammarus lacustris* native to lakes in the vicinity which were shallower, and therefore not as effectively buffered against daily and seasonal fluctuations in temperature. In addition to intraspecific variations in thermal tolerances over a spatial scale, gammarids show temporal variations in susceptibility to thermal stress. The freshwater amphipod *Gammarus limnaeus* has been shown to demonstrate seasonal variation in thermal tolerances, exhibiting upper critical limits of 26 °C in winter, rising to 30-32°C in summer (Krog, 1954).

Small aquatic ectotherms, like the gammarids, are expected to show little variation between body and environmental temperature (Helmuth & Hofmann, 2001; Huey & Kingsolver, 1989), and therefore are highly susceptible to thermal stress. Despite this, behavioural mechanisms still exist for the regulation of body temperature and avoidance of thermal stress. The polar amphipod *Gammarus wilkitzkii* shows a lower critical temperature of around -4 °C, but despite this increase in tolerance to lower temperatures compared with more temperate gammarids it does not tolerate freezing into solid ice (Aarset & Aunaas, 1990). Although *G. wilkitzkii* is a dominant species in the Barents Sea ice community (Aarset & Aunaas, 1987), it is likely that individuals exhibit behavioural thermoregulation, showing seasonal migration with the advancing ice front to avoid

freezing. Although studies of intertidal snails (Kuo & Sanford, 2009) and *Drosophila* species (Huey et al., 1991) have indicated a genetic basis to differences in thermal tolerances among populations with latitude, studies have also observed interspecific variation in the plasticity of upper critical limits in response to temperature acclimation (Calosi et al., 2008; Stillman, 2003; Vernberg, 1959), it has even been suggested that there is a trade-off between the height of upper thermal limits and the plasticity of the trait (Stillman, 2003). Measures of thermal tolerance should therefore include both the breadth of the thermal window and the ability to adjust thermal limits. In addition to mechanisms controlling the ability to survive transient heat stress, measures of tolerance may include traits which impact long-term tolerance to temperature, such as growth, fecundity and costs of living. Currently it is unknown whether gammarid populations distributed along a latitudinal cline show variations in thermal tolerances and whether such changes are associated with differences in the acclimatory capacity of the trait.

The temperature coefficient (Q_{10}) describes the proportional increase in rate function over a 10 °C rise in temperature, and is used to measure the thermal sensitivity of a physiological rate process, such as metabolic rate determined indirectly as changes in rates of oxygen uptake. Metabolic rate is expected to double or triple over a 10 °C rise in temperature (Clarke & Fraser, 2004). Higher Q_{10} values than this indicate increased thermal sensitivity and may be associated with stenothermal species adapted to respond quickly to small variations in their environment (Pörtner et al., 2005), or a failure to acclimate (Clarke, 1983). The oxygen and capacity limitation hypothesis suggests critical thermal limits are determined by the demand and availability of oxygen (Frederich & Pörtner 2000), and therefore thermal limits may be shifted by adjustments of aerobic capacity (Pörtner et al., 2001). At optimal temperatures oxygen availability and therefore the scope for aerobic activity is at its maximum, but as temperatures rise or fall toward extremes (pejus temperatures) the partial pressure of oxygen in the haemolymph declines as aerobic scope reduces (Frederich & Pörtner 2000). At critical temperature thresholds the declining oxygen supply becomes insufficient to supply demand and a transition to anaerobic mitochondrial metabolism occurs (Sommer et al., 1997). Adjustment of aerobic capacity in the cold has been suggested to occur through increases in mitochondrial density and capacity (Johnston, 1982; Pörtner et al., 2000), while adjustment to high temperatures is achieved through a reduction in thermal sensitivity, as seen in the reduction in the Michaelis-Menten constant of cytosolic malate dehydrogenase in invertebrates inhabiting deep-sea hydrothermal vents (Dahlhoff & Somero, 1991).

1.2. Total energy demand

The metabolic cold adaptation hypothesis dates back as far as a prediction by Munro Fox (1939) that at a common acclimation temperature a higher latitude species will exhibit higher metabolic rates than their more southern counterparts. This is considered a necessary adaptation to meet the elevated costs of protein synthesis, growth and development during the shorter growing seasons at higher latitudes (Jose et al., 2009) and to compensate for reductions in locomotor ability (Clarke, 1991) and enzyme catalytic rates with reductions in habitat temperature (Powers et al., 1991). Metabolic rate may alter with temperature by one of two mechanisms (Clarke & Fraser, 2004). The first is a direct effect of temperature on metabolic rate, caused by increases in catalytic activity with temperature leading to higher ATP synthesis. The second is indirect, and a more likely explanation according to Clarke & Fraser (2004). This explanation suggests that basal metabolic rate is the result of trade-offs between enzyme structure and function (thermal sensitivity and activation energy) according to temperature (Clarke, 1993). A reduction in the Arrhenius activation energy of metabolism with latitude indicates alteration in the specific activity of metabolic enzymes and is considered a typical response to cold compensation (Johnston et al., 1975; Lucassen et al., 2006; Sommer & Portner, 2002).

Metabolic cold adaptation has been supported by several studies, including evidence from experimental populations of *Drosophila melanogaster* (Berrigan & Partridge, 1997), a global review of metabolic rates in insects (Addo-Bediako et al., 2002) and in latitudinally distinct populations of the lugworm *Arenicola marina* (Sommer & Pörtner, 2004). Dittman (1997) has shown latitudinal compensation in the oyster *Crassostrea virginica* has a genetic basis, but unfortunately few multigenerational studies such as this and the study by Berrigan & Partridge (1997) exist. Other authors have argued that it represents a fitness cost and so the metabolic cold adaptation hypothesis is still highly controversial. In addition, other mechanisms such as latitudinal variations in enzyme isoforms render metabolic cold adaptation unnecessary (Guderley & Johnston, 1996). Many recent studies have shown that when extrapolated or acclimated to common temperatures, higher latitude populations or species show reduced metabolic rates compared to their temperate counterparts (Pörtner et al., 2007). Although comparisons between Antarctic and temperate species are problematic due to their relatively high phylogenetic separation, the less isolated Arctic has still provided evidence contrary to the theory of metabolic cold adaptation, for example *Gammarus* populations across a latitudinal gradient (Rastrick &

Whiteley, 2011). The authors observed no evidence for compensation of metabolic rate in the circumpolar species *G. setosus*, and saw a decline in energy consumption of the subarctic/boreal species *G. oceanicus* with latitude. However two species with a more southerly range, *G. locusta* and *G. duebeni*, conserved relatively high metabolic rates. *G. oceanicus* exhibited a lack of metabolic diversity between populations, indicating phenotypic causes for differences in thermal tolerances between populations, in comparison to *G. locusta* which showed high metabolic diversity, suggesting local genetic adaptation. This supports earlier work by Bulnheim (1979) and Tedengren *et al.* (1988), which demonstrated *G. oceanicus* to be less metabolically tolerant to fluctuations in environmental conditions than *G. duebeni*, although Bulnheim (1979) did find *G. oceanicus* to be more metabolically resistant than *G. locusta*.

As opposed to previous studies, which have focused on polar species, studies on gammarids distributed along a temperate to sub-arctic gradient suggest the eurythermal nature of the temperate environment promotes conservation of metabolic rate. The stable, but harsher polar conditions select for a reduction in metabolic rate with temperature in response to the energy limited environment (Clarke, 1991, 1993). The experimental methodology of early studies in support of metabolic cold adaptation has come under heavy criticism, due to issues with unaccounted increases in metabolic rate from the stress of handling, and with the extrapolation of metabolic rates with temperature (Clarke, 1991). Although metabolic cold adaptation in polar populations has been largely disproved (Pörtner et al., 2007), comparisons between temperate species and populations have shown interesting compensatory trends with thermal gradient, and therefore local adaptation and phenotypic plasticity in temperate and sub-arctic organisms warrants further investigation.

In addition to the latitudinal range of an organism, seasonality and resource availability may also have an effect on the prevalence of metabolic cold adaptation. For example, Sokolova & Pörtner (2003) observed that sub-arctic populations of *Littorina saxatilis* exhibited higher metabolic rates than populations from lower latitudes, but they suggested that this plasticity was limited, and metabolic depression was still likely in the sub-arctic populations during the winter months. The authors attributed metabolic depression to resource limitation driving reductions in basal turnover and ionic work (Clarke, 1993). Seasonal increases in metabolic rate in gammarids have also be linked to indirect effects of temperature, such as oxygen content of the water (Krog, 1954) and the availability of food

(Wijnhoven et al., 2003). Age too has an effect on metabolic rates, with cold compensated metabolic rates in the mussel *Mytilus edulis* recorded only up to eight years, after which northern populations showed no significant difference in metabolic rate compared to more southerly populations (Sukhotin et al., 2006). This variation with age was attributed to the increased cost of living associated with growth and maturation of young individuals.

Citrate synthase is a key metabolic enzyme (Vetter, 1995a), responsible for regulating the citric acid cycle, and is often used as a proxy for mitochondrial volume and aerobic capacity (Berges & Ballantyne, 1991). Citrate synthase has been widely utilised to study long term acclimation of mitochondria, particularly in fish (St-Pierre et al., 1998), as upregulation of citrate synthase activity is likely to be indicative of changes in the properties and density of the mitochondria (Lucassen et al., 2003). The rate of ATP generation in mitochondria has been shown to decrease with latitude in ectotherms (Johnston et al., 1994), demonstrating the existence of constraints on compensatory increases in the aerobic capacity of mitochondria through structural changes. In teleost fish, this reduction in ATP generation at higher latitudes has been linked to reductions in enzyme activity in the cold (Guderley & Johnston, 1996), and to the need for an increase in unsaturated fatty acids to maintain mitochondrial membrane fluidity (Lurman et al., 2009), leading to increased proton leak (Brand, 1990).

To counteract the effect of low temperatures on aerobic capacity, ectotherms may exhibit several compensatory mechanisms: expression of enzyme isoforms as a means of increasing catalytic rates at low temperature (Guderley & Johnston, 1996); or modifications to the structure and abundance of the mitochondria in the cells (Clarke, 2003). Studies have shown polar species of fish to possess greater mitochondrial volume densities than their temperate counterparts (Archer & Johnston, 1991; Johnston & Dunn, 1987), with one of the highest mitochondrial volume densities in the vertebrate kingdom present in the aerobic muscles of Antarctic silverfish, *Pleuragramma antarcticum* (Johnston et al., 1998). Studies within a single species have also shown that acclimation to lower temperatures can result in an increase in the number of mitochondria per cell (Egginton & Johnston, 1984; Johnston & Maitland, 1980), resulting in partial metabolic rate compensation.

Although increased mitochondrial density with latitude has been well documented in the teleosts, few studies have been conducted into mitochondrial abundance in marine invertebrates. In recent years, studies have shown higher mitochondrial densities in

brachiopod species from the Antarctic compared to temperate congeners (Lurman et al., 2009), in latitudinally distinct populations of the lugworm *Arenicola marina* (Sommer & Portner, 2002) and the mussel *Mytilus edulis* in association with an increase in metabolic rate (Sukhotin et al., 2006). In the Antarctic limpet *Nacella concinna*, cold acclimation resulted in an increase in mitochondrial cristae surface density, but no change in mitochondrial volume density (Morley et al., 2009). This does not necessarily contradict previous work, as Lurman et al. (2009) found that although intraspecific variation between the temperate and polar brachiopods was marked by alterations in abundance of mitochondria, seasonal acclimatisation in *Liothyrella neozelanica* was marked by surface density change. Increasing the surface density of mitochondrial cristae may provide compensation without the costs of mitochondrial synthesis (Lurman et al., 2009), but may result in an increase in the basal metabolic rate (Guderley & Johnston, 1996).

An increase in abundance of mitochondria has been observed in *G. oceanicus* gill cells following a few weeks acclimation to reduced salinity, in conjunction with an alteration in their conformation and an increase in their proximity to the plasma membrane (Milne & Ellis, 1973). This evidence that *G. oceanicus* exhibits plasticity in the abundance, conformation and placement of mitochondria within the gills in response to changes in salinity (and therefore the rate of sodium transport across the gill) suggests that similar mitochondrial plasticity may be possible in response to temperature change. Although it is yet to be formally investigated, it does appear that mitochondrial densities increase with latitude in *G. locusta* (Nia Whiteley, unpublished data).

1.3. Protein synthesis and associated variables

Protein synthesis is a costly process (Storch & Pörtner, 2003), which serves to synthesise new proteins for growth as well as the turnover and repair of existing proteins (Waterlow, 1995). Protein synthesis is represented by the continuous synthesis and degradation of proteins. As with other biological processes, temperature has a strong direct influence on rates of protein synthesis, but also an indirect influence through the correlation of protein synthesis with other temperature-dependent factors such as food consumption rates (McCarthy et al., 1993). During short-term exposure to rising temperatures protein synthesis rate is expected to increase until an optimal value is reached, due to the effect of temperature on RNA activity levels (Intanai et al., 2009; Robertson et al., 2001; Whiteley &

Faulkner, 2005). The correlation between temperature and protein synthesis rates has been demonstrated by intraspecific studies into the effect of temperature acclimation, for example in the Atlantic wolfish *Anarchichas lupus* (McCarthy et al., 1999).

Protein synthesis has been linked to thermal tolerance and acclimatory ability in ectothermic organisms. A loss of capacity for temperature acclimation has been observed in the carp, *Cyprinus carpio*, following starvation and the resultant net protein loss (Gerlach et al., 1990), and in the European bitterling, *Rhodeus amarus*, following treatment with the protein synthesis inhibitor actinomycin D (Kunnemann, 1973). High rates of protein turnover may be associated with a broad thermal tolerance and ability to rapidly respond to environmental change (Koehn & Bayne, 1989) as a less stable protein pool may favour replacement and renewal, however high rates are also energetically expensive. Hawkins et al. (1987) observed that mussels, *Mytilus edulis*, with high rates of turnover were characterised by greater temperature sensitivity and reduced survival to acute thermal stress. The energy demand associated with high rates of protein turnover may prove too costly when temperatures deviate from the optimal, therefore resulting in a species with high rates of turnover exhibiting a greater variability in physiological performance with temperature (Hawkins, 1991).

Unlike the theory of metabolic cold adaptation, cold-adapted ectotherms are presumed to have lower rates of protein synthesis than their temperate counterparts (Fraser & Rogers, 2007) due to a smaller energy budget resulting from thermal (Pörtner, 2001) or resource (Fraser et al., 2002) limitations at higher latitudes. This has been supported by a recent observation of a lower rates of protein synthesis in a high latitude population of subarctic/boreal gammarid amphipod *Gammarus oceanicus* in comparison to more southerly populations. However, another species, the temperate *G. locusta*, did exhibit compensation of protein synthesis rates between populations (Rastrick & Whiteley, 2013). The cost of protein synthesis in isopods has been shown to be four times higher in the Antarctic species *Glyptonotus antarcticus* compared to the temperate species *Idotea rescale*, resulting in lower protein synthesis rates in the polar species (Whiteley et al., 1996). A later study by Whiteley & Faulkner (2005) observed that the temperate isopod *Lignia oceanica* exhibited similar rates of protein synthesis to *G. antarcticus*, with no correlation between synthesis rates and temperature. This may indicate the ability of temperate crustaceans to show environmental canalisation of protein synthesis rates, to prevent a net loss of protein due to low turnover rates at low temperatures or to limit

energy requirements at high temperatures (Whiteley & Faulkner 2005). This ability to regulate the rate of protein synthesis may not be present in stenothermal ectotherms, and as with metabolic rate, the ability to show compensation of protein synthesis may be restricted to temperate species and populations. Though costly, conservation of protein synthesis rates in temperate species and populations from different thermal regimes (Fraser et al., 2002; Fraser & Rogers, 2007; Marsh et al., 2001), may be an adaptive strategy to conserve growth rates or a necessary expense to counter high rates of protein degradation in the cold, as indicated by high levels of ubiquitin-conjugated proteins in Antarctic notothenioid fish (Place & Hofmann, 2005; Place et al., 2004). Relatively high rates of protein synthesis rates in cold-adapted species or populations may be achieved by upregulating RNA concentrations to compensate for low RNA activities in the cold (Foster et al., 1992; Fraser et al., 2002; McCarthy et al., 1999; Robertson et al., 2001; Storch et al., 2003). However RNA synthesis is also energetically expensive (Fraser et al., 2002) unless there is an increase in RNA stability (Storch et al., 2003; Storch et al., 2005).

1.4. Growth, protein synthesis retention efficiency, and body size

Although growth rates in marine invertebrates have been shown to have a genetic basis, high growth rates are also achieved through a combination of high rates of feeding, reduced basal metabolic rates and low metabolic costs of growth (Bayne, 2004), and by increasing the capacity for growth through a negative relationship between growth efficiency and temperature (Hawkins & Day, 1996; Heilmayer et al., 2004). The cost of protein synthesis may increase with decreasing temperatures, impacting growth rates in polar environments (Pannevis & Houlihan, 1992), but cold-adapted species may compensate for a smaller energy budget by diverting available energy in favour of growth, to the detriment of other costly processes such as reproduction (Childress et al., 1980). Studies of growth in benthic amphipods of the family Ampeliscidae have found moult frequencies increased with increasing temperature, regardless of the rate of growth, reaching maturity after a fixed number of moults and therefore a lower size (Highsmith & Coyle, 1991). In crustaceans such as the gammarids, growth rate is thought to be constrained by moult frequency (Hauton et al., 2009), therefore a reduced growth rate in polar crustaceans may be due to a combination between a reduced rate of protein synthesis, and the constraints on growth resulting from an extended period between moults. Increases in growth rates with temperature have been recorded in *Gammarus*

locusta (Neuparth et al., 2002*;* Table 1.1.) and *G. salinus* (Skadsheim, 1989), and the Arctic *G. wilkitzkii* exhibits the low growth rates expected to occur in polar organisms when compared to temperate congeners (Poltermann, 2000). *G. salinus* was also associated with a smaller size at maturity (Skadsheim, 1989), although moult frequency was not measured.

When fed a protein rich diet, *Gammarus lawrencianus* exhibits higher rates of growth and matures earlier and at a smaller size than when fed on algae alone (Vassallo & Steele, 1980). This increase in growth rate was, however, associated with a decrease in longevity, and as the growth rate slowed after maturation no significant differences in the maximum body size reached by mature adults fed different diets. The increased costs associated with elevated growth rates may be responsible for the trends in mortality rates seen during this study, and although diet may affect juvenile growth rates, diet and growth rate were not seen to affect maximum size after maturity. This suggests body size has a genetic basis or is controlled by longevity, while growth rate is linked to the trade-off between the costs of protein synthesis for growth and the costs of living. One such trade-off has been recorded in bivalve molluscs between growth in protein and in lipid storage utilised during reproduction (Bayne, 2004). At higher latitudes, slower growth rates may therefore allow for an increase in reproductive rate, providing a higher overall fitness. Across a latitudinal gradient, species are therefore likely to optimise growth rate, rather than maximise it at the cost of reproductive fitness, resulting in plasticity of growth rates with respect to latitude.

Despite these findings, some species appear to show the opposite trend. Increases in growth rate with decreasing culture temperature have been documented in *Drosophila melanogaster* (James & Partridge, 1995). Although this appears counterintuitive given the proposed increase in the cost of protein synthesis, and therefore growth, with latitude (Whiteley et al., 1996), there is a strong adaptive explanation for maximising protein synthesis rates during the short growing season of the Arctic, when temperatures are higher and food resources more abundant. In addition to this, it has been suggested that the cost of protein synthesis is determined by both the temperature and the rate itself, with higher rates of protein synthesis proving less costly (Pannevis & Houlihan, 1992). This would suggest that organisms exhibiting high levels of protein synthesis at high temperatures are operating the most efficiently, and may help to mitigate the increased costs of protein synthesis at low temperatures. However, the suggestion that metabolic costs vary with rates of protein synthesis has been disputed and several studies have

recently demonstrated that costs remain unchanged (Bowgen et al., 2007; Whiteley & Fraser, 2005).

Bergmann (1847) coined a theory that states that endothermic species inhabiting a colder thermal environment tend to have a larger body size than species inhabiting warmer environments, due to the requirement for a smaller surface area to volume ratio to conserve heat at high latitudes. Although the extension of this rule to ectotherms by Ray (1960) has been supported by many studies of, mostly terrestrial, invertebrates (Arnett & Gotelli, 1999; Atkinson & Sibly, 1997; Partridge & Coyne, 1997; Van Voorhies, 1996), some authors have proposed that ectotherms reverse the trend (Blanckenhorn & Demont, 2004; Mousseau, 1997). A third theory, countergradient variation, states that body size is maintained throughout the latitudinal gradient due the primary influence of genetic factors (Conover & Schultz, 1995).

It has been argued that ectotherms should not demonstrate Bergmann's rule, as small species of marine invertebrates would be expected to show no difference between ambient and body temperatures (Helmuth & Hofmann, 2001). Instead Bergmann's rule in ectotherms may represent negative selection on body size at high temperature, due to factors such as reduced energy available for growth at high temperatures or the risk of hypoxia associated with increased body or cell size (Blanckenhorn & Demont, 2004), rather than the positive selection on body size at low temperatures. An alternative suggestion is that as cell size increases with latitude, total body size increases in accordance (Van Voorhies, 1996, 1997). The argument for the converse Bergmann rule is based on the slower growth rates and constrained growing seasons mentioned earlier in this introduction, and has been shown to have a genetic component in terrestrial invertebrates (Mousseau, 1997). Countergradient variation is based upon similar grounds, and it too possesses a genetic element (Conover & Schultz, 1995). This is related to the outcomes of the study by Vassallo & Steele (1980) mentioned earlier, in which faster growth rates were counteracted by a shorter period of growth. This was due to reduced longevity and reductions in growth after maturity causing convergence of adult size, but in polar species this may also be due to the discrepancy between the high growth rates of the summer season and low growth rates in winter compared to the constant intermediate rate of growth of a temperate species. Due to the differences in mechanisms underlying each theory, Blanckenhorn & Demont (2004) have suggested that despite the controversy the three hypotheses are not mutually exclusive. The trend in body size over a latitudinal

gradient may depend on the species studied and on the interplay of selection pressures such as those associated with body size and fecundity.

Table 1.1 shows that in the three *Gammarus* species reviewed, variation in body length shows interspecific variation and a few studies have suggested intraspecific variation exists in body size with latitude (e.g. *G. oceanicus* and *G. locusta* in Rastrick & Whiteley, 2013). Some of the lack of increase in body length seen in the table may be due to differences in sampling periods for each study, and therefore variation in the age of individuals sampled. Given the apparent slower rate of growth in *Gammarus* species from high latitudes (Table 1.1), it seems paradoxical that these organisms would exhibit a larger body size. Larger body size at high latitudes may instead be linked to the longevity of the species or population. *Gammarus wilkitzkii*, an Arctic species, is up to 50% greater in maximum body length than the temperate *G. oceanicus*, but has a maximum age of around five years, twice that of *G. oceanicus* (Poltermann, 2000), and *G. oceanicus* appears to show an increase in longevity with decreasing environmental temperature (Table 1.2.). This larger size may therefore be simply a function of a longer period for growth after maturation, or a result of the delayed maturation at lower temperatures (Angilletta, 2004) seen in *G. locusta* (Table 1.2; Neuparth et al., 2002). Although metabolic rate depression is thought to result in reduced rates of protein synthesis and therefore growth, a reduction in metabolism may support a larger body size by increasing longevity. It has been shown that an overall reduction in metabolic rate decreases the rate of superoxide anion radical generation, reducing oxidative damage and prolonging the life span of an organism (Van Voorhies, 2002). The low metabolic rate of *G. wilkitzkii* compared to temperate congeners (Werner et al., 2002) may therefore be an underlying cause of its large body size.

The mechanisms for the adaptive nature of plasticity of body size with latitude outlined above are simplistic in that they focus on the effects of body size on one aspect of fitness. In the gammarids, the link between temperature and body size may be controlled by a number of factors, including the limits to maximum body size at temperature (Kingsolver, 2009), the effect of temperature on growth rate from embryo to mature adult (Neuparth et al., 2002), increases in brood size and therefore fecundity with size, and the associated effects of latitude and size on juvenile mortality rates (Skadsheim, 1984). Factors other than temperature which show variation over the latitudinal gradient, such as food availability, photoperiod or predation, may also exert an effect over body size.

Table 1.1. Body size and growth rates of *Gammarus* species according to average holding temperature. Location indicates the latitude (°N) and country or continent of origin for each species studied (CA = Canada, DK = Denmark, EUR = Europe, NO = Norway, PL = Poland, PT = Portugal, SE = Sweden, UK = United Kingdom, US = United States of America). Temperatures during rearing experiments (RT) are given in °C, as are mean winter and summer temperatures (T, °C) for field populations (NOAA OI SST V2 Data for all references). Values are given for mean and maximum female body length (FL) in mm, weight (W) in mg, and growth rate (GR) in mm.week^{-1} according to sex where specified. F represents females and M, males.

Species	Location	T		FL		W	GR		Reference
		W	S	mean	max		F	M	
G. duebeni	51 UK	9	13.5	-	-	24.3	-		Dunn & McCabe (1995)
	52 UK	9	13.5	-	15	-	-		Sheader (1983)
	53 UK	15-20 RT		10.5	-	-	~0.2		Hynes (1954)
	56 UK	15 RT		10.2	-	-	0.65		Naylor et al. (1988)
	57 DK	6	13.5	-	15	-	-		Kolding & Fenchel (1979)
	47 CA	1	12	14	18	-	-		Steele & Steele (1969)
G. locusta	38 PT	20 RT		10.2	-	-	1.31	1.66	Neuparth et al. (2002)
	38 PT	15 RT		99	-	-	0.94	1.23	Neuparth et al. (2002)
	38 PT	15	18	-	-	33	-	-	Rastrick & Whiteley (2011)
	38 PT	15	18	8.6	15.7	-	-	-	Costa & Costa (1999)
	43-52 EUR	12	18	≈10	-	-	-	-	Kolding & Fenchel (1981)
	50 UK	10	13.5	-	-	-	-	-	Hauton et al. (2009)
	52 UK	9	13.5	11	20	-	-	-	Spooner (1947)
	53 UK	8	13.5	-	-	92	-	-	Rastrick & Whiteley (2011)
	41 US	7	19	6.4	13.2	-	-	-	Crozier & Snyder (1923)
	57 DK	6	13.5	≈10	15	-	-	-	Kolding & Fenchel (1979, 1981)
	59 SE	3	13.5	≈10	-	-	-	-	Kolding & Fenchel (1981)
G. oceanicus	47-58 CA	12 RT		-	22	-	-	-	Steele & Steele (1972)
	59 NO	10 RT		15.7	19.7	-	-	-	Skadsheim (1989)
	58 UK	8	12	-	-	81	-	-	Rastrick & Whiteley (2011)
	41 US	7	19	18-23		110-220	-	-	Werntz (1961)
	42 US	6	16	14.1	19	-	-	-	Croker & Gable (1977)
	57 DK	6	13.5	≈15	-	-	-	-	Kolding & Fenchel (1981)
	57 DK	6	13.5	-	23	-	-	-	Kolding & Fenchel (1979)
	47-58 CA	3 RT		-	25	-	-	-	Steele & Steele (1972)
	59 SE	3	13.5	≈15	-	-	-	-	Kolding & Fenchel (1981)
	47 CA	1	12	≈15	22	-	-	-	Steele & Steele (1972)
	79 NO	1	3	-	-	166	-	-	Rastrick & Whiteley (2011)
	79 NO	1	3	26.4	32.5	331	-	-	Węsławski & Legezynska (2002)
	58 CA	-1	1.5	≈20	28	-	-	-	Steele & Steele (1972)
G. setosus	47 CA	1	12	26	30	287	-	-	Steele & Steele (1970)
	58 CA	-1	1.5	18	23	-	-	-	Steele & Steele (1970)

1.5. Life history strategies

Gammarid crustaceans exhibit a range of life history strategies, allowing them to tolerate and prosper in environments with a variety of thermal regimes. These life history strategies include variations in the number and size of broods per year, the duration of embryonic development and the size of eggs and hatched juveniles (Sheader, 1996). It is expected that gammarids will compensate for adverse conditions at high latitudes or during winter breeding by increasing the allocation of resources to offspring (Dunn & McCabe, 1995). This has been supported by several studies in the gammarids which have demonstrated a significant negative relationship between egg size and temperature (*G. duebeni*, Dunn & McCabe, 1995; *G. insensibilis*, Sheader, 1996; *G. lacustris*, Wilhelm & Schindler, 2000; *G. locusta*, Kolding & Fenchel, 1981; *G. oceanicus*, Kolding & Fenchel 1981, Skadsheim, 1989), independent of the effects of female size or the number of eggs produced per brood (Wilhelm & Schindler, 2000). A larger egg size extends the brooding period (Sheader, 1996), which is characterised by maternal control over the thermal environment of the embryos, and results in larger juveniles, reducing juvenile mortality rates at low temperatures and the time to maturity (Skadsheim, 1984).

The ability to exhibit these plastic responses in egg size with temperature has shown to vary with latitude. Although southern populations of *G. duebeni* express seasonal variation in egg size, this plasticity is lacking in northern populations, which show a constant response to the colder temperatures at higher latitudes (Dunn & McCabe, 1995). The response of egg size to season may however show a greater effect of interspecific genetic difference, rather than phenotypic plasticity within populations, as northern populations of *G. insensibillis* have a larger relative variation in egg size between season than populations of *G. duebeni* at the same latitude, but at the southernmost edge of the species' range (Sheader, 1996). Although egg size has been linked to temperature, evidence suggests that brood size is controlled by the energy available for reproduction (Skadsheim, 1989). Table 1.2 shows little evidence of the expected trend of decreasing brood sizes with latitude corresponding to the increase in cost of living discussed earlier, however there has been little study of latitudinal differences in brood size within a species, and discrepancies in methods of measuring brood sizes may result in the effects of early juvenile mortality confounding the results.

Table 1.2. Reproductive outputs of *Gammarus* species from populations inhabiting coastal sites fringing the Atlantic and Arctic Oceans, arranged according to mean habitat temperature. Location indicates the latitude (°N) and country where the population originated (see Table 1.1. for an explanation). Mean winter and summer temperatures (T, °C) are given for each population (NOAA OI SST V2 Data for all references). For gammarids reared in the laboratory, rearing temperatures (RT) are given. Life history measurements are the size at maturity (MS, mm) and time to maturity (MT, weeks). For females, generation time (GT, weeks), brood size (BS, nº juveniles released), egg volume (ES, mm3), broods per year (B/yr, broods female-1 year-1), female life span (LS, months), maximum broods produced over life span (Bmax, for winter and summer breeders if applicable) are also given.

Species	Location	T		MS	MT	BS	ES	B/yr	LS	B_{max}	Ref[1]
		W	S								
G. duebeni	51 UK	9	13.5	-	-	24.3	-	-	-	-	1
	41 US	9	13.5	-	-	9.9	-	-	-	-	2
	52 UK	9	13.5	8.7	25.7	-	0.1	-	12	-	3
	53 UK	8	13.5	-	-	18.1	-	-	-	-	4
	54 UK	8	13.5	9	31	21	-	-	-	-	5
	54 DE	8	13.5	12	-	36.5	-	-	-	-	6
	54 PL	8	13.5	-	-	40.5	-	-	-	-	7
	56 UK	8	12	-	-	25	0.7	-	-	-	8
	56 UK	8	12	8	21.4	23.8	-	-	-	-	9
	57 DK	6	13.5	12	-	15.3	0.67	1.8	12	-	10, 11
	59 SE	3	13.5	12	-	17.0	0.82	2.7	12	-	10, 11
	47 CA	1	12	11	-	25.4	0.25	5	12	5	12
G. locusta	38 PT	20 (RT)		-	5	35.44	-	-	-	-	13
	38 PT	15 (RT)		-	7	33.16	-	-	-	-	13
	38 PT	15	18	7	-	41.1	-	-	-	-	14
	43-52	12	18	-	-	65.4	0.25	-	-	-	11
	50 UK	10	13.5	-	-	-	-	-	-	-	15
	52 UK	9	13.5	8	-	62.1	0.36	-	-	-	16
	57 DK	6	13.5	7	>9	41	0.26	-	-	3.8-7.8	11
	59 SE	3	13.5	-	-	36.3	0.37	-	-	3.8-7.8	11
G. oceanicus	59 NO	20 (RT)		-	-	-	0.47	-	-	-	17
	47-58 CA	12 (RT)		12	33	-	-	1-3	-	-	18
	59 NO	10 (RT)		-	-	-	0.52	-	-	-	17
	59 NO	7	12	11.4	-	-	-	-	15	-	19
	42 US	6	16	-	-	38.7	-	-	-	-	20
	57 DK	6	13.5	-	-	27.3	0.76	-	-	-	11
	57 DK	6	13.5	10	-	-	-	1	15	-	10
	47-58 CA	3 (RT)		12	47	-	-	1-2	-	4	18
	59 SE	3	13.5	-	-	29.3	0.81	-	-	-	11
	47 CA	1	12	≈ 11	-	46.5	-	1-3	36	-	18
	79 NO	1	3	18	-	78	-	1	-	-	21
	58 CA	-1	1.5	≈ 13	-	-	-	-	<48	-	18

Table 1.2. continued

Species	Location	T		MS	MT	BS	ES	B/yr	LS	B_{max}	Ref[1]
		W	S								
G. setosus	47 CA	12 (RT)		-	-	-	-	>1	-	-	22
	47 CA	10 (RT,		-	-	-	-	>1	-	-	23
	47 CA	10 (RT, NL)		-	-	-	-	1	-	-	23
	47 CA	3 (RT)		12	49	-	-	1	-	-	22
	47 CA	1	12	-	-	-	-	1	-	-	24
	47 CA	1	12	-	-	25	-	1	-	3	22
	79 NO	1	3	19	-	70	-	1	>36	-	21
	58 CA	-1	1.5	-	-	-	-	1	-	-	24
	58 CA	-1	1.5	-	-	67	-	1	-	-	22
	64 CA	-1	1.5	-	-	-	-	1	-	-	24
	67 +	-1		-	-	-	-	1	-	-	23
	75 CA	-1	1.5	-	-	-	-	1	-	-	24

[1]References: 1) Dunn & McCabe, 1995; 2) Ginn et al., 1976; 3) Sheader, 1983; 4) Cheng, 1942; 5) Hynes, 1954; 6) Kinne, 1953, from Sainte-marie, 1991; 7) Jazdzewski, 1973; 8) McCabe and Dunn, 1994; 9) Naylor et al., 1988; 10) Kolding & Fenchel, 1979; 11) Kolding & Fenchel, 1981; 12) Steele & Steele, 1969; 13) Neuparth et al., 2002; 14) Costa & Costa, 1999; 15) Hauton et al., 2009; 16) Spooner, 1947; 17) Skadsheim, 1989; 18) Steele & Steele, 1972; 19) Skadsheim, 1984; 20) Croker & Gable, 1977; 21) Węsławski & Legezynska, 2002; 22) Steele & Steele, 1970; 23) Steele & Steele, 1986; 24) Steele et al., 1977

1.6. Salinity tolerance and osmoregulation

The intertidal is characterised not only by a high degree of temporal and spatial variation in temperature, but also in salinity. As with temperature, salinity is a key environmental variable in determining the distribution of estuarine and intertidal species. Within the intertidal, species differences in salinity tolerance are likely to vary according to the niche inhabited by an organism. In gammarid amphipods for example, low shore species such as *G. locusta* and *G. oceanicus* are expected to experience little variation in salinity from full strength seawater for relatively short periods of time at low tide by the diluting effect of freshwater run off or precipitation or the concentrating effect of evaporation, whilst for high shore species such as *G. duebeni*, more frequently found in estuarine habitats and in freshwater runoff, fluctuations in salinity are expected to be more rapid and extreme (Bolt, 1983). Euryhaline organisms therefore require flexibility in their regulatory responses as ambient salinities show high temporal and spatial variation.

The regulation of internal ionic concentrations serves to buffer osmotic stress; therefore salinity tolerance is in part determined by the capacity for this regulation (Sornom et al., 2010). High haemolymph osmolality as a result of a failure to osmoregulate at high salinities can result in mortality (Hart et al., 1991) ultimately because of the problems associated with cell volume regulation (in this case cell shrinkage) (Whiteley et al., 2001). Similarly exposure to dilute seawater can cause the opposite problem, with the passive uptake of water along an osmotic gradient leading to cell swelling. Temperature and salinity have also been shown to have a combined effect on mortality. In the gammarids *G. tigrinus* and *G. pulex* for example, changing ion levels in brook water have been shown to affect thermal tolerances (Wijnhoven et al., 2003).

Acclimation to salinity change may occur via physiological adjustments of gill ultrastructure, changes in permeability of the general body surface, changes in urine production, mobilisation of small molecular weight osmolytes, or by changes in gill Na^+/K^+-ATPase activities (Brooks &Lloyd Mills, 2006; Freire & McNamara, 1995; Henry et al., 2012; Milne & Ellis 1973). In osmoregulators exposed to dilute seawater, the passive diffusive loss of Na^+ ions to the environment is compensated by an increase in gill Na^+/K^+-ATPase activities and is usually associated with an increase in the number of epithelial cells associated with active ion transport as wells as by increased urine production (e.g. Henry et al., 2012; Siebers et al., 1982). In addition to these physiological adjustments organisms may show behavioural avoidance, for example by shell valve closure in the sessile blue mussel (*Mytilus edulis*; Riisgård et al., 2012), or, in the case of mobile organisms such as the gammarid amphipods, by actively avoiding areas of extreme salinity levels.

Species may show variation in their iso-osmotic point and window of ionoregulation according to current or historical habitat conditions. Estuarine crustaceans such as prawns of the Macrobrachium genus, which are recent colonisers of freshwater, tolerate low salinities by maintaining high haemolymph osmolarities in freshwater (Freire et al., 2003; Ordiano et al., 2005). The iso-osmotic point of the estuarine shrimp *Crangon crangon* (Weber & Spaargaren, 1970) and prawn *Palaemon langirastris* (Campbell & Jones, 1989) shifts according to temperature as an adaptive response to migration from areas of low salinity in the summer to high salinity in the winter. More efficient osmoregulation at higher temperatures may confer an advantage of an increased tolerance to low salinity through the ability to maintain higher haemolymph osmolarities (Campbell & Jones, 1989).

The euryhaline amphipod *G. duebeni* is capable of hyperosmotic regulation at low salinities, however when exposed to full strength seawater *G. duebeni* is unable to ionoregulate and becomes an osmoconformer (Brooks &Lloyd Mills, 2006; Tedengren et al., 1988). This osmoregulatory pattern appears however not to apply to all life stages in *G. duebeni*. There is evidence late stage (stages 5 to 7) *G. duebeni* embryos follow a pattern of hyper-hypo-regulation in comparison to the hyper-isosmotic osmoregulation demonstrated by adults (Morritt & Spicer, 1995). Species differences exist within the gammarid amphipods according to the salinity of their natural habitat. An investigation by Werntz (1961) into the relationship between the osmotic concentration of the blood and that of the external medium found the marine species of gammarids studied (*Marinogammarus finmarchium* and *G. oceanicus*) regulated their blood concentration at a higher level than the brackish *G. tigrinus*, which in turn exhibited a higher level than the freshwater *G. fasciatus*.

An interaction between temperature and salinity tolerance in gammarid amphipods has been suggested by Dorgelo (1974), however interspecific differences exist in the nature of this interaction. *Gammarus tigrinus* exhibits a greater tolerance to higher salinities at high temperatures, whilst it is suggested that low temperatures enhance tolerance to high salinities in *G. fossarum*, however interactions were slight and confined only to exposure to extreme conditions outside of expected habitat values for temperature or salinity (Dorgelo, 1974).High salinity acclimation in the copepod *Tigriopus brevicornus* results in an extension of its thermal window (Damgaard & Davenport, 1994). Although the mechanisms for higher heat tolerance at high salinities is unclear, it can be suggested to be an adaptive response given that in the intertidal high temperatures and high salinities are stressors which often co-exist. The increase in cold tolerance following acclimation to high salinities may afford the advantage of lowering the melting point of the body fluids and therefore avoiding freezing during ice formation. This response may be seen in cold water amphipods, for example at temperatures of 1 to 2 °C and salinities above 34 psu, the Arctic gammarid *G. wilkitzkii* osmoconforms to the ambient salinity (Aarset & Aunaas, 1987).

Osmoregulation is one of the most important regulatory functions (Morritt & Spicer, 1995), but is also one of the more costly. Osmoregulation is thought to account for 11-21 % of the energy budget in *G. pulex* (Sutcliffe, 1984). Cost estimates for maintenance of Na+/K+-ATPase mediated transport have not been estimated in gammaridean amphipods and show too much variation in other species to generalise. Maintenance of Na+/K+-ATPase mediated transport has been estimated to account for 2.8 % of energy expenditure in

isolated hepatocytes in the rainbow trout Oncorhynchus mykiss (Pannevis & Houlihan, 1992) and 40% of metabolic rate in the sea urchins Strongylocentratus purpuratus and Lytechinus pictus (Leong & Manahan, 1997). Osmoregulation has further costs associated with protein synthesis, as a result of the production of non-essential amino acids used by invertebrates, including crustaceans, as small organic osmolytes for cell volume control during hyper-osmotic exposure (Gilles, 1980). In the blue mussel, *Mytilus edulis*, an increase in the intracellular concentration of ninhydrin-positive substances (primarily free amino acids) during hyperosmotic exposure serves to adjust osmotic pressure and regulate cell volume (Hawkins & Hilbish, 1992). When exposed to dilute seawater the euryhaline crab *Eriocheir sinensis* increases production and efflux of amino acids from the tissues (Whiteley et al., 2001). Despite this, Itanai et al. (2009) observed no effect of salinity on rates of protein synthesis in the prawn *Macrobrachium rosebergii*, but there are very few studies on the effect of salinity on rates of protein synthesis and the relationship between the two requires further investigation.

The energetic costs of osmoregulation are expected to be at their lowest during exposure to a salinity that is iso-osmotic with the haemolymph (Intanai et al, 2009; Wang et al., 2004). The arctic amphipod *Onisimus glacialis* exhibits a three-fold increase in oxygen consumption following transfer from full strength seawater to dilute seawater with a salinity of 5 psu (Aarset & Aunaas, 1990). Similarly, intraspecific differences in talitroidean amphipods exposed to hyposaline conditions have shown the species with a greater tolerance of low salinity conditions, *Talitrus saltator*, exhibits an increase in heart rate, while *Talorchestia ugolinii* exhibits a significantly lower heart rate and reduced survival (Calosi et al., 2007). Brook and Lloyd Mills (2006) observed a decrease in Na+/K+-ATPase activities with increasing salinity after the iso-osmotic point (approximately 20 psu) in *G. duebeni*, although this increase was not significantly different from the value observed at 10 psu. The ability to vary Na/K-ATPase activity levels appears to be higher in euryhaline than freshwater species of gammarids (Brooks &Lloyd Mills, 2003).

Although osmoregulation is an effective strategy to tolerate high salinities in the short term, over a longer term the energy expenditure involved with osmoregulation and the maintenance of ion homeostasis could prove too costly a process to maintain. An organism which is able to osmoregulate during short term fluctuations in salinity may therefore behave as an osmoconformer when subjected to longer term exposure to a high salinity. In addition to potential effects on physiological processes, acclimation to high salinities may

also affect life history traits. A review by Sainte-Marie (1991) of life history traits in gammaridean amphipods according to habitat found a tendency toward r-selected life history traits such as a greater reproductive potential and potential fecundity but a shorter lifespan in brackish water species compared to species from either freshwater or marine habitats. No evidence was found for variation in life history according to salinity within the Gammaroidea superfamily, however the number of observations was low. Salinity may however have an effect at the intraspecific level, for example the reduced viability at low salinity of *Echinogammarus marinus* embryos (Egilsdottir et al., 2009; Vlasblom & Bolier, 1971) and *G. locusta* embryos and juveniles (Neuparth et al., 2002). Low viability of embryos during low salinity exposure may be due to the egg swelling as the extra-embryonic fluid rapidly becomes isotonic (Steele and Steele, 1991). Vlasblom & Bolier (1971) suggest optimal salinity range for egg production in *E. marinus* is narrower than that for other physiological functions; however a study by Maranhão and Marques (2003) found no measurable effect of salinity on fecundity or the duration of embryonic development in the species. The effects of low salinity on embryo and juvenile mortality may be mitigated by maternal effects. The crab *Chasmagnathus granulatus,* for example, shows an increase in larval mortality at low salinities, correlated with a lower biomass at hatching (Giménez, 2006). To counter the greater loss of biomass during embryogenesis, females exposed to lower salinities produce larger eggs.

1.7. Gammarid amphipods

The *Gammarus* genus comprises 204 species (Väinölä et al., 2008). The majority of species are freshwater, however *Gammarus* are also found in estuarine and marine habitats. Amphipods in the genus *Gammarus* are typically described as shredders (Gerhardt et al., 2011), but may also function as detritivores (Walter, 1980), herbivores (Kolding & Fenchel, 1981), predators (Macneil et al., 1997) and cannibals (Andersson et al., 2009; Dick et al., 1993; Wilhelm & Schindler, 2000). In addition to this plastic feeding behaviour, they are an important ecosystem constituent as prey for terrestrial and aquatic predators and are often found in dense “swarms” (Costa et al., 2009; Weslawski et al., 1994). Gammarid amphipods are frequently employed as the subject of ecotoxicology (Brooks & Lloyd Mills, 2003; Lieb & Carline, 2000; MCAahon & Pascoe, 1988; Neuparth et al., 2002; Wallace & Estephan, 2004) and parasitology (Ponton et al., 2005, 2009; Wilkinson et al., 2011) studies, and more recently in studies relating to range size, environmental tolerance and distribution along

thermal gradients (Gaston & Spicer, 2001; Rastrick & Whiteley, 2011; Rastrick & Whiteley, 2013). This thesis focuses on four species from the *Gammarus* genus, with an established phylogeny (Costa et al., 2009), the circumpolar *G. setosus*, the subarctic/boreal *G. oceanicus*, the boreal/temperate *G. duebeni duebeni* and the temperate *G. locusta*. Three of the species are marine and found in the low intertidal, the last, *G. d. duebeni*, is a euryhaline species commonly found in the high intertidal.

G. d. duebeni is the marine haplotype of the species (Rock, 2007) and was chosen for this study due to its wide tolerance of temperature and salinity (Gaston & Spicer, 2001; Rock et al., 2007). *G. d. duebeni* is tolerant of both hypo and hyper-saline water, but has an iso-osmotic point around a salinity of 20 psu (Morritt & Spicer, 1995) and is often seen inhabiting areas of the shore with a freshwater influence (Ironside et al., 2003). *G. d. duebeni* inhabits a large range across both sides of the Atlantic, and is distributed within Europe from the English Channel to Northern Norway (Gaston & Spicer, 2001). Two populations of *G. d. duebeni* are utilised in this study from near the southern (Wales, 53 °N) and northern (Norway, 70 °N) edges of its range. In Wales, *G. d duebeni* will be compared with the lower shore species *G. locusta*, which has a more southerly distribution (from southern Spain to northern Norway, (Gaston & Spicer, 2001) and a lower salinity tolerance, preferring higher salinities (Kolding & Fenchel, 1979). In Norway *G. d. duebeni* will be compared to a different low shore species, *G. oceanicus*, which has a more northerly distribution and is found as far north as Svalbard (Węsławski & Legezynska, 2002).

1.8. Aims and objectives

Although studies have highlighted the relationships between thermal tolerance and metabolism; thermal sensitivities and protein synthesis; and natural thermal gradients and growth and life history in marine ectotherms, the interactions and relative importance of these relationships are unknown. Responses to temperature in marine ectotherms are complex and highly variable and there is strong evidence of adjustments of thermal tolerance limits according to thermal habitat (Calosi et al., 2010), and according to season (Pörtner, 2010). Physiological adjustments to temperature are likely in the first instance to be phenotypic (Skadsheim, 1989), but have an underlying genetic basis (Dittman, 1997) with evidence of local adaptation among populations distributed along a natural thermal gradient (Kolding, 1985; Kuo & Sanford, 2009; Schulte, 2001).

Previous research has shown interesting inter- and intra-specific physiological variation of key biological rate processes (metabolic rate and protein synthesis rate) in acclimatised gammarid amphipods straight from the shore (Rastrick & Whiteley, 2011; Rastrick & Whiteley, 2013). This thesis aims to expand on these initial studies by examining the specific effects of temperature on metabolism and protein synthesis in a range of gammarid species with differing thermal histories and thermal tolerances. The thesis will use this information to establish acclimatory capacities within the *Gammarus* genus and to relate any observed differences to the ecologically relevant factors of growth and life-history traits. Improving our understanding of the thermal responses of these key biological rate processes will lead to a better understanding of the role of temperature in determining distribution patterns on the shore and also physiological capacities for further change. Investigations will focus on three gammarid species with varying latitudinal ranges along the west coast of Europe to include a subarctic and temperate population of *Gammarus oceanicus*, a boreal and temperate population of *G. duebeni*, and a temperate population of *G. locusta*.

The main objectives are to:

1) Fully explore the plasticity of thermal tolerances by determining upper thermal tolerances in the three gammarid species in field acclimatised amphipods, and by comparing response in the laboratory after temperature acclimation according to species, latitude, season and experimental conditions (Chapter 3).

2) Determine whether the effects of temperature on whole animal rates of oxygen uptake (proxy for metabolic rates) and aerobic capacities differ with species, latitude and season, and in the case of *G. duebeni*, in response to changes in salinity (Chapter 4).

3) Determine whether the effects of temperature on whole animal rates of protein synthesis differ with species, latitude and season, and in the case of *G. duebeni*, in response to changes in salinity (Chapter 5).

4) Compare the influence of temperature on growth rates and life history traits among and within the three gammarid amphipod species, and in the case of *G. duebeni*, in response to differing salinities (Chapter 6).

The studies outlined in the chapters above will be discussed in the context of *in situ* measurements of temperatures at the collection locations for two *G. duebeni* populations from Anglesey, Wales, 53 °N, and Tromsø, Norway, 70 °N (Chapter 2).

Chapter 2

General methodology

2.1. Collection sites

Gammarid amphipods were collected from four coastal sites in total spanning temperate to polar latitudes along the coasts of Western Europe. Amphipods were collected on three different occasions spanning different seasons (Table 2.1). The four *Gammarus* species collected comprised the circumpolar *Gammarus setosus*, the subarctic-boreal *G. oceanicus*, the boreal-temperate species and marine haplotype *G. duebeni duebeni* (Rock et al., 2007; referred to in the rest of the text as *G. duebeni*) and the temperate species *G. locusta*. The four species studied differ both in latitudinal distribution patterns and in the niche occupied on the shore, but have overlapping distributions (Figure 2.1). *G. duebeni* inhabits areas with a freshwater influence in the mid to high intertidal, while the remaining three species inhabit the low intertidal (Rock et al., 2009). The British species, *G. duebeni, G. locusta* and *G. oceanicus*, were identified according to Lincoln (1979); *G. oceanicus* and *G. setosus* from Svalbard were distinguished according to Klekowski & Weslawski (1992).

Collection sites (Figure 2.1.) ranged from Anglesey, Wales (53 °N) and the Isle of Skye, Scotland (59 °N) in the UK, to Tromsø (70 °N) and Ny-Ålesund, Svalbard (79 °N) in Norway. The two main collections in 2012 were made in Anglesey and Tromsø, to represent a temperate and boreal habitat, respectively. Ny-Ålesund in Svalbard can be considered a subarctic climate due to the warming influence of the Atlantic water of the West Spitsbergen Current (Weslawski et al., 2010). Temperatures at the time of collection are given below in the text, and in Table 2.1.

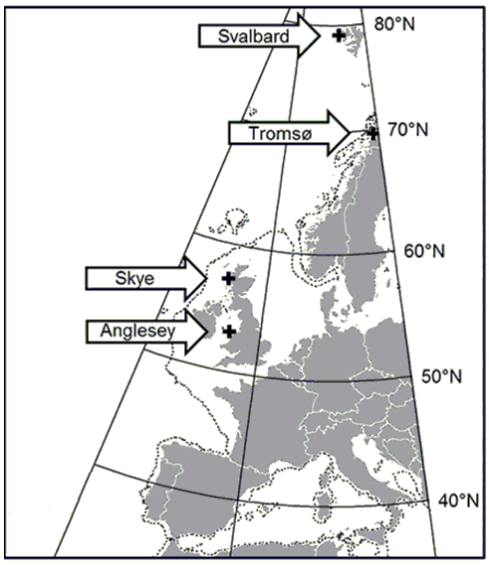

Figure 2.1. Map to show the locations of the four collection sites used in this thesis. See Table 2.1. for further details

All collections were made with hand nets at low tide when amphipods were found under seaweed at the water's edge (*G. locusta,*) or under stones submerged in freshwater inputs on the mid to high shore (*G. duebeni*), or beneath rocks and seaweed on the lower shore (*G. oceanicus* and *G. setosus*).

Microhabitat temperature at the time of collection was recorded using a K-type thermocouple (HI91532K, Hanna Instruments, UK) in areas in which individuals were abundant. HOBO® pendant temperature data loggers (UA-001-64, Onset Computer Corporation, Massachusetts, USA) were deployed to take temperature readings at 20 to 30 minute intervals *in situ* at several sites. Data loggers were attached under rocks in areas with an abundance of amphipods at low tide. Temperature readings were taken in September 2010 for 24 hours prior to collection at two sites in Tromsø, one in Svalbard and two in Skye. Each of the loggers were then redeployed and left to record. The logger from the *G. duebeni* site in Skye was recovered in July 2011 and the logger from the *G. duebeni* site in Tromsø was recovered in October 2012 and recorded 13 months of data before the battery ran out. Unfortunately, none of the lower shore loggers from all three were locations recovered.

Table 2.1. Location of the collection sites used and *Gammarus* species sampled. Temperatures shown were measured at time of collection.

Date	Collection site	Latitude and longitude	Species	Temperature (°C)
September 2010	Ny-Ålesund, Svalbard	78.92°N, 11.92° E	*G. oceanicus* *G. setosus*	2
	Tromsø, Norway	69.61°N, 18.9°E	*G. duebeni* *G. oceanicus*	9
	Skye, Scotland	57.66°N, 5.33°W	*G. oceanicus*	12
	Anglesey, Wales	53.23°N, 4.51°W	*G. duebeni*	15
May, 2012	Anglesey, Wales	53.23°N, 4.51°W	*G. duebeni* *G. locusta*	15
October, 2012	Tromsø, Norway	69.61°N, 18.9°E	*G. duebeni* *G. oceanicus*	7
	Anglesey, Wales	53.23°N, 4.51°W	*G. duebeni* *G. locusta*	11

2.1.1. Anglesey, Wales, 53 °N

2.1.1.1. Collection site

Gammarus locusta were collected from the shore at Rhosneigr, Anglesey. At low tide individuals were located in a small lagoon formed at low tide, and were found primarily under seaweed on rocks in the middle of the pool and under seaweed at the edge in the coarse sand. *Fucus spiralis* and *vesiculosus* and *Ulva lactuca* and *intestinalis* were dominant.

In May 2012 *G. locusta* was found in abundance, with evidence of both brooding pairs and juveniles (under 88 mm; Spooner, 1947), however in October individuals were harder to locate, indicating either a reduction in population size compared to the summer months, or a shift in position on the shore. The salinity at the collection site was the same as the seawater below the low tide line, 32 psu.

G. duebeni were collected from Rhoscolyn, Anglesey at low tide. Individuals were found near the high tide mark in a freshwater stream that runs onto the shore. Salinity at low tide was 0 psu, however the location was completely submerged by seawater at high tide. Amphipods were collected under rocks and freely swimming in the flowing water. In May 2012 *G. duebeni* were abundant and the high shore was dominated by growth of *U. intestinalis*, and in October 2012 *G. duebeni* was still abundant even though there was a marked reduction in the presence of *U. intestinalis*. During the winter *G. duebeni* were difficult to find, possibly indicating a shift in their distribution on the shore due to the lack of productivity in the high shore. Pairs and brooding females were present in the October population, while juveniles, late brooding females and older, non-reproductively active adults were present in the May population.

2.1.1.2. Temperature profile

At the time of collection in May 2012, a high degree of temperature variation was recorded at the *G. locusta* site at Rhosneigr. The temperature of the lagoon water ranged from 20.0 °C in the shallows at the edge of the pool to 15.2 °C in the deeper water. Under the damp seaweed at the edge of the pool temperatures were as low as 9.3 °C. *G. locusta* was collected from both of the cooler areas, and was observed actively swimming in the

warmer shallow water. See Section 2.1.5. for a more detailed discussion of the temperatures in Rhoscolyn.

Figure 2.2. A: Rhosneigr, Anglesey. Collection site for *G. locusta. G. locusta* were found clinging to the seaweed in the areas to the middle of the photograph which was a shallow seawater lagoon in contact with the sea. B: Rhoscolyn, Anglesey. Collection site for *G. duebeni*. *G. duebeni* were abundant in the small freshwater stream flowing from right to left in the forefront of the image. The red arrow indicates the placement of the data logger in the freshwater stream. Both sites photographed at low tide in May 2012.

2.1.2. Isle of Skye, Scotland, 58 °N

Gammarus duebeni and *G. oceanicus* were found occupying two different niches within one shore near Duntulm Castle, Isle of Skye, with no population overlap. *G. duebeni* inhabited a higher shore area characterised by smaller stones and pebbles but dominated by a freshwater input. Salinity at low tide was 0 psu, however the location was under marine influence when the tide was in. *G. oceanicus* was located beneath much larger rocks and

boulders covered in *F. vesiculosus* and *spiralis* on the lower shore near the low tide line, where salinity was 32 psu. Although temperatures showed a similar variation corresponding to the timing of low tide, temperatures were generally lower at the *G. oceanicus* site and showed a more abrupt change than the *G. duebeni* site (Figure 2.3.). As with previous sites, although *F. spp*. were abundant in both niches, the *G. duebeni* site was characterised by a larger presence of *Ulva. spp*.

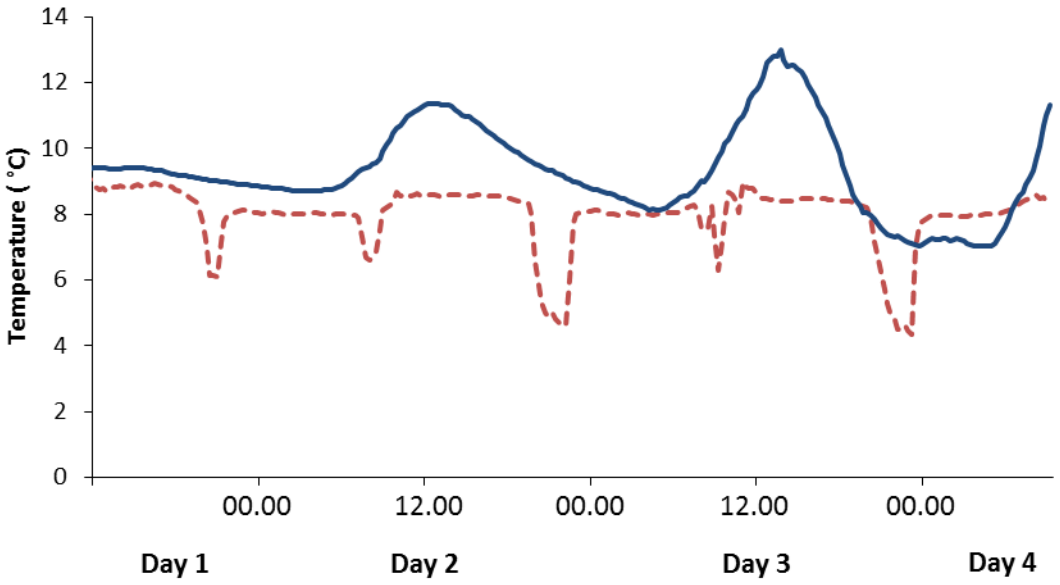

Figure 2.3. *In situ* temperatures recorded in the *G. duebeni* (high shore, blue, solid line) and *G. oceanicus* (low shore, red, dashed line) habitats within one shore on the Isle of Skye, Scotland, 58 °N. Temperatures were recorded at 15 minute intervals at both sites simultaneously in September 2010 using HOBO data loggers.

2.1.3. Tromsø, Norway, 70 °N

2.1.3.1. Collection site

Gammarus oceanicus were collected from Telegraph Bay, Tromsø, within a few metres of the low tide line. Amphipods were located in coarse sediment and small rocks beneath a shelter of large seaweed covered rocks, and were submerged or dampened despite being above the tide line. The salinity was 32 psu. *Fucus spp.* were dominant near the low tide line.

G. duebeni were collected from Finnvika, Kvaløya, overlooking the west side of Tromsø island, from an estuary formed by a small river flowing into the fjord. Individuals were found in areas with coarser sediment rather than the silt further from the freshwater influence. *G. duebeni* were more active than *G. oceanicus* at low tide, and were observed actively swimming between clumps of seaweed below the tide line and cannibalising other individuals. The salinity in the collection location was 0 psu. Between the collections in September 2010 and October 2012 there was a build-up of silt at the mouth of the river, and *G. duebeni* were shifted further upstream into the estuary, in an area with coarser sediment. *F. vesiculosus* was dominant along the water's edge.

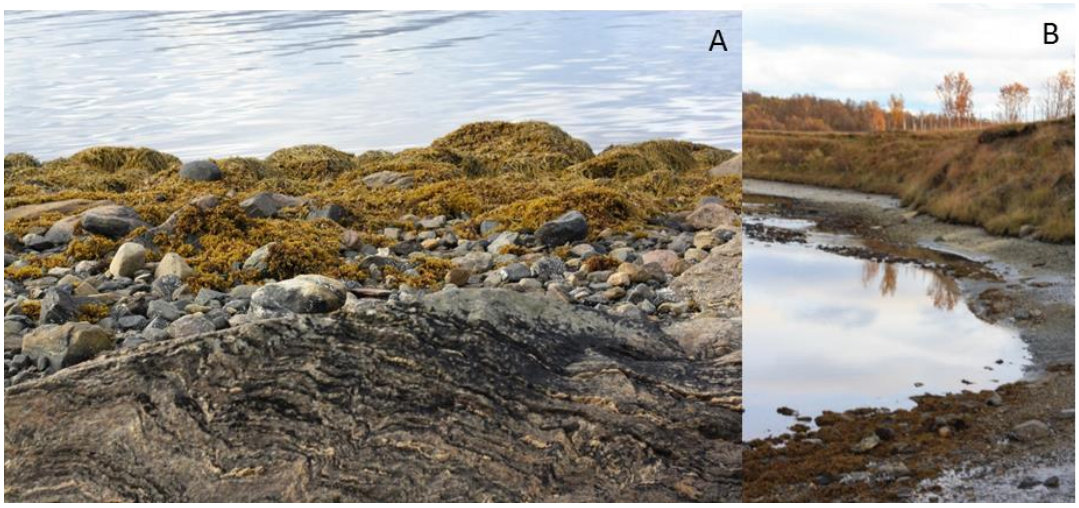

Figure 2.4. Sites near Tromsø, Norway. A: Telegraph Bay, collection site for *G. oceanicus*. *G. oceanicus* were found beneath the large seaweed covered boulders at the low tide line in this seawater site. B: Finnvika, Kvaløya, collection site for *G. duebeni*. This site was estuarine, bordering on marine. The photo shows the bend of the estuary as it entered the fjord. *G. duebeni* were found in the shallows of the estuary in the foreground of the photo.

2.1.3.2. Temperature profile

At the *G. oceanicus* site in Telegraph Bay local temperature variation was low. At the time of the collection in 2010, the temperature of the shallow seawater beneath rocks where the amphipods were located was 8.4 °C, whilst both air and sea temperatures were 8.9 °C. In contrast, at the *G. duebeni* site at Finnvika, air temperatures on the same day were 9.6 °C, while temperatures in the shallows and higher up on the shore in pools beneath rocks were 10.7 and 10.4 °C, respectively. Figure 2.5. shows the difference in the magnitude of daily *in situ* temperature variation between the two sites. Although the mean temperature

at the *G. duebeni* site was only 0.29 °C higher than that recorded at the *G. oceanicus* site, significant differences were observed between the two sites (ANOVA: $F_{2,539}$ = 3773.503, P = 0.000; LSD test: P = 0.001).

2.1.4. Ny-Ålesund, Svalbard, Norway, 79 °N

Gammarus setosus and *G. oceanicus* were identified and distinguished from one another and *G. wilkitzkii* by comparison of the setae on the telsons according to Klekowski & Weslawski (1992). *G. setosus* was more abundant than *G. oceanicus* at the collection site in Ny-Ålesund and had a higher relative abundance than in previous years (Nia Whiteley, pers. comment). *G. oceanicus* were generally located closer to the low tide mark and buried deeper in the gravel, although the two species occurred as a mixed population. *G. setosus* showed more evidence of a breeding population, with pairs evident in early September 2010, while *G. oceanicus* had a higher proportion of juveniles (assuming that a length of 11 and 12 mm was an indicator for 50 % maturity in *G. oceanicus* and *G. setosus* respectively; Klekowski & Weslawski, 1992). Although the brooding period has been stated as being October to May for both species (Klekowski & Weslawski, 1992), the varying status and size of the two species observed at the time of collection suggests delayed reproduction in *G. oceanicus* in comparison to *G. setosus*. For the purpose of the study an attempt was made to sample a range of sizes for both populations. *In situ* temperatures in Svalbard (79 °N) were significantly different from those measured in Tromsø (70 °N) within the same week (ANOVA: $F_{2,539}$ = 3773.503, P = 0.000; LSD test: P = 0.000 for comparisons to both sites).

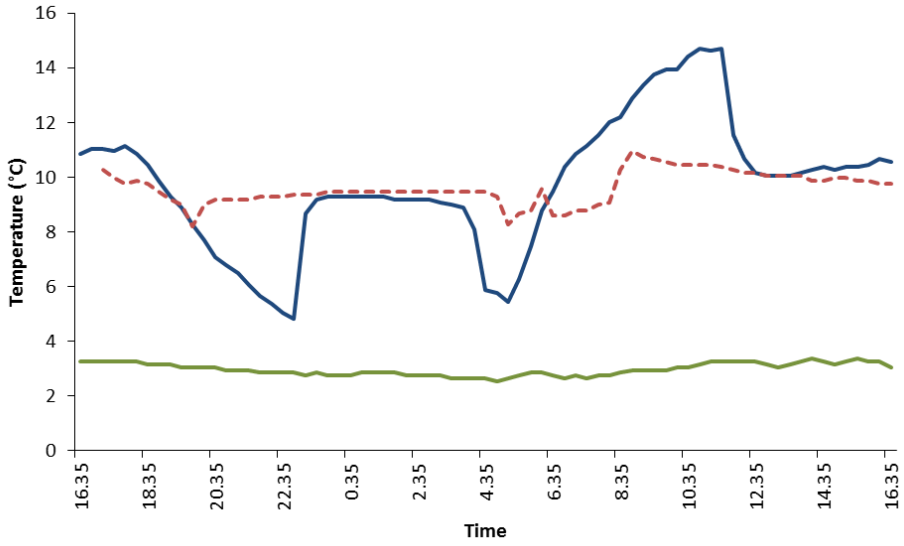

Figure 2.5. *In situ* 24 hour temperature readings of the habitats occupied by the three species from Norway. The blue solid line represents the relatively high temperature variation recorded at the collection site for *G. duebeni*, the high shore species from the estuarine habitat in Finnvika, Kvaløya (70 °N), in comparison to the low shore collection site for *G. oceanicus* at Telegraph Bay (red dashed line, 70 °N). Temperatures for both sites were collected in the same 24 hour period. The green line represents temperature recordings in Ny-Ålesund, Svalbard (79 °N) within the same week as measurements in Tromsø. All three sites exhibited significant differences (ANOVA: $F_{2,539}$ = 3773.503, P = 0.000). Temperatures in Ny-Ålesund were recorded in the low shore, where *G. setosus* and *G. oceanicus* inhabit a colder and less variable habitat than the two species collected around Tromsø (70 °N) (LSD test: P = 0.000). All data collected *in situ* in September 2010 using HOBO data loggers situated below rocks and seaweed at the low tide line where an abundance of amphipods could be found.

2.1.5. Comparison of the thermal regimes of Rhoscolyn and Tromsø

Data loggers recorded over a 12 months of habitat temperature data *in situ* at the high shore *G. duebeni* collection sites in Rhoscolyn, Wales (53 °N) in 2012 to 2013, and in Finnvika, Kvaløya near Tromsø , Norway (70 °N) in 2010 to 2011. Figure 2.6. provides a summary of *in situ* mean monthly temperatures, along with the maximum and minimum temperatures recorded each month. Recorded temperatures were significantly higher in Rhoscolyn than Tromsø (t-test: t_{41647} = -126.174, P = 0.000).

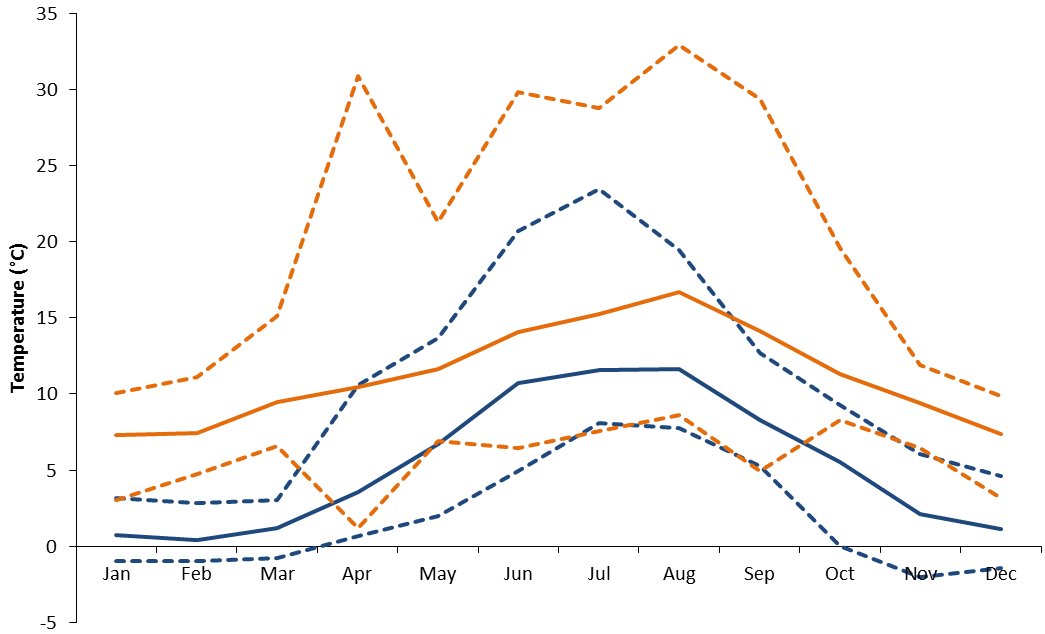

Figure 2.6. Monthly *in situ* temperatures for the *G. duebeni* populations in Rhoscolyn, Wales (53 °N, orange) and Finnvika, Kvaløya, Tromsø, Norway (70 °N, blue). Solid line indicates monthly mean temperatures, dashed lines the minimum and maximum temperatures recorded in each month. Data recorded using HOBO data loggers in 2010 to 2011 in Norway and 2012 to 2013 in Wales. Temperatures recorded in Rhoscolyn were significantly higher than those recorded in Tromsø (t-test: t_{41647} = -126.174, P = 0.000).

The maximum *in situ* temperature reached in Tromsø was 23.5 °C in July, compared to 32.9 °C in August in Rhoscolyn. Monthly minimum temperatures were closer between the two shores than monthly maximum temperatures, but the lowest temperature recorded in Tromsø was -1.0 °C (January and February) while in Rhoscolyn temperatures did not go below 1.2 °C (April). The range between minimum and maximum temperatures within each month was greater in Rhoscolyn than Tromsø. In Rhoscolyn, temperatures for June showed the greatest variability, with a 23.4 °C range, and November the lowest with a 5.4 °C range. The greatest variability in Tromsø was a 15.8 °C difference in temperature in June, while the lowest variation was 3.8 °C in February and March.

Table 2.2. *In situ* temperatures recorded at the two *G. duebeni* collection sites in Finnvika, Kvaløya, Tromsø, Norway (70 °N) and Rhoscolyn, Wales (53 °N) using HOBO data loggers. Temperatures listed are mean daily minimum and maximum temperatures, the mean daily variation in temperatures and the monthly mean, plus the standard error of each value.

Month	Site	Temperature (°C)			
		Minimum	Maximum	Variation	Mean
May	Rhoscolyn, Wales, 53 °N	8.6 ± 0.3	16.0 ± 0.6	7.4 ± 0.8	11.7 ± 0.2
	Tromsø, Norway, 70 °N	4.5 ± 0.2	10.0 ± 0.3	5.5 ± 0.4	6.7 ± 0.2
October	Rhoscolyn, Wales, 53 °N	10.4 ± 0.3	13.1 ± 0.5	2.7 ± 0.3	11.3 ± 0.3
	Tromsø, Norway, 70 °N	3.6 ± 0.3	7.1 ± 0.2	3.4 ± 0.2	5.5 ± 0.3

Temperatures were relatively stable on the shore in Tromsø (70 °N). Temperatures were recorded at twenty minute intervals, and in 38 % of these twenty minute periods no temperature change was observed from one measurement to the next. The highest recorded temperature changes were of up to a 1.4 °C increase or 1.7 °C decrease over a twenty minute interval (changes of 4.2 and 5.1 °C.hr^{-1} respectively). In Rhoscolyn (53 °N) temperatures were much more variable between time intervals, although this was not reflected in the difference between minimum and maximum temperatures in a given month (e.g. Rhoscolyn compared to Tromsø in October, Table 2.2). Temperatures remained constant between measurements in 27 % of the twenty minute periods, but in 29 % of the time periods temperature changes of ≤ 1.5 °C (4.5 °C.hr^{-1}) were observed from one measurement to the next.

2.2. Husbandry

2.2.1. Transport

For transport to marine aquaria at Bangor University from field sites outside of Wales amphipods were laid in plastic boxes between layers of paper dampened seawater from the collection sites, and packed in polystyrene boxes between freezer blocks to maintain low and constant temperatures during transit (maximum transit time 36 h). Temperatures during transport from Tromsø, Norway to the laboratory were 10.6 ± 0.1 °C. A minimum of 1 litre of water was collected from each site and transported in a separate container to allow a gradual transfer from the water at the collection site to the water available in the

marine aquarium, Bangor University. Amphipods from local collection sites were transported back to Bangor University in small volumes of the water taken from the collection site, and maintained at a constant temperature while in a cool box. Transport times from local sites were approximately 30 to 40 min. Mortality rates were approximately 20 % within one week of capture and transfer from remote field sites, but were minimal in animals from Wales.

Measurements on acclimatised amphipods (upper thermal tolerances and resting rates of oxygen uptake) were conducted within 24 hours of capture on animals held in fully aerated water from the collection site at ambient temperatures. These measurements were conducted at Bangor University, Wales where possible, however thermal tolerance experiments were conducted in Skye, Scotland, and the determination of thermal tolerances and rates of oxygen uptake were conducted on species collected at 79 °N at the NERC Arctic Research Station in Ny-Ålesund, Svalbard.

2.2.2. Husbandry conditions

Acclimation temperatures were chosen to correspond to the range of *in situ* temperatures measured across the collection sites (Table 2.1 and Figure 2.2.). The 5 °C minimum was the lowest temperature the temperature controlled facilities would allow and was chosen to minimise stress in amphipods collected in Ny-Ålesund at temperatures of between 2 and 4 °C (Table 2.1, Figure 2.5.). Temperatures recorded *in situ* near Tromsø, Norway at the *G. duebeni* site showed a mean annual temperature of 5.3 °C with temperatures remaining below this for four months of the year, and a mean summer temperature of 11.3 °C (Section 2.1.5.). In Rhoscolyn, Wales the average minimum *in situ* temperature was 5.7 °C, the mean annual temperature was 11.2 °C and the mean summer temperature was 15.4 °C, however temperatures regularly climbed above 20 °C in the summer months. Acclimation temperatures of 5 and 10 °C were therefore selected for Norwegian populations to correspond to autumn and summer mean *in situ* temperatures, with 5 °C most closely representing the mean temperature of 5.5 °C measured in Tromsø during the month of collection (Table 2.2.). Acclimation temperatures of 5 to 20 °C were chosen for Welsh populations, with mean winter temperatures falling between 5 and 10 °C, and 15 °C equivalent to the mean summer temperature. Mean temperatures during the months of

collection (May and October) fell nearest to 10 °C (11.7 and 11.3 °C respectively, Table 2.2.).

Amphipods were maintained at their respective acclimation temperatures by placing a series of tanks per population and species into 4 separate controlled temperature rooms, held at either 5, 10, 15 or 20 °C. These rooms were maintained within 1 °C of the intended temperature, but seldom varied by more than 0.5 °C. Unfortunately the 5 °C cold room malfunctioned in December 2010, raising the temperature to 12 °C overnight. This resulted in the mortality of over half of *G. setosus* and both Norwegian *G. oceanicus* populations within 48 hours. Mortality rates continued to be high in these three populations, greatly reducing the number of possible physiological determinations. Some measurements of rates of oxygen uptake were made more than six weeks after this temperature shock, but only on individuals that were in good condition. Condition was judged based on willingness to move if prodded with a paintbrush and checked after determination of metabolic rates by transferring amphipods to separate tanks. If individuals died within a fortnight their metabolic rates were not included in any further analysis. An acclimation period of six weeks was judged sufficient for the acclimation of many physiological variables to a given temperature.

Table 2.3. Acclimation temperatures and salinities. In 2010-2011 an initial study into the effect of long term acclimation was conducted, with amphipods acclimated to 5 °C for a period of 9 months. In 2012 species and populations collected in two seasons were acclimated to a shorter term of 6 weeks, the time interval required for the acclimation of many physiological variables to a given temperature.

Date	Collection site	Species	Acclimation		
			Temp (°C)	Sal (psu)	Period
Sep, 2010	Ny-Ålesund, Svalbard	*G. setosus*	5	35	9 months
		G. oceanicus			
	Tromsø, Norway	*G. duebeni*			
		G. oceanicus			
	Skye, Scotland	*G. oceanicus*			
	Anglesey, Wales	*G. duebeni*	5 - 20		
May, 2012	Anglesey, Wales	*G. duebeni*	5 - 20	18 and 35	6 weeks
		G. locusta	5 - 15	35	
Oct, 2012	Tromsø, Norway	*G. duebeni*	5 - 10	18	
		G. oceanicus	10	35	
	Anglesey, Wales	*G. duebeni*	5 - 15	18	
		G. locusta	10	35	

On arrival at Bangor University, amphipods were initially transferred to tanks containing seawater from their respective collection sites in the controlled temperature room closest to their habitat temperature at the time of collection and left for 48 hours. After this period individuals were gradually acclimated to the conditions stated in Table 2.3. Individuals were divided into tanks for the respective treatments, held at a maximum density of 50 individuals per six litres of water, and these tanks were transferred between controlled temperature rooms by ± 5 °C every 48 hours until they reached the correct temperature in order to minimise thermal stress. In the case of *G. duebeni*, which were collected in freshwater, a third of the holding water was changed every 48 hours with full strength seawater until the appropriate salinity was achieved. All animals were therefore held in their respective acclimation conditions within twelve days, from which point the acclimation period commenced.

Tanks were provided with a layer of stones and gravel to offer hiding places and encourage natural behaviour. The seawater was continuously aerated. The salinity of seawater available in the marine aquarium, 35 psu, was slightly above that of seawater, 32 psu. A salinity regime of 18 psu was chosen for *G. duebeni* to match the brackish preference of the species (Tedengren et al., 1988) and a regime of 35 psu to investigate the situation on the shore at high tide, and to allow comparisons between *G. duebeni* and *G. locusta* under common acclimation temperatures. The controlled temperature rooms were maintained in a 12:12 light regime. Tanks were visually checked daily and dead individuals removed. Animals were fed ad libitum once every 48 hours on Aquarian® sinking pellets (protein 36.0 %, fat 9.0 %, ash 9.5 %, fibre 3.5 % , Vitamin A 10,000 IU/kg, Vitamin D 2,600 IU/kg, Vitamin E 500 IU/kg). Any uneaten pellets remaining the next day were siphoned out of the tanks. Salinity was monitored once every 48 hours and freshwater added as necessary to counter the effect of evaporation. A third of holding water was changed once per week, or as needed. Tanks were checked once a fortnight for nitrate, nitrite and ammonia levels using API® test kits, or if problems were observed (high mortality rates, amphipods unresponsive or clustered along water line etc.).

Chapter 3

Upper critical limits and the effect of temperature acclimation

3.1. Abstract

In order to investigate the effects of temperature on survival, upper thermal tolerances were determined in four species of intertidal gammarid amphipod with varying latitudinal ranges. In acclimatised amphipods, thermal tolerances were negatively correlated with latitude. Following temperature acclimation, *G. locusta* from Wales (53 °N) and populations of *G. duebeni* from Wales (53 °N) and Norway (70 °N) exhibited an upward adjustment of thermal limits as acclimation temperature increased but the effect of acclimation temperature on thermal limits varied between species. In Wales, *G. locusta* exhibited a greater acclimatory capacity of thermal tolerance but *G. duebeni* had a higher upper thermal limit overall, suggesting a broader tolerance and lower vulnerability to temperature change. Acclimated *G. duebeni* from Wales (53 °N) had a higher thermal tolerance than the population from Norway (70 °N), but the population from Wales inhabited an area with temperatures far closer to the upper thermal limit than the population from Norway. After acclimation to 20 °C, *G. duebeni* from Wales had an upper thermal limit of 36.2 °C, while temperatures on the shore peaked at 33.4 °C. Temperatures from the shore in Norway peaked at 23.5 °C on one of only four days of the year in which temperatures climbed above 20 °C. Despite *G. duebeni* from Norway having a lower thermal limit (31.6 °C following acclimation to 10 °C), the population appears less vulnerable to warming than the population from Wales if susceptibility is defined by a population's proximity to threshold temperatures. Salinity acclimation had an interesting effect on thermal tolerance in *G. duebeni*, interacting with acclimation temperature to increase mortality rates at 20 °C and 35 psu.

3.2. Introduction

Temperature is considered an important determining factor in the distribution of species (Stevens, 1989) due to the influence it has over biological processes from the whole organism (Peck et al., 2009) to molecular level (Mermillod-Blondin et al., 2013). Exposure to stressful temperatures is especially critical in small aquatic ectotherms such as amphipods, for which body temperature closely mirrors environmental temperature (Helmuth & Hofmann, 2001). Species and conspecific populations may show genetic adaptations to extreme or variable temperatures (Bennett & Lenski, 1993), such as a broad thermal window, or adaptive plasticity, i.e. acclimatisation and acclimation (Hazel &

Prosser, 1974). Variations in thermal tolerance may therefore be used to help identify the relative vulnerability of species and populations to climate change (Somero, 2010).

Range shifts have been reported in a variety of aquatic organisms in response to increasing temperatures (Barry et al., 1995; Calosi et al., 2008; Parmesan, 2006), and recent predictions of climate change (Parry et al., 2007) have lent importance to the investigation of the role physiological temperature tolerance has on species distribution patterns. A recent ecological study has noted that warming of air and sea temperatures in Svalbard over the last 20 years has coincided with an increase in the relative abundance of the boreal amphipod *Gammarus oceanicus* while the arctic *G. setosus* has retreated toward the cooler areas close to the glaciers (Węsławski et al., 2010). To predict the susceptibility of species and populations to climate change, it is important to understand the link between range size and thermal tolerance, and to investigate the physiological mechanisms underlying critical limits and the capacity for acclimatory ability in the face of rapid change. Upper temperature limits are close to optimal temperatures for physiological processes (Jobling, 1981) and are a useful measure for investigating the influence that natural thermal gradients exert over species distributions. The climate variability hypothesis, or Rapoport's rule (Stevens, 1989) suggests thermal tolerance breadth, and hence range size, increases with latitude in the northern hemisphere due to the selective pressure for eurythermal species in the more variable conditions of the temperate zone. Vulnerability to climate change may therefore be higher in polar and tropical regions, where species are largely stenothermal.

The intertidal is characterised by a high degree of temporal and spatial variation in temperature and salinity, exerting a high degree of stress on the organisms that inhabit this zone. Over the course of a tidal cycle an intertidal species is likely to experience a greater range of temperatures than a subtidal species from the same shore (Stillman & Somero, 1996), and local factors such as the timing of low tide can exert a strong influence on the extent and magnitude of thermal variation (Helmuth et al., 2006). Intertidal invertebrates are therefore expected to have the physiological or behavioural capacity to maintain performance in variable environments (Overgaard et al., 2011). This may be achieved through expression of relatively high levels of phenotypic plasticity, a trait lacking from some stenothermal species due to the high energetic costs incurred (Ghalambor et al., 2006) and the theory of "jack of all trades, master of none" (Huey & Hertz, 1984). Not all studies have supported the theory of increasing tolerance according to shore height. For

example, slippershell snails, *Crepidula fornicata*, from the intertidal experience temperatures 15 °C higher than conspecific individuals from the subtidal, but very little difference is seen between the two populations in upper thermal tolerance (Diederich & Pechenik, 2013). Intertidal invertebrates may therefore be operating closer to lethal limits than generally assumed, even those individuals lower down on the shore. The theory of the cost of tolerance as a limiting factor is also under debate. A study into the effect of selection for increased temperature tolerance in the copepod *Tigriopus californicus* found upper lethal limits plateaued rapidly (Kelly et al., 2013), however as selected lines showed increases in fitness in other areas the authors concluded that the limited adaptive ability of *T. californicus* was due to limited variation within populations, rather than high costs associated with broad tolerance.

Temperate species are thought to have broader thermal windows than for restricted polar or tropical species (Calosi et al., 2010; Deutsch et al., 2008; Gilchrist, 1995), possibly due to variations in cold tolerance rather than heat tolerance, which is thought to be relatively conserved (Addo-Bediako et al., 2000). Past thermal history may, however, have a lingering influence over the current physiological temperature tolerance of a species. Recently Mermillod-Blondin et al. (2013) demonstrated species differences between subterranean isopods from the *Proasellus* genus from a relatively thermally stable habitat. Although some species exhibited the expected narrow thermal window of stenothermal organisms, one species exhibited eurythermal characteristics which the authors attributed to continued dispersal and gene flow between populations from thermally contrasting habitats.

Stillman (2003) has proposed that a trade-off exists between the magnitude of a species' thermal tolerance and the acclimatory capacity of this tolerance. Studies in porcelain crabs, genus *Petrolisthes*, (Stillman & Somero, 2000) and *Drosophila spp.* (Overgaard et al., 2011) have suggested that species distribution is more closely linked to the breadth of thermal windows, rather than the plasticity of limits. However, Calosi et al. (2008) saw a positive correlation between upper thermal tolerance and the acclimatory ability of the trait in European diving beetles of the genus *Deronectes*. Clearly further studies are required in a range of aquatic invertebrates before firm conclusions can be drawn about the relationship between acclimatory capacity and thermal tolerances.

Tolerance may be investigated through limits for coma (Overgaard et al., 2011), aerobic scope (Giomi & Pörtner, 2013; Portner, 2001), sterility (Chakir et al., 2002), locomotory

activity (Mermillod-Blondin et al., 2013), immune response (Díaz et al., 2013; Mermillod-Blondin et al., 2013) or survival (Calosi et al., 2010). This Chapter focuses on upper thermal tolerances, measured in terms of survival, in four congenic species of gammarid amphipods with varying latitudinal distributions and shore heights as a means of elucidating species differences in range size and local niche. This Chapter also attempts to predict their relative susceptibility to climate change. Laboratory acclimation of three species to a series of temperatures was used to compare upper thermal limits at common acclimation temperatures and to investigate the phenotypic plasticity of upper thermal tolerances. Acclimation experiments are useful as they can be used to compare species and populations according to distribution patterns across latitudinal and tidal gradients, and to assess the plasticity of tolerance limits (e.g. Calosi et al., 2010). Two populations of *G. duebeni* were examined from Anglesey in Wales and Tromsø in Norway to investigate whether the same species from habitats with differences in mean temperature, as well as seasonal and daily variability, would show differences in upper thermal limits.

Ramping assays, in which organisms are exposed to gradually increasing or decreasing temperatures, have been suggested to have a greater ecological relevance than exposure to a static preselected temperature (Overgaard et al., 2012; Terblanche et al., 2011). A gradual rate of change may allow individuals to express plastic responses (Terblanche et al., 2007). In this Chapter attempts were made to partially address concerns over the often rapid rates of temperature change used in such studies by performing an additional experiment into the effect of rate of warming on upper thermal limits. Although laboratory experiments were limited in scale (both in the number of variables considered and the time frame of the study) in comparison to studies that attempt to correlate range shifts to temperature change in the field, they complement field observations and represent an important initial investigation of the physiological mechanisms underlying thermal tolerance in confamilial gammarid amphipods.

3.3. Methodology

3.3.1. Thermal tolerance of field-acclimatised gammarids

In September 2010, three species from the *Gammaridae* family were collected from three latitudes; *G. setosus* and *G. oceanicus* from Svalbard, *G. oceanicus* from Tromsø and *G. duebeni* from Wales (Table 2.1.). Amphipods were held for 24 hours in fully aerated

seawater at the salinity and temperature recorded at the time of collection (Table 2.1.). During this time, amphipods were held without feeding prior to the start of the thermal tolerance experiment to allow recovery from handling and to exclude the influence of recent feeding events. Brooding or egg-bearing females and individuals undergoing a moult were excluded from the study. All thermal tolerance determinations were carried out close to the site of collection.

Individual amphipods were transferred into individual 15 ml tubes open to seawater but divided by a layer of mesh suspended within small 6 litre tanks held in a water bath controlled to the nearest 0.1 °C (Grant Instruments, Cambridge, UK). Temperatures within individual compartments were measured using a K-type thermocouple (Hanna Instruments, Leighton Buzzard, UK), with starting temperatures equivalent to collection temperatures. The tanks were aerated throughout the experiments and frequent measurements were taken of seawater oxygen partial pressures (PO_2) to ensure the water did not fall below 16kPa and become hypoxic. The temperature was raised by 1 °C every 20 minutes (3 °C per hour) to match observed rates of warming on the shore (see Chapter 2). This ramping rate also allowed for comparisons between thermal tolerance experiments and the study on the effect of acute temperature change on oxygen uptake rate in Chapter 4. As the temperature of the tubes lagged behind the temperature of the water bath by up to 10 minutes, readings of the temperature at the time of mortality were taken from within tubes containing amphipods and the equipment was monitored frequently between temperature increases to ensure a steady 3 °C per hour increase within tubes. Every 20 minutes amphipods were visually monitored, PO_2 measurements of the seawater within tubes were taken and the temperature adjusted. Amphipods showing no pleopod movement were gently prodded with a small paintbrush, and if no response was observed then this was taken as the end point as the amphipod was considered moribund. Moribund amphipods were transferred to cooler aerated seawater and monitored for signs of recovery. The temperature at which individual amphipods reached the end point (morbidity, no recovery) of the experiment was recorded as the value for each individual's upper thermal limit.

3.3.2. Thermal tolerance of acclimated gammarids

The same thermal tolerance experiment was repeated twice on three species of gammarid amphipods (*G. duebeni, G. locusta* and *G. oceanicus*) collected from two latitudes and acclimated to a range of temperatures and salinities. Amphipods were collected in May and October 2012 (Table 2.1.). Amphipods from Tromsø, Norway, were transported to Bangor University between layers of paper soaked in seawater and maintained at a constant low temperature during transit. All amphipods were returned to aerated seawater in the cold rooms at Bangor University at the appropriate temperature and left for 48 h to recover before transfer to acclimation conditions as described in Chapter 2. Amphipods were acclimated to the conditions outlined in Table 3.1. for six weeks. Acclimation temperatures and salinities were selected as described in Section 2.2.2.

Table 3.1. Acclimation conditions for six weeks prior to measurements of thermal tolerance. Shaded boxes indicate amphipods were held at the specified temperature.

Date of collection	Collection site	Species	Salinity (psu)	Temperatures (°C)			
				5	10	15	20
May, 2012	Anglesey, Wales, 53 °N	*G. duebeni*	18	■	■	■	■
			35	■	■	■	
		G. locusta	35	■	■	■	
October, 2012	Tromsø, Norway, 70 °N	*G. duebeni*	18	■	■		
		G. oceanicus	35		■		
	Anglesey, Wales, 53 °N	*G. duebeni*	18	■	■	■	
		G. locusta	35		■		

Individuals that were not brooding, egg-bearing or undergoing a moult were selected from each acclimation treatment and added to tubes in series as the acclimation temperature was reached. Amphipods were not fed for 24 h before experiments commenced. Upper thermal tolerance limits were measured at the respective acclimation salinities. Ramping rates were 3 °C per hour, and the experiment was conducted and monitored as in Section 3.3.1.

3.3.3. The influence of rate of warming on upper thermal limits

As the rate of warming influences the results of thermal tolerance studies (Peck et al., 2009), a further experiment was conducted to investigate the effect of slower rates of warming on the upper thermal limit (UTL) of *G. duebeni*. *G. duebeni* collected in October 2012 from Norway and Wales and acclimated to 5 and 10 °C for six weeks (Table 2.1. and 3.1.) were subjected to a 3 °C increase in temperature per day in a modified version of the previous experiments.

Ten individuals from each treatment were selected as in Section 3.3.2. and transferred to two aquaria of approximately 2 litres volume each held in water baths (Grant Instruments, Cambridge, UK) controlled to the nearest 0.1 °C. Each of the eight tanks was individually aerated for the duration of the experiment. Amphipods were provided with hiding places, fed daily *ad libitum* as in acclimation treatments (see Chapter 2) and salinity was monitored and adjusted during daily 30 % water changes to maintain values at 18 psu. Measurements of nitrate (API® test kit) and PO_2 were conducted daily to ensure water quality. Actual temperatures were measured within aquaria with a K-type thermocouple (Hanna Instruments, Leighton Buzzard, UK) and were increased by 1.5 °C every 12 hours from starting acclimation temperatures to 32 °C, the temperature at which 100% mortality was recorded. In the 12 hours each day between temperature ramping, amphipods were visually monitored hourly as in previous experiments and moribund individuals were removed.

3.3.4. Statistical analysis

Upper thermal limits (UTLs) were calculated as the mean temperature at which the end point (morbidity) occurred in amphipods from each experiment. Means were compared by one or two way analysis of variance (ANOVA) according to the number of factors, or t-tests when comparing only two groups after the Kolmogorov-Smirnov test for normality and Levene's test for homogeneity of variances to satisfy the criteria for the tests. Multiple comparisons were made using the least significant difference post hoc test (LSD test). Values are given as means ± S.E.M, and results are considered significant at the 5 % confidence interval ($P < 0.05$). Statistical analyses were performed using SPSS (SPSS version 21; SPSS Inc., Chicago, IL, USA).

3.4. Results

3.4.1. Thermal tolerance of field-acclimatised gammarids

In response to increasing temperatures at 3 °C.h^{-1} gammarids showed the same sequential behaviours regardless of species or latitude. After an initial period settled at the bottom of the tube, individuals became increasingly active, circling the inner surface of the tubes and moving up and down within the water column. They then ceased swimming and returned to the bottom of the tubes but increased the rate of pleopod beating. The rate of pleopod beating slowed and became more erratic approaching lethal limits. Amphipods showing no response to prodding also showed no sign of recovery within 60 minutes of incubation at starting temperatures, demonstrating morbidity. Individuals generally died in uncurled positions with pereopods splayed outwards, suggesting an attempt to increase water flow over the pleopods. Both between and within species, upper thermal limits (UTLs) of gammarids measured within 24 hours of collection from the shore were significantly higher at lower latitudes (ANOVA: $F_{1,201}$ = 17.425, P = 0.000) as seen in Figure 3.1. The highest UTL was at the lowest latitude. *G. duebeni* from Anglesey, Wales at 53 °N had an UTL of 32.7 ± 1.64 °C (N = 66), which was 6.4 °C higher than the UTL of *G. setosus* from Ny-Ålesund, Svalbard at 79 °N (26.3 ± 0.21 °C, N = 74). In *G. oceanicus,* the population at the lower latitude (Skye, Scotland at 57 °N) had an UTL 2.2 °C higher than the population from Ny-Ålesund, Svalbard at 79 °N (t-test: t_{63} = -6.127, P = 0.000). In a comparison of the two species collected from the same shore in Ny-Ålesund at 79 °N, the boreal/sub-arctic species, *G. oceanicus,* had a significantly higher UTL (28.6 ± 0.15 °C, N = 55) than the circumpolar species, *G. setosus* (26.3 ± 0.21 °C, N = 74, t-test: t_{127} = -9.225, P = 0.000).

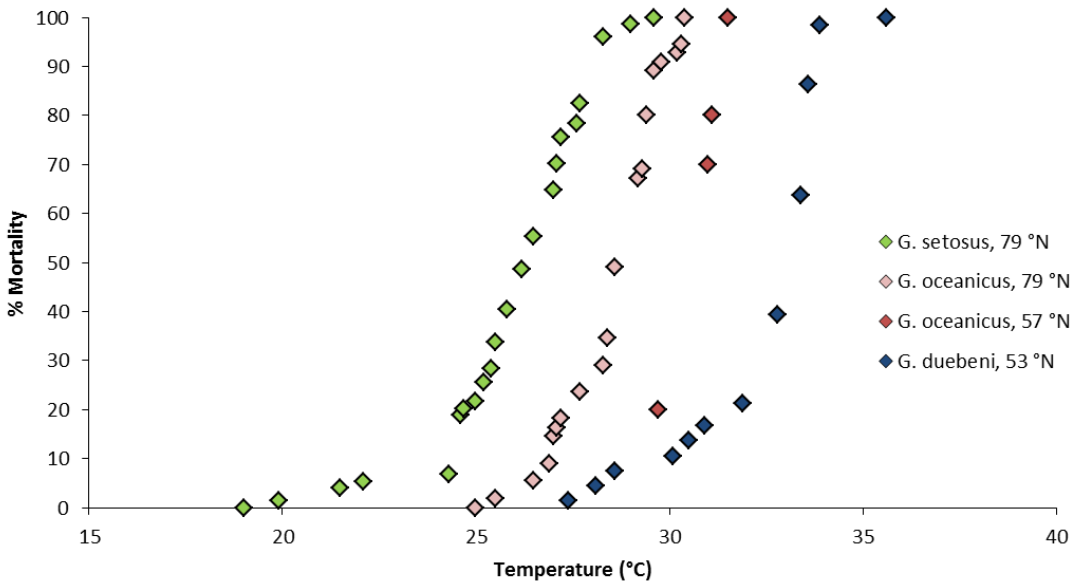

Figure 3.1. Percentage mortality of three *Gammarus* species from three latitudes during exposure to increasing temperature. From L-R green represents *G. setosus* from Svalbard (79 °N), light and dark red *G. oceanicus* from Svalbard (79 °N) and Scotland (58 °N) respectively, and blue *G. duebeni* from Wales (53 °N). All measurements taken within 24 hours of collection from the field and at a rate of 3 °C increase per hour. Starting temperatures corresponded to measurements taken on shore at the time of collection. *G. setosus* and *oceanicus* from 79°N (*N* = 74 and 55 respectively) had a starting temperature of 2 °C, *G. oceanicus* from 57°N (*N* = 10) had a starting temperature of 12 °C, and *G. duebeni* (N = 66) had a starting temperature of 15 °C. Upper thermal limits (UTLs) were significantly higher in lower latitude populations (ANOVA: $F_{1,201}$ = 17.425, *P* = 0.000), and at 79°N the boreal *G. oceanicus* showed a significantly higher UTL than the circumpolar *G. setosus* (t-test: t_{127} = -9.225, *P* = 0.000).

3.4.2. Thermal tolerance of acclimated gammarids

Acclimated amphipods demonstrated the same sequential behaviours in response to warming as described in Section 3.3.1. Acclimation temperature had a significant effect (ANOVA: $F_{3,36}$ = 8.292, *P* = 0.001). In *G. duebeni* acclimated to 18 psu, the mean UTL was 2.2°C higher in individuals acclimated to 20 °C rather than 5 °C (Figure 3.3., LSD test: *P* = 0.000). In *G. duebeni* from Wales, temperature during the six week acclimation period rather than the temperature at time of collection was the critical factor in determining thermal tolerance, as season had no significant effect on UTL (ANOVA: $F_{1,54}$ = 2.921, *P* = 0.093). Despite this, significant variation in thermal tolerance was observed between the two *G. duebeni* populations from different latitudes (Figure 3.2., ANOVA: $F_{1,36}$ = 43.403, *P* =

0.000). UTLs were 3.2 °C higher in the population from Wales (53 °N) than the population from Norway (70 °N) after acclimation to a common temperature of 5 °C (LSD test: P = 0.000), and 3.1 °C higher at a common temperature of 10 °C (LSD test: P = 0.000), as the two populations showed a similar increase in UTL with acclimation temperature (mean UTL increased by 1.6 °C between 5 and 10 °C acclimation in the population from 53 °N, and by 1.7 °C from 70 °N).

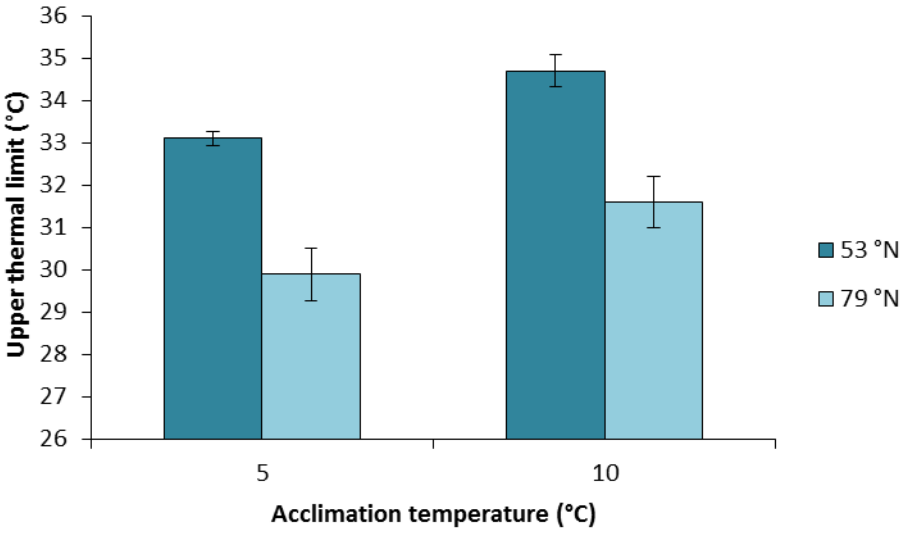

Figure 3.2. Variation in upper thermal limits between populations of *Gammarus duebeni* from Wales (dark blue, 53 °N) and Norway (light blue, 70 °N) after acclimation for six weeks at 5 and 10 °C. Values are means ± 1 S.E.M. N = 10 for all data points. Upper thermal limits show a significant increase with an increase in acclimation temperature (ANOVA: $F_{1,36}$ = 11.909, P = 0.001) or decrease in latitude (ANOVA: $F_{1,36}$ = 43.403, P = 0.000). All values are significantly different at the P < 0.05 level (ANOVA: $F_{3,36}$ = 18.441, P = 0.000).

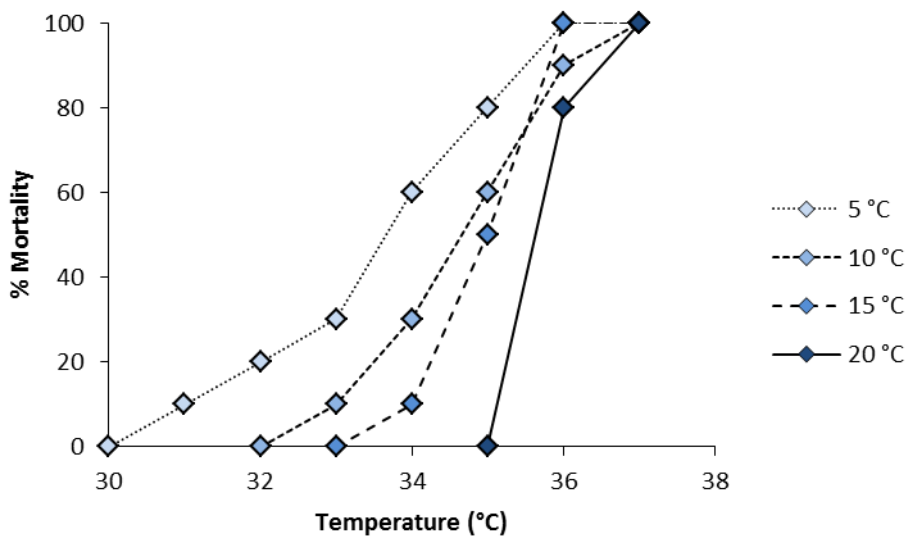

Figure 3.3. Percentage mortality of *Gammarus duebeni* from Wales (53 °N) after exposure to increasing temperatures at a rate of 3 °C per hour (N = 10 for each acclimation temperature). Amphipods were acclimated for six weeks to 18 psu salinity and temperatures of 5, 10, 15 and 20 °C. Upper thermal limits (UTLs) vary significantly according to acclimation temperature (ANOVA: $F_{3,36}$ = 8.292, P = 0.001), increasing from an UTL of 34.0 ± 0.51 °C in 5 °C acclimated *G. duebeni* to 36.2 ± 0.13 °C when acclimated to 20 °C.

G. locusta exhibited a lower thermal tolerance than *G. duebeni* from Wales (Figure 3.4., ANOVA: $F_{1,48}$ = 61.512, P = 0.000), with the difference in UTL when acclimated to common temperatures varying between 2.3 °C at 15 °C and 5.5 °C at 5 °C. *G. locusta* acclimated to 15 °C had a mean UTL equivalent to *G. duebeni* acclimated to 5 or 10 °C (LSD test: P = 0.354 and 0.234 respectively). Although *G. duebeni* has a higher UTL than *G. locusta*, *G. locusta* shows a greater acclimatory capacity. With a 10 °C increase in acclimation temperature, *G. locusta* exhibited a 4.7 °C increase in UTL. This change in UTL is almost three times higher than the 1.6 °C increase observed for *G. duebeni* held at a common salinity of 35 psu. No interspecific difference was detected at 70 °N between *G. duebeni* and *G. oceanicus* at a common acclimation temperature of 10 °C (t-test: t_{18} = 1.490, P = 0.154), however the two species were measured at different salinities (18 and 35 psu, respectively).

Populations of *G. duebeni* from Wales exhibited no difference in UTLs with salinity (ANOVA: $F_{1,54}$ = 0.015, P = 0.904) when acclimated to half strength or full strength seawater (salinities of 18 and 35 psu, respectively), however thermal tolerance measurements are

lacking for *G. duebeni* acclimated to 35 psu and 20 °C because of the high mortality rates under these conditions.

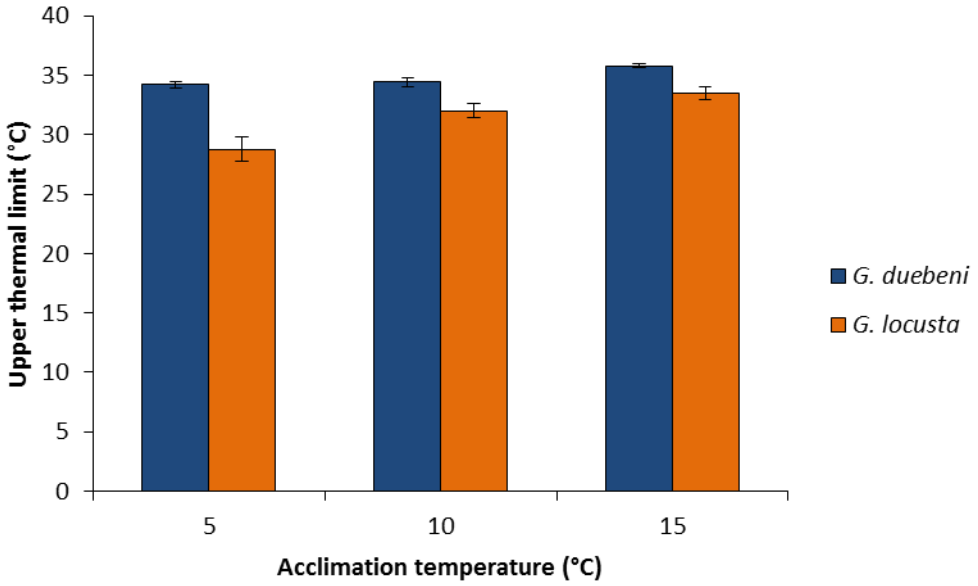

Figure 3.4. Species differences in upper thermal limits between *G. duebeni* and *G. locusta* from Wales (53 °N) after acclimation for six weeks to 35 psu salinity and 5, 10 and 15 °C. Values are means ± 1 S.E.M. N = 10 for all data points. Upper thermal limits show significant variation according to acclimation temperature (ANOVA: $F_{2,48}$ = 18.102, P = 0.000), species (ANOVA: $F_{1,48}$ = 61.512, P = 0.000) and the interaction between the two (ANOVA: $F_{2,48}$ = 5.742, P = 0.006).

3.4.3. The influence of rate of warming on upper thermal limits

When exposed to a slower rate of temperature increase, *G. duebeni* from both 53 and 70 °N showed a reduction in UTL (Figure 3.5., t-tests: t_{18} = 3.812-5.163, P = 0.000-0.002) in the range of 4.5 to 6.2 °C. Normal feeding and activity levels were maintained at least until the day before mortalities were recorded. Although *G. duebeni* from 53 °N maintained a significantly higher UTL after acclimation to 10 °C compared with 5 °C (t-test: t_{18} = 2.305, P = 0.033), the population from 70 °N no longer showed this response (t-test: t_{18} = 0.824, P = 0.421). In addition, a significant difference between the two populations during the slower

rate of warming was only observed at a common acclimation temperature of 10 °C (t-test: t_{18} = 2.546, P = 0.020), not at 5 °C (t-test: t_{18} = 1.1018, P = 0.322).

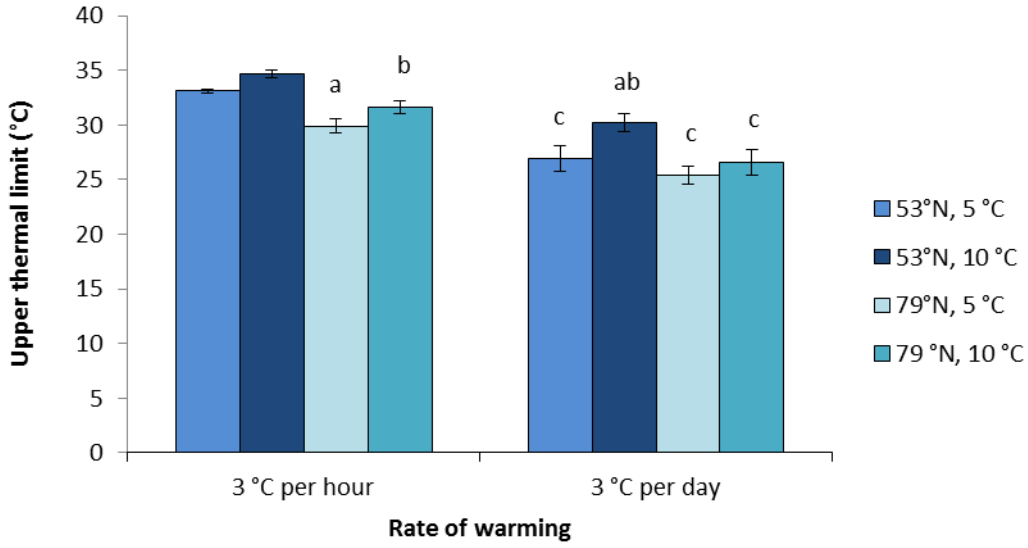

Figure 3.5. The effect of the rate of warming on upper thermal limits (UTLs) in two populations of *G. duebeni* from Wales (53 °N) and Norway (70 °N) acclimated to 5 and 10 °C and 18 psu salinity. Values are means ± S.E.M. N = 10 for all data points. Letter groups indicate values with no significant differences between them. (t-tests: P < 0.05). A slower rate of temperature increase results in a significant reduction in UTLs for both latitudes and acclimation temperatures, but also reduces differences between populations and acclimation temperatures.

3.4.4. Summary

Upper thermal limits in acclimatised gammarids were significantly higher at lower latitudes. In Ny-Ålesund, the boreal/sub-arctic species *G. oceanicus* had a higher UTL than the circumpolar species *G. setosus*. After acclimation to common temperatures, *G. duebeni* from Wales (53 °N) exhibited a higher UTL than a population of the same species from Norway (70 °N). Acclimation to a series of temperatures resulted in a significant variation in UTL according to acclimation temperature in both populations of *G. duebeni* and in *G. locusta*, demonstrating that UTL is a relatively plastic response. A higher increase in UTL with acclimation temperature was observed in *G. locusta*, indicating a greater acclimatory capacity, however *G. duebeni* had a higher UTL at all temperatures. A reduction in the ramping rate from 3 °C per hour to 3 °C per day reduced upper thermal limits in *G. duebeni*,

however at a common acclimation temperature of 10 °C intraspecific differences persisted between the two populations from Wales and Norway.

3.5. Discussion

Thermal tolerances varied between and within gammarid species, were influenced by thermal acclimation and were influenced by the rate of warming. In acclimatised *G. oceanicus*, thermal tolerances were lower in the population inhabiting the Arctic latitude (79 °N) compared with the population at the cold-temperate latitude (58 °N). At the same latitude, however, temperature acclimation had a different effect depending on species, whereas salinity acclimation had a profound effect on thermal tolerances in *G. duebeni*.

3.5.1. Effect of latitude

In the acclimatised amphipods, i.e. those representative of animals on the shore, the southern population of *G. oceanicus* had a higher thermal tolerance demonstrating intra-specific differences. The two populations have distinct thermal habitats, with northern polar habitats characterised by consistently low temperatures, and southern cold-temperate habitats characterised by higher and more variable temperatures at certain times of the day (Chapter 2, Sections 2.1.3. and 2.1.5.). The northern population is considered to be stenothermal and displays some cold-water characteristics. For example, acclimatised individuals within this population have limited maximal aerobic threshold temperatures and relatively low, whole-animal rates of metabolism and protein synthesis (Rastrick & Whiteley 2011; Rastrick & Whiteley 2013). Acclimatised individuals from the southern population, occupying more variable thermal environments have higher maximal aerobic thresholds, higher whole-animal rates of metabolism and relatively high protein synthesis rates. It is currently unknown whether these intraspecific differences in *G. oceanicus* are due to phenotypic plasticity or genetic differences between populations, however phylogenetic analysis has shown relatively low genetic diversity within *G. oceanicus* across Europe. It appears that thermal tolerances in acclimatised amphipods are negatively correlated with latitude, although this remains to be tested with a wider range of gammarid species and populations.

A consistent response was observed in amphipods acclimated to common temperatures, as the southern population of *G. duebeni* continued to have a higher thermal tolerance than the northern population. Acclimation to a given temperature gives a clearer indication of the differences between the populations, as it helps to define the role of temperature by isolating it from changes in other abiotic factors that may be occurring simultaneously in the field. A negative correlation in thermal tolerances with latitude has also been reported after temperature acclimation in other aquatic ectothermic species, such as freshwater diving beetles (Calosi et al., 2010); killifish (Fangue et al., 2006); dog whelks (Sorte & Hofmann, 2005), and marine snails of the genus *Tegula* (Tomanek & Somero 1999). Collectively these studies demonstrate local adaptation among populations of the same species distributed along natural thermal gradients.

Changes in thermal limits with latitude have been used to predict latitudinal range extents (Calosi et al. 2010; Gaston & Spicer 2001). It is possible that the data obtained here for thermal limits in acclimatised gammarid amphipods supports this hypothesis. For example, *G. oceanicus* inhabiting the same shore line as *G. setosus* in Ny-Ålesund had a higher upper thermal limit. *G. oceanicus* has the widest geographical range of the five marine and estuarine *Gammarus* species reviewed by Gaston and Spicer (2001) and has a wider latitudinal range and more southern distribution than *G. setosus*. Evidence suggests that *G. setosus* is already being out-competed on the western shores of Svalbard, with dominance now held by *G. oceanicus* while populations of *G. setosus* have shifted to the lower salinity but cooler areas at the foot of the glaciers (Węsławski et al., 2010). It appears that despite the fall in salinity, *G. setosus* is intolerant of the general increase in sea surface temperature reported in the Kongsfjorden over the last ten years (Ikko & Lyubina, 2010).

3.5.2. Effect of temperature acclimation

Despite evidence of seasonal variability in upper thermal limits and thermal sensitivity of the gammarid *G. limnaeus* (Krog, 1954), the Pacific oyster *Crassostrea gigas* (Hamdoun, Cheney, & Cherr, 2003) and the intertidal isopod *Ligia oceanica* (Whiteley & Faulkner 2005), season had no significant effect on upper thermal tolerances in *G. duebeni* or *G. locusta* from Wales following temperature acclimation. Both species, however, demonstrated variation in upper thermal limits according to acclimation temperature. This suggests that in addition to previous thermal history, thermal tolerance limits are also

subject to acclimation demonstrating that there is significant plasticity in this response. Recovery time from cold shock in *Drosophila* has been shown to be a plastic trait affected by temperature during development (Ayrinhac et al., 2004). Heat shock responses in a range of intertidal species are also reported to be plastic and subject to acclimation, for example heat shock responses in natural populations of the mussel, *Mytilus trossulus* (Hofmann & Somero, 1995), and in two species of eurythermal goby fishes (Dietz & Somero, 1992). As elevated endogenous levels of heat shock proteins are thought to increase the chances of survival they have been referred to as the molecular mechanisms underlying thermal tolerances (Hofmann & Somero, 1995; Somero, 2010). Brief thermal experiences can also affect thermal tolerances, for example a higher thermal tolerance can be induced in *Artemia franciscana* by short exposure to thermal stress, possibly due to the production of heat shock proteins (Miller and McLennan, 1988).

The effect of acclimation temperature on thermal limits varied between species. *G. duebeni* exhibited a higher thermal tolerance than *G. locusta* at all three common acclimation temperatures, however *G. locusta* exhibited a greater acclimatory capacity at a salinity of 35 psu. Both species exhibited an upward adjustment of thermal limits in response to acclimation to higher temperatures, however the magnitude of the variation in thermal tolerances of amphipods over a 10 °C difference in acclimation temperature was more than twice as high in *G. locusta* than *G. duebeni*. This response could be explained by *G. locusta* being at its preferred salinity whilst *G. duebeni* was in hyper-osmotic conditions and suffering from an increase in haemolymph [Na+] (Brooks & Lloyd Mills, 2006). A similar increase in upper thermal limit with acclimation temperature was seen in both *G. duebeni* populations, with an increase in acclimation temperature from 5 to 10 °C giving a corresponding increase in thermal tolerance of 1.6 °C for the population from Wales and 1.7 °C for the population from Norway.

The higher upper thermal limit in *G. duebeni* compared to *G. locusta* may reflect the ability of *G. duebeni* to occupy a wider environmental niche, and occupy higher shore locations with greater fluctuations in temperature. However it may also indicate that *G. locusta* possesses a higher maximum performance. It has been suggested that a trade-off exists between performance breadth and maximum performance, meaning *G. duebeni* may be able to tolerate a wider range of temperatures than *G. locusta*, at a cost of a reduction in maximal fitness (Huey & Kingsolver, 1993). This may explain why despite its ability to

occupy a wide niche, when several *Gammarus* species are present on one shore, *G. duebeni* tends to be outcompeted (Gaston & Spicer, 2001).

Stillman (2003) found a negative correlation between maximal habitat temperature and acclimatory capacity of Porcelain crabs of the genus *Petrolisthes*. He proposed the existence of a trade-off between tolerance to high temperatures and ability to adjust upper thermal limits through acclimation, and theorised that warm adapted, tropical *Petrolisthes spp*. are not only less able to adjust upper thermal limits, but are closer to their thermal maxima than temperate species (Stillman & Somero 2000). In contrast, a study of European diving beetles (*Deronectes spp*.) showed a positive correlation between upper thermal tolerance and acclimatory ability (Calosi, Bilton, & Spicer, 2008). Stillman's (2003) view may apply to *G. duebeni* and *G. locusta*, with the former trading high absolute thermal tolerance for a lower acclimatory capacity. However, despite the acclimatory ability of *G. locusta* with regards to thermal tolerance, 5 °C acclimated individuals exhibited a comparable upper thermal limit to *G. duebeni* acclimated to 15 °C. Within these two species, upper thermal tolerance seems to hold a greater importance than the capacity to adjust thermal tolerance with acclimation. As with the diving beetles (Calosi, Bilton, & Spicer, 2008) it may be that the magnitude of thermal tolerance rather than its acclimatory ability determines range size, explaining why *G. locusta* exhibits more phenotypic plasticity but occupies a much narrower geographical range and niche breadth than *G. duebeni* (Gaston & Spicer, 2001). In general the importance of acclimatory ability is still debated, with Stillman (2003) arguing acclimation is the most critical factor determining susceptibility to climate change while Peck et al. (2009) contends that this may not always be the case, and species and populations are likely to respond differently based on a host of other factors, including performance windows and how close habitats are to the thermal limits of the individuals residing in them.

It is also important to appreciate that problems can arise from assuming latitudinal populations originate from different thermal regimes, with temperature decreasing predictably with latitude. Helmuth et al. (2006) pointed out the oversimplification of using latitudinal gradients when discussing thermal habitats and climate change, as local tidal regimes (both the timing and magnitude of low tide) and wave splash can affect temperatures, creating complex thermal mosaics. When Sorte & Hofmann (2005) acclimated five *Nucella spp*., they found thermal tolerance was affected by both latitude and tide height. Species from the high intertidal at low and mid-latitudes were more

thermotolerant than species from the low intertidal, or the high intertidal but at a high latitude. Similar results have been found in studies of marine snails from the genus *Tegula* from tropical, temperate, subtidal and intertidal habitats (Tomanek & Somero 1999). The complexity of the thermal environment may be highest in the temperate zone, as evidenced by greater variability of lethal temperatures within the same field site for temperate than tropical porcelain crabs (Stillman 2003). Consequently it is important to measure the thermal microclimates experienced by the species of interest as carried out for the gammarid species in the current study (Chapter 2).

The highest temperature measured over 12 months in Tromsø was 23.5 °C in July. However this was one of only four days in the year in which temperatures climbed above 20 °C (Section 2.1.5.). In Anglesey temperatures of 20 °C and over were recorded for six months of the year, and peaked at 33.4 °C in August. The upper thermal limit of *G. locusta* acclimated to 15 °C was 33.5 °C. If susceptibility to warming was defined by a population's proximity to threshold temperatures the most vulnerable would be *G. locusta*, while populations of *G. duebeni* and *G. oceanicus* from Norway (70 °N) would be the least vulnerable. The population of *G. duebeni* from Wales (53 °N) inhabits an area of the shore far closer to the upper thermal limit of 36.2 °C measured after acclimation to 20 °C than *G. duebeni* from Norway (70 °N), which after acclimation to 10 °C have a thermal tolerance 8 °C higher than the maximum recorded temperature on the shore. In a review of terrestrial insects, Deutsch et al. (2008) concluded that populations from temperate habitats not only have a broader thermal tolerance than populations from the tropics, but may also be operating below their thermal optima. If *G. duebeni* at the northern extent of their range are residing below their optimum temperatures then they may experience an increase in fitness as a result of climate change. Temperatures in Wales and Norway were, however, only recorded in the mid to high intertidal, where *G. duebeni* were abundant, as data loggers from the low shore were not recovered (Chapter 2).

As *G. locusta* has the most southerly range of the three species from the acclimation study (Costa et al. 2009; Gaston & Spicer 2001; Rock et al. 2009), similar latitudinal differences as in *G. duebeni* are likely. Unlike intertidal sessile invertebrates, gammarid amphipods are highly mobile and likely to show behavioural thermoregulation by seeking cooler areas on the shore; therefore temperatures from a static data logger are unlikely to be an accurate prediction of body temperature. When temperatures in tide pools on Anglesey reached above 20 °C *G. locusta* were generally found underneath rocks and vegetation where

temperatures were lower, and individuals of *G. duebeni* and *G. locusta* have been observed emerging from pools and crawling on land where they can take advantage of evaporative cooling as described for the semi-terrestrial isopod, *Ligia oceanica* (Edney, 1953).

Kuo & Sanford (2009) reared the intertidal snail *Nucella canaliculata* for two generations, discovering that differences in lethal temperatures between populations persisted and therefore were likely to have a genetic basis. The latitudinal populations of *G. duebeni* acclimated to common temperatures for six weeks showed the same significantly lower UTL in the northern populations as acclimatised *G. oceanicus*. This could indicate genetic differences and possibly differences in underlying physiology; however it may also be the influence of thermal history during development. Interestingly both populations showed a very similar increase in UTL between an acclimation temperature of 5 and 10 °C, with a greater difference seen between populations than between acclimation temperatures.

3.5.3. Effects of salinity acclimation on *Gammarus duebeni*

Salinity acclimation had an interesting effect on thermal tolerances in *G. duebeni*, interacting with acclimation temperature to influence mortality rates. *G. duebeni* is considered to be highly tolerant to a wide range of environmental variables including salinity, and can therefore be found occupying marine, estuarine and freshwater habitats (Gaston & Spicer, 2001). Previous research has already suggested that *G. duebeni* is a strong osmoregulator, showing relatively low stress responses to acute salinity change in comparison to other *Gammarus* species (Tedengren et al., 1988), although survival of *G. duebeni* in laboratory studies has tended to be highest when cultured in brackish environments (Kinne 1952). Mortality rates of *G. duebeni* during acclimation were too high at 20 °C and 35 psu for inclusion in this study, but despite this, upper thermal limits at the remaining temperatures remained unchanged across the acclimation salinities of 18 and 35 psu. This suggests that although salinity is a stressor for *G. duebeni*, the species is tolerant enough to be unaffected by salinity provided exposure to thermal stress is short-lived. Although the changeable conditions of the intertidal requires physiological flexibility, this high tolerance for salinity in conjunction with temperature is unusual in even high shore species. The copepod *Tigriopus brevicornus* for example, found in rock pools above the high tide mark, has a thermal window which increases with salinity (Damgaard & Davenport, 1994).

The regulation of internal ionic concentrations is crucial to survival in gammarid amphipods (Sornom et al., 2010). If osmoregulation is disrupted as salinitity increases, mortality may occur as a result of high haemolymph osmolality (Hart et al., 1991), as occurs in *G. roeseli* exposed to high salinities (Sornom et al., 2010). *G. duebeni* are thought to prefer a salinity between 5 and 22 psu (Kinne, 1952; Tedengren et al., 1988), and are close to their iso-osmotic point at 50 % seawater (Brooks & Lloyd Mills, 2003). Gammarid amphipods have been shown to acclimate to low salinities through physiological adjustments of gill ultrastructure (Milne & Ellis, 1973b), and by increasing gill Na^+/K^+-ATPase activities to counteract the loss of Na+ ions to the environment (Brooks & Lloyd Mills, 2006). Consequently, *G. duebeni* is capable of hyperosmotic regulation below 50 % seawater, but struggles to ionoregulate in full strength seawater, becoming an osmoconformer at higher salinities (Brooks & Lloyd Mills, 2006; Tedengren et al., 1988).

The iso-osmotic point has been shown to reduce with temperature in the estuarine shrimp *Crangon crangon* (Weber & Spaargaren, 1970) and prawn *Palaemon longirostris* (Campbell & Jones, 1989); the authors suggest this is an adaptive response in estuarine crustaceans to maintain osmotic homeostasis during seasonal migrations from areas of low salinity in summer to areas of higher salinity in winter. During the winter months in Wales *G. duebeni* were scarce in the high-intertidal. Although a survey was not carried out into distribution patterns according to season, individuals found during the colder months were lower down on the shore. Kinne (1952) related increased tolerances to low salinities at cold temperatures in *G. duebeni* to higher haemolymph osmolarity. A similar interaction between salinity and temperature was seen in *G. roeseli*, which showed a decrease in salinity tolerance with temperature (Sornom et al., 2010). *G. duebeni* may exhibit acclimation to low salinities, but may only be able to tolerate high salinities for relatively short periods of time. At 20 °C and 35 psu salinity *G. duebeni* exhibited almost 90 % mortality over the six week acclimation period in comparison with less than 10 % mortality in all other conditions. This may be due to both high temperature and high salinity acting as individual stressors, or it could be that elevated temperature exacerbates the osmoregulatory ability of *G. duebeni*.

3.5.4. Effect of rate of warming

The rate of warming is known to affect critical thermal limits (Peck et al., 2009). *G. duebeni* has already been shown to be affected by the duration as well as the magnitude of the thermal stress (Ginn et al., 1976). Ginn et al. (1976) subjected freshwater *G. duebeni* from New York to an 8.3 °C acute increase in temperature. When mortality was plotted against exposure time a shift in slope was observed around 300 minutes, with a lower mortality rate observed before this time. The authors suggested that the rate change indicated differences in the cause of death during exposure for up to 300 minutes and the cause of death after this time point. When exposed to a slower rate of temperature increase, *G. duebeni* from Norway (70 °N) no longer showed a significant difference in upper thermal limit between 5 and 10 °C acclimated individuals, and the two populations no longer differed at a common acclimation temperature of 5 °C. It may be that the faster rate of warming magnified differences between populations and acclimation treatments, making them more apparent, or it may be that the slower rates of warming enabled the colder amphipods to adjust physiologically to the elevation in temperature.

Further investigations are required on the underlying physiology involved in determining thermal tolerance in the *Gammaridae*. Variation in the expression of heat shock protein (Hsp) in the inducible Hsp70 family has been suggested as a measure of the susceptibility of species to thermal stress and a possible mechanism underlying changes in thermal tolerances (Hamdoun et al. 2003, Hoffmann et al. 2003, Tomanek & G. Somero 1999). In freshwater gammarid amphipods exposed to thermal stress, small heat shock protein (sHsp, molecular mass of 15-30 kDa) expression in the euryecious *G. lacustris* peaks after 12 hours then declines, suggesting acclimation, while sHsp expression in the stenoecious *Eulimnogammarus cyaneus* steadily increases (Timofeyev et al., 2009).The heat shock response can however be complex, as although Fangue et al. (2006) found higher levels of Hsp70-2 induction in the more northern of two latitudinal populations of the killifish *Fundulus heteroclitus* in conjunction with a lower thermal tolerance, the southern killifish population was characterised by an elevation of heat shock cognate 70 (Hsc70) mRNA in response to heat shock.

Although the heat shock response may play a role in determining upper thermal limits assumptions cannot be made about the expression of heat shock proteins in this study. The fast rate of warming involved in the main part of this study may have precluded the influence of the heat shock response. The induction of Hsp70 in *Mytilus edulis* has a

delayed response, not occurring until six hours after initial exposure to thermal stress (Chapple, Smerdon, & Hawkins, 1997) and has been shown to be lacking entirely in the Antarctic gammarid *Paraceradocus gibber* (Clark, Fraser, & Peck, 2008). The heat shock response is energetically costly and therefore species from stenothermal environments or species able to undergo behavioural thermal regulation may lack the heat shock response or not show the same plasticity in response demonstrated by sessile intertidal invertebrates (Tomanek & Somero 1999).

The oxygen and capacity limitation hypothesis (Frederich & Pörtner 2000) suggests critical thermal limits are determined by an interaction between the demand and supply of oxygen. When exposed to increasing temperatures the *Gammarus spp.* studied showed a pattern of an increase in pleopod beat frequency, indicating an increase in ventilatory activity followed by a subsequent decrease leading to mortality. This response has been recorded before in gammarid species by Wijnhoven et al. (2003), and reflects the findings of Frederich & Pörtner (2000) who found the important factor in the spider crab, *Maja squinado*, to be the supply of oxygen (ventilation rate) rather than the ability to distribute it (heart rate). Stillman & Somero (1996) observed a similar rise followed by decline in the heart rate of porcelain crabs (*Pestrolisthes spp*.) exposed to acute rises in temperature. The strong link between the temperature associated with loss of cardiac function and thermal tolerance limits of the whole organism in porcelain crabs lends further support for this argument (Somero 2010). Increasing mitochondrial density and therefore aerobic capacity is thought to be one of the primary means of avoiding oxygen limitation during less rapid or extreme change (Pörtner et al. 2001) and would be an interesting area of further study in gammarid amphipods.

3.5.5. Conclusion

Thermal tolerances in acclimatised amphipods were negatively correlated with latitude. The boreal/sub-arctic *G. oceanicus* exhibits population differences in upper thermal tolerance, and has a higher tolerance at 79 °N than the circumpolar *G. setosus* from the same shore. *G. duebeni* and *G. locusta* both exhibit adjustment of thermal limits in response to temperature acclimation, however, *G. duebeni* has a higher overall thermal tolerance while *G. locusta* has a greater acclimatory capacity. If vulnerability to climate change is determined by habitat proximity to threshold temperatures then *G. locusta* from

Wales (53 °N) will be the most susceptible while the populations of *G. duebeni* and G. *oceanicus* from Norway (70 °N) should show the least vulnerability.

G. duebeni from Wales exhibited the highest acclimatised and acclimated thermal tolerance of the species and populations studied, and was unaffected by salinity at acclimation temperatures up to 15 °C. Despite this apparent adaptation to intertidal conditions and the species' relatively large geographical range it is generally out-competed by other *Gammarus* species on the shore (Gaston & Spicer, 2001). Studies into the physiological responses to temperature are required to determine the critical factors responsible for setting thermal limits, and to examine relative fitness levels of populations to fully understand how gammarid amphipod populations will respond to warming.

This study only takes into account the effect of thermal stress on gammarid amphipods, whereas in the intertidal these animals are exposed to multiple stressors such as temperature, salinity, emersion and anoxia. In a study of the freshwater gammarids *G. fossarum* and *G. pulex*, the velocity of the stream was deemed a more critical factor than a range of other biotic factors including temperature in determining the distribution of the two species (Peeters & Gardeniers, 1998). In a study of invasive and indigenous gammarid species, it was concluded that the invasive *G. tigrinus* has a competitive advantage in ion-rich water, while the indigenous *G. pulex* is likely to dominate in relatively ion-poor brook water due to the negative influence of low ion levels on thermal tolerance in euryhaline invasive species (Wijnhoven et al., 2003). Although salinity acclimation had no effect on thermal tolerance at the acclimation temperatures used here, high mortality in *G. duebeni* held at 35 psu salinity and 20 °C suggests that exposure to full strength seawater is detrimental to *G. duebeni* when combined with elevated temperatures.

Chapter 4

The effect of acclimation on aerobic metabolism: rates of oxygen uptake and citrate synthase activity

4.1. Abstract

Whole-animal rates of oxygen uptake (MO_2, proxy for metabolic rates) and tissue citrate synthase activity (proxy for aerobic capacity) were determined in gammarid amphipods with varying latitudinal range extents following temperature acclimation. The boreal-temperate *G. duebeni* exhibited compensation of rates of both oxygen uptake and citrate synthase activity, however intraspecific differences existed between populations from Norway (70 °N) and Wales (53 °N). The population from Norway had lower rates of oxygen uptake and citrate synthase activity levels than the population from Wales, and a greater thermal sensitivity of MO_2 indicating metabolic diversity between populations and suggesting local adaptation. Although *G. duebeni* exhibited temperature independence following acclimation to salinities equivalent to half strength seawater, the ability to show metabolic plasticity was compromised when held in salinities equivalent to full strength seawater due to the high metabolic costs associated with osmoregulation. The warmer water species, *G. locusta*, exhibited a higher rate of oxygen uptake when acclimated to 5 °C than higher temperatures, possibly as a compensatory response to maintain their higher energy lifestyle in the cold.

4.2. Introduction

Inter- and intraspecific variations in the thermal tolerance of ectothermic organisms have been related to distribution patterns along natural thermal gradients, according to both large-scale latitudinal variations in temperature and to local effects such as shore height. Thermal tolerances are therefore a useful measure to identify the relative susceptibility of an organism to climate change (Somero, 2010). The ability to cope with change may therefore be dependent on the ability of an organism to exhibit temperature compensation in cellular and whole-animal processes (Clarke, 2003). Such responses are likely to hold a great importance in gammarid amphipods, due to both the close relationship between environmental and body temperature (Helmuth & Hofmann, 2001) and the relatively high temporal and spatial temperature variation experienced in their intertidal habitats.

Metabolic rate is an important whole-animal response which has been shown to positively correlate to temperature, typically showing a Q_{10} of approximately 2-3 (Clarke & Fraser, 2004), and provides an estimate for the cost of living and an organism's total energy expenditure. Investigations into the relationship of metabolic rate and temperature have a

long history (Krogh, 1916) and significant interspecific variations in resting metabolic rate have been observed (Careau et al., 2008) along with variation in the thermal sensitivity of rates of oxygen uptake according to species, population and the temperatures considered (Whiteley & Taylor, 1998). In a comprehensive review of the underlying physiology of temperature dependent biogeography in ecotherms, Pörtner (2002) suggested that limitations in oxygen availability and aerobic scope were critical in determining thermal tolerances. Metabolic adjustments to compensate for declining oxygen supply and increasing oxygen demands at high temperatures can therefore be related to thermal tolerance, and hence temperature dependent distribution patterns and tolerances to climate change (Pörtner et al., 2007).

Metabolic cold adaptation (MCA) assumes polar species will show an elevation of metabolic rate at the low temperatures experienced in their environment in comparison to warmer-water counterparts exposed to the same cold temperatures (Clarke, 1983). MCA has been supported by a large review of terrestrial insects (Addo-Bediako et al., 2002) and by studies on mitochondrial proliferation in the cold (Egginton & Johnston, 1984; Johnston et al., 1998), however the theory has received criticism and evidence to the contrary is prevalent (Chapelle & Peck, 1995; Werner et al., 2002; Whiteley et al., 1996). For example, it has been argued that MCA in polar ectotherms would incur a significant fitness cost, and that early studies showing an elevated metabolic rate in polar organisms may have failed to account for limitations in the methodology, such as the stress of handling, and to adequately extrapolate metabolic rate with temperature (Clarke, 1991). Instead polar organisms exhibit low metabolic rates and a low-energy lifestyle, although it is unknown whether the selective pressure for reduced resting metabolic rates is due to the cold temperatures themselves, the relative stability of the environment or limited resource availability (Clarke, 1993; Clarke, 2003; Lonsdale & Levinton, 1989; Pörtner, 2002)

Although the existence of MCA in polar species is controversial, elevations of metabolic rate in lower latitude populations have been observed in response to the cold (Sommer & Pörtner, 2002). It may be that the more variable nature of the temperate environment exerts a stronger selective pressure on the conservation of metabolic rate. A few multigenerational studies have indicated these intraspecific differences persist through several generations of rearing at common acclimation temperatures, indicating a genetic basis and suggesting local adaptation according to differences in thermal regime between populations (Dittman, 1997; Lonsdale & Levinton, 1989).

Citrate synthase is a key metabolic enzyme responsible for initiating and regulating the citric acid cycle and is in turn regulated by the supply and demand of ATP (Vetter, 1995b). Citrate synthase activity is often used as a proxy for mitochondrial volume and aerobic capacity (Berges & Ballantyne, 1991). Despite the temperature dependency of enzyme function and activity (Salomon & Buchholz, 2000) compensatory responses in citrate synthase activity have been observed according to seasonal temperature variations in the horse mussel *Modiolus modiolus* (Lesser & Kruse, 2004), and among populations of the lugworm *Arenicola marina* (Sommer & Pörtner, 2004) and the limpet *Nacella concinna* (Morley et al., 2009).

The present study investigates oxygen uptake rates following temperature acclimation in gammarid amphipods with varying but overlapping latitudinal distributions (Rock et al., 2009), differences in environmental tolerance (Gaston & Spicer, 2001) and an established phylogeny (Costa et al., 2009). Previous research has shown evidence of metabolic compensation with latitude in two acclimatised amphipods, the temperate *G. locusta* and temperate/boreal *G. duebeni*, but not in the subarctic/boreal *G. oceanicus* (Rastrick & Whiteley, 2011). This study intends to expand on those findings by exploring inter- and intraspecific differences in the phenotypic plasticity of the metabolic response through temperature acclimation, including long term acclimation to the cold (5 °C). This is supported by an investigation into the effect of temperature acclimation on citrate synthase activity in gammarid amphipods. *G. duebeni* is a high shore, euryhaline gammarid with a broad environmental tolerance (Rock et al., 2007) and therefore the interacting effects of temperature and salinity on the plasticity of rates of oxygen uptake and aerobic capacities were explored to investigate how tolerance may be affected by osmoregulation.

4.3. Methodology

4.3.1. Rates of oxygen uptake

4.3.1.1. Field-acclimatised juveniles

Juveniles of *Gammarus setosus* and *oceanicus* were collected from under rocks and stones on the shore-line in Ny-Ålesund, 79 °N in September 2010 by using hand nets. Amphipods were maintained in fully-aerated seawater from the collection site at 5 °C for 24 hours

without feeding to allow them to recover from handling stress and prevent artificial elevation of oxygen uptake rates due to recent feeding.

Oxygen uptake rates (MO_2) were measured in six *G. setosus* and five *G. oceanicus* juveniles in a small volume respirometer (RC350, Strathkelvin Instruments Limited, Scotland) connected to a 781 meter (Strathkelvin Instruments, Scotland) maintained at the appropriate temperature by water flowing through the surrounding water jacket from a temperature-controlled water bath (Grant Instruments, UK). The respirometer contained a stirrer beneath a pierced platform to prevent the formation of oxygen partial pressure gradients (PO_2). The oxygen electrode was zeroed and calibrated according to the manufacturer's instructions. Amphipods were placed individually into the respirometer and the sea water aerated for ten minutes. The electrode holder was then inserted into the respirometer, ensuring no air bubbles remained in the chamber. PO_2 levels were determined immediately after inserting the electrode, then again after a ten minute interval to allow stabilisation and provide a starting value. PO_2 levels were then determined at regular intervals for a further 10 minutes to give a measurement of oxygen uptake rates. Whole animal rates of oxygen uptake were calculated as in Section 4.3.1.3. Ten minutes was deemed sufficient to view a change in PO_2 levels without causing hypoxia, i.e. drop in oxygen levels below 16 kPa.

4.3.1.2. Acclimated amphipods

In September 2010 adult *G. setosus* from Ny-Ålesund, 79 °N and adult *G. oceanicus* and *G. duebeni* from Skye, 58 °N were collected and returned to marine aquaria at Bangor University (Table 2.1.). Individuals were acclimated to a common temperature of 5 °C for nine months (Table 2.2) as described in Chapter 2, Section 2.2.2 and starved for 24 hours prior to the determination of oxygen uptake rates.

Amphipods from two seasons, species and latitudes were collected in 2012 (Table 2.1.). Individuals were acclimated to a range of temperature and salinity conditions (Table 4.1. for summary) and held for six weeks under the conditions described in Chapter 2 then starved for 24 hours prior to measurements of resting oxygen uptake rates and rates after an acute change, as described below.

Table 4.1. Acclimation conditions for six weeks prior to measurements of oxygen uptake rates. Shaded boxes indicate amphipods were held at the specified temperature. Oxygen uptake rates were measured for amphipods from all acclimation conditions. Citrate synthase activity was measured in all but in *G. duebeni* at 18 psu and 20 °C.

Date of collection	Collection site	Species	Salinity (psu)	Temperatures (°C)			
				5	10	15	20
May, 2012	Anglesey, Wales, 53 °N	*G. duebeni*	18	■	■	■	■
			35	■	■	■	
		G. locusta	35	■	■	■	
October, 2012	Tromsø, Norway, 70 °N	*G. duebeni*	18	■	■		
		G. oceanicus	35		■		
	Anglesey, Wales, 53 °N	*G. duebeni*	18	■	■	■	
		G. locusta	35		■		

The determination of MO_2 in acclimated individuals was undertaken in stop flow respirometers maintained at the appropriate temperature by water flowing through the surrounding water jacket from a temperature-controlled water bath. The respirometers (final volume was 8 ml each) contained a stirrer beneath a mesh platform to prevent the formation of a PO_2 gradient within the respirometers, and several beads to encourage the amphipods to keep activity levels to a minimum. Each chamber was supplied with a continuous flow of fully aerated seawater at a controlled temperature and salinity. Amphipods were placed individually within respirometers and allowed to settle overnight to determine resting MO_2 levels. The respirometer was covered for the duration of the settling period and the experiment to minimise disturbance to the amphipods.

Water samples were taken for the determination of oxygen partial pressure (PO_2) levels before and after sealing the respirometers for 20 minutes, a time judged sufficient in preliminary experiments to view a change in PO_2 levels without causing hypoxia, i.e. drop in oxygen levels below 16 kPa. To measure the response to acute changes in temperature, the water flow to each respirometer was re-established and the temperature was increased or decreased at a rate of 3 °C per hour. Water samples were removed for the determination of PO_2 measurements at intervals of 5 °C. Each time the respirometer was resealed, water

PO_2 was checked to ensure that the seawater within the respirometer was 100 % saturated with air.

PO_2 readings were taken by collecting water samples from directly within each chamber and passing them over a Strathkelvin oxygen electrode maintained at experimental temperatures by means of a water jacket, connected to a Strathkelvin 781 meter (Strathkelvin Instruments Limited, Scotland). The oxygen electrode was zeroed and calibrated to fully aerated seawater according to the manufacturer's instructions. Amphipods which had recently undergone a moult or moulted while in the respirometer were excluded from the data analysis, as metabolic rates in gammarid amphipods are known to be elevated during the moulting period (Halcrow & Boyd, 1967).

4.3.1.3. Calculation of MO_2 and Q_{10} values

Whole animal rates of oxygen uptake were calculated as the change in PO_2 per hour, multiplied by the solubility coefficient for oxygen at the appropriate salinity and temperature (Harvey, 1957) and adjusted according to the volume of water within the respirometer. Values were standardised to SDTP and then converted into nmol. Rates of oxygen uptake standardised to a wet body mass of 1 g was calculated using a mass exponent of 0.62 (Rastrick & Whiteley, 2011) and expressed as a whole animal value for MO_2 with the units $nmol.O_2.g^{-1}.h^{-1}$.

The mean temperature coefficient (Q_{10}) was calculated using the van 't Hoff equation:

$$Q_{10} = R2/R1^{(10/T2-T1)}$$

Where T1 is the initial temperature (acclimation temperature of 10 °C) and T2 is the temperature following an acute change (20 °C). R1 and R2 are the whole-animal MO_2 values ($nmol.O_2.g^{-1}.h^{-1}$) at the respective temperatures.

4.3.2. Citrate synthase activity in acclimated gammarid amphipods

Acclimated amphipods from the six week study (Table 4.1.) were sacrificed, the gut removed and the remaining carcass frozen in liquid nitrogen in preparation for the citrate synthase assay as described by Bergmeyer (1978). Individual amphipods were homogenised in 150 µl Tris-HCl buffer, then sonicated briefly to break up the mitochondria.

300 µl of assay cocktail (25.5 ml 50 mM imidazole-HCl pH 8.2, 30 ml 15 mM $MgCl_2$, 1.2 mg DTNB and 3 mg Acetyl Co.A) and 20 µl of each sample was pipetted into a microplate in triplicate. The plate was read each minute for five minutes in a SpectraMax® M2e microplate reader at 412 nm to provide a background reading. The reaction was started by adding 4 µl of 5.3 $mg.ml^{-1}$ oxaloacetate dissolved in imidazole-HCl (pH 8.2) to each well. The increase in absorbance due to the reduction of DTNB was recorded each minute for a further five minutes at 412 nm.

Citrate synthase activity was calculated in International Units of activity (micromoles of substrate converted to product per minute) according to the formula:

$$Activity = \left(\frac{\Delta Absorbance}{minute}\ x\ volume\right) / Extinction\ coeffecient$$

Where *ΔAbsorbance* is the change in absorbance during the background reading subtracted from the change in absorbance after the addition of oxaloacetic acid and the micromolar extinction coefficient of DTNB is 13.6 moles μl^{-1} cm^{-1}. Activity levels were expressed as micromoles of citrate formed per minute, per gram fresh weight of tissue ($\mu mol.min^{-1}.g^{-1}$).

4.3.3. Statistical analysis

Mean whole-animal MO_2 ($nmol.O_2.g^{-1}.h^{-1}$) and citrate synthase activity levels (per gram of tissue) were compared by analysis of variance (ANOVA) or t-tests after performing the Kolmogorov-Smirnov test for normality and Levene's test for homogeneity of variances to satisfy the criteria for the tests. Multiple comparisons were made using the least significant difference post hoc test (LSD test). Values are given as means ± S.E.M, and results are considered significant at the 5 % confidence interval ($P < 0.05$). Statistical analyses were performed using SPSS (SPSS version 21; SPSS Inc., Chicago, IL, USA).

4.4. Results

4.4.1. Rates of oxygen uptake in gammarid amphipods

4.4.1.1 Field acclimatised juveniles and long term acclimation of adults

When measured within 24 hours of collection from the shore in Svalbard, 79 °N, rates of oxygen uptake (MO_2) in juveniles of the circumpolar *G. setosus* and the subarctic/boreal *G. oceanicus* showed no significant differences (t-test: t_9 = -1.856, P = 0.096). Rates of oxygen uptake (MO_2) were 48.0 ± 4.6 and 32.8 ± 7.1 nmol.O_2.g^{-1}.hr^{-1} in *G. setosus* and *G. oceanicus*, respectively, when scaled to a body mass of 1 g (Figure 4.1.). When adults from the same two species were acclimated to a common temperature of 5 °C for nine months, *G. setosus* from 79 °N showed no differences in MO_2 compared with *G. oceanicus* from a latitude of 70 °N (Figure 4.2., t-test: t_8 = 0.929, P = 0.377). At the same latitude of 70 °N, *G. duebeni* showed a significantly higher MO_2 at 40.1 ± 4.5 nmol.O_2.g^{-1}.hr^{-1} than *G. oceanicus* at 25.6 ± 2.0 nmol.O_2.g^{-1}.hr^{-1} (Figure 4.2., t-test: t_8 = 3.152, P = 0.014). The latter was not significantly different to the mean of 29.4 ± 3.3 nmol.O_2.g^{-1}.hr^{-1} measured in *G. setosus* at 79 °N (t-test: t_9 = 1.487, P = 0.171).

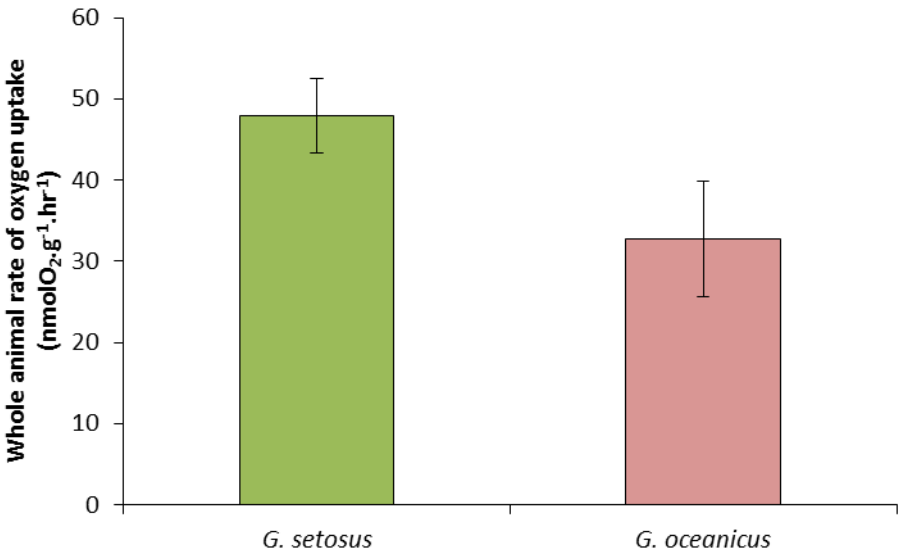

Figure 4.1. Whole animal rates of oxygen uptake in juvenile *Gammarus setosus* (N = 6) and *G. oceanicus* (N = 5). Amphipods were collected in Svalbard, 79 °N, and measured within 24 hours of collection at 5 °C. Values are means ± S.E.M. Rates are not significantly different (t-test, t_9 = 1.856 P = 0.096).

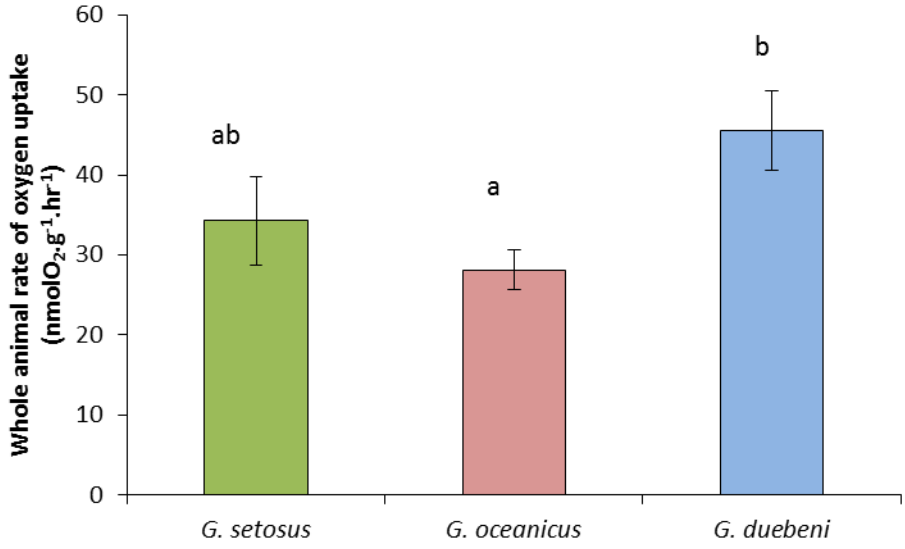

Figure 4.2. Whole animal rates of oxygen uptake in *Gammarus setosus* from 79 °N (N = 6) and *G. oceanicus* and *G. duebeni* from 70 °N (N = 5) acclimated to 5 °C and a salinity of 35 psu over a period of 9 months. Values are means ± S.E.M. Letter groups indicate values with no significant differences (P > 0.05). *G. duebeni* has a significantly higher rate of oxygen uptake than *G. oceanicus* (t-test: t_8 = 3.152, P = 0.014).

4.4.1.2. Six week acclimation of adults

Rates of oxygen uptake in G*. duebeni* collected from Wales in summer showed significant differences according to acclimation salinity, but not with temperature, although, there was an interaction between the two factors (Figure 4.3., ANOVA: $F_{2,59}$ = 11.607, P = 0.000). Although MO_2 showed no variation with temperature in *G. duebeni* acclimated to a salinity of 18 psu (ANOVA: $F_{3,32}$ = 0.963, P = 0.422), individuals acclimated to 35 psu showed a change in MO_2 with temperature (ANOVA: $F_{2,27}$: 18.464, P = 0.000). *G. duebeni* acclimated to 35 psu exhibited the highest mean MO_2 value at 15 °C (LSD test: P = 0.003 and 0.000 compared to 5 and 10 °C acclimated individuals, respectively), but also showed a value for MO_2 which was 70% lower than individuals held at 18 psu and a common acclimation temperature of 10 °C (LSD test: P = 0.000).

Rates of oxygen uptake in *G. duebeni* approximately halved with an increase in latitude from 53 °N to 70 °N, decreasing from 44.2 ± 2.5 to 23.1 ± 2.5 nmol.O_2.g^{-1}.hr^{-1} at a common acclimation temperature of 5 °C (t-test: $t_{6.658}$ = 7.851, P = 0.000), and 49.0 ± 2.1 to 23.9 ±

1.2 nmol.O_2.g^{-1}.hr^{-1} at a common acclimation temperature of 10 °C (Figure 4.4., t-test: t_{22} = 10.314, P = 0.000). Seasonal populations of *G. duebeni* showed no variation in MO_2 at any acclimation temperature (ANOVA: $F_{1,54}$ = 0.130, P = 0.720).

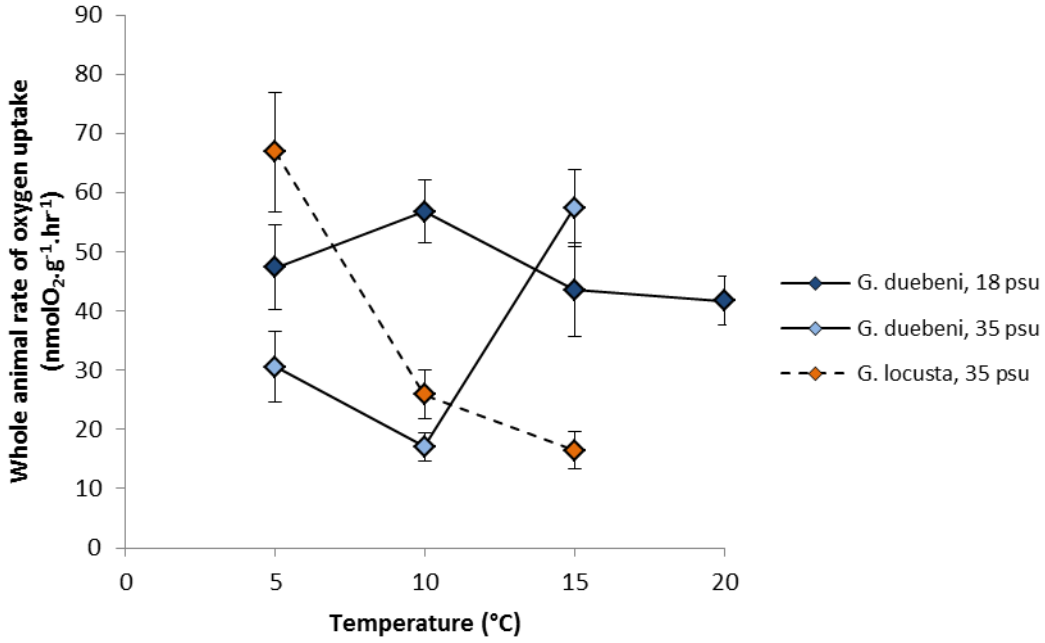

Figure 4.3. Whole animal rates of oxygen uptake in *Gammarus duebeni* and *G. locusta* from 53 °N in summer after acclimation to temperatures between 5 and 20 °C and salinities of 18 and 35 psu. Values are means ± S.E.M. N = 6 for all data points. *G. duebeni* showed no significant difference in oxygen uptake rate with acclimation temperature at a salinity of 18 psu (ANOVA: $F_{3,32}$ = 0.963, P = 0.422), however the ability to maintain a constant oxygen uptake rate with temperature is lost when held at 35 psu salinity (ANOVA: $F_{2,27}$ = 18.464, P = 0.000), as oxygen uptake rates were higher when acclimated to 15 °C than to lower temperatures (LSD test: P = 0.003 and 0.000 compared to 5 and 10 °C respectively). There was a significant interaction between species and acclimation temperature (ANOVA: $F_{2,59}$ = 21.441, P = 0.000).

The three species studied showed significant differences in MO_2 after six weeks acclimation. In Wales, MO_2 was affected by a significant interaction between species and acclimation temperature (Figure 4.3., ANOVA: $F_{2,86}$ = 21.441, P = 0.000). When individuals of *G. duebeni* and *G. locusta* were acclimated to a common salinity of 35 psu, *G. locusta* exhibited a higher rate of oxygen uptake at 5 °C (66.8 ± 10.0 versus 25.9 ± 4.1 nmol.O_2.g^{-1}.hr^{-1}, LSD test: P = 0.000) and a lower rate at 15 °C (16.4 ± 3.2 versus 57.4 ± 6.4 nmol.O_2.g^{-1}.hr^{-1}, LSD test: P = 0.000). In contrast to *G. duebeni*, the elevation in MO_2 value at 5 °C in *G.*

locusta (Figure 4.3., ANOVA: $F_{2,27}$ = 21.588, P = 0.000) was more than double the mean values observed at the higher acclimation temperatures (LSD test: P = 0.000).

In Norway species differences were also observed, although to a lesser extent. At a common acclimation temperature of 10 °C, *G. duebeni* had a mean MO_2 value of 23.9 ± 1.2 $nmol.O_2.g^{-1}.hr^{-1}$, and *G. oceanicus* a mean MO_2 of 19.7 ± 1.5 $nmol.O_2.g^{-1}.hr^{-1}$ (t-test: t_{22} = 2.214, P = 0.0387).

4.4.2. Thermal sensitivity of oxygen uptake in six week acclimated gammarid amphipods

When exposed to an acute increase in temperature to 20 °C from an acclimation temperature of 10 °C, significant increases in MO_2 were observed at the $P < 0.05$ level for all populations of *G. duebeni* and *G. locusta* (Table 4.2.), except for summer *G. duebeni* held at a salinity of 18 psu (t-test: t_{16} = 2.064, P = 0.056). In contrast, winter *G. duebeni* acclimated to the same temperature and salinity did show a significant increase (t-test: $t_{5.960}$ = 2.949, P = 0.026). Q_{10} values between 10 and 20 °C for *G. duebeni* were lower for individuals held at 18 psu (1.55 in summer and 1.44 in winter) than at the higher salinity (3.05 in summer).

Of the two latitudinal populations of *G. duebeni* studied, the population at the higher latitude (70 °N) showed a higher Q_{10} value of 2.49 compared with 1.44 obtained from the population at 53 °N (Figure 4.4.). This was due to the lower MO_2 at 10 °C in the population from 70 °N, as there was no observed difference in oxygen uptake rates following the 10 °C acute temperature increase (t-test: t_{10} = 1.163, P = 0.272).

Despite observed species differences in MO_2, the responses to acute temperature change in *G. duebeni* and *G. locusta* were similar, although *G. locusta* showed a higher oxygen uptake rate when exposed to 20 °C (t-test: t_{10} = 2.383, P = 0.038). In Wales at 53 °N, *G. duebeni* had a Q_{10} value of 3.05 compared to 3.20 for *G. locusta* (Figure 4.5.). Q_{10} values were not calculated for *G. oceanicus*, as although there were significant differences in MO_2 after an acute change in temperature (ANOVA: $F_{3,26}$ = 3.345, P = 0.034), the response over the 10 °C was a significant increase between 15 and 20 °C only, but not overall (LSD test: P = 0.014 and 0.203, respectively).

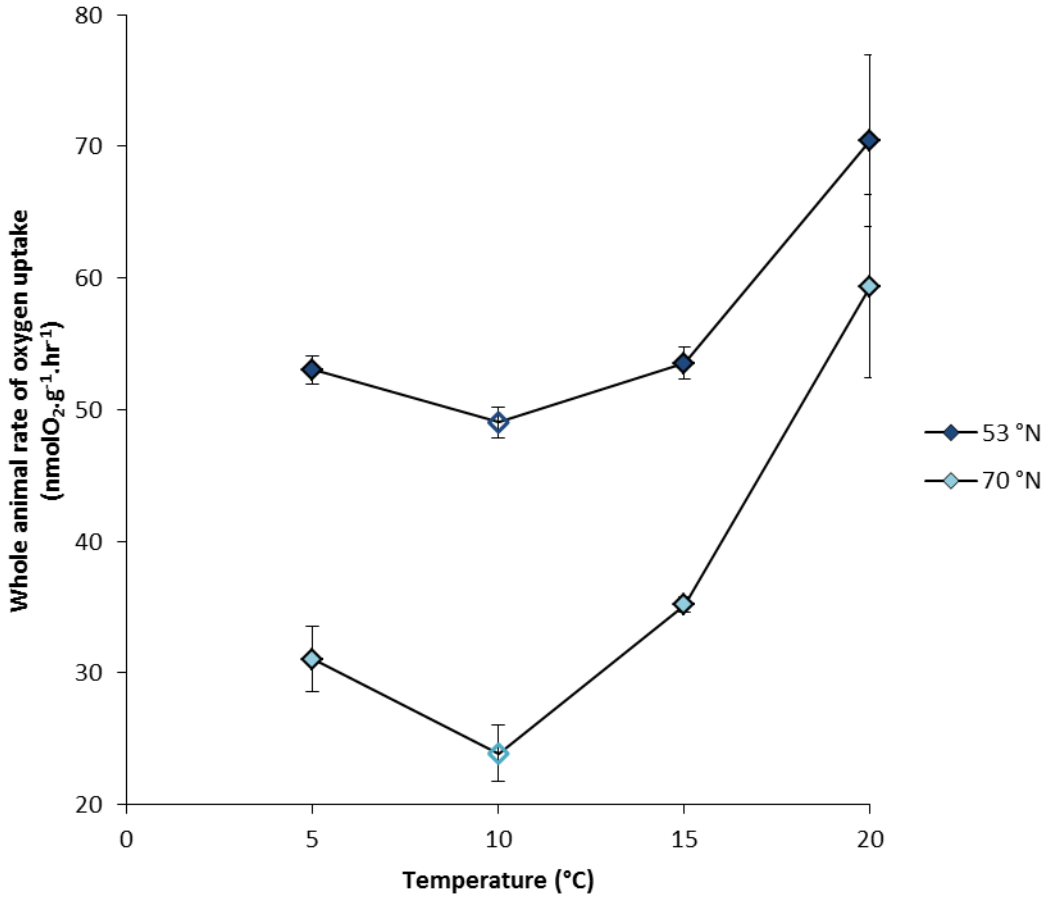

Figure 4.4. Metabolic response to an acute increase and decrease in temperature in *Gammarus duebeni* from two latitudes and acclimated to a temperature of 10 °C and a salinity of 18 psu. Values are means ± S.E.M. N = 6 for all temperatures except 10 °C, where N = 12. Q_{10} values between 10 and 20 °C are 2.49 at 70 °N and 1.44 at 53 °N. A significant increase in oxygen uptake rate was observed between 10 and 20 °C in both populations (t-test: $t_{5.960}$ = -2.949, P = 0.026 at 53 °N, $t_{5.336}$ = -5.341, P = 0.003 at 70 °N). The northern population of *G. duebeni* shows a significantly lower oxygen uptake rate at 10 °C (t-test: t_{22} = 10.314, P = 0.000), but a higher Q_{10} value between 10 and 20 °C.

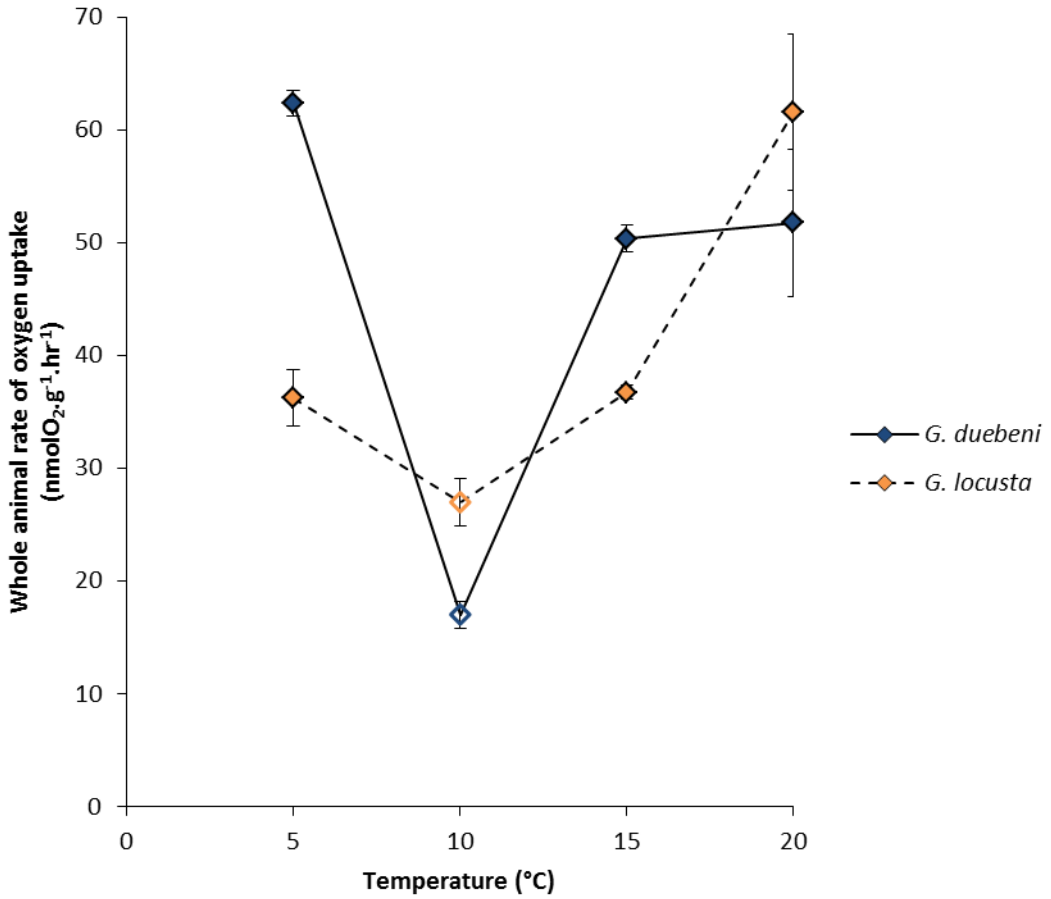

Figure 4.5. Metabolic response to an acute increase and decrease in *G. duebeni* and *G. locusta* held at 35 psu salinity and acclimated to a temperature of 10 °C. Values are means ± S.E.M. N = 6 for all temperatures except 10 °C, where N = 12. Q_{10} values between 10 and 20 °C were 3.05 for *G. duebeni* and 3.20 for *G. locusta*. A significant increase in oxygen uptake rate was observed between these temperatures in both species (t-test: t_{16} = -7.929, P = 0.000 for *G. duebeni*, t_{16} = -5.503, P = 0.000 for *G. locusta*). Although there was no significant difference in resting rates of oxygen uptake between the species (t-test: t_{22} = -1.858, P = 0.077), *G. locusta* shows a significantly higher rate of oxygen uptake at 20 °C (t-test: t_{10} = -2.383, P = 0.038).

Table 4.2. Temperature coefficients (Q_{10}) for whole animal rates of oxygen uptake (scaled to a body mass of 1 g) during an acute increase from a resting temperature of 10 °C (N = 12) to 20 °C (N = 6). All populations were acclimated to a common temperature of 10 °C and showed a significant increase in rates of oxygen uptake after an acute rise in temperature to 20 °C (t-tests: *t* values and *P* values shown) at the $P < 0.05$ level except for *G. duebeni* at 18 psu and *G. oceanicus.* MO_2 values ($nmol.O_2.g^{-1}.hr^{-1}$) are means ± S.E.M. D represents the month of collection, Sal is the acclimation salinity (psu) and Lat the latitude of the collection site (°N).

D	Species	Sal	Lat	MO_2		t-test			Q_{10}
				10 °C	20 °C	*t*	d.f.	*P*	
May	*G. duebeni*	18	53	56.8 ± 5.3	87.3 ± 18.9	2.064	16	0.056	1.55
		35	53	17.0 ± 2.4	51.8 ± 4.0	7.929	16	0.000	3.05
	G. locusta	35	53	25.9 ± 4.1	61.6 ± 6.2	5.503	16	0.000	3.20
Oct	*G. duebeni*	18	53	49.0 ± 2.1	70.4 ± 0.6	2.949	5.960	0.026	1.44
		18	70	23.9 ± 1.2	59.4 ± 6.5	5.341	5.336	0.003	2.49
	G. locusta	35	53	27.0 ± 1.1	61.6 ± 6.2	5.483	5.324	0.002	2.28
	G. oceanicus	35	70	19.7 ± 1.5	22.7 ± 1.6	1.247	16	0.230	1.15

4.4.3. Aerobic capacity in 6 week acclimated gammarid amphipods

G. duebeni showed sensitivity to salinity levels, exhibiting an overall variation in citrate synthase activity (proxy for aerobic capacity) with salinity (Figure 4.7., ANOVA: $F_{1,24}$ = 16.676, P = 0.000). Citrate synthase activity levels at 10 °C were over 1.5 times higher in *G. duebeni* when acclimation salinity levels were doubled (LSD test: P = 0.001).

Citrate synthase activity was lower in the more northern population of *G. duebeni* (ANOVA: $F_{1,24}$ = 6.892, P = 0.016) at both acclimation temperatures (Figure 4.6.). At 5 °C, *G. duebeni* from Norway (70 °N) had a mean activity level of 0.25 ± 0.05 $\mu mol.min^{-1}.g^{-1}$, compared to an activity level of 0.19 ± 0.04 $\mu mol.min^{-1}.g^{-1}$ in the population from Wales (53 °N). No significant variation with acclimation temperature was seen in either the winter population of *G. duebeni* over the three temperatures measured (ANOVA: $F_{2,14}$ = 1.388, P = 0.253), or in the same species from Wales in summer (Figure 4.7., ANOVA: $F_{2,12}$ = 0.699, P = 0.507). The two seasonal populations showed no difference in citrate synthase activity (ANOVA:

$F_{1,26}$ = 3.081, P = 0.091) even after taking acclimation temperature into account (ANOVA: $F_{2,26}$ = 2.846, P = 0.076).

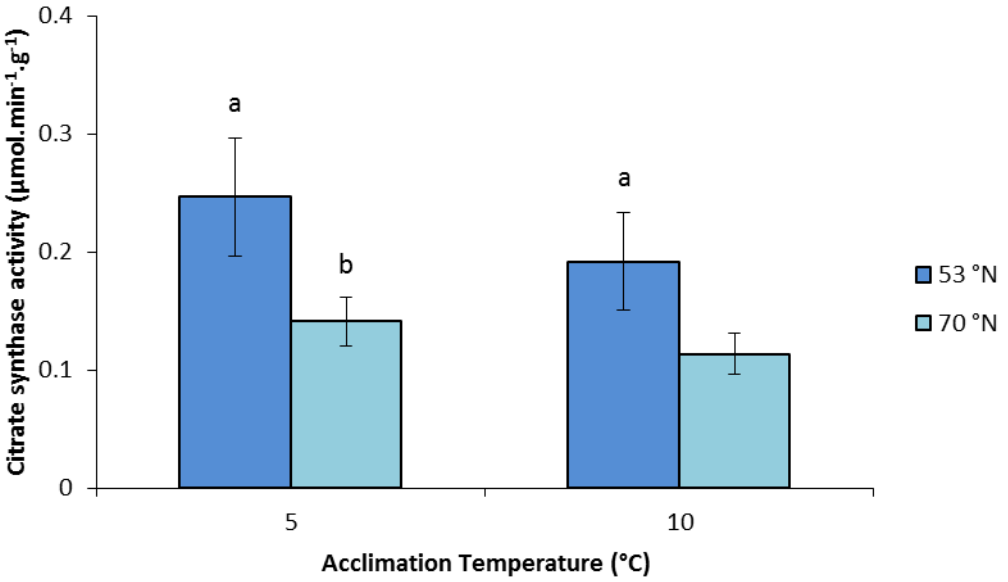

Figure 4.6. Citrate synthase activity per gram of tissue (µmol.min^{-1}.g^{-1}) in two latitudinal populations of *Gammarus duebeni* according to acclimation temperature. Values are means ± S.E.M. N = 5 for both populations at 5 °C and 7 at 10 °C. Means with the same letter are not significantly different (P > 0.05).

At a common acclimation salinity of 35 psu, *G. locusta* exhibited differences in citrate synthase activity levels to *G. duebeni* (Figure 4.7., ANOVA: $F_{1,24}$ = 9.523, P = 0.005). Again, the significant difference was observed at 10 °C, with activity levels for *G. locusta* significantly lower than *G. duebeni* (LSD test: P = 0.019) and comparable to *G. duebeni* acclimated to the lower salinity. The two Norwegian species, *G. oceanicus* and *G. duebeni*, showed similar levels of citrate synthase activity (t-test: t_{10} = 1.673, P = 0.125) at salinities of 35 and 18 psu, respectively, with activity levels of 0.19 ± 0.05 µmol.min^{-1}.g^{-1} and 0.11 ± 0.02 µmol.min^{-1}.g^{-1} respectively. As with *G. duebeni*, *G. locusta* showed no significant effect of acclimation temperature on citrate synthase activity (Figure 4.7., ANOVA: $F_{2,12}$ = 0.715, P = 0.509).

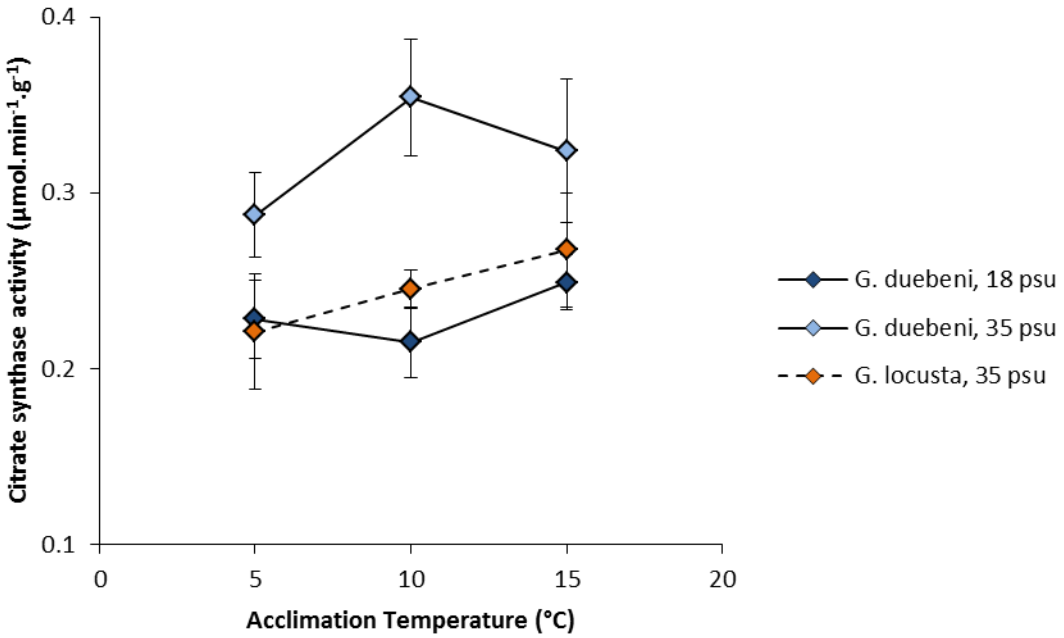

Figure 4.7. Citrate synthase activity per gram of tissue (µmol.min^{-1}.g^{-1}) in *G. duebeni* and *G. locusta* according to acclimation temperature. Values are means ± S.E.M. N = 5 for all data points. *G. duebeni* exhibits a significant variation in citrate synthase activity according to acclimation salinity (ANOVA: $F_{1,24}$ = 16.676, P = 0.000). At the salinity of 35 psu there were significant differences in citrate synthase activity levels between species (ANOVA: $F_{1,24}$ = 9.523, P = 0.005), as *G. duebeni* exhibited a higher citrate synthase activity than *G. locusta* at 10 °C (LSD test: P = 0.019).

4.5. Discussion

4.5.1. Resting metabolic rates and thermal sensitivity

Acclimation temperature had a different effect on rates of oxygen uptake in *G. duebeni* depending on salinity. In amphipods held at the higher salinity of 35 psu, equivalent to full strength seawater, MO_2 increased between acclimation temperatures of 5 °C to 15 °C, but at salinities equivalent to 50% seawater (18 psu), there was no variation in MO_2 between acclimation temperatures. Such differences were reflected in the Q_{10} values calculated for both groups when subjected to an acute increase in temperature between 10 and 20 °C, with Q_{10} values between 10 and 20 °C nearly twice as high in *G. duebeni* acclimated to full strength versus dilute seawater. Collectively, these results indicate that *G. duebeni* showed a greater thermal sensitivity when held in full strength versus dilute seawater. As the rate of temperature change in the acute temperature experiments was equivalent to changes

measured out on the shore, this data suggests that *G. duebeni* is better able to cope with short-term temperature fluctuations in brackish conditions than in salinities equivalent to full strength seawater. An explanation for these marked differences in response is provided by the acclimation experiments. By acclimating *G. duebeni* at both salinities to three different temperatures, it was revealed that *G. duebeni* in dilute seawater had consistently higher MO_2 values than those held in full strength seawater. Even though it is well documented that *G. duebeni* can tolerate a wide range of salinities from freshwater through to hypersaline conditions (Lockwood, 1992), *G. duebeni*, in terms of its ability to osmoregulate, is more characteristic of a brackishwater rather than a marine crustacean (Brooks & Lloyd Mills, 2003; Lockwood, 1992).

G. duebeni is hyper-osmotic in diluted seawater but struggles to maintain Na^+ concentrations of the bodily fluids against a large inward concentration gradient when exposed to full strength seawater (Bolt, 1983; Whitton, 1975) and has an iso-osmotic point around 20 psu salinity (Morritt & Spicer, 1995). At lower salinities and in freshwater, *G. duebeni* is capable of hyper-regulating haemolymph [Na^+] via the increased activity of gill Na^+/K^+-ATPases (Brooks & Lloyd Mills, 2006). This ion transporting enzyme facilitates the active uptake of Na^+ to counteract the passive loss of Na^+ to the environment and to maintain osmotic gradients across the gill. Gill Na^+/K^+ ATPase activities decline when salinities increase because of the reduced need for active Na^+ uptake (Brooks &Lloyd Mills 2006). As a result, haemolymph [Na^+] gradually increases at 20 psu, reaching values that are double those in freshwater at 40 psu. In this respect, *G. duebeni* shows similarities to those crustaceans that are recent colonisers of freshwater (e.g. freshwater prawns of the *Macrobrachium* genus) as they maintain high haemolymph osmolarities in freshwater and are able to tolerate a range of salinities (Freire et al., 2003; Ordiano et al., 2005).

The elevation in metabolic rates at 5 and 10°C in *G. duebeni* at 18 psu as opposed to 35 psu is difficult to explain. In *Machrobrachium* species, energetic costs of osmoregulation are at their lowest when the animals are at a salinity that is isosmotic with the haemolymph (Intanai et al., 2009; Wang et al., 2004). A similar response is expected here in *G. duebeni* at the salinity (18 psu) closest to the isosmotic point of the haemolymph. However, it is possible that Na^+/K^+-ATPase activities were higher in the gills of *G. duebeni* at 18 psu than those kept at 35 psu which may account for this discrepancy. Brook and Lloyd Mills (2006) observed a downward trend in Na^+/K^+-ATPase activities with increasing salinity even though this fall was not significant from the value observed at a salinity of 10 psu.

In addition, it would be expected that *G. duebeni* acclimated to 35 psu are experiencing a reduction in the production of non-essential amino acids which are used by crustaceans and other invertebrates as small organic osmolytes for cell volume control during hypo-osmotic exposure (Gilles, 1980). In dilute seawater there is an increase in the production and efflux of amino acids from the tissues in order to decrease intracellular osmotic concentrations (Whiteley et al., 2001). Such a response would have a distinct disadvantage during hyper-osmotic exposure in *G. duebeni* when haemolymph [Na^+] is also rising further, increasing the osmotic potential of the extracellular compartment. Such a situation would draw water out of the cells along an osmotic gradient and result in cell shrinkage. A reduction in the production of amino acids in the tissues and haemolymph of *G. duebeni* at 35 psu could signify a reduction in protein degradation rates and hence turnover rates, and presumably an associated reduction in metabolic costs. However, these changes remain speculative as the degradation rates for the two groups are unknown, and protein synthesis rates remained the same (Chapter 5). Moreover, metabolic rates were higher in amphipods at 35 psu rather than 18 psu when acclimated to 5 and 10°C. In addition, the mechanisms responsible for counteracting cell shrinkage in crustaceans after hyperosmotic exposure and may be metabolically costly.

Over the short term, *G. duebeni* is probably able to tolerate the increases in haemolymph [Na^+] observed in full strength seawater, but over the longer term, the amphipods are probably expending energy in an attempt to osmoregulate and maintain ion homeostasis. Such responses are known to be energy demanding and could account for the increase in MO_2 observed in *G. duebeni* held in full strength seawater for six weeks at 15 °C. Tedengren et al. (1988) and Kinne (1952) both found that respiration rates were raised in *G. duebeni* exposed to suboptimal salinities below 5 psu, although Kinne (1952) measured decreased rates in supraoptimal salinities of 22 psu and above, whilst Tendengren *et al.* (1988) found a slight increase.

The theory of metabolic cold adaptation (MCA) assumes polar species will compensate for low temperatures in their environment by elevating their metabolic rate (Clarke, 1983). MCA has been shown to occur in insects from both hemispheres (Addo-Bediako, Chown, & Gaston, 2002), and higher mitochondrial densities in the aerobic muscle fibres of polar (e.g. Johnston et al., 1998) or cold acclimated (Egginton & Johnston, 1984) fish is well documented as a mechanism of increasing aerobic capacity at colder temperatures. Despite this, the relationship between latitude and oxygen uptake rates is still contended

and it appears that the theory does not apply to all species or populations. In a study of two populations of *Littorina saxatilis*, for instance, the temperate population was able to increase metabolic rate during cold acclimation, but the sub-arctic population was unable to do so (Sokolova & Portner, 2003). This suggests that during extreme cold the sub-arctic population experiences metabolic depression rather than showing MCA. The significantly lower MO_2 values in *G. duebeni* from 70 °N compared to 53 °N at common acclimation temperatures indicates that this species does not show temperature compensation in the form of MCA. Instead, *G. duebeni* from Tromsø have a low rate of energy uptake, as seen in several species of polar crustaceans (Chapelle & Peck, 1995; Whiteley et al., 1996) and may be adapted to low temperatures in a different way by reducing energy demands through means such as a reduction in protein turnover (Whiteley & Taylor, 1998) or reproductive output (Clarke, 1987) rather than by increasing the supply of energy. *G. wilkitzkii*, an Arctic gammarid, has been reported to demonstrate a low metabolic rate in conjunction with a low activity lifestyle (Werner et al., 2002). Following long term acclimation to the cold (5 °C), the circumpolar *G. setosus* (79 °N) exhibited a similar rate of oxygen uptake to a population of the boreal-temperate *G. duebeni* from a lower latitude (70 °N), further supporting the lack of metabolic cold adaptation in cold water gammarid amphipods. Future work should include a study of the relationship between MO_2 and age in the two latitudinal populations, as MCA has been linked to life stage in the mussel *Mytilus edulis* as an adaptation during peak energy requirements for growth and reproductive output (Sukhotin et al., 2006). *G. locusta* was the only species studied that exhibited a higher oxygen uptake rate when acclimated to 5 °C compared to higher temperatures. *G. locusta* is a warmer-water species, more closely related to Mediterranean gammarids (Costa et al. , 2009) and would therefore be expected to have higher metabolic rates than cold-water gammarids (Rastrick & Whiteley, 2011). A higher rate of oxygen uptake at 5 °C may therefore be a compensatory response to cold temperatures to maintain their higher energy life style (Rastrick & Whiteley, 2013).

At a common acclimation temperature of 10 °C and salinity of 18 psu, *G. duebeni* from Norway (70 °N) had a lower rate of oxygen uptake than the population from Wales (53 °N) but exhibited a higher Q_{10} value for oxygen uptake rates during exposure to a 10 °C acute increase in temperature. Clarke (1983) has suggested that low Q_{10} values in warm-adapted species are an adaptation to stabilise metabolic rates. The *G. duebeni* population from Norway would not normally be exposed to this acute increase in temperature; temperature measurements from the collection site only reached 20 °C and above on four days of the

year (Chapter 2). *G. duebeni* from Wales, however, are regularly exposed to higher temperatures up to a maximum of 32.9 °C, almost 10 °C higher than the peak temperature recorded in Norway. The magnitude of temperature variation was also higher on the shore in Wales than Norway; the mean monthly temperature variation in Wales was 15.2 °C compared to 8.9 °C in Norway. The population of *G. duebeni* from Norway would therefore be expected to be more sensitive to the 10 °C increase in temperature, while the population from Wales is likely to show compensatory adjustment for higher summer temperatures and variability. A relatively high thermal sensitivity of metabolism is considered to be a characteristic feature of cold-water, sub-polar eurytherms. A sub-polar population of the lugworm *Arenicola marina* exhibits elevated ATP synthesis capacities in the mitochondria compared to a boreal population of the same species, leading to an elevated metabolic rate (Pörtner et al, 2005; Sommer & Pörtner, 2002; Sommer & Pörtner, 2004).

G. duebeni showed no variation in MO_2 after acclimation to common temperatures in both seasons, unlike the significantly higher MO_2 levels seen in winter compared with summer isopods *Ligia oceanica* after acclimation (Whiteley & Faulkner, 2005). Six weeks acclimation therefore appears sufficient to override the effects of previous thermal history and the acute temperature change after collection, and allow oxygen uptake rates to adjust to a new steady state. Although rates of oxygen uptake fully acclimated to the temperature change, *G. duebeni* may still show seasonal differences on the shore as observed in the amphipods *G. limnaeus* (Krog, 1954) and *Monoporeia affinis* (Lehtonen, 1996) due to seasonal factors other than temperature. Brockington & Clarke (2001) estimated that an increase in metabolic rate during the summer months in the urchin *Sterechinus neumayeri* was more strongly influenced by the higher levels of physiological activity, such as reproduction and growth, than directly by the rise in temperature, meaning seasonal differences in metabolic rate were pronounced even in the relatively stable annual temperatures of the Antarctic (Brockington & Peck, 2001). Variations in other factors such as growth, reproductive output, locomotory activity and food availability with season need to be investigated, as temperature does not appear to have the greatest influence on oxygen uptake rates as often assumed.

Regardless of season, *G. duebeni* showed no variation in oxygen uptake rates with acclimation temperature. Whole animal physiological processes such as resting rates of metabolism in invertebrates are expected to have a Q_{10} of approximately 2-3 (Andrew

Clarke & Fraser, 2004), depending on the range of temperatures investigated and the species or populations considered (Whiteley & Taylor, 1998). Within the thermal window considered in this experiment, *G. duebeni* showed a high level of plasticity in terms of metabolic rate and was able to regulate MO_2 independently of temperature. Acclimation to higher temperatures shifts the temperature at which maximum rates of oxygen consumption occur in the winkle *Littorina littorea*, suppressing the rate of oxygen consumption at higher temperatures and therefore maintaining metabolic rate with temperature (Newell & Pye, 1970). Both *G. duebeni* and *L. littorea* are therefore able to fully acclimate to temperature change. This is an adaptive strategy for the fluctuating environment of the intertidal, as high thermal sensitivities within habitat temperatures experienced on the shore are likely to be associated with high long term metabolic costs and a reduced tolerance to temperature extremes (Hawkins et al., 1987). *G. duebeni* may show a similar adaptation, maintaining a constant MO_2 with temperature by extending its thermal window during acclimation to higher temperatures. The lack of a significant increase in MO_2 between 5 and 15 °C demonstrates the rapid compensatory ability of *G. duebeni*. Bulnheim (1979) found that *G. duebeni* was able to reach a new steady state for metabolic rate following an acute increase in temperature from 5 to 15 °C within four hours, i.e. faster than the rate of warming used in this experiment. In comparison, *G. oceanicus* required five hours and *G. locusta* over 19 hours (Bulnheim, 1979).

The only acute temperature increase between which all populations of *G. duebeni* and *G. locusta* showed a significant increase in MO_2 was from an acclimation temperature of 10 °C to an acute temperature of 20 °C. 10 °C was also the temperature at which differences were detected between the two acclimation salinities for *G. duebeni*. Temperatures at the time of each collection were within 5 °C of this temperature for all populations, therefore it could be that 10 °C is a temperature to which all populations are adapted and differences in response are more apparent. The response to acute temperature change can also depend on the direction and rapidity of the change. In *Idotea baltica* (Bulnheim, 1974), warm acclimated individuals took 3 hours to stabilise their metabolic rate after an acute decrease in temperature from 15 to 5 °C, however cold acclimated individuals required 15 hours when exposed to the same temperature change in reverse. As previously stated, Bulnheim (1979) found *G. duebeni* and *G. oceanicus* adjusted to a new steady state of oxygen consumption following an acute 10 °C temperature increase from 5 °C faster than the rate of warming used during this study, therefore in this experiment it is likely that only

G. locusta was still be showing the initial overshoot in metabolic rate following acute temperature increase.

The range at which oxygen uptake rates in *G. oceanicus* are temperature independent has been linked to habitat temperature (Einarson, 1993). Rates of oxygen uptake in *G. duebeni* would likely be temperature dependent if higher temperatures were used than those in this study, as the temperatures chosen were intended to reflect relevant habitat temperatures of the amphipods studied (see Chapter 2). Studies on oxygen demand in gammarid amphipods have shown relatively low rates of increase with temperatures below 15 °C, for example in *Chaetogammarus marinus* between 5 and 15 °C (Dorgelo, 1974) and *G. pulex* and *fossarum* between 10 and 15 °C (Roux, 1975 in Bulnheim, 1979).

Species differences were more pronounced in Wales than in northern Norway. *G. locusta* showed a significant variation in whole-animal MO_2 with acclimation temperature, and had a significantly higher rate of oxygen uptake when acclimated to 5 °C (as previously discussed with respect to MCA) and a significantly lower rate when acclimated to 15 °C compared with *G. duebeni*. *G. duebeni* and *G. locusta* had similar Q_{10} values with an acute increase from 10 °C to 20 °C (3.20 and 3.05 respectively) when acclimated to a common salinity of 35 psu, although *G. locusta* had a significantly higher rate of oxygen uptake at 20 °C. The thermal sensitivity of metabolic rate in *G. locusta* has previously been assumed to be higher than *G. duebeni* (Bulnheim, 1979), however the study in question performed metabolic rate measurements at a common salinity of 10 psu. At 18 psu *G. duebeni* is able to maintain a metabolic rate independent of temperature between 5 and 20 °C, and has a lower sensitivity to acute change, therefore the similarities in thermal sensitivities of *G. locusta* and *G. duebeni* at 35 psu may be due to *G. duebeni* being at a salinity above the level for optimal performance. *G. locusta* generally inhabits a lower position on the shore than *G. duebeni*, and would therefore be expected to have a greater dependency of MO_2 on temperature, as it usually experiences a relatively stable environment. Branch et al. (1988) demonstrated that a subtidal species of limpet, *Patella oculus*, had a Q_{10} value approximately double that of the intertidal conspecifics *P. granularis* and *cochlear*.

Previous research has shown a decrease in acclimatised metabolic rates with latitude, with no evidence of metabolic cold adaptation in the circumpolar species *G. setosus* (Rastrick & Whiteley, 2011). Further examination of this response in the present study by long term acclimation to a common temperature of 5 °C demonstrated that there was no difference between *G. setosus* and a lower latitude population of the temperate species *G. duebeni*,

supporting the lack of metabolic cold compensation in the polar species. The two species are thought to be closely related (Costa et al., 2009), with a similar tolerance to dilute seawater. Of the two species from 70 °N *G. oceanicus* had a lower rate of oxygen uptake compared to *G. duebeni* following long term acclimation to 5 °C and a slightly lower rate after six weeks acclimation to 10 °C, although *G. duebeni* at 70 °N exhibited a consistently lower rate of oxygen uptake than *G. duebeni* from 53 °N. Previous research by Rastrick & Whiteley (2011) suggested that *G. oceanicus* shows no variation in metabolic rate with latitude when acclimated to a common temperature, and may not show population differences as with *G. duebeni*. A Q_{10} value was not measured for *G. oceanicus* as 10 °C acclimated animals did not show a significant increase in oxygen uptake after an acute increase in temperature to 20 °C. This lack of temperature dependence is curious, as it was expected that *G. oceanicus* would show a Q_{10} value similar to the other species studied. *G. oceanicus* has previously been reported to show greater metabolic sensitivity to temperature fluctuation than *G. duebeni* (Bulnheim, 1979), although it is expected that resting rates of oxygen uptake in *G. oceanicus* would have shown little variation with temperature had individuals been acclimated to multiple temperatures (Halcrow & Boyd, 1967). The lack of a significant increase in MO_2 with an acute increase from 10 to 20 °C is unlikely to indicate that *G. oceanicus* was close to its upper critical temperature as a significant increase was seen with acute change between 15 and 20 °C, with a Q_{10} value of 2.08.

Care was taken to ensure amphipods were as inactive as possible during measurements to provide a resting metabolic rate and a therefore a better indication of basal metabolism. Not only does oxygen consumption increase with locomotory activity (Torres & Childress, 1983), but Halcrow and Boyd (1967) have shown that in *G. oceanicus*, the thermal sensitivity of oxygen consumption also increases with activity level.

4.5.2. Aerobic capacity

Citrate synthase is a mitochondrial enzyme involved in the citric acid cycle, and can be used to provide an indicator of mitochondrial volume and aerobic capacity (Berges & Ballantyne, 1991). As with MO_2, citrate synthase (CS) activity in *G. duebeni* was significantly affected by salinity. CS activity was lower at 10 °C in *G. duebeni* acclimated to dilute (18 psu) compared to full strength seawater (35 psu), indicating that amphipods in lower salinities had reduced

mitochondrial numbers and subsequently aerobic capacities. This observation contradicts the elevation in whole-animal rates of oxygen uptake measured in *G. duebeni* held at 18 compared with 35 psu. At the same salinity, CS activities were higher in *G. locusta* compared to *G. duebeni*, but again the higher aerobic capacity of *G. locusta* was not accompanied by an elevation in MO_2. Salomon & Buchholz (2000) found no link between CS activity and metabolic rate in the isopod *Idotea baltica*, as an increase in metabolic rate with temperature did not correspond to a change in CS activity levels. Similarly, no correlation was discovered between CS activity and oxygen consumption in a study of 30 species of deep sea copepod (Thuesen et al., 1998). Donnelly et al. (2004) compared a range of biochemical measures including CS activity to oxygen consumption rates in nine species of micronektonic crustaceans, and found that activity levels of malate dehydrogenase was the best overall predictor for respiration rate (although the correlation varied with species), whilst CS activity showed little correlation. For the deep-sea copepods, lactate dehydrogenase was correlated to oxygen consumption rates, but only weakly (Thuesen et al., 1998). Although CS activity may not be a good proxy for metabolic rate in crustaceans, it can be used to examine changes in aerobic capacity at the tissue level. The higher CS activities or aerobic capacities in *G. duebeni* in full strength as opposed to dilute seawater is likely to be associated with the increased energetic costs associated with osmoregulation. Brooks & Lloyd Mills (2006) recorded maximal enzyme activity of gill Na^+/K^+-ATPase in gammarid amphipods acclimated to dilute seawater for 7 days. As the sodium gradient between the the haemolymph and external media decreased, so did the Na^+/K^+-ATPase activity levels. The euryhaline species studied, including *G. duebeni*, exhibited a greater ability to vary gill Na^+/K^+-ATPase activity levels than the freshwater species. This suggests that at a salinity of 35 psu Na^+/K^+-ATPase activity levels are significantly lower in *G. duebeni* than at 18 psu. Osmoregulation is a costly process, estimated to account for approximately 11-21% of the energy budget in *G. pulex* (Sutcliffe, 1984).

MO_2 and CS activity corresponded more strongly with latitude, with both MO_2 and CS activity lower in the higher latitude population of *G. duebeni*. This too is contrary to the theory of MCA. Somero & Siebenaller (1979) have shown that deep-water fish have low metabolic rates in conjunction with reduced activity levels of the metabolic enzyme lactate dehydrogenase in comparison to shallower-water species. Although a reduction in activity rates of CS with latitude in *G. duebeni* may seem counter-adaptive, lower energy requirements in the cold may be an effective survival strategy as previously discussed. As

with *G. duebeni*, lower CS activity is seen in a sub-polar population of the lugworm *Arenicola marina* in comparison to a boreal population (Sommer & Pörtner, 2004), although in both populations CS activity also varied according to acclimation temperature. The lower CS activity in the sub-polar population occurred despite an increase in mitochondrial capacity with cold adaptation; Sommer and Pörtner (2004) believe this indicates a shift in metabolic control within the mitochondria.

Season and acclimation temperature had no effect on CS activity, as with MO_2. This was unexpected, as cold acclimation is expected to lead to mitochondrial proliferation (Egginton & Johnston, 1984) and an associated increase in CS activity, as in the eelpout *Zoarces viviparous*, for example (Lucassen et al., 2003). The horse mussel *Modiolus modiolus* has been shown to be able to compensate for seasonal temperature variation, as CS activities were the same in summer and winter mussels if measured at their respective temperatures, but were highest in winter animals measured at its reciprocal seasonal temperature (Lesser & Kruse, 2004). The thermal sensitivity of CS, yet maintenance of activity levels with season in *M. modiolus,* was linked to an increase in the concentration of CS. The response of CS activity levels to an acute temperature change was not measured in *G. duebeni*, but it would be interesting to discover whether CS levels show the same temperature independency between 5 and 15 °C as with MO_2, or whether CS activity is more thermally sensitive. In the Southern Ocean, only the most northern of three populations of the limpet *Nacella concinna* was able to maintain CS activity after acclimation (Morley et al., 2009), showing latitudinal variation in plasticity. This study showed that the northern population of *G. duebeni*, was able to maintain CS activity levels across the two acclimation temperatures used in addition to maintenance of MO_2, indicating the ability to show plasticity in the response of CS to temperature despite the overall depression of CS activity levels in comparison to the southern population. Both populations therefore exhibit acclimatory ability.

As CS activity was maintained across the three acclimation temperatures in *G. duebeni* it appears that this species is able to maximise enzyme activity within the thermal window studied. It has been suggested that upregulation of CS activity in the cold may be achieved through an increase in enzyme-substrate affinity, as with the metabolic enzyme lactate dehydrogenase in the loach *Misgurnus fosslilis* (Ozernyuk et al., 1994). However, Salomon and Buchholz (2000) found that for the crustacean *Idotea emarginata*, low acclimation temperatures resulted in a low substrate affinity for CS. Alternatively, a study of temperate

and polar crustaceans by Vetter (1995a) discovered that although the temperature at which peak CS activity occurred did not correspond to habitat temperature, Antarctic krill *Euphausia superba* had a lower activation energy than the temperate species. Marine invertebrates may also show differences in regulation of CS activity based on their lifestyle; in the polar benthic isopod *Serolis polita* ATP concentrations had little regulatory effect on CS activity, while in the more active, pelagic euphausiids *Euphasia superba* and *Meganyctiphanes norvegica* CS was activated by low levels of ATP, with accumulating ATP inhibiting the citric acid cycle causing a feedback loop (Vetter, 1995b).

4.5.3. Conclusions

Overall, a greater difference in rates of oxygen uptake and citrate synthase activity and their relationship with temperature was seen between *G. locusta* and *G. duebeni* in Wales than in *G. oceanicus* and *G. duebeni* in Norway. *G. duebeni* is able to maintain both metabolic rate and citrate synthase activity with acclimation temperature; however population differences persist after acclimation. The population from Norway (70 °N) has lower rates of oxygen uptake and CS activity levels than the population from Wales (53 °N), further supporting the absence of MCA, and a greater thermal sensitivity of MO_2. Overall *G. duebeni* shows a high degree of metabolic plasticity and shows a remarkable independence from temperature after acclimation, although this ability is compromised during exposure to salinities equivalent to full strength seawater because of the metabolic costs associated with osmoregulation. CS activities in temperate, boreal populations decreased with latitude in keeping with a reduction in MO_2.

Chapter 5

Thermal responses of protein synthesis and growth

5.1. Abstract

Rates of protein synthesis and growth were determined in gammarid amphipods following temperature and salinity acclimation. Protein synthesis rates exhibited temperature dependence in *G. duebeni* acclimated to a salinity of 18 psu, and in *G. locusta* acclimated to a salinity of 35 psu. Rates of growth and protein synthesis were relatively low in the boreal/temperate *G. duebeni* in comparison to the warm-temperate species *G. locusta*. Species variation in protein synthesis rate appeared to be associated with differences in RNA translational efficiency rather than an increased capacity for protein synthesis. Lower rates of protein synthesis and growth in *G. duebeni*, suggests a relatively rapid turnover of proteins enabling rapid adjustments of the protein pool, which may be an advantage when exposed to highly fluctuating environmental conditions. The relatively high rates of protein synthesis in *G. locusta* and the faster rates of growth indicate lower rates of protein turnover, greater thermal sensitivities and reduced ability to maintain physiological performance with limited abilities to turn over the protein pool. The northern population of *G. duebeni* from Norway (70 °N) exhibited higher rate of protein synthesis and RNA concentrations than *G. duebeni* from Wales (53 °N) following cold acclimation to 5 °C. *G. oceanicus* from Norway (70 °N) at 10 °C exhibited the similar relatively low rates of protein synthesis to *G. duebeni*. *G. duebeni* acclimated to 35 psu salinity exhibited thermal independence of protein synthesis rate, possibly due to problems caused by the failure to maintain haemolymph [Na^+] homeostasis.

5.2. Introduction

Protein synthesis is an energetically expensive process (Storch & Pörtner, 2003; Whiteley & Fraser, 2009) with the purpose of synthesising new proteins for growth and turning over and repairing existing proteins (Waterlow, 1995). Temperature may exert a direct effect on rates of protein synthesis according to the pervasive influence of temperature on physiological rate processes or an indirect effect according to increasing food consumption with temperature. Food availability is known to positively correlate with protein synthesis rates (McCarthy et al., 1993), therefore confounding the interrelationship between latitudinal variations in temperature and food availability in marine ectotherms (Fraser & Rogers, 2007). In addition, temperature effects may be influenced by simultaneous changes in other environmental variables, such as salinity, which may also influence protein synthesis rates via the energetic demands of osmoregulation (Itanai et al., 2009).

Cold-adapted ectotherms are expected to have a smaller energy budget due to thermal limitations on the supply of oxygen (Pörtner, 2001) and possible resource limitation (Fraser et al., 2002), and are therefore expected to exhibit lower whole-animal rates of protein synthesis than their temperate counterparts (Fraser & Rogers, 2007). Fractional rates of protein synthesis have been seen to increase with temperature after temperature acclimation, for example in the Atlantic wolfish *Anarhichas lupus* (McCarthy et al., 1999), and in response to acute changes in temperature in the acclimatised temperate isopod *Ligia oceanica* in both winter and summer (Whiteley & Faulkner, 2005). There are, however, known exceptions, as evidenced by the relatively high protein synthesis rates observed in some Antarctic species, for example during development in the sea urchin *Sterechinus neumaryeri* (Marsh et al., 2001), and the intertidal limpet *Nacella concinna* (Fraser et al., 2002). In constrast, whole-animal fractional rates of protein synthesis were lower in the Antarctic isopod *Glyptonotus antarcticus* (Whiteley & Faulkner, 2005), compared with the temperate species, *Idotea rescata*, and costs of protein synthesis were considerably higher (Whiteley et al., 1996). When measured at their respective habitat temperatures, acclimatised rates of whole-animal protein synthesis showed various intraspecific responses with a decrease in synthesis rates observed in *G. oceanicus* with latitude and a lack of response in *G. locusta* and *G. duebeni* (Rastrick & Whiteley, 2013). The authors concluded that these contrasting responses result from differing local temperatures and their variability, as well as changes in other environmental factors.

Despite the high intrinsic cost of protein synthesis, the maintenance of high rates at low temperatures can be considered an adaptive strategy to conserve growth rates, or alternatively a necessary expense to counter elevated rates of protein degradation in the cold, as evidenced by high levels of ubiquitin-conjugated proteins (an indicator of denatured proteins) seen in Antarctic notothenioid fish (Place & Hofmann, 2005; Place et al., 2004). Protein synthesis rates may be maintained by direct compensation of translational capacities of RNA (RNA activity levels) with temperature, or by upregulating RNA concentrations in the cold to compensate for the trend of decreasing RNA activity levels as temperatures fall (Foster et al., 1992; Fraser et al., 2002; McCarthy et al., 1999; Robertson et al., 2001; Storch et al., 2003). However, the latter is likely to be energetically expensive (Fraser et al., 2002) unless the increase coincides with an increase in RNA stability (Storch et al., 2003; Storch et al., 2005). Provided the costs of protein synthesis are not a limiting factor, high levels of protein turnover may be associated with an ability to

respond to rapid environmental change (Koehn & Bayne, 1989) as protein synthesis is considered an important element of temperature acclimation (Hazel & Prosser, 1974).

Hawkins (1991) has suggested that low rates of protein turnover are a cause rather than a consequence of high rates of growth. Knowledge of an organism's protein synthesis rate and growth rate can therefore provide an estimate of rates of protein turnover. Growth is expected to be highest at the temperature at which maximum rates of protein synthesis occur (Pannevis & Houlihan, 1992), however, rates of protein degradation are also temperature dependent and are elevated and at supra-optimal temperatures (McCarthy et al., 1999). In the wolfish *A. lupus*, protein synthesis rates increased linearly up to 14 °C, but the optimum temperature for growth was between 10 and 11 °C due to the negative effect of increased protein degradation on protein synthesis retention efficiency, which represents protein growth divided by the fractional rate of protein synthesis (McCarthy et al., 1999). A decrease in growth efficiency at higher temperatures has been observed in pectinid bivalves (Heilmayer et al., 2004), and in the mussel *Mytilus edulis* where individuals with relatively slow rates of growth were characterised by high rates of protein turnover (Hawkins et al., 1987).

Provided the associated costs are not a limiting factor, northern species or populations may exhibit an elevated growth rate compared to a warmer-water counterpart exposed to the same temperatures, rather than a decrease in growth rate with a decrease in habitat temperatures. High latitudes are associated with a reduction in temperatures and growing season, and therefore some northern populations compensate for lengthening development periods caused by slow growth by lowering the optimal temperature for growth (Conover & Present, 1990; Gotthard et al., 2000; Levinton, 1983; Levinton & Monahan, 1983; Schultz et al., 1996). If high latitude, stenothermal populations or species are characterised by lower protein turnover and maintenance costs then they may theoretically have higher growth efficiencies and so exhibit countergradient variation in growth (Conover & Present, 1990; Conover & Schultz, 1995; Lonsdale & Levinton, 1985). A shorter juvenile period has advantages in reducing the effect of the high mortality rates experienced during that period (Lonsdale & Levinton, 1985), and a larger body size has a fitness advantage in terms of factors such as the increase in brood size in gammarid amphipods according to female body length (Kolding & Fenchel, 1981; Steele & Steele, 1969).

The present chapter aims to investigate variations in whole-animal rates of protein synthesis and its relationship to temperature in three species of gammarid amphipod following temperature acclimation, including an intraspecific comparison between populations of *G. duebeni* from Norway (70 °N) and Wales (53 °N) to examine whether the higher latitude population of this highly tolerant species shows any evidence for compensation of protein synthesis rates in the cold. Measurements of growth rates will be used as an indicator of relative rates of protein turnover. To further investigate the physiological tolerance and acclimatory capacity of the temperate, high-intertidal species *G. duebeni*, rates of protein synthesis and growth were determined at two acclimation salinities equivalent to full and half strength seawater. *G. duebeni* is hyper-osmotic in freshwater and iso-osmotic in salinities of approximately 50 % seawater (Morritt & Spicer, 1995). As marine crustaceans have to maintain osmotic gradients and use an increased production of non-essential amino acids to function as osmolytes in freshwater and dilute seawater, protein synthesis rates may change in response to salinity and further affect thermal relationships (Whiteley et al. 2001). Overall this Chapter aims to further characterise the relationship between environmental temperature and protein synthesis rates by studying the response of a highly tolerant intertidal amphipod, *G. duebeni*, to various acclimation temperatures and salinities, as well as any differences observed as a result of season or latitude. Comparisons between *G. duebeni* and other gammarid amphipods from similar latitudes are also provided to include species that are less resilient to temperature change.

5.3 Methodology

5.3.1. Determination of protein synthesis rates

Protein synthesis rates were investigated in *Gammarus* species collected in 2012 from various latitudes and in different seasons (summarised in Table 2.1.) after six weeks acclimation to various temperatures, and in *G. duebeni* after acclimation to two different salinities (summarised in Table 5.1.). Individuals were starved for 24 hours prior to the determination of protein synthesis rates to avoid the effects of specific dynamic action.

Table 5.1. Acclimation conditions for six weeks prior to measurements of rates of protein synthesis and growth. Shaded boxes indicate amphipods were held at the specified temperature. Growth rate was not measured in *G. duebeni* at 20 °C or in *G. oceanicus* (see Section 5.3.2.).

Date of collection	Collection site	Species	Salinity (psu)	Temperatures (°C)			
				5	10	15	20
May, 2012	Anglesey, Wales, 53 °N	*G. duebeni*	18	■	■	■	■
			35	■	■	■	
		G. locusta	35	■	■	■	
October, 2012	Tromsø, Norway, 70 °N	*G. duebeni*	18	■	■		
		G. oceanicus	35		■		
	Anglesey, Wales, 53 °N	*G. duebeni*	18	■	■	■	
		G. locusta	35		■		

5.3.1.1. Injection regime

Whole-animal fractional rates of protein synthesis were determined using the flooding dose methodology (Garlick, McNurlan, & Preedy, 1980), modified for use in gammarid amphipods (Rastrick & Whiteley, 2013). Individual amphipods were weighed to determine wet body mass, then held in place in aerated seawater beneath a stereomicroscope (Vickers) with the dorsal surface emersed and facing upwards and lit by fibre optic lights (Leica CLS 150) to allow internal structures to be viewed. Each amphipod was injected with 1 µl of crab saline (Pantin, 1934) containing 150 mmol.L^{-1} of unlabelled L-phenylalanine and 3.7 MBq.ml^{-1} of L-[2,3,4,5,6,-^{3}H] phenylalanine (specific activity 37 MBq.ml^{-1}) per 50 mg wet body mass.

The injection cocktail was injected into the body cavity of the amphipods using a micro-droplet manipulation system modified from Tomos et al. (1994). The micromanipulator was used to insert the tip of a pulled glass microcapillary containing the injection solution at an oblique angle between the 7th pereon segment and 1st pleopod segment. Small volumes of haemolymph were withdrawn into the microcapillary before slowly injecting the cocktail. The microcapillary was held in place for 10 s before removal from the amphipod. After injection the amphipods were incubated at their respective acclimation temperatures and salinities in fully aerated seawater. Incubation time was 2 hours at 5 °C and 1 hour at 10 and 15 °C as validated by previous work on protein synthesis rates in the species measured (Rastrick & Whiteley, 2013). The incubation time for the 20 °C acclimation treatment was validated as described in the following section. Following incubation, amphipods were sacrificed, frozen in liquid nitrogen and stored at – 80 °C for later analysis.

5.3.1.2. Validation of the flooding dose methodology

Validation of the determination of protein synthesis rates via a single flooding dose of [^{3}H] phenylanine is necessary to ensure that certain assumptions have been met. To summarise, the intracellular free-pools are completely flooded by the injection of unlabelled phenylalanine, the specific radioactivities of the free-pools increase rapidly and then remain stable and finally the increase in the specific radioactivity of the protein-bound fraction is significant and linear. To validate the flooding dose methodology for *G. duebeni* acclimated to 20 °C, individuals were incubated for 30, 60 or 90 minutes post-injection to determine the optimum time course for incorporation of the labelled phenylalanine into the protein fraction. Five additional *G. duebeni* were injected with crab saline for the analysis of free-pool phenylalanine levels in the absence of phenylalanine to test the assumption that the intracellular free-pools were completely flooded by phenylalanine in those amphipods injected with the labelling cocktail.

5.3.1.3. Analysing protein synthesis rates

Individual amphipods were pooled depending on wet body mass, to a target of 100 mg wet body mass per pooled sample. Analysis proceeded as described by Garlick et al. (1980) and Rastrick & Whiteley (2013). Samples were homogenised under liquid nitrogen, vortexed with 3 ml of 2 % perchloric acid (PCA) then centrifuged at 3500 rpm for 15 minutes at 4 °C

(Multispeed refrigerated centrifuge, PK 121R, ALC International Ltd., Italy). The intracellular free-pools were separated by removing the supernatant from the precipitated protein and RNA and storing at –20 °C for later analysis of free-pool specific activities. The pellet was then washed twice more in 3 ml 2 % PCA before being re-suspended in 4 ml 0.3 N NaOH and incubated for an hour at 37 °C to re-dissolve the protein. After incubation a 20 µl subsample was taken for protein quantification using the Pierce BCA protein assay kit (Thermo Scientific, Pierce Biotechnology). Protein was precipitated from the alkali digest before adding 2.5 ml 12 % PCA and leaving the samples on ice for 10 min. After centrifugation at 3500 rpm for 15 minutes at 4 °C the pellets were washed in 2.5 ml 2 % PCA, and the supernatants combined for determination of RNA levels. The protein pellets were hydrolysed in 2 ml of 6 N HCl for 18-24 hours at 110 °C, before the acid was allowed to evaporate over a period of days at a temperature of 85 °C.

Hydrolysates were re-suspended in 1.1 ml of 0.5 M citrate buffer (pH 6.3) and adjusted to a pH between 5.5 and 6.5 by the addition of 3 N NaOH if necessary. In preparation for enzymatic conversion of the intracellular free-pool (supernatants) and protein bound (hydrolysate) phenylalanine fractions into β-phenylethylamine (βPEA) using tyrosine decarboxylase (Tyr-D, Worthington Biosciences, 44C335A), the supernatants were neutralised with the addition of 0.5 ml saturated tri-potassium-citrate per ml of supernatant. Enzyme mix (1.4 units.ml^{-1} Tyr-D and 1 mg pyridoxal phosphate in 1 ml 0.5 M sodium citrate buffer, pH 6.3) was added to 0.5 ml of each hydrolysate or hydrolysate blank (0.5 M citrate buffer), and 0.25 ml of the enzyme mix to 1 ml of each supernatant or supernatant blank (1 ml 2 % PCA neutralised by 0.5 ml tri-potassium citrate). Samples were then sonicated to increase enzyme efficiency, before being incubated for 15 hours at 52 °C. To test the efficiency of the enzymic conversion of phenylalanine, two sets of phenylalanine standards were prepared (0, 2, 4, 8, 16, 24, 32 and 40 nM β-phenylethlamine in 0.01 H_2SO_4) and taken through the enzyme conversion step at the same time as the sample hydrolysates and supernatants.

β-phenylethlamine was extracted into heptane, and then into 0.01 M H_2SO_4. Subsamples were used to determine the specific radioactivities of the protein bound and intracellular free-pool fractions using Optiphase Hisafe scintillant and scintillation counting (Wallac, WinSpectral ™ 1414 Liquid Scintillation Counter, Perkin Elmer, Massachusetts, USA) at a counting efficiency of 37 %. β-phenylethlamine concentrations were determined fluorometrically by enzyme conversion as described by Garlick et al. (1980) using a

multilabel counter (Wallac, Victor^{2} ^{TM} 1420 Multilabel Counter, Perkin Elmer, Massachusetts, USA) at an excitation of 355 nm and emission of 460 nm. Specific radioactivities were expressed as disintegrations per minute (dpm) per nmol of β-phenylethlamine. RNA levels were measured by ultraviolet absorption at 232 and 260 nm (SpectraMax® M2e microplate reader) and verified using known standards (RNA, Sigma R 8508).

5.3.1.4. Calculations

Whole-animal fractional rates of protein synthesis were calculated according to Garlick et al. (1980):

$$k_s = \frac{S_b}{S_a}\, x \frac{24}{t} x100$$

Where k_s is the percentage protein mass synthesised per day (% day^{-1}), S_a is the free-pool specific radioactivity of phenylalanine (dpm.nmol phe^{-1}), S_b is the protein-bound specific radioactivity of phenylalanine (dpm.nmol phe^{-1}) and t is the incubation time in hours.

Absolute rates of protein synthesis (A_s; mg protein.day^{-1}) were calculated by the equation:

$$A_s = k_s/100\ x\ mass\ of\ protein$$

RNA concentrations are expressed using RNA to protein ratios (µg RNA.mg protein^{-1}). RNA activity (K_{RNA}; mg protein.mg RNA^{-1}.day^{-1}) was calculated according to Preedy et al. (1988):

$$K_{RNA} = \frac{10\ k_s}{RNA{:}\,protein}$$

All data was standardised to 1 g fresh body mass to account for variation in size between species and populations. The regression coefficient 0.7 was used for whole animal values (A_s) and -0.2 was used for weight-specific values (k_s, k_{RNA} and RNA:protein) (Houlihan et al., 1990). The standardisation equation was calculated according to Hawkins et al. (1986):

$$Y_s = (W_s/W_e)^b x Y_e$$

Where Y_s is the physiological rate of a standard animal, W_s is the weight of a standard animal (1 g), W_e is the weight of an experimental animal, Y_e is the uncorrected physiological rate and b is the corresponding weight exponent (0.7 or -0.2).

5.3.1.5. Statistical analysis

The Kolmogorov-Smirnov test for normality and Levene's test for homogeneity of variances were performed on all data to satisfy the criteria for the parametric testing. Non-parametric data was subjected to a $\log_{10}$ transformation, after which the data satisfied the assumptions of the tests. For the validation of the flooding dose methodology, intracellular free-pool specific radioactivity of phenylalanine and phenylalanine concentration was examined over time by analysis of variance (ANOVA) and the variation in the protein-bound specific activity of phenylalanine over time was determined by performing a least squares regression. Values for k_s, A_s, K_{RNA} and RNA:protein levels were compared by ANOVA or t-tests after performing the Kolmogorov-Smirnov test for normality and Levene's test for homogeneity of variances to satisfy the criteria for the tests. Multiple comparisons were made using the least significant difference post hoc test (LSD test). For the relationship between acclimation temperature and rates of protein synthesis, a least squares regression was utilised. Values are given as means ± S.E.M, and results are considered significant at the 5 % confidence interval ($P < 0.05$). Statistical analyses were performed using SPSS (SPSS version 21; SPSS Inc., Chicago, IL, USA).

5.3.2. Adult growth rates

5.3.2.1. Measurements of adult growth rates

Adult growth rates were measured in *Gammarus* species collected in 2012 (Table 2.1.) and held at the acclimation temperatures and salinities specified in Table 5.1. No measurements were made for *G. duebeni* held at 20 °C or *G. oceanicus* due to lack of numbers. Initial length measurements were taken after two weeks acclimation to minimise the effect of thermal and feeding history prior to collection, then measurements were repeated once every two to three weeks for up to 12 weeks as preliminary work indicated rates of adult growth were relatively slow. Individuals used for growth measurements were held separately from the amphipods used for other experiments to allow for identification, at a density of 6 to 10 amphipods per 3 litre tank. Animal husbandry methods were identical to those described in Chapter 2.

To measure changes in body length, amphipods were dried and placed on a stage graticule with a scale with divisions of 0.1 mm under a dissecting microscope (Vickers). Amphipods were gently encouraged to uncurl, and a photograph taken with a camera (Nikon Coolpix

p1500) attached to the microscope via a Brunel unilink and 37 mm adapter ring (Brunel Microscopes Ltd, Chippenham, Wiltshire, UK). Care was taken to ensure amphipods were undamaged and not emersed for more than two minutes to minimise the effect of repeated handling stress. Total body length was measured as the distance between the anterior end of the rostrum and the posterior end of the telson. The uropods were excluded as individuals often showed damage in this area, making estimation of length difficult. Body length measurements were made using ImageJ software (Wayne Rasband, National Institutes of Health, USA; for further detail see Chapter 6).

5.3.2.2. Statistical analysis

Rates of growth (mm.week^{-1}) were calculated as the mean growth in length over the 12 week period. Comparisons were made using one or two way analysis of variance (ANOVA) according to the number of factors, or t-tests when comparing only two groups, after the Kolmogorov-Smirnov test for normality and Levene's test for homogeneity of variances to satisfy the criteria for the tests. Multiple comparisons were made using the least significant difference post hoc test (LSD test). Values are given as means ± S.E.M, and results are considered significant at the 5 % confidence interval ($P < 0.05$). Statistical analyses were performed using SPSS (SPSS version 21; SPSS Inc., Chicago, IL, USA).

5.4. Results

5.4.1. Protein synthesis rates

5.4.1.1. Validation of the flooding dose methodology

Validation was necessary for the flooding dose technique for use in gammarids acclimated to 20 °C. In *G. duebeni* at 20 °C, the specific radioactivity of phenylalanine in the intracellular free-pools showed no significant variation between 30 and 90 minutes (Figure 5.1.; ANOVA: $F_{2,9} = 0.309$, $P = 0.744$) whilst the specific radioactivity of phenylalanine in the protein-bound fraction exhibited a significant linear increase over the same time period (Figure 5.1.; least squares regression: $b = 0.007 \pm 0.001$, $P_b = 0.002$, $a = -0.079 \pm 0.088$, $P_a = 0.397$, $N = 10$) from 0.11 ± 0.03 to 0.50 ± 0.06 dpm.nmol phe^{-1}.

Phenylalanine concentrations in the intracellular free-pools of individuals injected with crab saline were 207.5 ± 36.8 nmol.g^{-1} while individuals injected with the injection cocktail and incubated for 30 minutes exhibited concentrations of 1267.9 ± 314.9 nmol.g^{-1} (Figure 5.1C.). Injection with the labelled phenylalanine therefore resulted in a six-fold increase in free-pool phenylalanine levels, confirming that the free pools were flooded. Following this significant increase, free-pool phenylalanine concentrations remained stable with incubation from 30 to 90 minutes (ANOVA: $F_{3,12}$ = 4.090, P = 0.044). The maintenance of stable phenylalanine concentrations and specific radioactivities in the intracellular free-pools over the incubation period plus the linear and significant incorporation of radiolabelled phenylalanine into protein demonstrated that the criteria for the flooding dose technique had been fully met.

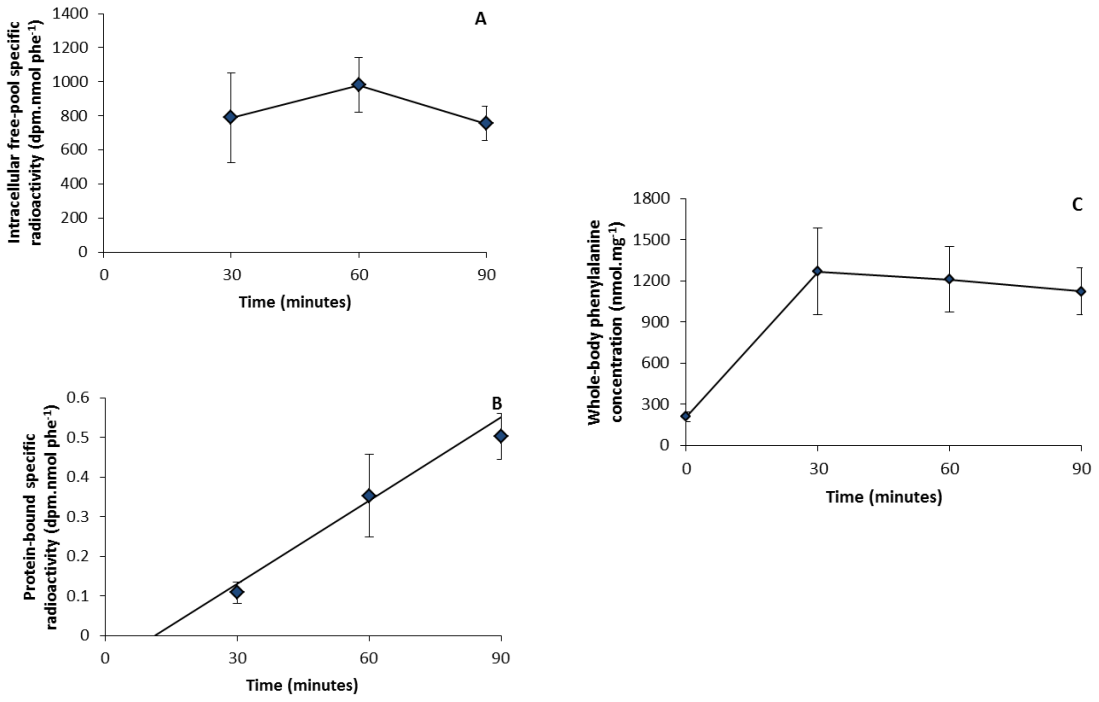

Figure 5.1. Validation of the flooding dose methodology in *G. duebeni* at 20 °C. The elevated intracellular free-pool specific radioactivity of phenylalanine (**A**) showed no significant variation over time (ANOVA: $F_{2,9}$ = 0.309, P = 0.744). The specific radioactivity of phenylalanine in the protein bound fraction (**B**) showed a significant, linear increase over time (Least squares regression: b = 0.007 ± 0.001, P_b = 0.002, a = -0.079 ± 0.088, P_a = 0.397, N = 10). Whole-body phenylalanine concentrations (**C**) increased significantly after injection, and remained stable over the 90 minute incubation period (ANOVA: $F_{3,12}$ = 4.090, P = 0.044). Values are means ± *S.E.M*. N = 3 for all data points except 30 minutes, for which N = 4.

5.4.1.2. Whole-animal fractional and absolute rates of protein synthesis

Acclimation temperature had a greater overall effect than salinity on k_s and A_s in *G. duebeni* (Fig. 5.2.; ANOVA: $F_{2,14}$ = 4.018 P = 0.032 for k_s; $F_{2,14}$ = 3.694, P = 0.041 for A_s). Mean k_s was more than twice as high at 5 °C in *G. duebeni* acclimated to a salinity of 35 psu than 18 psu (LSD test: P = 0.033), but A_s remained unchanged. There was a significant interaction between temperature and salinity on whole-animal fractional rates of protein synthesis (k_s) in *G. duebeni* (Figure 5.2.; ANOVA: $F_{2,14}$ = 4.259, P = 0.027).

Season had no effect on either k_s or A_s in *G. duebeni* from Wales. In summer *G. duebeni* from Wales acclimated to 18 psu salinity, k_s increased with acclimation temperature (Figure 5.2.; Least squares regression: b = 0.144 ± 0.028, P_b = 0.000, a = -0.691 ± 0.400, P_a = 0.099, N = 22), as did A_s (Figure 5.2.; Least squares regression: b = 0.386 ± 0.092, P_b = 0.000, a = -1.147 ± 1.318 P_a = 0.394, N = 22). However temperature had no effect on summer *G. duebeni* and *G. locusta* acclimated to a salinity of 35 psu, and only had an effect on A_s in *G. locusta*, where values increased from 2.80 ± 1.57 mg.day^{-1} in individuals acclimated to 5 °C to 8.99 ± 1.41 mg.day^{-1} in individuals acclimated to 15 °C (Figure 5.2.; ANOVA: $F_{2,23}$ = 4.955, P = 0.027; LSD test: P = 0.009). In general, k_s and A_s were higher in *G. locusta* than *G. duebeni* at common acclimation temperatures and a common acclimation salinity of 35 psu (Figure 5.2.; ANOVA: $F_{1,22}$ = 6.753, P = 0.016 and $F_{1,22}$ = 10.237, P = 0.004 for k_s and A_s respectively). The respective Q_{10} values for k_s between acclimation temperatures of 5 and 15 °C were lowest in *G. duebeni* at a salinity of 35 psu (1.48), intermediate in *G. locusta* at 35 psu (3.08) and highest in *G. duebeni* acclimated to a salinity of 18 psu (4.52). No differences in k_s or A_s were observed between *G. duebeni* and *G. oceanicus* in Tromsø at a common acclimation temperature of 10 °C (Table 5.2.).

Latitude had no effect on k_s or A_s in *G. duebeni* at a common acclimation temperature of 10 °C. At 5 °C, however, k_s was nearly three times as high in the northern population (Figure 5.3.; t-test: t_7 = 2.541, P = 0.039), but there was no differences in A_s (t-test: $t_{5.035}$ = 2.190, P = 0.080).

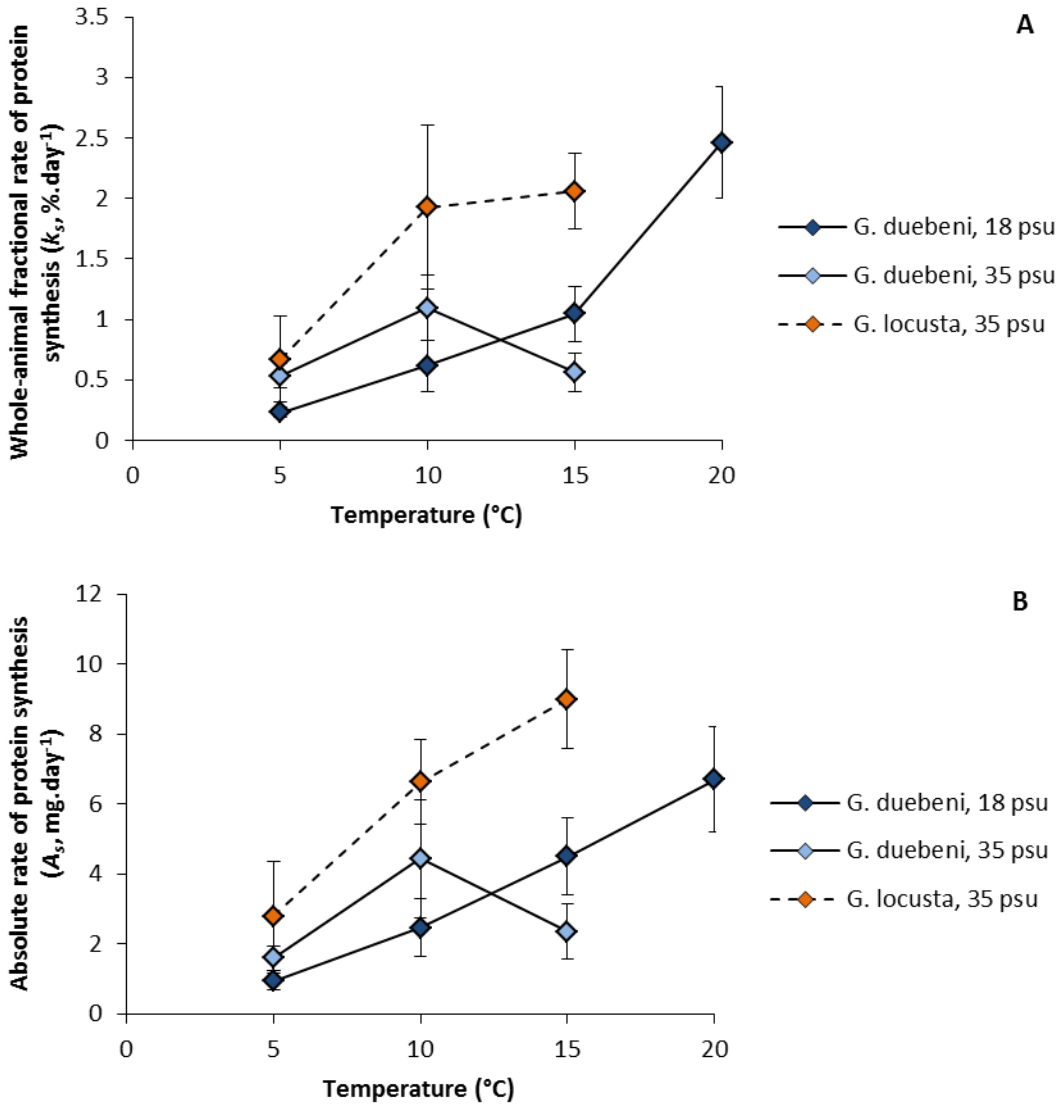

Figure 5.2. Whole-animal fractional rates of protein synthesis (A; k_s) and absolute rates of protein synthesis (B; A_s) in *G. duebeni* (solid line, dark blue indicates a salinity of 18 psu, light blue 35 psu) and *G. locusta (*dashed line, orange, salinity of 35 psu*)* according to acclimation temperature and salinity. Values are means ± *S.E.M*. *N* = 4 – 7. *G. duebeni* held at a salinity of 18 psu showed a significant increase in both fractional (k_s) (ANOVA, $F_{3,22}$ = 10.488, *P* = 0.000) and absolute rates (A_s) (ANOVA, $F_{3,22}$ = 5.328, *P* = 0.008) of protein synthesis with a rise in acclimation temperature. In *G. locusta*, only A_s shows a significant increase between 5 and 15 °C (ANOVA, $F_{2,14}$ = 4.955, *P* = 0.027), but in general both k_s and A_s are significantly higher in *G. locusta* than *G. duebeni* when acclimated to full strength seawater (35 psu) (ANOVA: $F_{1,22}$ = 6.753, *P* = 0.016 and F_1 = 10.237, *P* = 0.004 respectively).

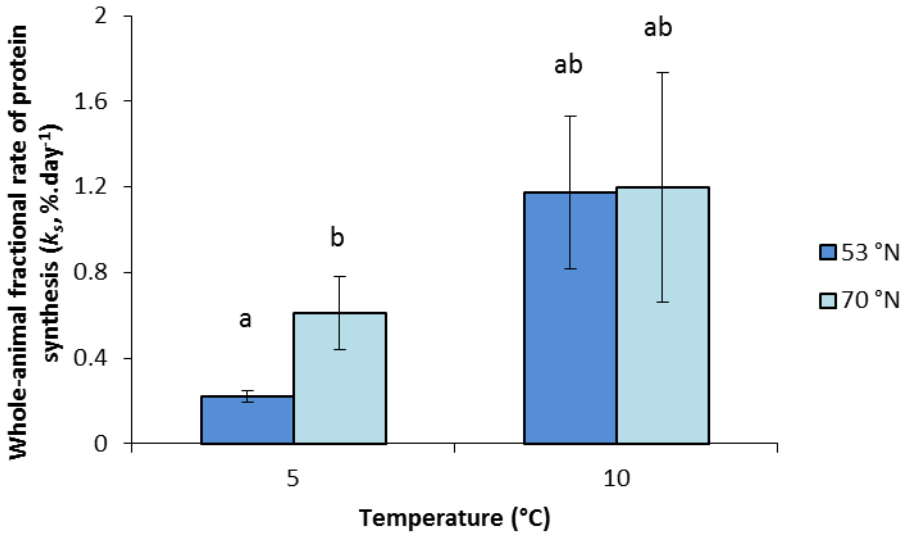

Figure 5.3. Whole-animal fractional rates of protein synthesis (k_s) in two populations of *G. duebeni* acclimated to 5 and 10 °C. Dark blue indicates *G. duebeni* from Wales (53 °N) and light blue the population from Norway (70 °N). Values are means ± *S.E.M*. Means with the same letter are not significantly different ($P > 0.05$). 53°N $N = 4$ for 5 °C acclimated amphipods and 6 for 10 °C. At 70 °N, $N = 5$. Although 10 °C acclimated *G. duebeni* showed no variation in protein synthesis rates with latitude, when acclimated to 5 °C the northern population of *G. duebeni* showed significantly higher fractional protein synthesis rates (t-test: $t_7 = 2.541$, $P = 0.039$).

Table 5.2. Whole-animal fractional (k_s) and absolute (A_s) rates of protein synthesis in three *Gammarus* species, collected from two latitudes in winter and acclimated to a common temperature of 10 °C. *G. oceanicus* and *G. locusta* acclimated to a salinity of 35 psu, *G. duebeni* to 18 psu. Values are means ± *S.E.M*.

Latitude	Species	k_s (%.day^{-1})	A_s (mg.day^{-1})	*N*
70 °N	*G. oceanicus*	2.73 ± 1.44	11.85 ± 6.78	5
	G. duebeni	1.20 ± 0.53	4.70 ± 2.14	5
53 °N	*G. duebeni*	1.17 ± 0.36	4.72 ± 1.13	6
	G. locusta	2.81 ± 1.13	14.13 ± 6.19	5

5.4.1.3. RNA concentrations and activities

Acclimation temperature had no significant effect on RNA concentrations (RNA:protein levels) in *G. duebeni* collected from Wales or from Tromsø, but RNA:protein levels were almost twice as high in the northern population at 5 °C (LSD test: $P = 0.014$; values are 6.79 ± 1.30 versus 2.50 ± 1.02 ug.mg^{-1}). In contrast, K_{RNA} varied only with acclimation temperature (ANOVA: $F_{1,17} = 4.752$, $P = 0.044$), but not with latitude. Salinity had no

significant effect on RNA:protein levels and activities (K_{RNA}), and there was no interaction between temperature and salinity in *G. duebeni* from either population.

Neither season nor temperature alone had a significant effect on RNA:protein levels in *G. duebeni*, but there was a significant interaction between the two factors (ANOVA: $F_{2,26}$ = 5.953, P = 0.007). At a common temperature of 5 °C, RNA:protein levels were 4.88 ug.mg^{-1} higher (LSD test: P = 0.004) in summer versus winter amphipods, but K_{RNA} was unaffected. K_{RNA} values in *G. duebeni*, however, changed significantly with acclimation temperature (Figure 5.5.; ANOVA: $F_{3,22}$ = 4.982, P = 0.015), with K_{RNA} in amphipods acclimated to 5 °C significantly lower than those held at higher acclimation temperatures (LSD test: P = 0.066 when comparing 5 and 10 °C, 0.006 for 5 and 15 °C, and 0.005 for 15 and 20 °C).

Significant differences existed between *G. duebeni* and *G. locusta* from Wales in terms of K_{RNA} (Figure 5.6; ANOVA: $F_{1,22}$ = 4.492, P = 0.046) but not RNA:protein levels. Both species showed an increase in K_{RNA} with an increase in acclimation temperature from 5 to 15°C (P = 0.027 for *G. duebeni*, 0.001 for *G. locusta*), but at 15 °C , *G. locusta* exhibited a higher level of K_{RNA} than *G. duebeni* (P = 0.004). As with k_s and A_s, no differences were seen between *G. duebeni* and *G. oceanicus* in Tromsø for either RNA:protein levels or K_{RNA}.

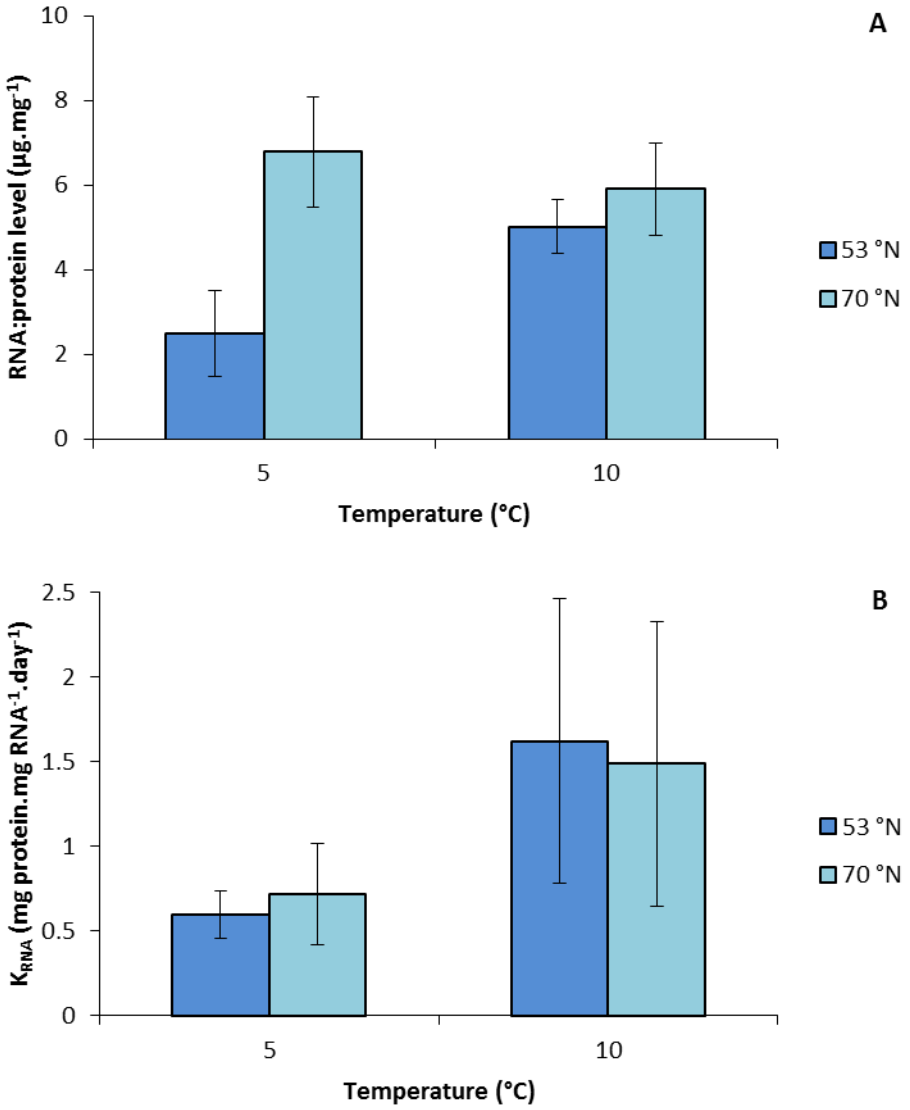

Figure 5.4. RNA concentration (A; RNA:protein levels) and RNA activity (B; K_{RNA}) in two populations of *G. duebeni* acclimated to 5 and 10 °C. Populations were from Wales, 53 °N (dark blue) and Norway, 70 °N (light blue). Values are means ± *S.E.M*. For 53° N, N = 4 and N = 6 for 5 and 10 ° C respectively, and for 70 ° N N = 6 and 5. RNA:protein ratios show significant differences between populations (ANOVA: $F_{1,17}$ = 5.846, P = 0.027), however K_{RNA} only varies with temperature (ANOVA; $F_{1,17}$ = 4.752, P = 0.044).

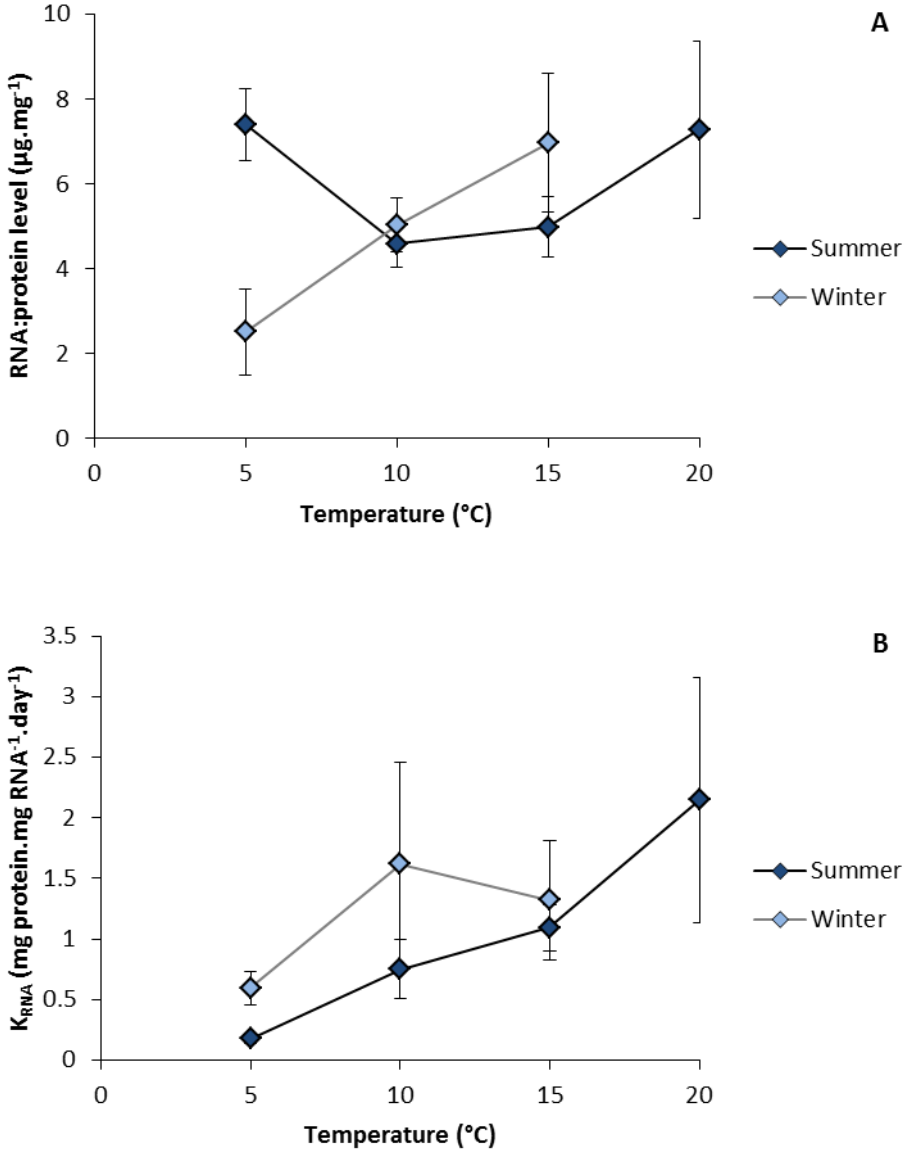

Figure 5.5. RNA concentrations (A; RNA:protein level) and RNA activity (B; K_{RNA}) according to acclimation temperature in *G. duebeni* collected from Wales (53 °N) in summer (dark blue) and winter (light blue) and acclimated to 18 psu. Values are means ± *S.E.M*. *N* = 4 for summer amphipods at 10 °C and winter amphipods at 5 °C, *N* = 7 for summer amphipods at 20 °C and *N* = 6 for all other data points. RNA:protein levels showed significant variation associated with an interaction between season and temperature (ANOVA: $F_{2,26}$ = 5.953, *P* = 0.007), however K_{RNA} varied only with acclimation temperature (ANOVA: $F_{2,26}$ = 4.982, *P* = 0.015).

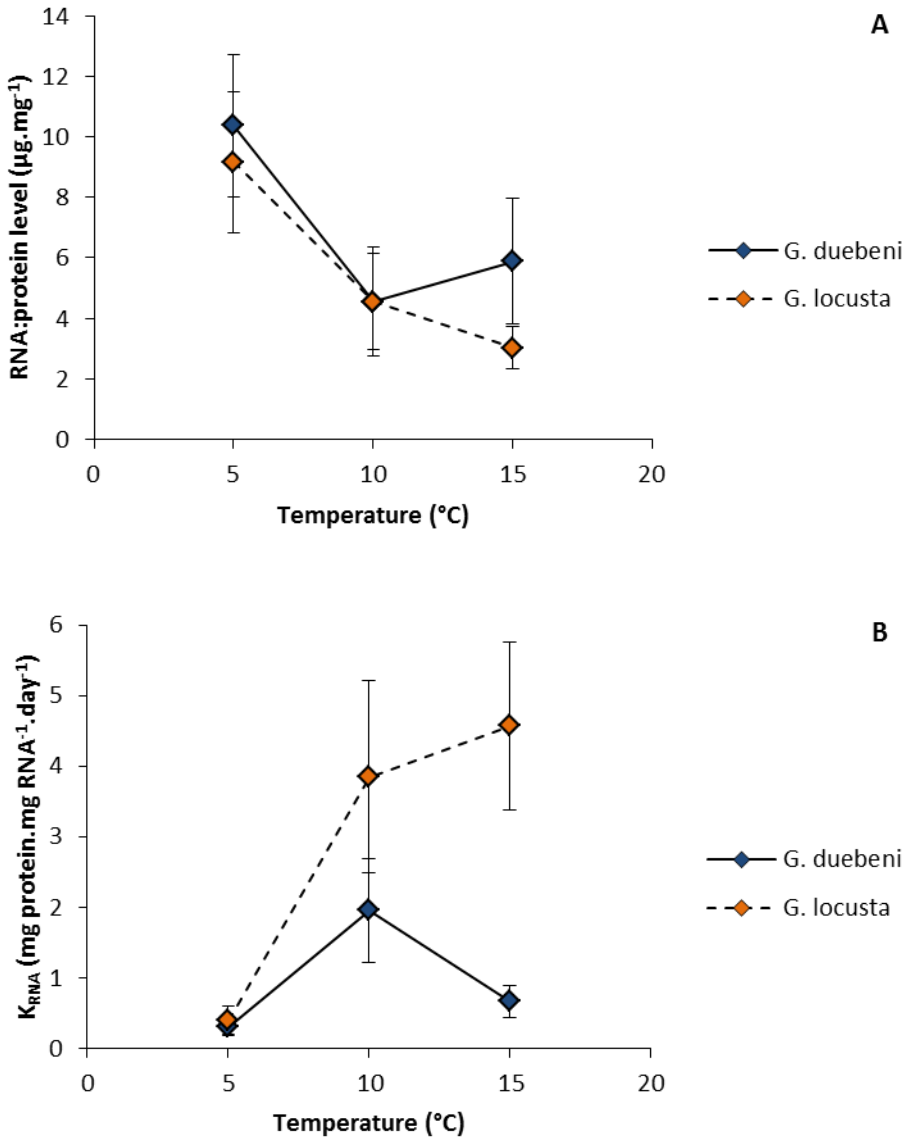

Figure 5.6. RNA concentrations (A; RNA:protein level) and RNA activity (B; K_{RNA}) in *G. duebeni* (dark blue, solid line) and *G. locusta* (orange, dashed line) collected from Wales (53 °N) and acclimated to a salinity of 35 psu and a range of temperatures. Values are means ± *S.E.M*. *N* = 4 for *G. duebeni* at 5 and 10 °C, and *N* = 5 for all other data points. Although RNA:protein levels showed a significant variation with acclimation temperature (ANOVA: $F_{2,22}$ = 5.044, *P* = 0.016) but not species, K_{RNA} showed significant differences according to temperature (ANOVA: $F_{2,22}$ = 11.558, *P* = 0.000) and species (ANOVA: $F_{1,22}$ = 4.492, *P* = 0.046). According to post hoc LSD tests, both species showed a higher value for K_{RNA} when acclimated to 10 than 5 °C (*P* = 0.027 for *G. duebeni*, 0.001 for *G. locusta*), but at 15 °C *G. locusta* exhibited a higher level of RNA activity than *G. duebeni* (*P* = 0.004).

5.4.2. Adult growth rates

Temperature but not salinity had an effect on adult growth rates in *G. duebeni* (ANOVA: $F_{2,15}$ = 18.952, P = 0.000). Growth rates showed a significant increase from 0.030 ± 0.007 mm.week^{-1} at an acclimation temperature of 5 °C, to 0.112 ± 0.014 mm.week^{-1} at 15 °C (LSD test: P = 0.000). Acclimation temperature also had a significant effect on growth rates in *G. locusta* (ANOVA: $F_{2,15}$ = 28.017, P = 0.000); as growth increased from 0.048 ± 0.018 to 0.209 ± 0.023 mm.week^{-1} with a 10 °C increase in acclimation temperature (LSD test: P = 0.000). Interspecific variation in growth rates was observed between *G. duebeni* and *G. locusta* from Wales (53 °N; ANOVA: $F_{1,164}$ = 15.954, P = 0.000). *G. locusta* exhibited significantly higher growth rates than *G. duebeni* at all three acclimation temperatures (Figure 5.7.; LSD test: P = 0.038 at a common acclimation temperature of 5 °C and 0.000 at 10 and 15 °C), however the magnitude of the increase in growth rate over the 10 °C thermal window was similar, roughly quadrupling in both species. In contrast, acclimation temperature had no effect on growth rates in *G. duebeni* from Norway (70 °N; t-test: t_{10} = 1.623, P = 0.136), and no interspecific variation in growth rate was observed between the two populations from Norway (70 °N) and Wales (53 °N).

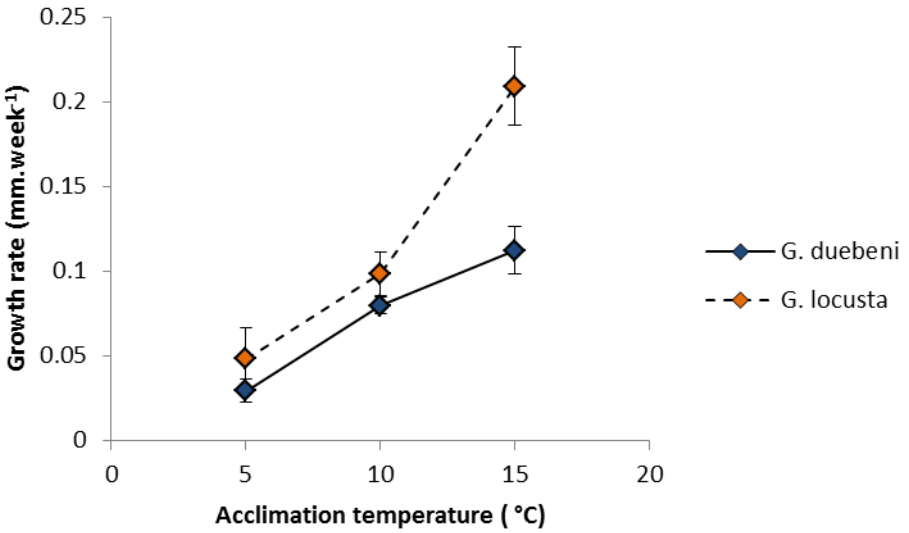

Figure 5.7. Adult growth rates (mm.week^{-1}) in *G. duebeni* at 18 psu (blue, solid line) and *G. locusta* at 35 psu (orange, dashed line) from Wales (53 °N) held at acclimation temperatures of 5, 10 and 15 °C. Values are means ± *S.E.M* and N = 6 for all data points.

5.5. Discussion

5.5.1. Whole-animal fractional and absolute rates of protein synthesis

Whole-animal fractional and absolute rates of protein synthesis were determined primarily in the highly tolerant species, *Gammarus duebeni*, in response to changes in acclimation temperature and salinity. Measurements were taken at two different times of the year to examine seasonal differences and also in individuals from two populations at different latitudes, in order to more fully understand the relationship between protein synthesis rates and environmental temperatures. Comparisons were also made between *G. duebeni* and one other species from the same location but with different thermal experiences and tolerance levels (*G. locusta* for a comparison in Wales and *G. oceanicus* for a comparison in Tromsø).

Whole-animal fractional rates of protein synthesis were over twice as high in 5 °C acclimated *G. duebeni* when held at a salinity of 35 psu than at 18 psu, however this was not reflected by any variation in absolute rates. The interactive effects of temperature and salinity were complex, as although the only significant difference between salinity treatments was at 5 °C, *G. duebeni* held at 35 psu did not show an effect of temperature on protein synthesis rates while *G. duebeni* held at 18 psu showed an increase in both fractional and absolute rates of protein synthesis with temperature. Although *G. duebeni* has a wide tolerance range for salinity (Beadle & Cragg, 1940; Gaston & Spicer, 2001; Hynes, 1954), the optimum salinity range for this species has been suggested to be between 5 and 22 psu (Ikko & Lyubina, 2010). *G. duebeni* is iso-osmotic in salinities of around 20 psu (Morritt & Spicer, 1995), and would be expected to increase rates of protein turnover in freshwater, facilitating the production and loss of amino acids from the intracellular pools to act as osmolytes (Hawkins, 1991), either by increasing protein synthesis at constant degradation rates, or by increasing protein degradation rates at constant synthesis rates. Also, it has been suggested that protein turnover may be reduced during hyper-osmotic exposure (Hawkins & Hilbish, 1992). In the present study, *G. duebeni* hyper-osmoregulating at a supra-optimal salinity (35 psu) exhibited higher rates of protein synthesis at 5 °C than their counterparts held in the salinity near the iso-osmotic point (18 psu). However, cost limitations associated with the maintenance of osmotic gradients through Na^+/K^+-ATPase mediated transporting pumps (Intanai et al., 2009) and a reduction in amino acid production at the higher salinity may account for the lack of an increase in protein synthesis rates with temperature in *G. duebeni* at 35 psu salinity. Studies into the

energetic costs of osmoregulation have given highly variable cost estimates for maintenance of Na+/K+-ATPase mediated transport, for example 2.8 % of energy expenditure in isolated hepatocytes in the rainbow trout *Oncorhynchus mykiss* (Pannevis & Houlihan, 1992) and 40 % of metabolic rate in the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* (Leong & Manahan, 1997). Regardless of absolute costs, it is likely that energy is re-allocated from protein synthesis towards osmoregulation in hyper-osmoregulating *G. duebeni*, which would account for the lack of temperature-dependent increases in protein synthesis at 15 °C but not 5 °C.

Earlier research found no significant difference in fractional rates of protein synthesis between populations of *G. duebeni* from 70 °N and 58 °N after acclimation to 10 °C (Rastrick & Whiteley, 2013). In support of these results no significant difference was seen in the current study between the populations from Norway (70 °N) and Wales (53 °N) after acclimation to 10 °C, however after acclimation to 5 °C the northern *G. duebeni* population exhibited fractional rates of protein synthesis nearly three times the magnitude of the southern population. This resulted in the maintenance of protein synthesis rates across temperatures of 5 and 10 °C in *G. duebeni* from Norway (70 °N). The higher rate of protein synthesis at 5 °C in the northern *G. duebeni* population may represent cold adaptation, suggesting that this boreal/temperate species is able to upregulate protein synthesis rates in the cold unlike its polar counterparts. Polar marine ectotherms generally have lower rates of protein synthesis than their temperate counterparts (Fraser & Rogers, 2007). This has already been demonstrated in the genus of gammarids used in this study; *G. oceanicus* from Svalbard (79 °N) have been shown to have a lower rate of protein synthesis than conspecifics from Scotland (58 °N) when measured at their respective capture temperatures (Rastrick & Whiteley, 2013), while the present study demonstrated that following acclimation to 10 °C, *G. oceanicus* from 70 °N exhibited the same relatively low rates of protein synthesis as *G. duebeni*. Protein synthesis is known to be an energetically expensive process (Storch & Pörtner, 2003; Whiteley & Fraser, 2009). As cold-adapted ectotherms may have thermal limitations on the supply of oxygen (Pörtner, 2001) and therefore lower rates of oxygen consumption and a smaller energy budget, it is assumed these organisms will also have low rates of protein synthesis, especially if prioritising other costly process such as reproduction (Clarke, 2003). There are, however, known exceptions to this rule; Storch et al. (2003) discovered the capacity for protein synthesis in the Antarctic scallop *Adamussium colbecki* at 0 °C was comparable to the temperate European scallop *Aequipecten opercularis*, achieved through a reduction in activation energy and high

levels of RNA, however this was measured in a cell free system. Other examples from the Antarctic include the sea urchin *Sterechinus neumayeri* (Marsh et al., 2001) and the limpet *Nacella concinna* (Fraser et al., 2002). While the Antarctic isopod *Glyptonotus antarcticus* has a lower rate of protein synthesis and higher associated cost than the temperate *Idotea rescala* (Whiteley et al., 1996), another temperate species (*Lignia oceanica*) has a similar rate to the polar species (Whiteley & Faulkner, 2005).

Upregulation of protein synthesis rates in the cold, although costly, may be necessary simply for protein maintenance. Cold temperatures may lead to high degradation rates of protein, and therefore high rates of protein turnover (Place & Hofmann, 2005; Place et al., 2004). Even if protein turnover rates are not elevated in the cold, conserving protein synthesis rates at lower temperatures confers benefits in terms of maintaining growth rates. In fish, high protein growth efficiency is linked to low rates of protein turnover and high retention of synthesised protein as growth (McCarthy et al., 1993). Compensation of growth rates with latitude can be beneficial, especially as a means to minimise the time required for juvenile growth, a life stage associated with a high predation risk (Lonsdale & Levinton, 1985). The two *G. duebeni* populations however showed no difference in growth rate at either acclimation temperature, suggesting the higher rate of protein synthesis in the northern population may be associated with a higher rate of protein degredation, as discussed in further detail in Section 5.5.3.

In *G. duebeni* protein synthesis rates were unaffected by season, but highly influenced by temperature. Between 5 and 20 °C, the Q_{10} values for fractional and whole animal rates of protein synthesis in *G. duebeni* were 4.8 and 3.7, respectively. It is possible that summer and winter protein synthesis rates are likely to be more pronounced on the shore than they are in lab acclimated animals. Seasonal variation in protein synthesis rates have been measured in the Antarctic limpet *Nacella concinna* (Fraser et al., 2002) and the blue mussel *Mytilus edulis* (Hawkins, 1985). Although protein synthesis rates in *G. duebeni* and *G. locusta* vary according to temperature but not season of collection, as with *N. concinna,* temperature is not necessarily the primary determining factor for protein synthesis rates because food availability is also important. Phytoplankton in the Menai Strait show one annual major bloom in spring, with no autumn bloom (Jones & Spencer, 1970). At the collection site in Anglesey, algal biomass on the high shore was less abundant in the winter (Chapter 2), however the presence of *G. duebeni* in the high intertidal was also noticeably reduced at this time of year, so it is possible that this species is able to maintain feeding

throughout the year by shifting its position on the shore. Resource limitation on the shore in winter may result in a more pronounced reduction in protein synthesis rates at lower temperatures than seen in acclimated *G. duebeni* fed *ad libitum* in the laboratory (Mueller & Diamond, 2001). Although little is known about the diet composition of gammarid amphipods due to their generalist nature (Cruz-Rivera & Hay, 2000), *G. lawrencianus* and *G. pulex* both exhibit higher growth rates, maturing faster and at a smaller size when fed a high protein diet in comparison to diets consisting predominately of vegetation alone (Sutcliffe et al., 1981; Vassallo & Steele, 1980). In fish the retention efficiency of protein synthesis has been shown to depend on activity levels and diet (McCarthy et al., 1999), which given the lifestyle of *G. duebeni* is likely to vary within the species dependent on season and other environmental conditions.

Acclimation temperature only had an effect on absolute whole-animal rates of protein synthesis in *G. locusta*. At a common acclimation salinity of 35 psu protein synthesis rates were higher overall in *G. locusta* than *G. duebeni* from the same latitude and season. This will be discussed in further detail in Section 5.5.3. with regards to growth rates and protein turnover. *G. locusta* shares a putative phylogroup with Mediterranean gammarids (Costa et al., 2009) and has been suggested to maintain a high energy, warm-water lifestyle across latitudinal populations (Rastrick & Whiteley, 2011). Higher rates of protein turnover in *G. locusta* may therefore be related to its warm-water ancestry in comparison to the boreal/temperate species *G. duebeni* (Rastrick & Whiteley, 2013).

5.5.2. RNA concentrations and activities

The higher fractional rates of protein synthesis observed in the northern versus southern population of *G. duebeni* at 5 °C was accompanied by an elevation in RNA concentrations at similar RNA activities. As RNA activity levels were significantly reduced at 5 °C, it appears the northern population of *G. duebeni* is able to compensate for the reduction in RNA activity levels observed at 5 °C by upregulating RNA concentrations and therefore protein synthesis rates in the cold. An increase in RNA:protein levels to compensate for the effect of low temperatures on protein synthesis rates and RNA activity has been observed in numerous ectotherms (Foster et al., 1992; Fraser et al., 2002; Goolish et al., 1984; McCarthy et al., 1999; Storch et al., 2003; Storch & Pörtner, 2003; Treberg et al., 2005; Whiteley & Faulkner, 2005) and may be influenced by low rates of RNA turnover in the cold

(Storch et al., 2005). Indeed, RNA translational capacities have been shown to be higher in Antarctic species of eelpout, *Pachycara brachycephalum* compared to the temperate *Zoarces viviparus* (Storch et al., 2005), and scallops, *Adamussium colbecki* compared to the temperate *Aequipecten opercularis* (Storch et al., 2003) when measured in cell-free *in vitro* systems, indicating cold compensation. Northern populations of *G. duebeni*, however, are still exposed to variable environmental conditions, and are more eurythermal than their polar counterparts. As a result they appear to be more capable of upregulating protein synthesis rates in the cold. Interestingly, *G. duebeni* are not distributed further north than the northern coasts of the Norwegian mainland. To date, they have not been described in Svalbard, living under sub-polar and polar conditions.

Summer and winter Welsh *G. duebeni* exhibited different relationships between RNA concentrations and acclimation temperature; RNA:protein levels within summer amphipods were constant across acclimation temperatures, but the winter amphipods had RNA concentrations more than 50 % lower at 5 °C compared to 15 °C. The difference occurred between the 5 °C acclimated groups, with higher RNA concentrations in the summer animals than winter. This response is unusual as RNA:protein ratios are generally higher in winter ectotherms. For example, *L. oceanica* show seasonal differences in upregulation of RNA concentrations in the cold, however unlike *G. duebeni* this increase was seen in summer animals only (Whiteley & Faulkner, 2005). Storch et al. (2005) found RNA concentrations showed cold compensation in the eurythermal *Zoarces viviparous* after cold acclimation, but not in the Antarctic *Pachycara brachycephalum*. Increasing RNA:protein levels to compensate for a reduction in RNA activity levels in the cold is likely to be energetically expensive (Fraser et al., 2002) unless the increase can be attributed to a greater stability of RNA (Storch et al., 2005). Winter *G. duebeni* may fail to upregulate RNA:protein levels in the cold due to having a smaller energy budget as previously discussed, or alternatively RNA stability in the cold may be high and therefore the six week acclimation period was insufficient to override the effect of a higher rate of RNA synthesis in summer animals prior to collection. Therefore, it is possible that *G. duebeni* required longer to fully acclimate to the change in temperature with regards to RNA concentrations. RNA:protein levels in winter *G. duebeni* at 5 °C were lower than levels previously observed in gammarids (Rastrick & Whiteley, 2013), isopods (Whiteley and Faulkner, 2005) and prawns (Itanai et al., 2009), while values in summer animals were more comparable.

RNA activity levels in *G. duebeni* were significantly depressed at 5 °C compared to individuals held at higher acclimation temperatures. This lower activity in 5 °C acclimated animals was witnessed across both seasonal and latitudinal populations and in *G. locusta*, the other species exposed to a series of acclimation temperatures. Unlike the differences between latitudinal populations of *G. duebeni* seen at 5 °C, RNA activity levels rather than concentrations appear to be responsible in determining protein synthesis rates when comparing acclimation temperatures within populations. Variations in RNA activity levels rather than RNA capacity as a determinant for protein synthesis rates has been documented in crustaceans (Intanai et al., 2009; Robertson et al., 2001), and is considered a common response to temperature in both ectotherms and endotherms (McCarthy et al., 1999).

G. locusta and *G. duebeni* exhibited differences in RNA activity levels but not in RNA concentrations. As observed in *G. duebeni,* RNA activity levels were significantly lower in 5 °C acclimated individuals of *G. locusta*, but overall RNA activity levels were higher in *G. locusta* than in *G. duebeni*. Rastrick & Whiteley (2013) found conservation of K_{RNA} between latitudinal populations of acclimatised *G. locusta* despite an 8 °C temperature difference, whereas this study found RNA activity levels to show a significant increase with a 10 °C increase in acclimation temperature. It would be interesting to assess the effect of acclimation on latitudinal populations, to determine whether colder populations show increased activity levels at equivalent temperatures and if both populations are equally thermally sensitive in terms of K_{RNA}.

5.5.3. Adult growth rates

High latitude gammarid species tend to be characterised by a large body size and a slower maturation rate (Sainte-Marie, 1991). Although Bergmann's (1847) rule suggested that the large body size of polar ectotherms is the result of an adaptive increase in body size with latitude, it has been proposed that a larger body size may instead be due to an absence of some of the selective pressures experienced by organisms from warmer latitudes (Blanckenhorn & Demont, 2004). Seasonal fluctuations in protein synthesis rates in the Antarctic limpet *N. concinna* correspond to growth rates, with winter animals experiencing reduced protein synthesis rates and negative growth rates suggesting high rates of protein degradation (Fraser et al., 2002). Relatively slow growth rates in polar ectotherms have

been documented due to latitudinal constraints on growth including the effects of temperature and shortened length of the growing season (Clarke et al., 2004; Fraser et al., 2002; Peck et al., 1997), as well as the effects of restricted moult frequencies at low temperatures (Hauton et al., 2009). In this study, however, no variation was seen between latitudinal populations of *G. duebeni* in terms of growth.

Countergradient variation in growth rate with latitude is a well-documented phenomenon (Conover & Present, 1990; Jonassen et al., 2000; Schultz et al., 1996) in which high latitude populations may exhibit compensation of growth rates and optimisation of growth during the shorter growing season (Conover & Present, 1990). This is achieved by increasing the capacity for growth through a negative relationship between growth efficiency and temperature (Hawkins & Day, 1996; Heilmayer et al., 2004). Lower maintenance costs in the cold could allow a greater allocation of the energy budget to growth (Wieser, 1994). High rates of growth have been observed in bathypelagic fish despite relatively low metabolic rates, hypothesised to be due to deferral of reproductive maturity (Childress et al., 1980).

The copepod *Scottolana canadensis* exhibits local adaptation of growth to temperature (Lonsdale & Levinton, 1985). Northern copepod populations had a faster rate of growth at the low temperatures commonly experienced at the higher latitude, but growth rates peaked and declined at a lower temperature than southern populations. This latitudinal compensation of growth is well documented within species and subspecies of intertidal polychaetes from the *Ophryotrocha* genus (Levinton, 1983; Levinton & Monahan, 1983). *G. duebeni* populations from both latitudes would need to be exposed to a wider range of temperatures to further investigate the lack of variation in adult growth rates at 5 and 10 °C. No evidence was observed to support local temperature adaptation, and a shift of the optimum temperature for adult growth as in *S. canadensis*; a higher maximum growth rate as in the countergradient variation hypothesis; or via a combination of the two as seen in the silversides *Menidia menidia* and *peninsulae* (Yamahira & Conover, 2002). However, as discussed in Chapter 6, growth in *G. duebeni* during the juvenile stages occurred at a higher rate in the northern population.

In addition to slow rates of adult and juvenile growth, *G. duebeni* has also been documented to have a lengthy development period, more comparable to the Arctic species *G. wilkitzkii* and *G. setosus* than to temperate congeners (Steele & Steele, 1975). It is possible that *G. duebeni* experiences a trade-off between growth performance and other

lifestyle features such as activity levels or reproductive rate (Pörtner et al., 2005). For example the trade-off seen in molluscs between growth in protein and in lipid storage for reproduction (Bayne, 2004). Steele & Steele (1969) expect latitudinal populations of *G. duebeni* to have a similar reproductive strategy. Kolding & Fenchel (1981), however, report that *G. duebeni* populations from the Baltic Sea and the Limfjord in Denmark have a yearly life cycle with winter breeding, but acknowledge reports of summer breeding in populations in northern France (den Beld, 1973 from Kolding & Fenchel, 1979), and therefore it is possible higher growth is at the expense of a more flexible life history.

As with protein synthesis rates, winter and summer *G. duebeni* had similar growth rates at a common temperature, but growth showed a significant positive correlation to acclimation temperature. As discussed with regards to protein synthesis rates, seasonal variation in food availability on the shore was not considered in this study but is known to be a factor in determining growth rates and time to maturity. The trend for increasing protein synthesis and growth rates with temperature is well known (Pörtner et al., 2005). If the cost of protein synthesis remains unchanged with temperature (Storch & Pörtner, 2003) and food availability is not a limiting factor then growth should increase with temperature to maximise growth during the summer months. It has been suggested that along with temperature, the rate of protein synthesis is itself a determinant of the energetic costs of synthesis (Pannevis & Houlihan, 1992), with species exhibiting high rates of protein synthesis at high temperatures operating the most efficiently. This response, however, depends on rates of protein degradation because costs of protein degradation are also expensive. In the mussel *Mytilus edulis* faster growth depends on a reduction in protein degradation and turnover, but also a decrease in whole-body rates of protein synthesis (Hawkins et al., 1986).

In this study, growth rates for adult *G. locusta* were significantly faster than for adult *G. duebeni* at equivalent temperatures. Kolding & Fenchel (1979) demonstrated both temperature dependent development in *G. locusta* and the relatively faster rate in *G. locusta* compared to *G. duebeni*. Field sampling of both species showed that the summer cohort of *G. locusta* juveniles reached maturity faster than summer *G. duebeni* from the same location, but that *G. locusta* juveniles produced in winter had a similar slower rate of growth to summer *G. duebeni*. Similarly the relationship between temperature and growth rate indicated growth is more thermally sensitive in *G. locusta* than *G. duebeni.* The

difference in growth rates between the species is therefore amplified at higher temperatures, supporting the observations of Kolding & Fenchel (1979).

Between acclimation temperatures of 5 and 15 °C, *G. duebeni* at a salinity of 18 psu exhibited Q_{10} values of 4.52 and 3.79 for whole-animal fractional rates of protein synthesis and growth in length, while at 35 psu salinity *G. locusta* exhibited values of 3.08 and 4.32, respectively. It is unknown whether protein synthesis retention efficiency (PSRE) is affected by acclimation temperature because the rates of protein degradation are unknown, but the differences in growth and protein synthesis with temperature suggests PSRE increased in *G. locusta* with temperature, but decreased in *G. duebeni* at 18 psu. At equivalent temperatures, fractional rates of protein synthesis were over twice as high in *G. locusta* compared to *G. duebeni*, however growth rates in *G. locusta* were only 1.5 times the magnitude of growth in *G. duebeni*. Although protein growth rates are unknown and therefore PSRE cannot be calculated, this suggests faster growth in *G. locusta* may be achieved through a faster rate of protein synthesis, rather than a change in protein degradation rates. Investigations into the correlation between growth efficiency and protein turnover have shown that faster growing individuals are characterised by low protein degradation rates and therefore low rates of protein turnover, leading to greater protein retention and lower energy expenditure (Hawkins, 1991; Hawkins et al., 1987). Slower growth has been associated with higher rates of protein turnover, higher degradation rates and higher maintenance costs, reducing the energy available for growth and reproduction (Hawkins et al., 1986; Hawkins & Day, 1996). Diehl, Gaffney, & Koehn (1986) observed that rates of oxygen uptake in *Mytilus edulis* was highest in individuals with low growth efficiencies, indicating that faster growth rates are associated with lower costs. According to Koehn & Bayne (1989), species such as *G. locusta* with high energy requirements may be less able to maintain physiological performance under fluctuating environmental conditions. Low rates of protein turnover are thought to be related to greater physiological stability (Hawkins, 1991) and a lower thermal sensitivity (Hawkins et al., 1987). The high rates of growth and protein synthesis in *G. locusta* indicate a more energetic lifestyle than *G. duebeni*, which may explain why this species has a more limited distribution, a generally lower tolerance to environmental change and is absent from the high intertidal (Bulnheim, 1979; Gaston & Spicer, 2001). *G. oceanicus* exhibited comparable protein synthesis rates to *G. duebeni* (70 °N). Although growth rates were not measured in adult *G. oceanicus*, and no previous study has attempted to estimate growth rates in this species, Chapter 6 showed that juvenile growth rates were higher in *G. oceanicus* than in

either *G. duebeni* or *G. locusta*. This suggests PSRE is significantly higher in *G. oceanicus* than *G. duebeni*, although this remains to be investigated.

5.5.4. Conclusions

The two populations of *G. duebeni* had a significantly different response after acclimation to 5 °C. The northern population was characterised by a higher protein synthesis rate, achieved through an elevation of RNA concentrations, and higher growth rates in the cold. Although the compensatory upregulation of protein synthesis rate in the northern population of *G. duebeni* was associated with elevated RNA concentrations, acclimatory experiments demonstrated that within populations protein synthesis rates and RNA activity levels increased with temperature. Salinity had some influence on protein synthesis rates in *G. duebeni*, but there was no variation in growth rate or RNA concentrations or activities between salinities.

Rates of growth and protein synthesis were relatively low in *G. duebeni* compared to the warm-temperate species *G. locusta*. This was due to a lower translational efficiency rather than a change in capacity for protein synthesis. The effect of short term fluctuations in temperature, as well as latitudinal and seasonal variation in terms of food availability requires further investigation to determine whether the low protein synthesis rates measured in *G. duebeni* in this study were a result of the constant acclimatory conditions or whether *G. duebeni* is able to maintain a low energy expenditure during conditions more representative of those on the shore.

Future research should also focus on the compensatory mechanisms involved in the upregulation of protein synthesis and growth in the cold, and attempt to define the relative cost of protein synthesis according to latitude and temperature.

Chapter 6

Life history of gammarid amphipods: brood size and juvenile growth rates

6.1. Abstract

G. locusta exhibited the large brood sizes and fast rate of juvenile growth typical of a warmer-water, southern gammarid species with a relatively energetically expensive lifestyle. *G. duebeni* had smaller brood sizes than either *G. locusta* or *G. oceanicus*, a previously suggested characteristic of brackish compared to marine amphipods. The lower reproductive potential of *G. duebeni* may explain why the species is out-competed when sharing a habitat with other gammarid species, and suggests *G. duebeni* populations may show a lower capacity for recovery following high mortality events. Juvenile growth rates were higher in *G. oceanicus* and *G. locusta* than in *G. duebeni*, suggesting a greater energy budget or higher allocation of available energy for growth, and showed an exponential increase with acclimation temperature in *G. duebeni* and *G. locusta*. Between populations, juvenile *G. duebeni* from Norway (70 °N) exhibited a faster rate of growth than juvenile *G. duebeni* from the southern population in Wales (53 °N), suggesting upregulation of growth to compensate for lower temperatures and a shorter growing season. Despite the broad salinity tolerance of *G. duebeni*, exposure to full strength seawater at high temperatures resulted in high mortality rates in the adult population, and a reduction in juvenile growth in comparison to *G. duebeni* held in half strength seawater. This is the first time that life history traits have been examined across gammarids in response to temperature.

6.2. Introduction

Amphipods are frequently used for ecological and ecotoxicological studies due to their ease of culture, widespread distribution and the wide environmental tolerance of some species (MCAahon & Pascoe, 1988). Studies of individual life history traits allow estimations of population level responses in the shorter term to transitory environmental perturbations and in the longer term to climate change. Temperature is one of the main environmental factors determining species distribution (Stevens, 1989) and exerts a strong influence on physiological rate processes (Peck et al., 2009). The three species of gammarid amphipod studied inhabit the intertidal and are therefore exposed to a relatively high degree of temporal and spatial variation in temperature (Stillman & Somero, 1996).

Studies of brood sizes in *G. duebeni*, *G. locusta* and *G. oceanicus* have highlighted the variation in reproductive strategy between the three species. *G. duebeni* is thought to have a relatively small brood size (Kolding & Fenchel, 1979; Kolding & Fenchel, 1981) in

comparison to the other two species, and unlike *G. locusta* shows a restriction of the timing of reproduction to only one season at higher latitudes (Steele & Steele, 1969; Table 6.3.). Discrepancies in methodology between studies, for example the development stage at which eggs or juveniles were counted, makes comparison between published data on brood sizes in populations of gammarids from different thermal regimes impractical. However, a review by Sainte-Marie (1991) indicates a trend within amphipods for greater brood sizes and larger female body size in the cold. Brood size correlates with female body size and embryo size, as it is limited by the capacity of the brood pouch, and appears to show a greater interspecific than intraspecific variation (Sainte-Marie, 1991), and is also dependent on oostegite structure which in turn is influenced by phylogeny and local evolution according to environment and reproductive strategy (Steele, 1991). The brood size and frequency of brood production does, however, also depend on the amount of energy available and the percentage devoted to reproduction (Clarke, 1987). The metabolic cold adaptation hypothesis (Clarke, 1983) has fallen out of favour in polar species (Chapelle & Peck, 1995; Werner et al., 2002; Whiteley et al., 1996) due to the suspected cost limitations, however conservation of metabolic rate with latitude has been shown to exist between northern and southern populations of marine ectotherms from temperate latitudes (Sommer & Pörtner, 2002). *G. duebeni* and *G. locusta* have been shown to exhibit conservation of rates of oxygen uptake with latitude (Rastrick & Whiteley, 2011) suggesting an ability to maintain high energy traits across thermal regimes in eurythermal species.

Data on intraspecific variation is relatively lacking, but *G. duebeni* is an interesting species for a study of population differences as it appears that this species alters reproductive strategy according to thermal habitat. Southern populations have been shown to produce variable egg sizes according to season (Dunn & McCabe, 2010) and have two annual peaks in juvenile production (Kolding & Fenchel, 1981), while northern populations exhibit a lower plasticity, showing no seasonality in egg size and brooding only in the winter (Table 6.3.). Two populations of *G. duebeni* were therefore employed for this study, one from northern Norway (70 °N) and one from Wales (53 °N), and brood sizes and juvenile growth rates are discussed with respect to temperature data recorded from their respective habitats.

Cold-adapted ectotherms are expected to have a smaller energy budget than their warmer-water counterparts (Pörtner, 2001), and have a lower rate of protein synthesis by association (Fraser & Rogers, 2007). There are, however, many examples of the existence

of conservation of growth rates with latitude (Conover & Present, 1990; Jonassen et al., 2000; Schultz et al., 1996). For example, the polychaete *Ophryotrocha costlowi* exhibits faster growth rates than the more southern *O. macrovifera*, despite inhabiting a colder habitat (Levinton, 1983). Upregulation of growth rates at low temperatures in colder-water species has been suggested to be a necessary adaptation in response to the restricted growing season experienced at higher latitudes (Conover & Present, 1990; Gotthard et al., 2000; Levinton, 1983; Levinton & Monahan, 1983; Schultz et al., 1996). This may be achieved by an increase in growth efficiency (Conover & Schultz, 1995; Conover & Present, 1990; Lonsdale & Levinton, 1985) or by a higher allocation of available energy to growth through a trade-off against other costly processes such as reproduction, as seen in bathypelagic fish (Childress et al., 1980). Rastrick & Whiteley (2013) observed a failure to compensate protein synthesis rates with latitude in *G. duebeni*, while *G. locusta* maintained significantly higher protein synthesis rates across populations. There are no examples of comparative growth experiments between these two species, however the few existing studies of growth in the two gammarid species suggest *G. locusta* exhibits faster growth (Hynes, 1954; Naylor et al., 1988; Neuparth et al., 2002) in addition to the higher rates of protein synthesis observed by Rastrick & Whiteley (2013), while growth data is lacking for the subarctic/boreal *G. oceanicus*.

The present chapter aims to investigate how populations of gammarid amphipod may be impacted by environmental change. The determination of brood sizes and juvenile growth rates in three species of gammarid amphipod, including two populations of *G. duebeni* from northern Norway (70 °N) and Wales (53 °N), will be used to explore the relative allocation of energy to growth and reproduction. The chapter will also assess the relative vulnerability of species and populations and their ability to respond to warming in positive ways, such as the increase in abundance of *G. oceanicus* in Svalbard in response to an increase in sea surface temperatures (Węsławski et al., 2010). The current diversity of the *G. oceanicus* population has been attributed to multiple colonising events (Krebes et al., 2011). This ability to colonise new habitats and the capability to recover from deleterious events which cause high mortality within a population, likely depends on life history traits, such as the time to maturity, brood size and the frequency of brood production. In addition to these measurements, the thermal sensitivity of juvenile growth rates in *G. duebeni* were examined at salinities equivalent to full and half strength seawater. *G. duebeni* is a brackish species with a wide salinity tolerance but a preferred range between 5 and 15 psu (Ikko & Lyubina, 2010). The cost of osmoregulation at supra-optimal salinities would be expected

to have a limiting effect on growth rates (Intanai et al., 2009), and an increase in oxygen uptake rates in *G. duebeni* at salinities above 22 psu by Tendengren *et al.* (1988) appears to support this view.

6.3. Methodology

6.3.1. Brood size

Brood size was determined in three species of gammarid amphipod, *G. duebeni*, *G. locusta* and *G. oceanicus*. Mature adult *G. duebeni* and *G. locusta* were collected from Wales (53 °N) in May and October 2012, and mature adult *G. duebeni* and *G. oceanicus* were collected from Norway (70 °N) in October 2012 (see Chapter 2, Section 2.1. for further details) and returned to the laboratory in Bangor within 48 h. Mature adult amphipods were maintained as described in Chapter 2. *G. duebeni* were held at a salinity of 18 psu as this is the optimal salinity for survival, while the low shore species *G. locusta* and *G. oceanicus* were held at a salinity of 35 psu.

Tanks were checked three times a week for the presence of breeding pairs, and once per week for the presence of brooding females. Brooding females were identified by the presence of eggs or juveniles in the brood pouch, clearly identifiable by a dark grey or orange mass beneath the oostegites when observed under a bright light (Steele, 1991). The presence of broods were confirmed under a dissecting microscope. Breeding pairs were transferred into smaller tanks (1 litre volume water, salinity of 18 psu for *G. duebeni*, 35 psu for *G. locusta* and *G. oceanicus*), at a density of one pair per tank, and monitored daily. Once the pair had separated, the male was removed and photographed under the dissecting microscope for the determination of total body length. This was achieved by removing excess water from individual amphipods before placing them on a stage graticule with a scale with divisions of 0.1 mm under a dissecting microscope. Each amphipod was gently encouraged to uncurl using a small paintbrush before an image was taken by a digital camera (Nikon Coolpix p1500) attached to the dissecting microscope via an adapter (Brunel unilink and 37 mm adapter ring, Brunel Microscopes Ltd, Chippenham, Wiltshire, UK). Care was taken to ensure amphipods were undamaged and not emersed for more than two minutes to minimise the effects of repeated handling stress and aerial exposure. Total body length was taken as the distance between the tip of the rostrum and the posterior margin of the telson. The uropods were excluded as individuals often showed

damage in this area, making estimation of length inaccurate. Measurements were made using ImageJ software (Wayne Rasband, National Institutes of Health, USA). Body length was determined using ImageJ software (Wayne Rasband, National Institutes of Health, USA). The software allows for measurement along a curve, and converts distances into mm according to the scale on the stage graticule. Males were then returned to the main holding tank and the brooding females were held in a separate tank to prevent cannibalisation of the female.

Brooding females were held in a maximum density of four per litre of water (at a salinity of 18 psu for *G. duebeni* and 35 psu for *G. locusta* and *G. oceanicus*) to minimise stress, maintained as described in Chapter 2, and monitored daily for the release of juveniles. After release from the brood pouch images of the juveniles and female were taken as described above in order to determine total body length. The total number of juveniles were also recorded and taken to represent total brood size. Juvenile growth rates were determined as described in the next section.

6.3.2. Juvenile growth rates

Juvenile growth rates were determined in three species of gammarid amphipod collected from populations at two different latitudes (Wales and northern Norway). *G. duebeni* was collected from both latitudes. Individual broods were divided across treatments to control for the effect of maternal size and thermal history on juvenile size and juveniles were transferred as soon as possible after release from the brood pouch to give an accurate portrayal of size at release and to reduce cannibalisation of juveniles by the female. The temperatures and salinities of each treatment are described in Table 6.1. Juvenile *G. duebeni* were not held at 20 °C and a salinity of 35 psu, as adults failed to survive under these conditions.

As high rates of juvenile mortality were experienced, especially within the first month following release from the brood pouch, the density of juveniles was strictly controlled to minimise cannibalism. Initially juveniles were maintained in 50 ml tubes with mesh bottoms in the main tank, but this method proved time consuming for animal husbandry. For the majority of the experimental period, juveniles were maintained up to a maximum density of 30 juveniles per litre of seawater. Juvenile amphipods were maintained as described for their respective adults (Chapter 2). The number of surviving juveniles was recorded once a

week, when juveniles were chosen at random and body length determined as described in the previous section.

Table 6.1. Acclimation conditions for juvenile gammarid amphipods during growth rate measurements. Collection site indicates the origin of mature adult amphipods, collected in October. Shaded boxes indicate juveniles were reared under the specified conditions.

Collection site	Species	Salinity (psu)	Temperatures (°C)			
			5	10	15	20
Anglesey, Wales, 53 °N	*G. duebeni*	18				
		35				
	G. locusta	35				
Tromsø, Norway, 70 °N	*G. duebeni*	18				
	G. oceanicus	35				

6.3.3. Statistical analysis

Brood sizes were compared within and between species using t-tests. Although female body length was recorded for comparison to brood size, only a very narrow range of total body lengths were seen in brooding females within a population rendering further analysis unreliable.

The increases in the total length of juvenile *Gammarus spp.* were linear over time during the measurement period, therefore least squares regressions were performed to provide growth rates. One-way analyses of covariance (ANCOVA) were conducted to compare growth rates (slope of the regression lines) according to salinity, temperature, population and species after a $\log_{10}$ transformation was applied to the data to achieve normality. A preliminary analysis was conducted to control for the effect of the vessel used to rear the juveniles on growth rates. Although the early use of tubes to rear juveniles gave a higher mortality rate, no significant effect of rearing vessel on growth rate was seen. Data acquired using both methods was therefore pooled for the main analysis. The measure of association (omega squared, ω^2) was calculated for the ANCOVA using the formula:

$$\omega^2 = \frac{SS_B - (df)MS_E}{SS_T + MS_E}$$

Where SS_B is the sum of squares for the independent variable, body length; MS_E is the mean square of the error and SS_T is the sum of squares for the total.

Statistical analyses were performed using SPSS (SPSS version 21; SPSS Inc., Chicago, IL, USA) and results were considered significant at the 5 % confidence interval. Post hoc comparisons for the effect of acclimation temperature on growth rates in *G. duebeni* and *G. locusta* were made using the Bonferroni procedure to control for Type 1 error across the six pairwise comparisons for *G. duebeni* (α' = 0.05/6 = 0.008) and three pairwise comparisons for *G. locusta* (α' = 0.05/3 = 0.016).

6.4. Results

6.4.1. Brood size

Of the two welsh species, *G. duebeni* had a significantly smaller brood size than *G. locusta* (t-test: t_{14} = 3.345, *P* = 0.005). *G. locusta* females produced 24.2 ± 2.6 juveniles in comparison to the 15.9 ± 1.2 juveniles produced per *G. duebeni* female, although brooding *G. locusta* were generally larger (t-test: t14 = 3.101, P = 0.008), with a mean total body length of 13.2 ± 0.3 mm in comparison to 12.2 ± 0.2 mm for *G. duebeni*. Brood size according to female body length was still higher in *G. locusta*, at 1.8 ± 0.2 juveniles.mm female^{-1} compared to 1.3 ± 0.1 juveniles.mm female^{-1} in *G. duebeni* (t-test: t_{14} = 2.643, *P* = 0.019). No significant difference was seen in juvenile size at the time of release between the two species, therefore total brood volume was larger in *G. locusta* than *G. duebeni*.

There was a significant difference in brood size between the two *G. duebeni* populations (t-test: t_{12} = 2.252, *P* = 0.044), with females from Norway (70 °N) producing almost 40 % more juveniles per brood than females from Wales (53 °N). This may be explained by the variation in size of brooding females between the two populations, as females from Norway were significantly larger (t-test: t_{12} = 7.273, *P* = 0.000; 16.4 ± 1.0 mm) and the two populations showed no significant difference if brood size were calculated according to female length (t-test: t_{12} = 0.136, *P* = 0.894). The sample size, however, was small; only three females from Norway released broods during captivity. In Norway, *G. oceanicus* had a brood size 50 % greater than *G. duebeni* at the time of release from the brood pouch (t-

test: t_{12} = 3.003, P = 0.022) despite being similar body lengths (average brooding *G. oceanicus* length was 16.5 ± 0.4 mm), as *G. oceanicus* produces more juveniles relative to length than *G. duebeni* (t-test: t_{12} = 2.557, P = 0.025; 1.3 ± 0.2 juveniles.mm female^{-1} in *G. duebeni*, 2.0 ± 0.1 juveniles.mm female^{-1} in *G. oceanicus*). Again, no intraspecific difference in juvenile size at time of release was noted; juvenile length at the time of first measurement was between 1.5 and 2.5 mm in all three species.

The two species from Norway (70 °N) were less reproductively active than those from Wales (53 °N). 25 to 35 % of individuals in tanks containing *G. duebeni* and *G. locusta* from Wales were brooding or paired at any one time during the treatments. In contrast, only 4 to 10 % of individuals were brooding or paired in tanks containing *G. duebeni* and *G. oceanicus* from Norway. The Norwegian populations released few juveniles over the course of the experiment (less than one juvenile produced per mature adult amphipod in the main holding tanks), however brooding females were present in both species and female *G. oceanicus* began to release juveniles at the very end of the experimental period, in March 2013. Juvenile production per mature adult amphipod in the holding tanks was 3.2 ± 0.8 in *G. duebeni*, and 7.4 ± 0.5 in *G. locusta*.

6.4.2. Juvenile growth rates

Salinity had a significant effect on growth rates in juvenile *G. duebeni* from Wales at 15 °C only, at lower acclimation temperatures salinity had no effect on juvenile growth rate. The growth rate was significantly higher in juveniles acclimated to a salinity of 18 psu than 35 psu, with salinity accounting for approximately 23% of the total variance in body length, controlling for the effect of time (ANCOVA: $F_{1,175}$ = 17.698, P = 0.000, ω^2 = 0.023). The respective rates of growth at salinities of 18 and 35 psu in juvenile *G. duebeni* held at 15 °C were 0.17 ± 0.01 and 0.114 ± 0.01 mm.week^{-1}.

At a salinity of 18 psu and temperature of 5 °C (ANCOVA: $F_{1,83}$ = 1.439, P = 0.000) growth rates were significantly faster in *G. duebeni* juveniles from Norway (70 °N) than from Wales (53 °N). Rates of growth in juveniles from females collected in Norway and Wales were 0.06 ± 0.03 and 0.04 ± 0.00 mm.week^{-1}, respectively at 5 °C. At a common temperature of 10 °C, the two populations of juveniles still varied significantly (ANCOVA: $F_{1,117}$ = 168.024, P = 0.000), however the difference between the populations in Norway and Wales was reduced from the 0.014 mm.week^{-1} variation in growth rate seen at 5 °C to only a 0.003 mm.week^{-1}

at 10 °C. Although Norway juveniles showed an increase in growth rate between 5 and 10 °C (ANCOVA: $F_{1,63}$ = 5.306, P = 0.025), the actual difference in growth rates between the two temperatures was only 0.006 mm.week^{-1}.

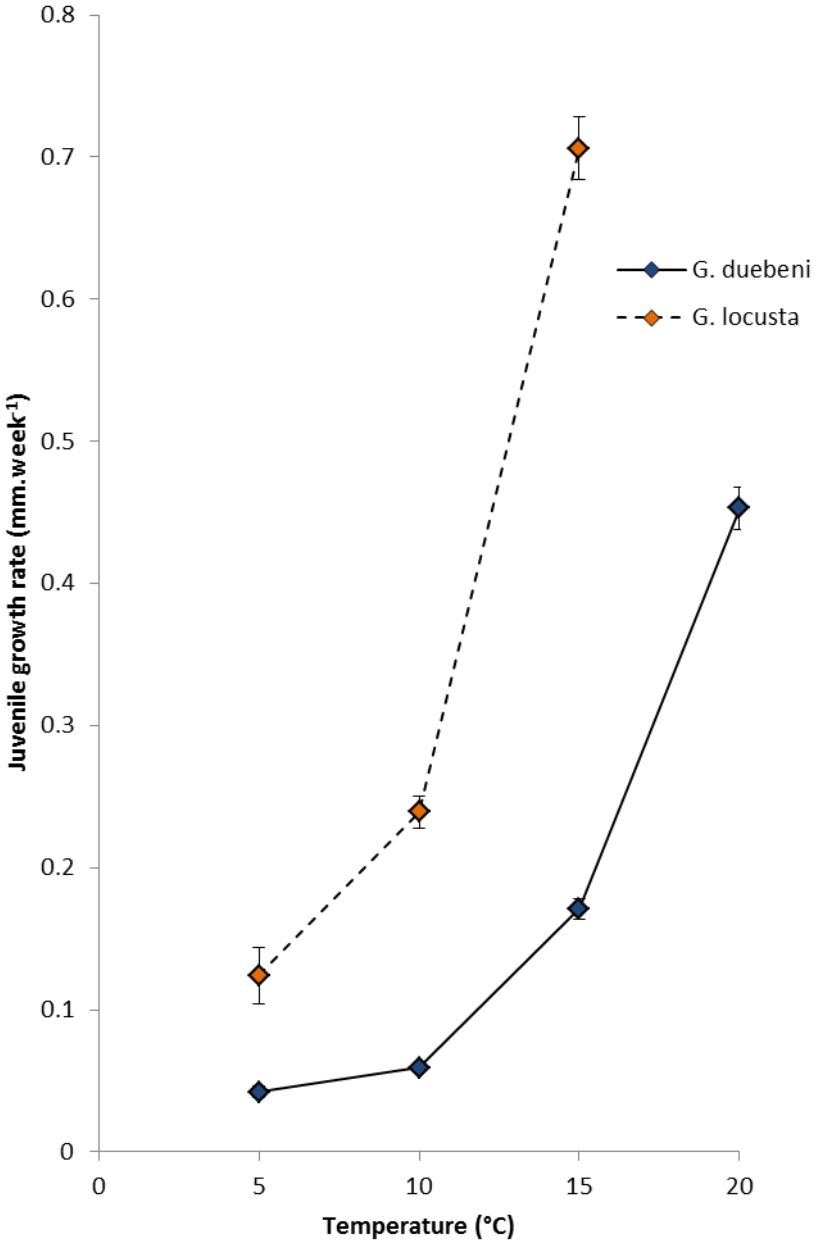

Figure 6.1. Juvenile growth rates in *G. duebeni* and *G. locusta* according to treatment temperature. Values are mm.week^{-1} ± *S.E.M*. *N* = 55, 83, 149 and 37 for *G. duebeni* at 5, 10, 15 and 20 °C respectively. For *G. locusta*, *N* = 29 at 5 and 10 °C, and 80 at 15 °C. Growth rates show a significant increase within each species according to temperature (ANCOVA: $F_{3,323}$ = 90.663, P = 0.000 for *G. duebeni*; $F_{2,137}$ = 236.314, P = 0.000 for *G. locusta*), and at all common temperatures growth in *G. locusta* occurs at a faster rate than in *G. duebeni* (ANCOVAs: P = 0.000).

Juvenile *G. duebeni* from Wales held at a salinity of 18 psu exhibited significant variations in growth rate according to acclimation temperature (ANCOVA: $F_{3,323}$ = 90.663, P = 0.000). Post hoc comparisons indicated significant differences between each acclimation temperature (P = 0.000 for all comparisons except between 5 and 10 °C, for which P = 0.005), with growth rates increasing exponentially between 5 and 20 °C from 0.04 ± 0.004= to 0.45 ± 0.02 mm.week^{-1} (Figure 6.1).

As for *G. duebeni*, acclimation temperature had a significant effect on juvenile growth rates in *G. locusta* held at a salinity of 35 psu (ANCOVA: $F_{2,137}$ = 236.314, P = 0.000). Growth rates increased from 0.12 ± 0.02 at 5 °C to 0.71 ± 0.02 mm.week^{-1} at 15 °C (P = 0.000; Figure 6.1). At all of the common temperatures measured, juvenile *G. locusta* had significantly higher growth rates than juvenile *G. duebeni* from Wales (Figure 6.1; ANCOVA: $F_{1,83}$ = 47.968, P = 0.000 at 5 °C; $F_{1,111}$ = 67.492, P = 0.000 at 10 °C; $F_{1,106}$ = 262.578, P = 0.000 at 15 °C). In Norway at a common acclimation temperature of 10 °C, *G. oceanicus* held at a salinity of 35 psu exhibited significantly faster rates of growth than *G. duebeni* held at a salinity of 18 psu (ANCOVA: $F_{1,56}$ = 46.033, P = 0.000). The growth rate of juvenile *G. oceanicus* was 0.59 ± 0.08 mm.week^{-1}, almost ten times as high as the rate of 0.06 ± 0.03 mm.week^{-1} in *G. duebeni*.

Table 6.2. Juvenile growth rates for *Gammarus spp.* according to treatment temperature and salinity, species and population. The results of the linear regression are shown, where *b* and *a* describe the slope and intercept, respectively. Growth rate (*b*) units are in mm.week^{-1}. Standard errors are given for both *b* and *a*. Values for *Pb* and *Pa* were all below 0.05, indicating that all results for *b* and *a* were significantly different from 0 at the $P < 0.05$ level. R^2 gives the coefficient determination, and *F* and *P* the results of the corresponding *F* test. Sp represents the species (*D*, *G. duebeni*; *L*, *G. locusta*; *O*, *G. oceanicus*), Loc represents the location of the collection site for mature adult amphipods (Tromsø, Norway, 70 °N or Anglesey, Wales, 53 °N), Sal and Temp the rearing salinity and temperature.

Loc	Sp	Sal (psu)	Temp (°C)	*b*	*a*	R^2 (%)	*F*	*P*
53 °N	*D*	18	5	0.042 ± 0.004	2.576 ± 0.069	62.7	$F_{1,55}$ = 90.93	0.000
			10	0.059 ± 0.003	2.037 ± 0.044	79.2	$F_{1,83}$ = 312.02	0.000
			15	0.171 ± 0.007	2.161 ± 0.051	80.4	$F_{1,149}$ = 608.90	0.000
			20	0.453 ± 0.015	1.599 ± 0.130	96.2	$F_{1,37}$ = 901.36	0.000
		35	15	0.138 ± 0.012	2.000 ± 0.069	84.6	$F_{1,27}$ = 143.18	0.000
	L	35	5	0.124 ± 0.020	2.681 ± 0.161	56.6	$F_{1,29}$ = 36.58	0.000
			10	0.239 ± 0.011	1.612 ± 0.084	94.7	$F_{1,29}$ = 498.51	0.000
			15	0.706 ± 0.022	1.745 ± 1.88	93.2	$F_{1,80}$ = 1078.05	0.000
70 °N	*D*	18	5	0.056 ± 0.026	5.255 ± 0.190	13.7	$F_{1,29}$ = 4.46	0.044
			10	0.062 ± 0.027	2.885 ± 0.190	14.0	$F_{1,35}$ = 5.52	0.025
	O	35	10	0.587 ± 0.081	2.547 ± 0.524	71.2	$F_{1,22}$ = 52.00	0.000

6.5. Discussion

6.5.1. Brood size

Brood sizes for all three species were generally low in comparison to those reported in the literature (Table 6.3.). Some of the discrepancy may be explained by the measures used for brood size. Spooner (1947) counted egg numbers in the brood pouch of *G. locusta* and *G. zaddachi,* and although his sample size was small he recorded a greater number of newly laid and early development stage eggs per female than the number of juveniles ready to hatch. In *G. oceanicus*, embryo and oogenia number are comparable in the summer, however in winter the number of embryos decreases relative to the oogenia, indicating variation in the percentage that develop according to season (Steele & Steele, 1972). Although the largest brood size in *G. duebeni* from Norway (70 °N) at the time of release from the brood pouch was 27 juveniles, one 17 mm brooding female removed from a tank after it expired was carrying a total of 38 eggs. The majority of previous research on brood

size has focused on counting egg numbers, but mortality rates in the brood pouch is unknown. Collectively, these data suggest that the number of eggs produced are greater than the number of juveniles released, meaning that the current determination of brood size are an under estimate. However, all of the species studied here were treated in the same way. As individual brooding females were so precious and as counting of the egg numbers would have sacrificed amphipods, the decision was made to count newly released juveniles from the brood pouch.

This study demonstrated population differences in brood size in *G. duebeni*, which could be a result of the variation in the size of brooding females between the two populations. Females from Norway released a larger number of juveniles, but had a longer body length than females from Wales. Female size is known to be a factor controlling brood size in *G. duebeni*, with larger females producing a greater number of eggs (Kolding & Fenchel, 1981; Steele & Steele, 1969). Although Kolding & Fenchel (1981) saw variation in *G. duebeni* brood size between locations even after controlling for the effect of body size, they did not attempt to explain the phenomenon and a review of the published literature shows no evidence for a latitudinal cline in brood size in *G. duebeni* (Table 6.3.). Brooding females from the two *G. duebeni* populations studied here experienced variations in thermal history prior to collection, and would therefore be expected to exhibit differences in reproductive effort according to temperature (Pockl et al., 2003). However, this did not appear to be the case. A review by Sainte-Marie (1991) concluded cold water amphipods produce a larger brood size than warm-water amphipods, but they also have larger body sizes. In the present study, differences in brood size within *G. duebeni* were determined primarily by body size confirming the observation by Sainte-Marie (1991) that the biggest differences in brood size are between species.

Table 6.3. Reproductive outputs of *Gammarus* species from populations inhabiting coastal sites fringing the Atlantic and Arctic Oceans, arranged according to mean habitat temperature. Loc indicates the latitude (°N) and country where the population originated (CA = Canada, DE = Denmark, NO = Norway, PL = Poland, SE = Sweden, UK = United Kingdom, US = United States of America). Mean winter and summer temperatures (T, °C) are given for each population (NOAA OI SST V2 Data for all references). For gammarids reared in the laboratory, rearing temperatures (RT) are given. Life history measurements are brood size (BS) in juvenile (J) or egg number (E), as a mean value or standardised to a female body length of 10 mm.

Species	Loc	T (°C)		BS/10 mm	Mean BS	Reference
		W	S			
G. duebeni	51 UK	9	13.5	-	24.3 E	Dunn & McCabe (1995)
	41 US	9	13.5	-	9.9 J	Ginn et al. (1976)
	53 UK	8	13.5	-	18.1 E	Cheng (1942)
	53 UK	8	13.5	-	15.9 J	This study
	54 UK	8	13.5	14.5 E	21 E	Hynes (1954)
	54 DE	8	13.5	-	36.5	Kinne (1953) from Sainte-marie (1991)
	54 PL	8	13.5	-	40.5 E	Jazdzewski (1973)
	56 UK	8	12	-	25 E	McCabe and Dunn (1994)
	56 UK	8	12	-	23.8 E	Naylor et al. (1988)
	57 DK	6	13.5	15.3 E	-	Kolding & Fenchel (1981); Kolding & Fenchel (1979)
	59 SE	3	13.5	17 E	-	Kolding & Fenchel (1981); Kolding & Fenchel (1979)
	47 CA	1	12	25.4 E	-	Steele & Steele (1969)
	70 NO	5	7	-	22.0 J	This study
G. locusta	38 PT	20 (RT)		35 E	-	Neuparth et al. (2002)
	38 PT	15 (RT)		34 E	-	Neuparth et al. (2002)
	38 PT	15	18	37 E	56 E	Costa & Costa (1999)
	43-52	12	18	65.4 E	-	Kolding & Fenchel (1981)
	52 UK	9	13.5	27.6 E	61.7 E	Spooner (1947)
	52 UK	9	13.5	24.3 J	43.2 J	Spooner (1947)
	53 UK	9	13.5	-	24.2 J	This study
	54 PL			-	49 E	Jazdzewski (1973)
	57 DK	6	13.5	41 E	-	Kolding & Fenchel (1981)
	59 SE	3	13.5	36.3 E	-	Kolding & Fenchel (1981)
G. oceanicus	42 US	6	16	-	38.7 E	Croker & Gable (1977)
	57 DK	6	13.5	27.3 E	-	Kolding & Fenchel (1981); Kolding & Fenchel (1979)
	59 SE	3	13.5	29.3 E	-	Kolding & Fenchel (1981)
	47 CA	1	12	24.2 E	46.5 E	Steele & Steele (1972)
	70 NO	5	7	-	33.1 J	This study
	79 NO	1	3	-	78 E	Węsławski & Legezynska (2002)

At the same latitude (Wales, 53 °N), *G. locusta* females produced a greater number of juveniles per brood than *G. duebeni*, as observed by Kolding & Fenchel (1981) and supported by a review of published brood sizes in the two species (Table 6.3.). *G. locusta* has also been reported to produce a greater number of broods per year on the shore, due

to year round breeding in comparison to the winter breeding strategy of *G. duebeni* reported in Northern Europe (Kolding & Fenchel, 1979; Kolding & Fenchel, 1981; Steele & Steele, 1969). Kolding & Fenchel (1981) reported almost twice as many broods per generation in winter breeders of *G. locusta* than *G. duebeni* in the Limfjord, Denmark. In the laboratory *G. duebeni* produced half the number of juveniles per adult compared to *G. locusta*, and broods from brooding *G. duebeni* females collected on the shore tended to be released from early spring to summer, while *G. locusta* produced broods in the laboratory throughout the year. The combination of a smaller brood size plus fewer potential broods per lifetime means that *G. duebeni* is less fecund than *G. locusta*. Lower reproductive output has been suggested to be typical of a colder water species like *G. duebeni* (Clarke, 1987) compared with *G. locusta* which has a more southerly distribution and Meditteranean origin (Costa et al., 2009; Sainte-Marie, 1991). The differences in breeding strategy between *G. duebeni* and *G. locusta* may be a mechanism by which the two species may minimise interspecies precopulation while co-existing on the shore (Kolding & Fenchel, 1979).

The two gammarid species from Wales exhibited higher rates of breeding activity in the laboratory than those from Norway. Although *G. duebeni* and *G. oceanicus* from Norway are expected to experience egg development over the winter and release broods in late spring (Kolding & Fenchel, 1981), they would therefore be unlikely to release juveniles during the course of the experiment which ran from October until March. Nevertheless, brooding females and pairs were present at the beginning of the acclimation period. Toward the end of the experiment, in March, *G. oceanicus* began to release juveniles; however *G. duebeni* from Norway were still relatively reproductively inactive. Only three broods in total were released by *G. duebeni* from Norway while *G. oceanicus* produced 11, despite initial adult numbers being similar and *G. oceanicus* experiencing a higher mortality rate during the acclimation period. The sex ratio of populations was not measured, however a representative range of sizes were collected and an obvious deviation from a sex ratio of 0.5 was not noticed when amphipods were sexed to select males for physiological experiments. *G. oceanicus* produced larger broods than *G. duebeni*, as previously demonstrated (Table 6.3.). The brood size of *G. oceanicus* measured in this study was similar to the values reported in the literature, except for a study which reported that egg numbers in *G. oceanicus* from Norway, 79 °N, were twice that of juvenile number reported here for the same species living at 70 °N (Węsławski & Legezynska, 2002). The mean length of females in their study was 10 mm longer than the mean length of females

from this study, and as previously discussed brood size is partially dependent on female body size. The authors also found that egg numbers in sampled females ranged ten-fold from 16 to 152. Although mortality during the development period would account for some of the difference between the numbers observed between the two studies, variations in female body size and the large range in egg number per female in the study by Węsławski & Legezynska (2002) also help to explain the observed difference. The difference in brood size between *G. duebeni* and *G. oceanicus* observed here could not be explained by female size; the difference in mean size between brooding females of the two species was only 0.1 mm, but brood size was 50 % larger in *G. oceanicus* than *G. duebeni*. Brood size has also been shown to be negatively correlated with embryo size and the frequency of brood production in amphipods (Sainte-Marie, 1991). Brood size therefore depends not only on the available space in the brood pouch, but the amount of energy devoted to reproduction (Clarke, 1987). The subartic/boreal species *G. oceanicus* would be expected to produce smaller broods than the boreal/temperate *G. duebeni*, as it has a more northern distribution and may be characterised by a lower energy lifestyle, with less available energy for reproduction. *G. duebeni* shows more flexibility in life history, with reports of summer breeding populations at lower latitudes (Kolding & Fenchel, 1981). The two species may exhibit different reproductive strategies, with *G. oceanicus* allocating a larger portion of the energy budget to reproduction and maintaining a constant strategy with latitude, while *G. duebeni* allocates a variable percentage dependent on available energy. A study of egg volume in *G. duebeni* from two locations within the UK showed the southern population bred twice during the year, producing larger eggs in the winter in reponse to colder temperatures, while the northern population produced consistently larger eggs with no seasonal variation (Dunn & McCabe, 1995). *G. duebeni* from Norway may therefore employ a strategy of fewer, larger eggs than *G. oceanicus* in response to the colder environment, but at lower latitudes exhibits a plasticity in brood volume in response to the more variable environment it encounters further south where it also occupies habitats higher up on the shore.

High reproductive capacity is indicative of a species or populations ability to mitigate the effects of high mortality. Gammarid amphipods would be expected to experience high rates of mortality during the juvenile period (Lonsdale & Levinton, 1985) and therefore large brood sizes would be an adaptive strategy to ensure some offspring reach sexual maturity. As the intertidal is a relatively unpredictable habitat, with a high degree of local variation and fluctuation in environmental conditions, the ability of a population to rapidly

increase in abundance given favourable conditions or following high mortality events would be key in determining its capability of colonising new habitats and maintaining its position on the shore. Of the species studied, *G. duebeni* would therefore be expected to be the most susceptible to climate change and short-term catastrophic events as based on the current observations of smaller brood size. Populations of this species would be slower to recover or to colonise new areas than either *G. locusta* or *G.oceanicus*. This may explain in part the reason *G. duebeni* tends to be out-competed when multiple *Gammarus* species are present on one shore, despite the species' broad geographical range and environmental tolerance (Gaston & Spicer, 2001).

6.5.2. Juvenile growth rates

Growth rates appeared linear over the time frame measured during this experiment, however it is likely that growth rates would have decreased as maturity was reached, as reported elsewhere for gammarid amphipods (Naylor et al., 1988; Vassallo & Steele, 1980). Growth rates in juvenile *G. duebeni* appear to be compromised in full strength seawater (35 psu), but only at higher temperatures. At 5 and 10 °C growth rates were equal in the brackish and full strength seawater conditions while at 15 °C juveniles held in full strength seawater had a slower rate of growth. Brooding females acclimated to 20 °C and full strength seawater failed to release any live juveniles, despite egg bearing females being present in the population prior to acclimation. Exposure to low salinity has been shown to reduce viability of *Marinogammarus marinus* embryos (Vlasblom & Bolier, 1971) and appears to increase embryo or juvenile mortality in *G. locusta* (Neuparth et al., 2002). Vlasblom & Bolier (1971) concluded that the optimal salinity range for egg production in *M. marinus* was narrower than that for other physiological functions in the adults. Although *G. duebeni* is highly tolerant of fluctuations in salinity, adult and embryo mortality in the 20 °C, 35 psu treatment indicates *G. duebeni* populations would be unlikely to be viable in areas with a combination of warm temperatures and high salinities. As the optimum salinity range for *G. duebeni* has been suggested to be between 5 and 15 psu (Ikko & Lyubina, 2010), growth at higher salinities may be compromised due to the unfavourable conditions presented by exposure to high salinities. *G. duebeni* is unable to regulate haemolymph [Na^+] levels during hyper-osmotic exposure (Brooks & Lloyd Mills, 2006) and the subsequent increase in haemolymph osmolarity may compromise cell volume control (Whiteley et al., 2001). Intanai et al. (2009) observed an increase in rates of oxygen uptake

in the freshwater prawn *Macrobrachium rosenbergii* with an increase in salinity, which was attributed to an increase in oxygen demand associated with increasing costs of osmoregulation. Tendengren *et al.* (1988) also observed an increase in rates of oxygen uptake *in G. duebeni* at supra-optimal salinities. However, rates of oxygen uptake in the present study were higher in *G. duebeni* at 18 psu compared with *G. duebeni* at 35 psu, at least at the lower acclimation temperatures. It appears that oxygen supply is limiting in *G. duebeni* at 35 psu, suggesting a reduction in available energy for growth (Pörtner et al., 2005). This may explain why growth rates in *G. duebeni* at a salinity of 35 psu were less sensitive to temperature than *G. duebeni* at 18 psu.

Female *G. duebeni* from Norway (70 °N) produced juveniles with faster rates of growth than those from Wales (53 °N) at the lower temperature of 5 °C, but not at 10 °C. Highsmith & Coyle (1991) state that both growth rate and moult frequency increase with temperature in amphipods, but that sexual maturity occurs after fixed number of moults rather than at a determined body size. This results in warmer water species achieving maturity earlier but at a smaller body size than colder water species. A similar response seems to be happening within species and among populations inhabiting different thermal regimes. This may account for the difference in body size of mature *G. duebeni* females collected in Norway compared to Wales, despite the same apparent growth rate at 10 °C. Upregulation of growth rates at cold temperatures in high latitude populations, though costly, may be an adaptive strategy to minimise the juvenile growth period given the high associated risk of predation (Lonsdale & Levinton, 1985). They may also represent increased growth rates during the summer season to take advantage of the more favourable conditions.

Acclimation temperature had a significant effect on juvenile growth rates in both *G. duebeni* and *G. locusta*. Growth rates increased ten-fold In *G. duebeni* between 5 and 20 °C, and six-fold in *G. locusta* between 5 and 15 °C. Increases in growth rate with temperature in gammarids has previously been observed in *G. locusta* by Neuparth et al. (2002), and also in *G. mucronatus* (Fredette & Diaz, 1986) and *G. tigrinus* (Chambers, 1977) (Table 6.4.). Variations in growth rate with temperature in *G. duebeni* and *G. locusta* were greater than those recorded in gammarids by Pockl (1992), who measured a five-fold increase in growth between 4 and 20 °C in juvenile *G. fossarum* and a seven-fold increase in *G. roeseli*. Neuparth et al. (2002) observed an acceleration of the life history of *G. locusta* with temperature. At 20 °C, individuals had a faster growth rate but a reduction in life span.

Previous studies have also come to the same conclusion on the effect of temperature on the life cycle of amphipods (Highsmith & Coyle, 1991; Sainte-Marie, 1991). Summer temperatures on the shore in Wales average around 15 °C and ranged between 6.5 to 32.9 °C. In contrast, summer temperatures in Norway averaged 11.3 °C and ranged between 4.9 and 23.5 °C. To reach the same body size at maturity, growth would therefore need to be accelerated in *G. duebeni* from the more northern population. In both locations temperatures showed a relatively high degree of variation in the summer compared with the winter (Chapter 2), therefore the rapid increase in growth with temperature may be an adaptation to divert energy towards fast growth at high temperatures to make use of brief periods of optimum growth temperatures, or may simply be a lack of temperature compensation.

Of the two species from Wales, juvenile *G. locusta* demonstrated faster rates of growth than *G. duebeni* at all three temperatures. At 15 °C, the temperature considered to be equivalent to average summer habitat temperatures in Wales (Chapter 2), growth rates were five times higher in *G. locusta* than *G. duebeni* at a common salinity of 35 psu. Although growth rates were depressed in *G. duebeni* at the higher salinity, brackish *G. duebeni* still grew at only a fourth of the rate of *G. locusta* at 15 °C. Although both species exhibited an elevation in growth rate with acclimation temperature, over a 10 °C rise in temperature, growth rates in *G. locusta* were more temperature sensitive. Growth in *G. locusta* increased by 0.59 mm.week^{-1} with a Q_{10} value of 5.9, compared to an increase of 0.13 mm.week^{-1} and Q_{10} value of 4.3 in *G. duebeni*. Growth efficiencies (expressed as the ratio between growth production and assimilation) in the congenic *G. mucronatus* are reduced during the warmest part of the year (LaFrance & Ruber, 1985). *G. duebeni* may also be constrained by low growth efficiencies at higher temperatures, resulting in the slower rate of growth in comparison to *G. locusta*. Lifestyle may have an effect on growth efficiency, as in non-insect invertebrates detritivores have a higher growth efficiency than carnivores, which in turn have a higher growth efficiency than herbivores (Humphreys, 1979). Although few studies have been performed on growth efficiencies in amphipods, *G. mucronatus* (LaFrance & Ruber, 1985) may have a relatively high efficiency in comparison to the non-insect invertebrates reviewed by Humphreys (1979). Growth efficiencies are also shown to be low in polar marine invertebrates (Fraser & Rogers, 2007), therefore could be a characteristic of cold water species.

The two species from Tromsø exhibited a larger difference in juvenile growth rate than those from Anglesey. *G. oceanicus* acclimated to 10 °C had a rate of growth nearly ten times higher than that of *G. duebeni*. According to Poltermann (2000) the large size of the Arctic gammarid *G. wilkitzkii* is due to its longevity rather than its growth rate, which is relatively slow and further constrained in females by the lack of moulting while reproductively active. The relatively slow growth rate in *G. wilkitzkii* is supported by evidence of a relatively low metabolic rate (Werner et al., 2002). *G. oceanicus* is thought to have a life span up to two years (Steele & Steele, 1972), while *G. duebeni* is only expected to live for 12 to 15 months (Hynes, 1954; Steele & Steele, 1969). Although the larger body size of the arctic-boreal *G. oceanicus* compared to the temperate *G. duebeni* may in part be attributed to the species longevity, in this study faster growth also appears to be a determining factor. Interestingly, *G. duebeni* is distributed along the coastal fringes of Western Europe from Portugal to Northern Norway at the northern limit of its range. *G. oceanicus*, however, has expanded north after the last glacial maxima (Costa et al., 2009) and has colonised shores as far north as Svalbard up to 79 °N. However, this has only been possible due to the warming influence of the North Atlantic current and the increase in SSTs along the western coasts of Svalbard (Węsławski et al., 2010), and is therefore not a typical cold-water, polar species.

Caution must be taken when extrapolating the results of this study to the situation on the shore. Fredette & Diaz (1986) found a discrepancy between growth rates of *G. mucronatus* in the laboratory in comparison to estimates of growth in the field. Laboratory experiments underestimated growth in the field, possibly due to suboptimal holding conditions. In addition, feeding in the laboratory may have the opposite effect and accelerate the life history of the amphipods. Gammarids were fed a high protein diet ad libitum (Chapter 2), and high food availability and a high protein intake can have the effect of increasing growth rates and decreasing the time to maturity (Sutcliffe et al., 1981; Vassallo & Steele, 1980). Also conditions on the shore are highly variable, especially for *G. duebeni*. It has previously been shown that intertidal invertebrates respond differently to variable versus constant temperatures, with limpets (*Cellana tramoserica*) from stable environments exhibiting seasonal compensation of rates of oxygen uptake while limpets from fluctuating environments show no such compensation (Sinclair et al., 2006). Exposure to constant temperatures in the laboratory may therefore have altered the amphipods metabolic response to temperature

Table 6.4. Growth rates of *Gammarus* species according to average temperature. Location indicates the latitude (°N) and country of origin for each population studied (see Table 6.3. for explanation). Temperatures during the rearing experiments are given in °C. Values are given juvenile growth rate in mm week^{-1} (GR), according to sex where specified. F represents females and M, males.

Species	Location	Temp	GR		Reference
			F	M	
G. aequicada	40 IT	18	0.60	0.69	Prato et al. (2006)
G. duebeni	53 UK	20	0.45		This study
	53 UK	15-20	~0.2		Hynes (1954)
	56 UK	15	0.65		Naylor et al. (1988)
	53 UK	15	0.17		This study
	70 NO	10	0.06		This study
	53 UK	10	0.06		This study
	53 UK	5	0.04		This study
	70 NO	5	0.06		This study
G. locusta	38 PT	20	1.31	1.66	Neuparth et al. (2002)
	38 PT	15	0.94	1.23	Neuparth et al. (2002)
	53 UK	15	0.71		This study
	53 UK	10	0.24		This study
	53 UK	5	0.12		This study
G. mucronatus	57 US	23	0.42		Fredette & Diaz (1986)
	57 US	14	0.35		Fredette & Diaz (1986)
	57 US	5	0.07		Fredette & Diaz (1986)
G. pulex	51 UK	13	0.52		MCAahon & Pascoe (1988)
G. oceanicus	70 NO	10	0.59		This study
G. tigrinus	53 NK	20	0.60	-	Chambers (1977)
	53 NK	16	0.56	-	Chambers (1977)
	53 NK	12	0.50	-	Chambers (1977)

6.5.3. Conclusions

G. locusta exhibited a high fecundity and a faster rate of juvenile growth typical of a warmer-water, southern gammarid species with relatively high metabolic and protein synthesis rates (Costa et al., 2009; Rastrick & Whiteley, 2011; Rastrick & Whiteley, 2013). *G. oceanicus* is typical of a cold water species with a large body size and brood number (Sainte-Marie, 1991), and relatively low rates of metabolism and protein synthesis (Rastrick & Whiteley, 2011; Rastrick & Whiteley, 2013). However *G. duebeni* exhibited a relatively slow rate of juvenile growth and lower fecundity in comparison to with *G. locusta* and *G. oceanicus*. *G. oceanicus* and *G. locusta* had similar large brood sizes despite differences in their latitudinal extents, in keeping with observations made in other studies (Table

6.3.).This has been suggested to be characteristic of amphipod species occupying marine habitats rather than brackish species such as *G. duebeni* (Sainte-Marie, 1991). In this study, larger brood sizes were associated with more stable habitats (low shore versus high shore, and Tromsø versus Wales).

The reproductive potential of *G. oceanicus* and *G. locusta* may be inferred to be higher than *G. duebeni* as not only do both species exhibit larger brood sizes, but *G. oceanicus* is known to have a greater longevity than *G. duebeni* (Steele & Steele, 1969; Steele & Steele, 1972) and *G. locusta* is known to have a shorter generation time than *G. duebeni* (Kolding & Fenchel, 1979). It is possible that populations of *G. duebeni* would be less able to recover from catastrophic events or to colonise new areas. Despite the broad salinity tolerance of *G. duebeni*, a combination of full strength seawater and high temperature resulted in high mortality rates of both adults and developing embryos. Although *G. duebeni* has a broad geographical range and environmental tolerance, a reduced reproductive potential and a restricted season for brooding may explain why *G. duebeni* is out-competed by *G. oceanicus* or *G. locusta* when two species are present on one shore (Gaston & Spicer, 2001). Further studies on age-specific mortality rates in the three species would help to give a more accurate picture of population growth (Levin et al., 1996) under varying thermal regimes and resolve the relative susceptibility of the *Gammarus* species to environmental change.

Chapter 7

General discussion

The main aim of this study was to more fully understand the effects of temperature on key physiological processes in a closely related taxa of gammarid amphipods with differing latitudinal distribution patterns and to examine some of the ecological consequences. Three species within the family Gammaridae were examined when present at latitudes spanning temperate to Arctic thermal regimes (53 to 79 °N). Species and populations were acclimated to common temperatures with the range normally encountered on the shore in order to determine the specific effects of temperature on metabolic rate (Chapter 4), and rates of protein synthesis (Chapter 5). Compensation can occur to allow for some independence from temperature which has several advantages, especially if the species live in highly variable and fluctuating environments. The ability to compensate for temperature was investigated an ecological context by studying the associated changes in thermal tolerances (Chapter 3), growth rates (Chapters 5 and 6) and life-history traits (Chapter 6). In addition to temperature, *G. duebeni* populations were acclimated to two salinities in order to further investigate whether the thermal effects on key physiological processes were compromised by the necessity for osmoregulation, and whether these interactions showed any seasonal variation. The main observations arising from this thesis are summarised and discussed for each species with an emphasis on *G. duebeni* and with specific attention to the changes observed with latitude, season and salinity.

7.1. Gammarus duebeni

7.1.1. Effects of temperature on thermal tolerances and biological rate processes

Upper thermal tolerances increased in *Gammarus duebeni* according to acclimation temperature (Chapter 3), demonstrating an ability to increase upper critical thermal limits according to thermal experience, and indicating some plasticity in the response. Morevoer *G. duebeni* had the highest thermal tolerance measured in this study.

An acute increase in temperature from 15 to 20 °C resulted in doubling or tripling of oxygen uptake rates in amphipods acclimated to temperatures between 5 and 15 °C, despite amphipods showing temperature independence of metabolic rate at temperatures below 15 °C (Chapter 4). The temperature dependence of MO_2 at higher temperatures suggests that oxygen limitation (Pörtner, 2002) may be a determining factor for upper critical limits. This is supported by the observation of a trend in the behavioural response to increasing temperatures of an initial increase in the frequency of pleopod beats followed by a sharp

decline when approaching the upper thermal limit of the individual (Chapter 3). The ability of *G. duebeni* to fully acclimate to temperatures between 5 and 20 °C within six weeks, combined with it having the highest upper thermal limit measured in this study suggests *G. duebeni* has a relatively broad thermal window.

In contrast, protein synthesis rates in *G. duebeni* were highly dependent on temperature, with a Q_{10} value of 4.8 and 3.7 for fractional and absolute rates, respectively (Chapter 4). Growth rates in both juveniles (Chapter 6) and adults (Chapter 5) increased with acclimation temperature; however both growth and protein synthesis rates (Chapter 5) in *G. duebeni* were the lowest of the three species studied, as observed in acclimatised amphipods by Rastrick & Whiteley (2013). This suggests *G. duebeni* experiences a faster rate of protein turnover (McCarthy et al., 1999), resulting in a reduced allocation to growth or poor growth efficiency. The mechanism for broad tolerance to temperature and salinity fluctuations in *G. duebeni* may involve high rates of protein turnover as a means to cope with rapid change because it supports faster replacement and repair of proteins (Koehn & Bayne, 1989). Although Hawkins et al. (1987) found a correlation between high individual rates of protein turnover and increased sensitivity and morbidity during acute temperature change in *Mytilus edulis*, the authors witnessed a positive relationship between oxygen uptake and protein synthesis rates which was lacking in the current study. Provided metabolic requirements are not limiting, high levels of protein turnover may therefore be an advantage rather than disadvantage for *G. duebeni* with regards to its ability to cope with rapid change. Given the relatively low Q_{10} of MO_2 with temperature in brackish acclimated *G. duebeni* and compensation of resting rates with temperature, this appears to be the case. Despite the high turnover of proteins, slow growth rates in *G. duebeni* in the cold may be related to low rates of oxygen uptake as seen in the population from Tromsø, Norway (Chapelle & Peck, 1995; Peck, 2002; Pörtner et al., 2007), and low protein synthesis retention efficiency and elevated protein degradation as suggested in polar and high latitude ectotherms (Fraser & Rogers, 2007).

7.1.2. Effects of temperature on growth

The increase in the rates of growth and protein synthesis with temperature in *G. duebeni* suggests that these biological rate processes do not compensate for temperature change within six weeks. Either compensation takes longer or there is some advantage in growth

and rates of protein synthesis being dependent of temperature. For example, faster growth at higher temperatures could possibly act to minimise the length of the juvenile growth period and decrease predation risk. If it is assumed that there is a close relationship between rates of oxygen uptake and protein synthesis, as the latter accounts for 11-42 % of the former (Fraser et al., 2002), then compensation of metabolic rates but not growth and protein synthesis rates with acclimation temperature suggests that energetic costs of protein synthesis may vary. Further research is required to investigate the cost of protein synthesis in *G. duebeni*, as although high rates of protein synthesis may incur a lower cost than expected due to an increase in efficiency according to rate (Pannevis & Houlihan, 1992) or due to interspecific differences in costs (Marsh et al., 2001), trade-offs in reproductive strategy, activity levels or other lifestyle features may occur. It was not possible to investigate brood sizes according to temperature in *G. duebeni*, as several months are required for females to reproduce and incubate broods at some of the lower temperatures considered (Steele & Steele, 1969) but it is possible the relative allocation of the energy budget to growth and reproduction in summer and winter may vary according to temperature. As *G. duebeni* is considered a winter breeder (Kolding & Fenchel, 1981b), it is possible this species allocates more of the energy budget to growth in the higher temperatures of the summer months following release from the brood pouch, and a larger portion of the energy budget to reproduction in the colder winter months following maturity. Between 15 and 20 °C the increase in growth with temperature occurs at a faster rate than the increase in protein synthesis rates, suggesting protein turnover is reduced at higher temperatures.

7.1.3. Effects of temperature on life history traits

In addition to slow growth rates, *G. duebeni* also exhibits smaller brood sizes than the other two species in this study (Chapter 6). This suggests *G. duebeni* may experience a trade-off in terms of energy expenditure between environmental tolerance and lifestyle features such as growth and reproductive output (Pörtner et al., 2001). *G. duebeni* therefore exhibits apparent K-selected low energy life history traits (Clarke, 1987) despite its temperate distribution, probably because of its ability to survive in highly fluctuating environments. Further research is required into potential variation in brood size with temperature in *G. duebeni*, as although smaller brood sizes may be a function of a smaller available energy budget for reproduction, they may also represent a plastic response due

to temperature or seasonal variation in primary productivity and therefore food availability during brood production (Reinikainen & Repka, 2003).

7.1.4. Effects of temperature plus salinity

The influence of the combined factors of salinity and temperature on MO_2, citrate synthase (CS) activity and the rate of protein synthesis in *G. duebeni* was complex. In amphipods acclimated to 18 psu salinity, MO_2 and CS activity were constant between 5 and 15 °C, suggesting full compensation with acclimation temperature (Chapter 4). This temperature independence was disrupted in the 35 psu salinity condition; at 10 °C *G. duebeni* exhibited a reduction in MO_2 but an increase in CS activity. The thermal sensitivity of MO_2 in 35 psu acclimated animals was higher, with Q_{10} values during an acute increase in temperature almost double that for individuals acclimated to 18 psu (Chapter 4). The negative compensation of MO_2 and overcompensation of CS activity with temperature at 35 psu is probably associated with osmoregulatory problems in this brackish water species during hyper-osmotic exposure (Gaston & Spicer, 2001; Sutcliffe, 1971; Tedengren et al., 1988). The opposite effect was seen on protein synthesis rates; in *G. duebeni* acclimated to a salinity of 18 psu protein synthesis rates showed a significant increase with acclimation temperature (Chapter 5). However, at 35 psu the same was not true. No variation was seen according to temperature due to a significantly higher rate of protein synthesis at 5 °C in the 35 psu treatment, possibly as a compensatory mechanism to counteract the elevated rates of protein degradation following cold acclimation (Hazel & Prosser, 1974). At higher temperatures in the full strength seawater condition, the lack of an increase in protein synthesis rates may have occurred due to cost limitations associated with maintenance of Na^+/K^+-ATPases via the Na^+/K^+-ATPase pump, as occurs in the prawn *Macrobrachium rosenbergii* (Intanai et al., 2009).

The conclusion from this study is that *G. duebeni* is better adapted to 18 psu salinity than 35 psu. Although the general consensus is that *G. duebeni* performs better in brackish conditions around 5 to 22 psu (Beadle & Cragg, 1940; Ikko & Lyubina, 2010; Sutcliffe, 1971; Tedengren et al., 1988), the tolerance of the species to freshwater is debated. Sutcliffe (1970) managed to establish a breeding population in low sodium water, but in his surveys was unable to find instances of *G. duebeni* breeding in freshwater above the high tide line. As the seawater supply in the laboratory was slightly hypersaline (35 psu) *G. duebeni* will

certainly not have been under optimal conditions. The brackish condition, a salinity of 18 psu, is considered close to the iso-osmotic point of this species (Morritt & Spicer, 1995), in hyper-osmotic conditions *G. duebeni* behaves as an osmoconformer, with increasing levels of Na+ ions in the haemolymph (Bolt, 1983; Whitton, 1975).

7.1.5. Effects of latitude

After acclimation to common temperatures, the northern population of *G. duebeni* from Tromsø, Norway (70 °N) showed significant differences when compared to the southern population from Anglesey, Wales (53 °N) in almost every physiological measurement considered during this study. Thermal tolerances (Chapter 3) in the three species of acclimatised gammarid amphipods from three latitudes showed a trend for a decrease in upper thermal limits with latitude as described by many similar studies of marine ectotherms (Calosi et al. 2010; Fangue et al. 2006; Sorte & Hofmann, 2005; Stillman & Somero 2000; Tomanek & Somero, 1999). After acclimation to common temperatures, a reduction in upper thermal limits persisted in the northern population of *G. duebeni*. Resting rates of oxygen uptake were lower in the Norwegian population however the Q_{10} value for MO_2 after exposure to a 10 °C acute increase in temperature was 2.49, compared to 1.44 for the Welsh population (Chapter 4). Despite the apparent reduction in metabolic rate with latitude, the greater thermal sensitivity of oxygen uptake in the northern population supports the oxygen limitation hypothesis as an underlying cause for the differences in upper thermal limit. Lower oxygen uptake at the higher latitude were associated with lower CS activities and therefore aerobic capacities (Chapter 4).

Regardless of the reduction in MO_2 with latitude, protein synthesis rates were elevated at 5 °C in the northern population (Chapter 5). This was achieved by an upregulation of the capacity for protein synthesis, as shown by other ectotherms in the cold (Foster et al., 1992; Fraser et al., 2002; Goolish et al., 1984; McCarthy et al., 1999; Storch & Pörtner, 2003; Storch et al., 2003; Treberg et al., 2005; Whiteley & Faulkner, 2005). Both adult and juvenile growth rates (Chapters 5 and 6 respectively) were fractionally higher in *G. duebeni* from Tromsø, Norway, suggesting little variation in rates of protein turnover at 10 °C. Further research is required to investigate whether higher rates of protein synthesis at 5 °C in *G. duebeni* from Tromsø are due to an increase in protein degradation rates and therefore turnover rates at low temperatures, as suggested in some polar marine

invertebrates (Place & Hofmann, 2005; Place et al., 2004). As the highest acclimation temperature considered for Tromsø *G. duebeni* was 10 °C, it is unknown how the higher latitude population would respond to higher temperatures than this. It is possible that to maximise growth in colder climates, Norwegian *G. duebeni* have downshifted the optimum temperature for growth (Yamahira & Conover, 2002), and would therefore be outperformed at higher temperatures by Welsh *G. duebeni*. As growth rates showed a typical temperature-dependent relationship with a greater increase in rate between 15 and 20 °C than at lower temperatures, it is likely that the optimum temperature for growth is higher than 10 °C in *G. duebeni* from Tromsø, despite this being within 1 °C of the mean summer temperature on the shore (Chapter 2). Although brood size was larger in *G. duebeni* from Tromsø, this may be explained by the larger body size of females collected from that location. It therefore appears that the species is characterised by a strategy of wide environmental tolerance at the cost of growth and reproduction. If however northern populations of *G. duebeni* currently inhabit thermal regimes below their optimum temperature for physiological processes such as growth, warming on the shore due to climate change may be beneficial to these populations and allow for range expansion at their northernmost limit. Bindoff et al. (2007) estimated a 0.6 °C rise in mean global sea surface temperatures since 1950, with further rises predicted along with associated warming of air temperatures in coastal areas (Parry et al., 2007).

7.1.6. Effects of season

Seasonal differences were only observed in one variable (RNA:protein levels) at one acclimation temperature (5 °C) between populations of *Gammarus duebeni* (Chapter 5). It can therefore be assumed that *G. duebeni* fully compensated for the effect of temperature after six weeks acclimation and showed little difference in response between summer and winter seasons. *G. duebeni* showed compensation of oxygen uptake rates (MO_2) and citrate synthase activity, as both rates were independent of acclimation temperature in animals held at 18 psu salinity (Chapter 4). Upper thermal tolerances, however, increased according to acclimation temperature (Chapter 3). This demonstrates that *G. duebeni* is able to increase upper critical thermal limits according to thermal experience, indicating some plasticity in the response.

Protein synthesis rates showed no correlation with oxygen uptake rates in any of the species studied. The northern *G. duebeni* population exhibited elevated protein synthesis rates but lower oxygen uptake rates to the southern population. This is the opposite to the expected result, as organisms with a high rate of protein synthesis would be expected to incur a high cost and therefore require greater energy expenditure as protein synthesis is expected to account for a substantial fraction of the energy budget (Houlihan et al., 1995). As discussed earlier, variations in the cost of protein synthesis with temperature could explain the discrepancy between rates of protein synthesis and oxygen uptake, or alternatively the partitioning of the energy budget into protein synthesis, growth, reproduction, activity levels and other lifestyle features may vary according to temperature to provide adaptive strategies to cope with temperature variation according to season and latitude (Bayne, 2004; Pörtner et al., 2005).

7.1.7. Summary

In conclusion, *G. duebeni* is able to occupy most regions on the shore thanks to a wide environmental tolerance and ability to adjust to rapid temperature change in the high intertidal, although the species prefers brackish conditions and is unable to survive prolonged periods of a combination of high temperature and high salinity. Although *G. duebeni* is broadly tolerant, it may be outcompeted if conditions are optimal for another gammarid species, as tolerance appears to occur at the cost of growth and reproductive output.

7.2. Gammarus locusta

Growth rates were significantly higher in *G. locusta* than *G. duebeni* and showed a greater increase in rate with temperature (Chapters 5 and 6). This suggests that *G. locusta* has a low rate of protein turnover and a more stable protein pool. The species also shows a greater investment in reproduction, with a larger brood size than *G. duebeni* (Chapter 6). Fast growth rates and high reproductive output are typical of a warmer-water, higher energy lifestyle. It has been proposed that warm-water lifestyles may have a limiting effect on maximum body size and life span due to constraints resulting from high energy demands (Atanasov, 2005; Sukhotin et al., 2006; Van Voorhies, 2002) and the reduction in

oxygen solubility with temperature (Chapelle & Peck, 1995), however the latter remains a controversial hypothesis. At 15 °C *G. locusta* exhibits lower rates of oxygen uptake than *G. duebeni* and at 10 °C there is no difference, suggesting variation between the species in energy partitioning rather than energy uptake (Chapter 4). The r-selected life history features of fast growth and high reproductive potential suggests that at 10 and 15 °C *G. locusta* would outperform *G. duebeni* given stable conditions.

Although *G. locusta* has a more southerly geographic range in comparison to *G. duebeni*, it had a lower upper thermal tolerance (Chapter 3). As upper thermal tolerance increased with acclimation temperature as in *G. duebeni*, a latitudinal trend in upper thermal limits likely exists for this species too. *G. locusta* showed a greater acclimatory capacity of upper thermal tolerance than *G. duebeni*, and would therefore be expected to show a higher plasticity between populations despite a potentially narrower thermal window. Thermal tolerance may therefore be more closely linked to shore height and geographical range than acclimatory ability, with the high shore species *G. duebeni* better able to tolerate fluctuations in temperature than the low shore species *G. locusta*. It has been suggested that acclimatory ability has little influence on range size in some species (Calosi et al. 2008), yet Stillman (2003) proposed that acclimatory capacity is a determining factor and that a trade-off occurs between upper thermal limits and the ability to adjust said limits. Further research in this area is required to expand the number of species considered to see if there is a negative trend between the size and plasticity of the thermal window and to determine whether acclimatory capacity or upper thermal tolerance has the greater influence on range size and susceptibility to climate change in gammarid amphipods.

As with *G. duebeni*, oxygen limitation may be the determinant of thermal tolerance in *G. locusta*. The Q_{10} value for the response of oxygen uptake rates to an acute increase in temperature was similar to *G. duebeni* at a common acclimation salinity (Chapter 4), however *G. locusta* is considered a marine species as opposed to the brackish water preference of *G. duebeni* (Gaston & Spicer, 2001). *G. duebeni* acclimated to brackish conditions had a Q_{10} value approximately half of the value for *G. locusta* in full strength seawater. When both species coexist on the same shore, *G. locusta* tends to dominate the lower shore while *G. duebeni* occupies a higher shore niche, usually at an area with a freshwater influence (Sutcliffe, 1970). This was the situation at collection sites in Anglesey, Wales; *G. locusta* was found in a tide pool close to the low tide mark in water of 32 psu

salinity, while *G. duebeni* was discovered at the site of a freshwater input near the high tide line, which although submerged at high tide at low tide had a salinity of 0 psu (Chapter 2).

Despite the ability of *G. locusta* to show plasticity in upper thermal limits according to acclimation temperature, the same species showed a greater temperature sensitivity to acute temperature change for rates of oxygen uptake and a lower upper critical limit than *G. duebeni*. Resting rates of oxygen uptake were elevated in *G. locusta* at 5 °C, suggesting overcompensation at this low temperature. Although *G. locusta* possesses acclimatory ability, it appears to require more time to adjust to change. The relatively high growth rates in *G. locusta* (Chapters 5 and 6) suggest low rates of protein turnover and a more stable protein pool, a possible explanation for the slower response to change than seen in *G. duebeni* and is therefore less able to cope with fluctuating temperatures (Koehn & Bayne, 1989).

The over-compensation of oxygen uptake rate in *G. locusta* at 5 °C appears to be an attempt to compensate for an increase in protein degradation in the cold as evidenced by the low growth rates of both adults and juveniles (Chapters 5 and 6 respectively). This strategy is unsustainable in the long term, as evidenced by the low rates of oxygen uptake and protein synthesis that characterise polar marine ectotherms (Pörtner et al., 2005). Although the Baltic clam *Macoma balthica* has been shown to survive translocation to warmer temperatures, individuals failed to acclimate and showed an elevated metabolic rate and gradual loss of fitness (Hummel et al., 2000); *G. locusta* would be expected to show a similar loss in fitness if held at 5 °C for a more prolonged period of time. *G. duebeni* is highly successful in the UK and although *G. locusta* is able to out-compete *G. duebeni* given stable, warmer conditions, *G. duebeni* has a broader tolerance and is better adapted to the fluctuating conditions of the high intertidal and to colder temperatures. The two species therefore differ in geographical range size, latitudinal distribution and shore height, and populations do not overlap on the shore (Kolding & Fenchel, 1979). Given short term adverse environmental conditions it would therefore be expected that *G. duebeni* would be better able to compensate, however if long term warming took place *G. locusta* could be the more successful of the two Welsh populations. If summer temperatures show a greater increase than winter temperatures, as predicted by Parry et al. (2007) in western Europe, *G. locusta* may not be as benefitted by climate change as it would be by an increase in minimum temperatures in winter.

7.3. Gammarus oceanicus

This study has shown *G. oceanicus* to be broadly similar to *G. duebeni* from the same latitude in terms of upper thermal tolerance (Chapter 3), protein synthesis rates (Chapter 5) and the thermal sensitivity of oxygen uptake rates during acute temperature change (Chapter 4). This suggests the two species have similar acclimatory capacities and tolerance to rapid temperature change. Growth rates and brood size in *G. oceanicus* were, however, significantly greater (Chapter 6). This points to a lower rate of protein turnover at 10 °C (McCarthy et al., 1999), the only acclimation temperature measured. This temperature is relatively high for Tromsø, equivalent to the mean summer temperature on the shore. It would be interesting to research protein turnover rates in this species, as well as expanding this study to a range of temperatures.

Unlike *G. duebeni*, *G. oceanicus* is thought to have a narrow salinity tolerance with a preference for salinity equivalent to full-strength seawater (Steele & Steele, 1972). Amphipods collected in 2010 showed a high susceptibility to temperature variation, with 80 % mortality in *G. oceanicus* from Tromsø (70 °N) and Ny-Ålesund (79 °N) within the weeks following a cold room fault which caused a rapid 7 °C increase in temperature. Thermal tolerance was higher in acclimatised *G. oceanicus* from Scotland (58 °N) than Svalbard (79 °N), suggesting an ability to adjust upper thermal limits, but although acclimated *G. oceanicus* and *G. duebeni* from Tromsø had similar upper thermal limits the mortality rate following the temperature fault was far higher in *G. oceanicus* than *G. duebeni*. This study hints at a lower rate of protein turnover in *G. oceanicus* given the species faster rate of growth relative to *G. duebeni*. This faster growth rate and the mortality increase following the temperature fault may indicate a lack of ability to respond to and recover from rapid change (Koehn & Bayne, 1989). *G. oceanicus* is already showing evidence of an increase in abundance in response to warming. In Svalbard, the relative abundance of *G. oceanicus* and *G. setosus* has altered over the last 20 years, as *G. oceanicus* has become the dominant of the two species and the distribution of *G. setosus* has shifted to lower salinity, cooler areas at the foot of the glaciers (Węsławski et al., 2010). *G. oceanicus* appears to be likely to continue to out-compete *G. duebeni* at latitudes inhabited by both species provided climate change results in gradual warming as has been observed in Svalbard, however if conditions become more instable, with greater fluctuations in temperature in terms of rapidity and magnitude, *G. oceanicus* may not be as tolerant as *G. duebeni* to the change in climate.

G. oceanicus and *G. locusta* were once considered the same species (Stock, 1967), have overlapping geographical ranges and occupy the same low shore niche but belong to separate putative phylogroups; *G. oceanicus* and *G. duebeni* are members of the temperate western European phylogroup, while *G. locusta* is grouped with warmer water Mediterranean and Black Sea gammarids (Costa et al., 2009). It appears that of the two species *G. oceanicus* has a broader thermal window but at a lower optimal temperature than *G. locusta*. If climate change resulted in a southerly range shift in *G. oceanicus* it would be interesting to see if a greater interaction between the two species resulted in a shift in dominance in favour of *G. oceanicus* in areas previously occupied by *G. locusta*, as is occurring in Svalbard with *G. setosus*.

7.4. Summary

This study shows interesting differences within confamilial species of gammarid amphipods, with a range of physiological and ecological responses to changes in temperature. *G. duebeni* appears to exhibit a broad thermal tolerance and high acclimatory ability at the cost of reduced growth and reproductive output and K-selected life history traits. *G. locusta* seems to have an alternative strategy of a narrower tolerance and reduced ability to adjust to rapid temperature change, but given stable conditions within its thermal window and at higher salinities, is able to out-perform *G. duebeni*. Despite K-selected life history traits in *G. duebeni* a broad tolerance, and therefore possibly reduced mortality in response to extreme or fluctuating conditions, may have an equal or greater importance in determining the success of a population. Detailed demographic data, especially with relation to age-specific mortality, may give a better estimate of the ability of a population to expand and recover and hence its susceptibility to climate change. Variations in juvenile growth rate between species according to acclimation temperature were observed, as growth rates increased with temperature in all species but were slower across temperatures in *G. duebeni*, however this study failed to do the same for brood size. The small brood size in *G. duebeni* may be an indication of a smaller available energy budget for reproduction, but could also prove to be a plastic response. *G. oceanicus* proved to be broadly similar to *G. duebeni* in terms of upper thermal tolerances and rates of oxygen uptake and protein synthesis suggesting similar tolerances to rapid temperature change, however *G. oceanicus* exhibited the r-selected life history traits of faster juvenile growth and larger brood sizes.

7.5. Future directions

Limitations on facilities meant 5 °C was the lowest acclimation temperature used in this study, however as varying responses to low temperatures were seen intraspecifically in *G. duebeni* and interspecifically between *G. duebeni* and *G. locusta* further work should concentrate on responses to lower temperatures. Temperatures fell below 5 °C in January and February in Rhoscolyn and in all but two months in Tromsø. A study of *Drosophila spp.* found interspecific differences in cold resistance showed greater variation than differences in heat resistance (Hoffmann et al., 2003), and this study appears to suggest compensatory ability in the cold may be variable among gammarids. Expanding research on the size and plasticity of the thermal window to include cold tolerance and a larger number of species and populations from varying latitudes may help deduce which has the greater influence over range size and susceptibility to climate change in gammarid amphipods. The effect of the rapidity of warming during thermal tolerance experiments was touched upon in Chapter 3, however further work is required into the underlying physiology involved in determining upper thermal limits, for example the accumulation of metabolic end products, especially from anaerobic respiration, and expression of heat shock proteins during exposure to varying magnitudes and rates of temperature change.

Although interesting differences appear to exist between rates of protein turnover and growth efficiencies in the three species, further research is required to more fully explain the role of protein turnover in determining growth rates and acclimatory capacities in gammarids, especially as temperature and thermal experience varies. The observed discrepancy between rates of protein synthesis (Chapter 5) and oxygen uptake (Chapter 4) requires further investigation by the direct determination of both variables in the presence of protein synthesis inhibitors. Future work could expand on current measurements such as those on growth and reproduction (Chapters 5 and 6) as a means of examining partitioning of the energy budget, or could focus on investigating the cost of protein synthesis in gammarid amphipods with different thermal histories and experience. Acclimation to fluctuating temperatures which better represent conditions on the shore may provide an insight into whether the low protein synthesis rates in *G. duebeni* are a result of an ability to fully compensate for the changes, or represent a depression of protein synthesis rates to reduce energy expenditure on the shore.

The two salinity regimes *G. duebeni* was exposed to provided interesting results in terms of the complex interacting effects of temperature and salinity on rates of oxygen uptake

(Chapter 4) and protein synthesis (Chapter 5). This study would benefit from expansion to include a larger range of salinities, including exposure to freshwater conditions.

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