THE OSMOREGULATION OF SELECTED GAMMARID AMPHIPODS

by

STEVEN JOHN BROOKS

A thesis submitted in partial fulfilment of the requirements of the Nottingham Trent University for the Degree of Doctor of Philosophy

May 2003

NOTTINGHAM TRENT UNIVERSITY
DEPARTMENT OF LIFE SCIENCE
Doctor of Philosophy
THE OSMOREGULATION OF SELECTED GAMMARID AMPHIPODS
by Steven John Brooks

ABSTRACT

The osmoregulation of gammarid amphipods from fresh, brackish and marine environments are investigated. Osmoregulation is determined from measurements of haemolymph ion concentration, half time of body water exchange ($t_{1/2}$), sodium flux and gill Na^+, K^+-ATPase, with respect to salinity acclimation. The effect of rapid salinity change on $t_{1/2}$ and sodium influx in some gammarids is also investigated. Gill Na^+, K^+-ATPase is characterised for the main ion and co-factor requirements in *G. pulex*. This transport enzyme is actively involved in the osmoregulation of all gammarids investigated, with enzyme activity strongly influenced by the salinity of the external medium.

The observed differences in the osmoregulatory physiology of gammarids are related to the adaptation of species to the ionic concentration of their natural habitat. The ability of freshwater adapted gammarids to maintain haemolymph ion concentrations significantly lower than more brackish water species provides them with an energetic advantage in dilute media. These differences in osmoregulation are suggested to influence competitive interactions between gammarids from Northern Ireland and The Netherlands.

Significant differences in $t_{1/2}$ and sodium flux are found between the freshwater adapted *G. duebeni celticus* and the more brackish water form *G. duebeni duebeni*. Such differences are suggested to be due to the evolutionary adaptation of these isolated subspecies to their contrasting natural habitats. Furthermore, the effects of a number of stressors on the osmoregulatory physiology of the freshwater amphipod *G. pulex* are investigated. These stressors include: *in vivo* and *in vitro* copper exposure, parasite infection, and low energy cave environments. Significant effects of all these stressors on the osmoregulation of *G. pulex* are found and discussed in relation to the mechanisms of effect.

DECLARATION

This work has not been accepted in substance for any other degree and is not being submitted in candidature for any other degree.

This is to certify that the work here was carried out by the candidate himself. Due acknowledgment is made for all the assistance received.

Signed .....
(Candidate)

Signed .....
(Director of Studies)

ACKNOWLEDGMENTS

Special thanks to my supervisor Chris Lloyd Mills who has provided me with great assistance during my PhD, with regular meetings and informal chats, as well as his invaluable comments on the thesis. To my second supervisor Neil MacFarlane for his helpful suggestions in the production of the thesis.

Thanks to Jamie Dick and his research group from Queens University, Belfast and to Dirk Platvoet from Amsterdam University for their assistance with fieldwork sampling in Northern Ireland and The Netherlands respectively. Without their co-operation the animal collections from these countries would not have been possible. Also, thanks to Paul Wood from Loughborough University for collection of the cave populations and information on the Peak cave system.

To my laboratory colleagues for providing great support and friendship throughout my PhD. To my girlfriend Lucy, for her love and support, and for finding the time to proof read the thesis.

Finally, thanks to Nottingham Trent University for providing me with a bursary to carry out the research.

PUBLICATIONS

Papers

Brooks, S. J. & Lloyd Mills, C. 2003. The effects of copper on osmoregulation in the freshwater amphipod *Gammarus pulex*. *Comparative Biochemistry and Physiology*. (Accepted).

Abstracts

Brooks, S. J., Foster, J. R., and Lloyd Mills, C. 2002. The effects of copper on heart rate, pleopod beat and osmoregulation in the freshwater amphipod *Gammarus pulex*. *Comparative Biochemistry and Physiology*. Vol. **132A**, supplement 1, p. 7.

Lloyd Mills, C. & Brooks, S. J. 2002. Variation in osmoregulation in isolated populations of the amphipod *Gammarus duebeni*. Differences in sodium fluxes and water permeability. *Comparative Biochemistry and Physiology*. Vol. **132A**, supplement 1, p. 55.

Lloyd Mills, C. & Brooks, S. J. 2001. Differences in the energetic cost of osmoregulation in the amphipods *Gammarus pulex* and *Gammarus tigrinus*. 2nd Symposium for European Freshwater Sciences (Toulouse, France).

Brooks, S. J. & Lloyd Mills, C. 2001. Characterisation of gill Na^+, K^+-ATPase in the freshwater amphipod *Gammarus pulex*. The effects of salinity and comparisons with the more euryhaline amphipod *Gammarus tigrinus*. (Society for Experimental Biology, Canterbury).

Brooks, S. J. & Lloyd Mills, C. 2001. The influence of acanthocephalan parasite infection on osmoregulation in *Gammarus pulex*. (British Ecological Society Meeting: Animal Physiological & Behavioural Ecology, University of Warwick 18-20 Dec 2001).

TABLE OF CONTENTS

CHAPTER ONE

General Introduction

1. 1. Gammaridae

The crustacean sub-order Gammaridae comprises over 4500 species, approximately 85% of the order Amphipoda (Bousfield, 1973). In contrast to the three other amphipod sub-orders (the Hyperiidae, Ingolfiellidea and Caprellidea), which are highly specialised and ecologically restricted, the Gammaridae are widespread throughout a range of marine, fresh water and terrestrial habitats (Karaman & Pinkster, 1987). Most if not all gammarids have restricted distributions in relation to salinity and even in continuous water bodies such as estuaries there are distinct zonations between the species. Gammarids are particularly susceptible to fluctuations in the environment, due to their small size (generally < 100mg wet weight) and correspondingly large surface area to volume ratio. This characteristic, combined with the observation that large numbers of related species occupy a wide range of salinities makes gammarids an excellent group for investigations into osmotic and ionic regulation. This can give an insight into how physiological tolerance and adaptation can account for the present distribution of members of the family in Western Europe.

1. 2. Crustacean gills

The body surfaces of crustaceans are mostly covered by a thick, hydrophobic cuticle, which restricts the exchange of gases and electrolytes across the body surface. In order for gaseous exchange to occur at a sufficient rate, thin specialised structures such as the gills are necessary. Consequently, the gills of crustaceans act as a selective interface capable of controlling the exchange interactions in relation to the external environment. In addition to their role in gaseous exchange, the gills have been well documented to be the principal sites of ion exchange, contributing to acid-base balance, ion-regulation and ammonia excretion (reviewed in Péqueux, 1995).

Crustacean gills are lined by a single layer of epithelial cells whose basement membrane at the serosal side is directly bathed by the haemolymph. The mucosal side is covered by a chitinous cuticle and faces the external medium. The thickness of the epithelium varies, depending on its physiological and biochemical function (Dunel-Erb *et al.*, 1997). Thinner regions of the epithelium (Type I epithelium, 1-5μm) are predominantly associated with gaseous exchange and passive diffusion of ions, whereas thicker regions (Type II epithelium, 10-20μm) are mainly concerned with ion transport mechanisms, such as sodium, potassium-adenosine triphosphatase (Na^+, K^+-ATPase) (Luquet *et al.* 2000; Haond *et al.* 1998).

The distribution of these two types of epithelia in crustacean gills differs markedly. In most hyper-regulating decapod crustaceans, the anterior gills are commonly lined with type I epithelia and solely perform a respiratory function. The posterior gills contain type II epithelia, as typified by the shore crab *Carcinus maenas* (Compère *et al.,* 1989), the Chinese crab *Eriocheir sinensis* (Barra *et al.*, 1983), the mangrove crab *Ucides cordatus* (Martinez *et al.* 1999) and the estuarine crab *Chasmagnathus granulatus* (Genovese *et al.*, 2000; Luquet *et al.*, 2000). In freshwater crustaceans, it is claimed that all gills are involved in ion-transport (Morris, 2001), as in the freshwater branchiopod *Caenestheriella gifuensis*, (Kikuchi & Shiraishi, 1997). However, all the gills of the brackish euryhaline amphipod *Gammarus duebeni* have also been found to be involved in ion regulation from silver staining experiments (Lloyd Mills personal observation). Therefore, this phenomenon is not restricted solely to freshwater species.

In hyper-regulating crustaceans the gill cuticle is often over a thousand times more permeable than that of the body surface (Lignon & Péqueux, 1990). The permeability of the gill cuticle varies between crustacean species due to the differences in their osmotic and ionic capabilities, being much higher in marine osmoconformers than more freshwater

hyper-regulators (Lignon & Péqueux, 1990). The reduced cuticular permeability in hyper-regulators has been suggested to reduce diffusional ion leaks when in dilute media (Péqueux, 1994). In addition, different parts of the same gill cuticle have been found to show selective ion permeabilities. For example, in the gills of the crayfish *Astacus leptodactylus*, the lamina cuticle was selectively permeable to Cl^- and OH^-, whereas, the filament cuticle was selectively permeable to Na^+ (Dunel-Erb *et al.*, 1997; Barradas *et al.*, 1999). The permeability of the cuticle can therefore be important in influencing the ionic and osmotic regulation in crustaceans, and acts as a complementary structure to the epithelium.

In gammarids, coxal gills (branchiae) are thin flattened oval plates called lamellae, formed from the epipodites of the limbs (pereopods) of the thoracic segments (Sutcliffe, 1984). Most gammarids possess six pairs of gills, each suspended by a short stalk on the inside base of the pereopod (see figure 1. 1.).

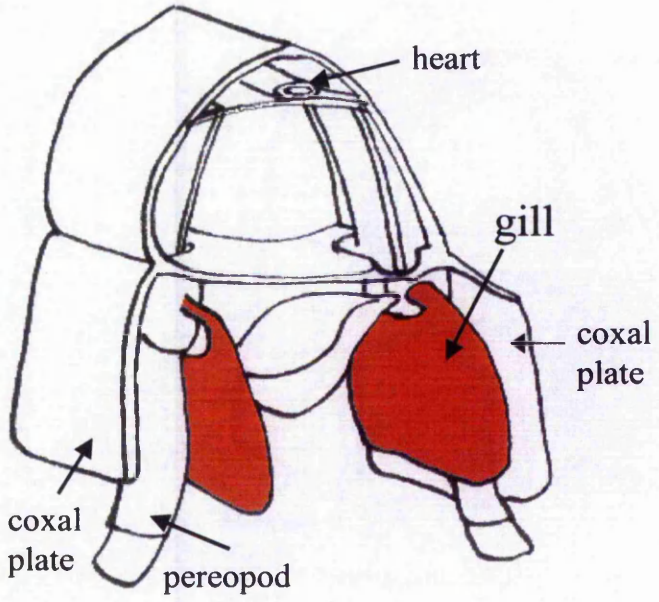


Figure 1. 1. Diagrammatic cross section through the body of a gammarid amphipod, gills highlighted. Adapted from Barnes, 1987.

The osmotic gradient between the internal body fluids and the external medium typically imposes a physiological stress on the structural integrity of gill epithelium. The movement of water following net ion fluxes will tend to change cell volume. This in turn may distance the junctions between cells potentially altering the permeability of the paracellular pathway. Well developed bundles of aggregated microtubules together with microfibrils have been observed to run in a basoapical direction in gammarids, and may serve to stabilise the gill structure against hydrostatic pressure (Kikuchi & Shiraishi, 1997; Shires *et al.*, 1994; 1995).

Shires *et al.* (1995) compared the microtubule systems associated with the septate junctions in the gills of four gammarid amphipods: *Chaetogammarus marinus*, *Gammarus locusta*, *G. pulex* and *G. duebeni*. The development of the septate junctions and the associated 'junctional' microtubules were correlated to the degree of osmotic stress experienced by the species. The least developed microtubule systems were found in the marine stenohaline species *C. marinus* and *G. locusta*, which are exposed to a relatively small degree of osmotic stress in their natural environment. A well ordered microtubule system was observed in the freshwater *G. pulex.* A higher degree of development was observed in the extremely euryhaline *G. duebeni duebeni*, with the most complex form reserved for the freshwater subspecies *G. duebeni celticus* when acclimated to full strength sea water. The euryhaline *G. d. duebeni* is isosmotic in 100% sea water.

In contrast, the freshwater form *G. d. celticus* is markedly hyperosmotic (this report, Chapters 4. 2 & 5). It was concluded that the 'junctional' microtubules played an important role in the mechanical buttressing of the septate junctions against disruption by osmotic stress (Shires *et al.*, 1995). It is likely that microtubule systems function in a similar way in other gammarids.

1. 3. Models of NaCl absorption in aquatic Crustacea

Sodium and chloride are the two principal ions responsible for over 90% of the ionic concentration in aquatic systems. Therefore, the regulation of these two ions is a functional necessity in aquatic Crustacea. The gills have been identified as the principal organs involved in ionic regulation (reviewed by Péqueux, 1995). The mechanisms of sodium and chloride transport in the gills of Crustacea have been shown to differ depending on the concentrations of these ions in the animals' natural habitat. Two models of NaCl uptake have been developed from fresh water and marine environments. However, these models are far from complete and there have been recent developments, which have raised doubts about the methods employed.

Marine Crustacea inhabit waters with relatively high and stable ion concentrations and typically maintain their haemolymph ion concentrations isosmotic with sea water. They are

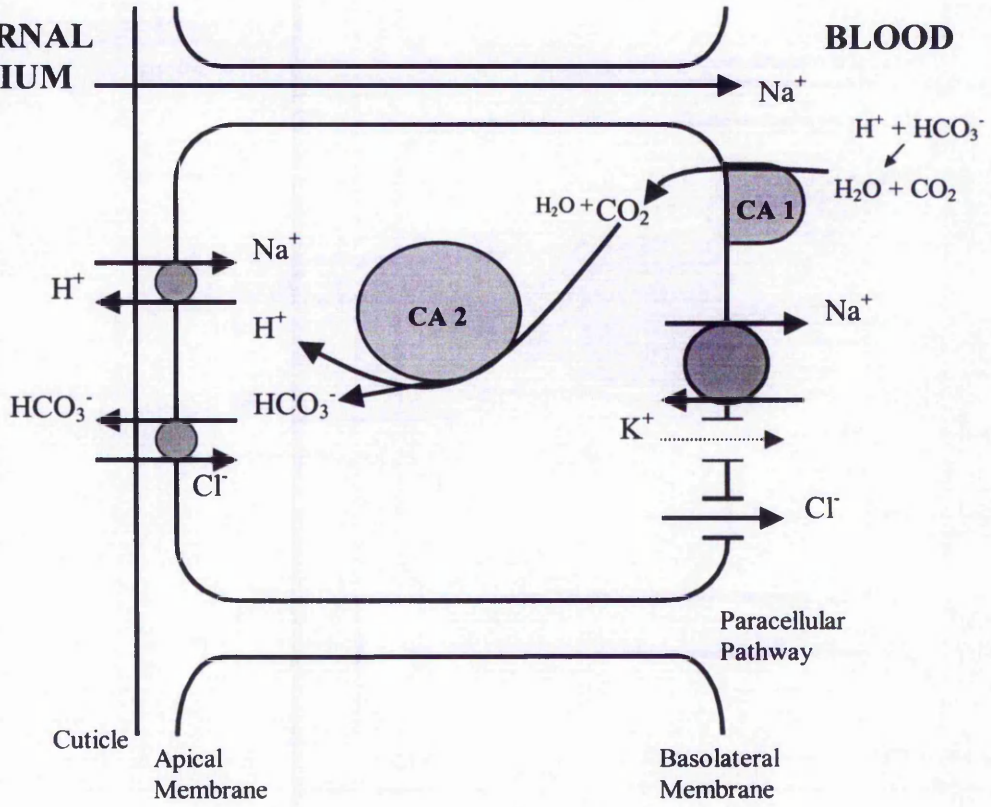


Figure 1. 2. Model of NaCl uptake across the gill epithelia of marine crustaceans (adapted from Onken & Riestenpatt, 1998)

strictly osmoconformers; movement of ions and water are mainly through passive forces and they do not require great powers of active ion regulation in order to maintain constant internal ion concentrations. However, some marine species have been able to invade estuarine waters of fluctuating salinities by actively hyper-regulating their internal ion concentrations. In hyper-regulating marine Crustacea, sodium and chloride uptake across the apical membrane of transporting epithelia is thought to be *via* the Na^+/ H^+ and Cl^-/ $HCO3^-$ antiporters (Fig. 1. 2.).

The inward movement of sodium and chloride is driven by Na^+, K^+-ATPase located on the basolateral membrane, which pumps sodium ions out from the cytoplasm into the haemolymph, thus keeping internal sodium concentrations low and maintaining an inward sodium gradient. A basolateral K^+ channel allows potassium to leak out of the cell, thereby recycling the potassium counterion for Na^+, K^+-ATPase. Involved in the process are two populations of carbonic anhydrase (CA). The first CA population is located on the basolateral membrane, which rapidly dehydrates HCO_3^- to CO_2. The gas diffuses through the basolateral membrane into the cytoplasm, where the second population of CA rehydrates CO_2 to form H^+ and HCO_3^-. Consequently, the counterions H^+ and HCO_3^- are provided for the apical Na^+ and Cl^- antiporters respectively. CO_2 is removed from the animal as HCO_3^-. (The role of CA in osmoregulation has been reviewed recently, Henry, 1998). The movement of chloride from the cytoplasm to the haemolymph proceeds *via* basolateral Cl^- channels.

Further insight into sodium and chloride transport has been achieved in recent years due to the development of new techniques, such as the mounting of split gill lamallae in modified Ussing chambers, which has allowed sodium and chloride transport to be measured as short-circuit current (reviewed in Onken & Riestenpatt 1998). From this technique

employing known inhibitors of sodium and chloride transporters in combination, a model for ion uptake in fresh water adapted crustaceans has been developed (Fig. 1. 3).

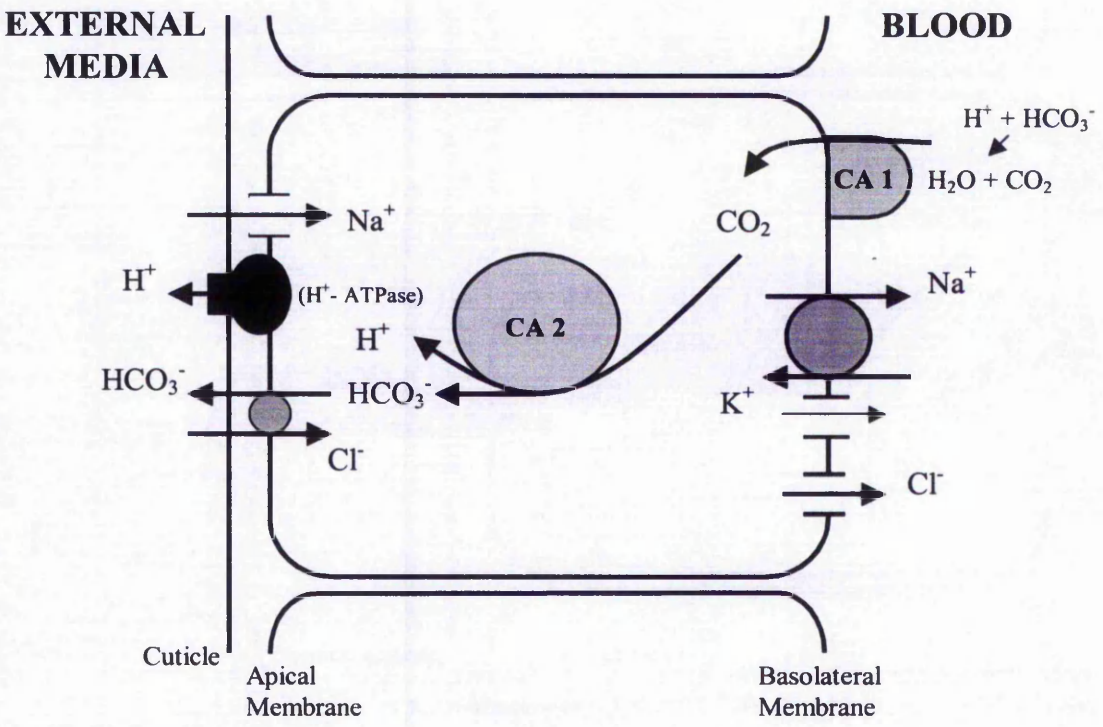


Figure 1. 3. Model of NaCl uptake across the gill epithelia of freshwater Crustacea (adapted from Onken & Riestenpatt, 1998).

The posterior gills of the Chinese mitten crab *Eriocheir sinensis* have been used extensively to demonstrate NaCl uptake in freshwater Crustacea (Onken, 1999; Riestenpatt *et al.*, 1996; Onken & Putzenlechner, 1996; Riestenpatt *et al.*, 1994). In *E. sinensis* sodium and chloride are absorbed independently of each other (Fig. 1. 3.). In fresh water, external sodium concentrations are significantly less than that of the gill epithelia cells; therefore the simple exchange of H^+ for Na^+ is unlikely to drive sodium inward. An apical V-type H^+-ATPase actively pumps H^+ ions outwards from the cytoplasm into the external medium, which creates an electrochemical gradient to drive Na^+ movement across the apical membrane *via* Na^+ channels. The basolateral Na^+, K^+-ATPase pumps sodium ions out of the cytoplasm into the haemolymph, maintaining low cellular sodium concentrations, thus contributing to the maintenance of the electrochemical gradient for the inward movement of sodium.

The apical V-type H^+-ATPase is also involved in driving the uptake of chloride in the posterior gills of *E. sinensis* (Onken, 1999; Riestenpatt *et al.*, 1996; Onken & Putzenlechner, 1996; Riestenpatt *et al.*, 1994). Uptake of chloride involves the apical Cl^-/HCO_3^- antiporter and basolateral Cl^- channels. The apical V-type H^+-ATPase aids the inward movement of chloride across both the apical and basolateral membranes. Firstly by energising the apical antiporter encouraging outward movement of bicarbonate and thus inward movement of chloride across the apical membrane, and secondly, through the hyper-polarisation of the cellular potential, which drives chloride against the concentration gradient from the cytoplasm to the haemolymph *via* Cl^- channels across the basolateral membrane (Onken & Riestenpatt, 1998).

In contrast to marine crustaceans, freshwater species such as the Chinese crab *E. sinensis* (Onken & Riestenpatt, 1998) and *D. pagei* (Onken & McNamara, 2002) have tight epithelia. The tightness of the gill epithelium relates to the restriction of paracellular pathways, which serves to minimise passive salt loss. The reduction in passive salt loss is of particular importance in freshwater species such as *E. sinensis*, which maintain large osmotic gradients between the external media and the internal body fluids.

A major point of contention in the uptake of sodium and chloride in the posterior gills of the Chinese crab *E. sinensis*, and in freshwater Crustacea in general, is whether or not sodium and chloride ions are absorbed independently or coupled. From studies using tracer fluxes or transepithelial short circuit currents (reviewed Onken & Riestenpatt, 1998) evidence for both sodium and chloride independent uptake has been reported. However, more recently by direct ion determination, no net uptake of sodium or chloride was achieved in the posterior gills of *E. sinensis* in the absence of the respective counter ion, or when uptake of one ion was blocked by a specific inhibitor (Rathmayer & Siebers, 2001). The contradictory evidence for the uptake kinetics of sodium and chloride raises doubt on

the reliability of transepithelial potentials in determining NaCl transport mechanisms. Rathmayer and Siebers (2001) have claimed that the net uptake of NaCl is independent of transepithelial potential and measurements using such techniques in determining mechanisms of NaCl transport are inappropriate. Further work is therefore needed to investigate the validity of tracer fluxes and transepithelial short circuit currents.

In addition to the fresh and sea water models, studies on split gill lamallae of posterior *C. maenas* gills, revealed active NaCl absorption to be electrogenic and proceed in a coupled mode with a stoichiometry of 2 Cl^- ions per 1 Na^+ ion (Riestenpatt *et al.* 1996; Onken & Riestenpatt, 2002). It was also found that Cl^- transporters in the apical membrane required sodium and potassium ions. This led to the suggestion that the passage of sodium and chloride across the apical membrane proceeded *via* a Na^+/ K^+/ $2Cl^-$ symporter (Riestenpatt *et al.* 1996).

Contrary to the studies on split gill lamellae described above, Towle *et al.*, (1997), have identified and sequenced the cDNA coding for a crustacean Na^+/ H^+ antiporter, starting with mRNA isolated from the gills of *C. maenas*. The absence of a Na^+/ H^+ antiporter from split gill lamallae preparations using the combination of inhibitors and absence of ions from the external medium, casts doubt on the effectiveness of this technique in identifying the mechanisms involved in ion regulation. As yet a gene coding for the apical Na^+/ K^+/ $2Cl^-$ symporter in gill epithelia of *C. maenas* has not been investigated. Until the presence of apical Na^+/ K^+/ $2Cl^-$ symporter has been confirmed, its role in NaCl transporter in *C. maenas* remains in question.

1. 4. Antennal glands

In addition to the gills, the antennal glands are believed to be involved in osmotic/ ionic regulation in crustaceans (Lin *et al.*, 2000). The antennal glands are the renal organs in

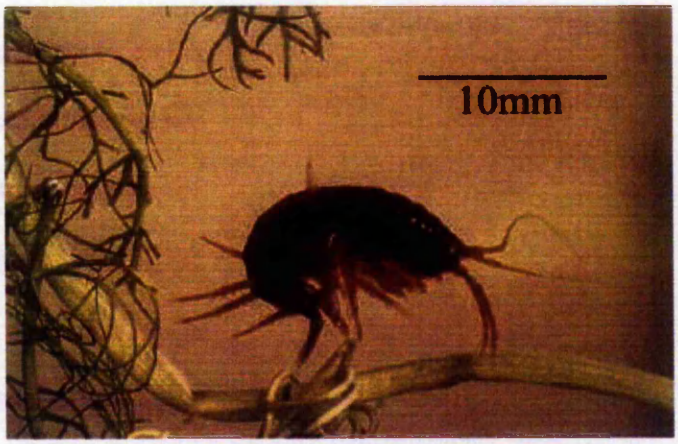
crustaceans. Located in the head, they excrete urine through an opening near the base of the second antennae (Potts & Parry, 1964). The antennal glands of crustaceans have been implicated in the reabsorption of sodium from the urine through the action of Na^+, K^+-ATPase (Sarver *et al.*, 1994; Horiuchi, 1980). Through the reclamation of sodium ions from the urine by Na^+, K^+-ATPase, animals are able to produce hypo-osmotic urine in relation to the external medium. This ability to produce hypo-osmotic urine enables hyper-regulating crustaceans to maintain haemolymph ion concentrations markedly hyper-ionic to the external medium. The production of hypo-osmotic urine has been found in the amphipods *Gammarus pulex* and the euryhaline *G. duebeni duebeni* (Lockwood, 1961). Although the antennary glands have not been investigated in this study, their involvement in the osmotic/ ionic regulation of the selected gammarid species has been considered.

1. 5. Experimental animals

The gammarid species used in this thesis were collected from fresh, brackish and sea water habitats. Animals were selected due to their differences in salinity tolerance and osmoregulatory capabilities.

Gammarus pulex

The freshwater amphipod *G. pulex* is a stenohaline species that occurs widely in the inland waters of Northern Europe (Sutcliffe, 1967b). Although *G. pulex* is normally confined to fresh water, it was found living at 25 salinity in the inland salt waters of Westphalia (Thienemann, 1913).



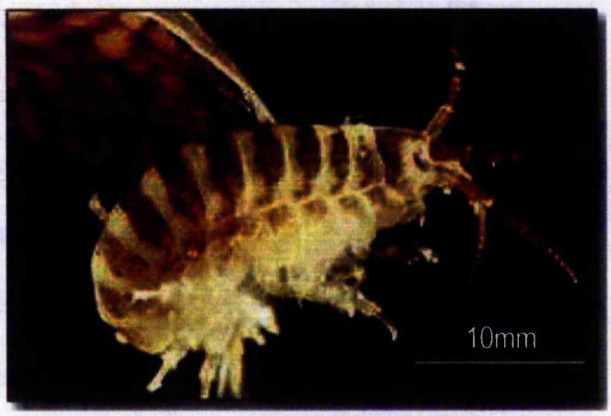
Source: www.medinavalleycentre.org.uk/images/wpe3.jpg

Figure 1. 5. 1. Photograph of the freshwater amphipod *G. pulex*.

Furthermore Sexton (1928) reported acclimation of *G. pulex* to undiluted sea water by gradually increasing the external concentration over a period of weeks. However, *G. pulex* is mostly confined to fresh waters and often dies on exposure to media more concentrated than about one-third sea water for reasons that are not fully understood (Sutcliffe, 1967b).

Dikerogammarus villosus

Dikerogammarus villosus has been recently characterised as a stenohaline amphipod (van der Velde *et al.*, 2000). In the natural environment *D. villosus* is found in salinities ranging from fresh water to 10 salinity (Jazdzewski, 1980) although can tolerate up to 20 salinity through gradual acclimation (Brujis *et al.*, 2001). It is native to the Ponto-Caspian region, although through the aid of human intervention has invaded most of Western Europe displacing many of the native gammarid species (Dick & Platvoet, 2001).



www.appliedvegetationdynamics.co.uk/IAAPwebsite/Freshwatanimsppintro.asp?ID=14

Figure 1. 5. 2. Photograph of the invasive amphipod *Dikerogammarus villosus*.

Gammarus tigrinus

Gammarus tigrinus is a euryhaline species predominantly found in brackish waters as well as fresh waters in which the ion content has risen due to pollution (Bulnheim, 1985). It can survive in waters varying in salinity from fresh to above sea water concentrations

www.hlug.de/.../gewaesserguete/bericht/alt/image69.jpg

Figure 1. 5. 3. Photograph of the euryhaline amphipod *G. tigrinus*.

(Pinkster *et al.*, 1977), although they have been found to prefer oligohaline waters around 5 to 15 salinity (Dorgelo, 1974). Originally native to the euryhaline coastal waters of Northern America (Bousfield, 1958; Reynolds, 1995), *G. tigrinus* has been introduced to many European waters, such as the German rivers Weser and Werra (Koop & Grieshaber, 2000) and the majority of the waterways of The Netherlands (Dick & Platvoet, 1996).

Gammarus zaddachi

Gammarus zaddachi is a euryhaline amphipod that often inhabits brackish waters of North Western Europe (Hough & Naylor, 1992). It can survive waters from fresh water to full strength sea water, although in its natural habitat is often found in oligohaline waters (Dennert *et al.*, 1969).

Gammarus duebeni

Gammarus duebeni is an extremely euryhaline amphipod that can tolerate salinities from fresh water to more than full strength sea water. They often inhabit brackish water environments of constant and/ or fluctuating salinities (Sheader, 1983). They are also found in fresh waters (Hynes, 1954) as well as the extremely hypersaline rock pools of Sweden, up to 73 salinity (Forsman, 1951). The subspecies *G. duebeni celticus* has been applied to the highly adapted freshwater form from Northern Ireland.

galleon.hispavista.com/craira/gammarus21.jpg

Figure 1. 5. 4. Photograph of *G. duebeni* in a precopula mating pair (female beneath male).

Gammarus locusta & Chaetogammarus marinus

The marine amphipods used in this thesis were *G. locusta* and *Chaetogammarus marinus*. Although they can both tolerate dilute sea water, they are more stenohaline than the brackish water species and are often restricted to marine intertidal habitats (Bolt, 1983).

livingthings.narod.ru/Clt/Ani/Art/Cru/Pcr/pcr001.jpg

Figure 1. 5. 5. Photograph of the marine amphipod *G. locusta*.

1. 6. General aims

The main aim of this thesis is to highlight the importance of the animals' natural environment in influencing the osmoregulatory mechanisms and geographical distribution of gammarid amphipods. Particular attention is given to the osmoregulatory enzyme Na^+, K^+-ATPase. This enzyme is important in hyper regulating crustaceans, with activity levels dependent on the salinity of the external environment. The activity of Na^+, K^+-ATPase in the gills of gammarid amphipods is determined with respect to the environmental salinity. The importance of this enzyme in the gills of a variety of gammarids from fresh, brackish and sea water is determined.

The invasive amphipod *D. villosus* has been found to displace the amphipods *G. d. duebeni* and *G. tigrinus* but not *G. pulex* in the waters of The Netherlands. Since osmoregulation is an energy demanding process, differences in the osmoregulatory capacities of these amphipods may provide one species with an energetic advantage over its competitors. The aim of this study is to compare osmoregulation in these amphipod species in order to provide a physiological explanation for the displacement of *G. d. duebeni* and *G. tigrinus* by *D. villosus*.

In the inland waters of Northern Ireland, the native amphipod *G. d. celticus* is being displaced by the invasive freshwater amphipod *G. pulex* (Dick, 1996a). It is proposed that *G. pulex* is more adapted to fresh water than *G. d. celticus*, which is influencing the competitive interactions between the species. The aim of this part of the study is to compare the osmoregulation of these two Irish amphipod populations in order to further understand their distributions in the Irish waters.

Long term adaptation to either fresh or brackish waters can lead to both morphological and physiological distinctions between isolated populations of the same species (Sutcliffe, 2000). The osmoregulation of isolated populations and subspecies of *G. duebeni* from waters of different ionic concentration are compared, in order to assess the effects of long term salinity adaptation on certain aspects of their osmoregulatory physiology.

In addition to the salinity effects on osmoregulation, the effects of chemical (copper toxicity), biological (endoparasite infection) and physical (cave environments) stressors on the osmoregulatory mechanisms of *G. pulex* are investigated.

CHAPTER TWO

Methods

All chemicals were obtained from Sigma, except where indicated.

2. 1. Collection, Storage and Acclimation of Gammarids

Gammarids were collected from water bodies of the British Isles and The Netherlands from locations shown in Table 2. 1. 1. Note that for *G. pulex*, *G. tigrinus* and *G. duebeni* more than one population was sampled. This included the sub-species of *G. duebeni*, namely *G. d. duebeni* and *G. duebeni celticus*.

In most cases animals were collected by the method of 'kick sampling', where disturbance of the substrate caused the animals to become dislodged from the stones, boulders and vegetation, which they usually inhabit. The animals were captured in a hand held net positioned downstream. Once captured, animals were placed in an enclosed container of river water for transport back to the laboratory. The marine amphipods *Chaetogammarus marinus* and *G. locusta*, were collected from the inter-tidal zone during low tide where they were found under algal canopies.

In all species, adults greater than 10mm in length were collected. On return to the laboratory, animals were placed in separate water tanks (12 litre capacity) of the appropriate media aerated and maintained at a temperature of 15 ± 0.5 ^{0}C. Water tanks were cleaned and replaced with clean media every 5 to 6 days. The animals were fed on wheatgerm (Jordans) once a week. All gammarids were acclimated for at least five days and starved for at least 3 days prior to experiments. All experiments, except where stated, were carried out at 15°C.

Table 2. 1. 1. List of gammarids used in this thesis, including sampling location and date of collection.

Gammarids	**Location of population**	**Date of collection**	**Grid Reference Ordnance survey for UK population**	**Location on Map Fig. 2.1.2**
G. pulex	Creswell Crags, Nottingham	Continual	SK533 741	1
G. tigrinus	River Poulter, Nottingham	Continual	SK649 757	2
G. pulex	Minnowburn, Belfast	Jan 2001	J442 781	8
G. tigrinus	Upperlands Reservoir, County Antrim	Jan 2001	C869 050	10
G. d. celticus	Lough Neagh, N. Ireland	Jan 2001	H960 793	9
G. d. duebeni	Totton Marsh, Southampton	Sept 2001	SU365 135	5
G. duebeni	Lizard Point, Cornwall	Oct 2001	SW83 339	7
G. zaddachi	Gibraltar Point, Skegness	June 2001	TF560 587	3
G. locusta	Hayling Island, Portsmouth	Sept 2001	SU718 040	6
Chaetogammarus marinus	Hayling Island, Portsmouth	Sept 2001	SU718 040	6
G. pulex	River Wye, Peak District	July 2001	SK164 732	4
G. pulex	Peak Caves, Peak District	July 2001	SK139 827	4
Dikerogammarus villosus	Monnickendam, Holland	April 2002	52° 25' 05" N 6° 05' 05" E	13
G. tigrinus	Ouestland, Holland	April 2002	52° 33' 27" N 4° 59' 40" E	12
G. pulex	Apeldoorn, Holland	April 2002	52° 51' 15" N 5° 42' 0" E	14
G. d. duebeni	Petten, Holland	April 2002	52° 45' 20" N 4° 39' 30" E	11

Source: www.ordsvy.gov.uk

www.freegk.com/wordatlas/netherlands.php

Figure 2. 1. 2. Outline Maps of *Great Britain and Northern Ireland* and *The Netherlands* showing the sample locations for the collection of the gammarids (site numbers correspond to the sites in table 2. 1. 1.).

2. 1. 1. Transportation of foreign gammarids

Irish and Dutch gammarids were transported to Nottingham by van and overnight ferry across the Irish and North Sea respectively. With the aid of battery-operated air pumps (Hagen), the animals' media was kept fully aerated in 25 litre food grade plastic bins throughout the journey. Mortality of gammarids was low during this transport phase.

2. 1. 2. Salinity acclimation

Sea water concentrations were made from dechlorinated Nottingham tap water and synthetic seawater salts (Instant Ocean, Aquarium systems), diluted to the appropriate salinity. Throughout this thesis 100% sea water consisted of 40 g. l^{-1} of seawater salts (i.e. 40 salinity). This is stronger than the normal sea water concentration of approximately 35 salinity. Care should be taken when comparing salinity effects on gammarids in this thesis with those of other studies, since the actual sea water concentration may differ. All sea

water media were fully aerated before the addition of the animals, and were replaced with fresh media every 5 - 6 days.

Table 2. 1. 2. 1. List of cation concentrations of 100% sea water (SW, 40g. l^{-1}) and fresh water (FW, dechlorinated Nottingham Tap water) media used in the acclimation experiments (mean ± SD). Ions were measured with Ion Chromatography (as in section 2. 6. 2.), and expressed in mM concentrations.

Cation	100%SW		FW	
Na	542.72	3.24	1.76	0.20
K	10.98	0.21	0.16	0.02
Mg	63.00	0.62	1.15	0.21
Ca	13.08	0.08	2.82	0.30

All *Gammarus pulex* populations, where appropriate, were acclimated to 0, 15 and 30% sea water concentrations. To aid survival, *G. pulex* were acclimated firstly to 15% sea water for 1 day before acclimation to 30% sea water. However, despite this gradual acclimation technique, attempts were unsuccessful in acclimating *G. pulex* to 50% sea water due to high mortality.

Populations of *Gammarus tigrinus*, *G. duebeni*, *G. d. duebeni*, *G. d. celticus* and *G. zaddachi* are able to tolerate a wide range of salinities and were successfully acclimated to 0, 25, 50, 75 and 100% sea water for one week. To aid survival these species were acclimated stepwise to 25, 50 and 75% sea water as appropriate for one day each, prior to one week acclimation in the final acclimation medium.

The marine amphipods *Chaetogammarus marinus* and *G. locusta* were unable to tolerate fresh water acclimation. Therefore, both species were acclimated to 25% sea water as the minimum concentration. Due to the small numbers collected, *G. locusta* were only acclimated to 100% and 25% sea water. *C. marinus* was acclimated to 25, 50, 75 and 100%

sea water. To aid survival these species were acclimated firstly in 75% and 50% sea water for one day each, prior to one week acclimation in 25% sea water.

Dikerogammarus villosus has a limited salinity tolerance range and can only withstand salinities up to 50% sea water (Brujis *et al.*, 2001). Therefore, *D. villosus* was acclimated to 0, 15, 25, 40 and 50% sea water. As in all gammarids, to increase survival *D. villosus* was acclimated gradually at each of the lower salinities for one day before acclimation to 50% sea water.

Disposal of *D. villosus*

Particular care was taken to prevent *D. villosus* from escaping into the field. All water containing *D. villosus* was sterilised using the appropriate concentration of chloride tablets (Boots plc). To ensure all animals were dead, the sterilised water containing animals was left standing for several days before disposal down the sink.

2. 1. 3. Cave populations of *G. pulex*

Cave populations of *G. pulex* were collected from the Peak cavern percolated underwater systems in the Peak District (O.S. Grid reference SK139 827). The cave water temperature was recorded as 8 ± 0.5°C. In the lab, the cave animals were acclimated in the dark to dechlorinated fresh water at 8°C in an incubator (LMS). All experiments concerning the cave populations of *G. pulex* were carried out at 8°C.

2. 1. 4. Parasitised *G. pulex*

Parasitised *G. pulex* were collected from the Creswell Crags population (O.S. Grid reference SK533 741). Approximately 5% of animals were infected with at least one cystacanth of the acanthocephalan parasite, *Polymorphus minutus*. The infected individuals were clearly distinguishable due to the presence of an orange spot in the thoracic region of

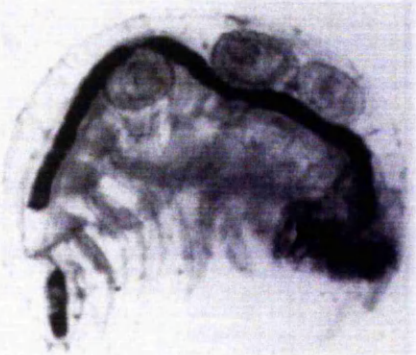


Figure 2. 1. 4. 1. *G. pulex* parasitised with three cystacanths of *P. minutus*. The cystacanths (spherical objects) are located in the haemocoel of the intermediate host.

the animal. Infected (one or more cystacanths) and non-infected animals were sorted and placed in separate water tanks before acclimation to appropriate sea water media.

2. 1. 5. Copper exposure

Gammarus pulex were exposed for up to 5 days in nominal concentrations of 100 & 1000 μg. l^{-1} copper, obtained from appropriate dilutions of a 10 mg. l^{-1} copper stock made with $CuCl_2.2H_20$ (VWR International) dissolved in dechlorinated fresh water. Copper has a tendency to attach onto the sides of glassware, causing reduction in the soluble copper concentration. To reduce this effect, copper media was replaced every 24 hours.

2. 2. Gill Na^+, K^+-ATPase

2. 2. 1. Excision & Homogenisation of gammarid gills

Animals were decapitated and pinned dorsal surface down on a wax dissection plate. A small amount of acclimation media was poured onto the animal allowing the gills to be suspended away from the ventral body surface. This enabled fast and simple excision of all six pairs of gills using a fine pair of forceps. Once excised the gills were immediately placed in ice-cold homogenisation media (100mM NaCl, 100mM imidazole, 0.1% sodium deoxycholate, pH 7.2). To gain enough enzyme for gill Na^+, K^+-ATPase activity to be measured, gills from several animals were pooled together. For best results a ratio of the gills from one animal for every 50μl of homogenate was used.

The gills were homogenised on ice with approximately 20 turns of a hand held 3ml ground glass homogeniser (Uniform). The crude homogenate was then centrifuged at 2000g for 15min at 4°C in a 'Harrier 18/80' refrigerated centrifuge (Sanyo). The supernatant was pipetted off into a 1.5ml micro centrifuge tube and used as the enzyme extract.

2. 2. 2. Na^+, K^+-ATPase assay

Gill Na^+, K^+-ATPase activity was determined by the liberation of phosphate from adenosine triphosphate (ATP) in the presence and absence of ouabain, a specific inhibitor of Na^+, K^+-ATPase. A 30µl sample of enzyme extract was placed in 2ml microcentrifuge tubes containing 500µl of either incubation media A (optimum media) or B (optimum media plus ouabain). Optimal ion, ouabain and ATP concentrations plus pH were determined from pilot experiments.

A	100mM NaCl	**B**	Solution A
	15mM KCl, 10mM $MgCl_2$		+ 10mM ouabain
	100mM imidazole		pH 7.2

N.B. **A** = measure of all ATPases present in the enzyme extract

B = all ATPases except Na^+, K^+-ATPase ("Mg^{2+} ATPase", Sola *et al.*, 1995)

A – **B** = measure of Na^+, K^+-ATPase

The mixtures were vortexed (Whirlmixer, R. W. Jennings & Co. Ltd) and incubated in a water bath at 4°C for 15min, which allowed time for ouabain to block the activity of gill Na^+, K^+-ATPase. After the 15min incubation, 50µl of 58mM Na_2ATP (pH to 7.2 with Imidazole buffer powder) was added to the incubation media giving a final concentration of 5mM ATP. The tubes were then vortexed and incubated in a water bath at 37°C for 20min. This temperature was chosen as only small amounts of sample tissue were available, and the Na^+, K^+-ATPase activity of *G. pulex* is greater at 37°C than at 15°C. Typically three replicate samples were used, although this was altered to two or four in some experiments

depending upon the availability of enzyme extract. A separate blank (incubation media + ATP only) was used for each treatment.

The reaction was stopped with 1ml Bonting's reagent (560mM H_2SO_4, 8.1mM ammonium molybdate, 176mM $FeSO_4$)*. Phosphate in the presence of Bonting's reagent produces a blue colour. The colour was allowed to develop for 20min at room temperature before absorbance was measured at 700nm using a photospectrometer (Cecil CE1011, 100 series).

Phosphate concentration was determined from a phosphate calibration graph (Fig. 2. 2. 2. 1.). Known phosphate concentrations were made from sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) to give a pH of 7.2. A phosphate calibration graph was produced for each set of experiments. Gill Na^+, K^+-ATPase activity was expressed as μmoles phosphate. mg $protein^{-1}$. $hour^{-1}$.

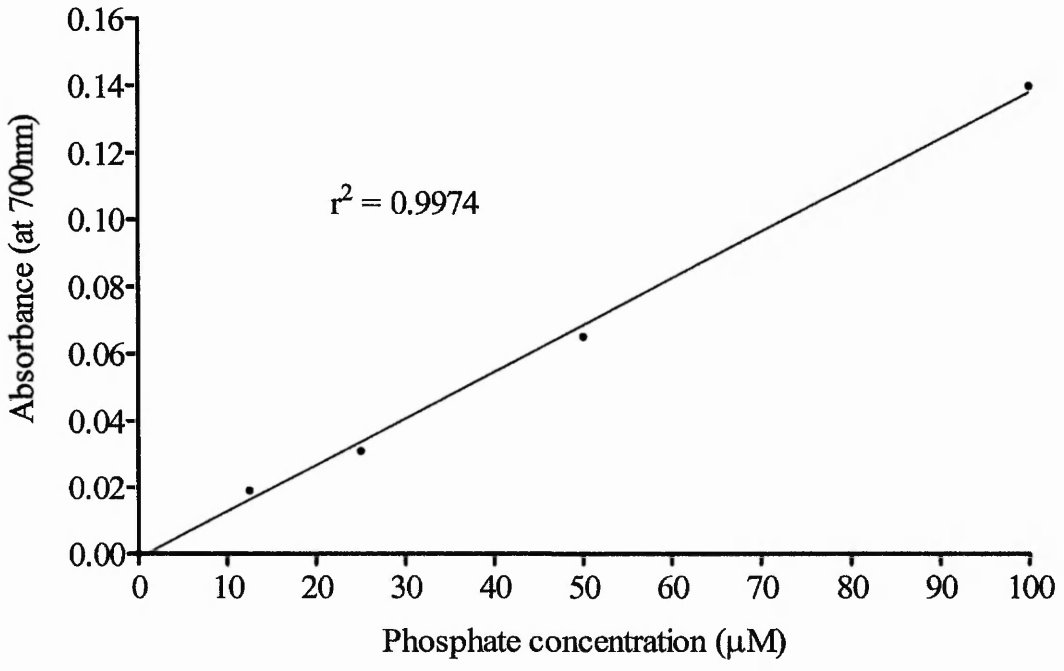


Figure 2. 2. 2. 1. Typical phosphate calibration graph used to determine phosphate concentrations from absorbance readings at 700nm.

* When making Bonting's reagent, ammonium molybdate must be added first and allowed to dissolve in the 560mM sulphuric acid mixture before ferrous sulphate is added, to produce a colourless liquid.

2. 2. 3. Characterisation of Na^+, K^+-ATPase assay

Characterisation of the assay involved the determination of the optimum conditions resulting in maximum levels of gill Na^+, K^+-ATPase activity. The essential ions of Na^+, K^+-ATPase are sodium, potassium and magnesium (Glynn & Karlish, 1975). The optimum concentrations of these ions in the incubation media were determined in order to increase gill Na^+, K^+-ATPase activity. Optimum co-factor concentrations, including pH of the incubation media, concentration of substrate ATP and the amount of ouabain required to totally inhibit gill Na^+, K^+-ATPase activity, were determined. The following ranges of concentrations were tested for all ion and co-factors:

i) ouabain (range, 0 – 10mM)
ii) sodium (range, 0 – 300mM)
iii) potassium (range, 0 –25mM)
iv) magnesium (range, 0 – 25mM)
v) ATP (range, 0 – 10mM)
vi) pH (range, 6 – 8.5).

To determine optimum ion and co-factor requirements, only one variable was changed per experiment. Experiments were repeated in triplicate for each variable.

2. 2. 4. The effects of copper on gill Na^+, K^+-ATPase

Experiments were carried out as described above (section 2. 2. 2.), except for the exclusion of imidazole. Imidazole is known to bind to copper (Verweij, *et al.*, 1990). Therefore, in copper experiments 100mM hepes was used as the pH buffer in the incubation media. In the preparation of the ATP solution, imidazole was replaced with 100mM hepes plus 1M KOH to correct the pH to 7.2.

Nominal copper concentrations of 10, 100, 300 and 1000 $\mu g.\ l^{-1}$ were included in the assay solutions A and B. These concentrations were obtained from the appropriate dilutions of a 10mg. l^{-1} copper stock made with $CuCl_2.2H_20$. Copper has a tendency to attach onto the side of glassware, causing reduction in the soluble copper concentration. To reduce this effect, freshly prepared copper media (< 2 days old) was used.

2. 2. 4. 1. Reversal of copper toxicity to gill Na^+, K^+-ATPase

To investigate the effect of the heavy metal chelators dithiothreitol (DTT) and diethylenetriaminepentaacetic acid (DTPA), the Na^+, K^+-ATPase protocol (section 2. 2. 2.) was modified slightly. As with all experiments including copper, imidazole was replaced with hepes in the incubation media. After the 15 minute incubation step with copper at 4°C, the chelators were then added to a final concentration of 1mM where appropriate, vortexed and left for 1 minute, prior to the addition of ATP and further vortexing. This was followed by the final incubation at 37°C for 20 minutes.

2. 3. Protein measurements

2. 3. 1. Protein assay 1

The protein concentrations of the supernatants used in the Na^+, K^+-ATPase assay (Section 2. 2.) were determined using the following protocol (adapted from Lowry *et al.*, 1951).

Solution A: 2% w/v sodium carbonate (Na_2CO_3)

0.1M sodium hydroxide (NaOH)

0.04% w/v sodium potassium tartrate (NaK tartrate).

Solution B: 1% w/v copper sulphate ($CuSO_4$)

Solutions A and B were combined in the ratio 50:1, before 750µl of the combined media was added to 25µl of supernatant sample in a 1.5ml microcentrifuge tube, vortexed and left for 10min at room temperature. After 10min 75µl of 50% Folin ciocalteau reagent was added, the mixture was vortexed and left for 30min at room temperature. Absorbance was recorded at 750nm using a spectrophotometer (Cecil, CE1011, 100 series).

Sample protein concentrations were determined from a protein/ absorbance calibration graph (see Fig. 2. 3. 1. 1.). Known protein concentrations were made with bovine serum

albumin (BSA) fraction V standards dissolved in distilled water between the range 0 to 160μg. ml^{-1}.

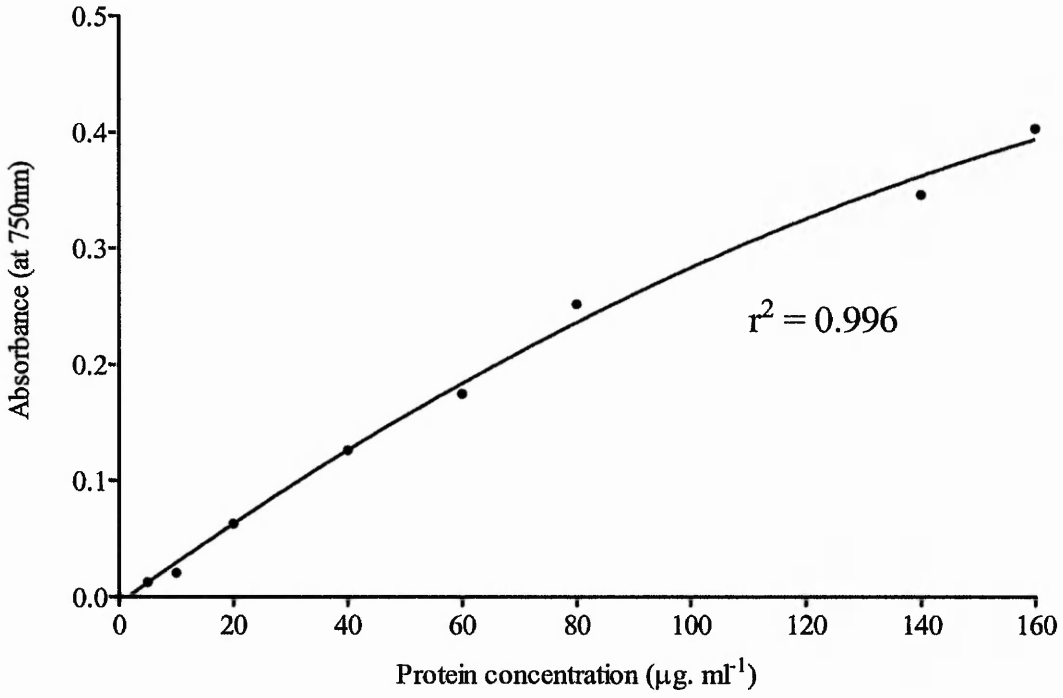


Figure 2. 3. 1. 1. Typical protein calibration graph to determine sample protein concentrations from absorbance readings at 750nm. Protein standards made using serial dilutions of bovine serum albumin fraction V.

2. 3. 2. Protein Assay 2

The protein assay described above is affected by hepes buffer in the sample. Therefore, when hepes was used in the incubation media (i.e. copper experiments in Na^+, K^+-ATPase assay (section 2. 2. 2.), the Bio-Rad Protein Assay was adopted. The Bio-Rad Protein assay is a dye-binding assay, in which the coomassie blue dye binds to primarily basic and aromatic amino acid residues. Protein standards were prepared from bovine serum albumin (fraction V). A typical standard curve can be seen in figure 2. 3. 2. 1.

The Protein assay Dye Reagent (Bio-Rad Laboratories) containing dye, phosphoric acid and methanol was diluted 1 part to 4 parts distilled water. 200μl of the dye reagent was

added to individual microtiter plate wells containing 10µl of the sample and standard solutions. The well contents were mixed by repeatedly withdrawing and dispensing the well fluid several times with an auto-pipette. The mixtures were incubated at room temperature for at least 5 minutes before absorbance was measured at 590nm on a micro-plate reader (Pericom).

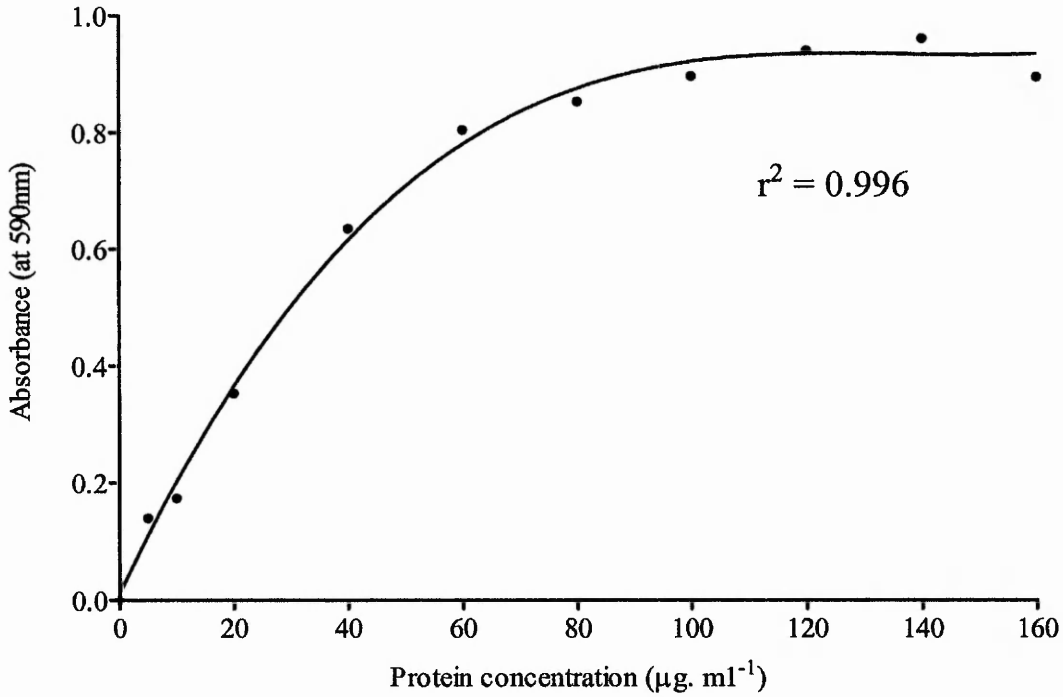


Figure 2. 3. 2. 1. Typical protein calibration graph to determine sample protein concentrations from absorbance readings at 590nm. Protein standards made using serial dilutions of bovine serum albumin fraction V.

2. 4. Efflux measurement of water permeability

Half time of exchange of body water ($t_{1/2}$) was determined in gammarids, with the efflux technique of Lockwood *et al.*, (1973) using tritiated water (THO, Emerson chemicals). Animals were acclimated for at least one week in the sea water concentrations described in section 2. 1. 2.

Prior to the experiment, animals were placed in a sealed vessel containing 50ml of THO at 1.85MBq. ml^{-1} of the same salinity as their acclimation media for 2 hours. The 2 hours was approximately six times the expected $t_{1/2}$ and allowed sufficient uptake of THO to approach a steady state. After the 2 hour loading period, animals were washed thoroughly in non-tritiated water to allow superficial tracer to be removed from the external surface. The animals were then transferred to a sealed vessel containing 10ml of unlabelled medium of the same salinity as the acclimation media. The vessel was sealed to prevent any exchange of water with water vapour in the air.

Duplicate 50µl aliquots of the unloading medium were then taken at regular time intervals over the first 20 minutes and again at 2 hours. The last sample reading (C_∞) at 2 hours was when the THO tended towards a steady state in the external medium. Each of the 50µl

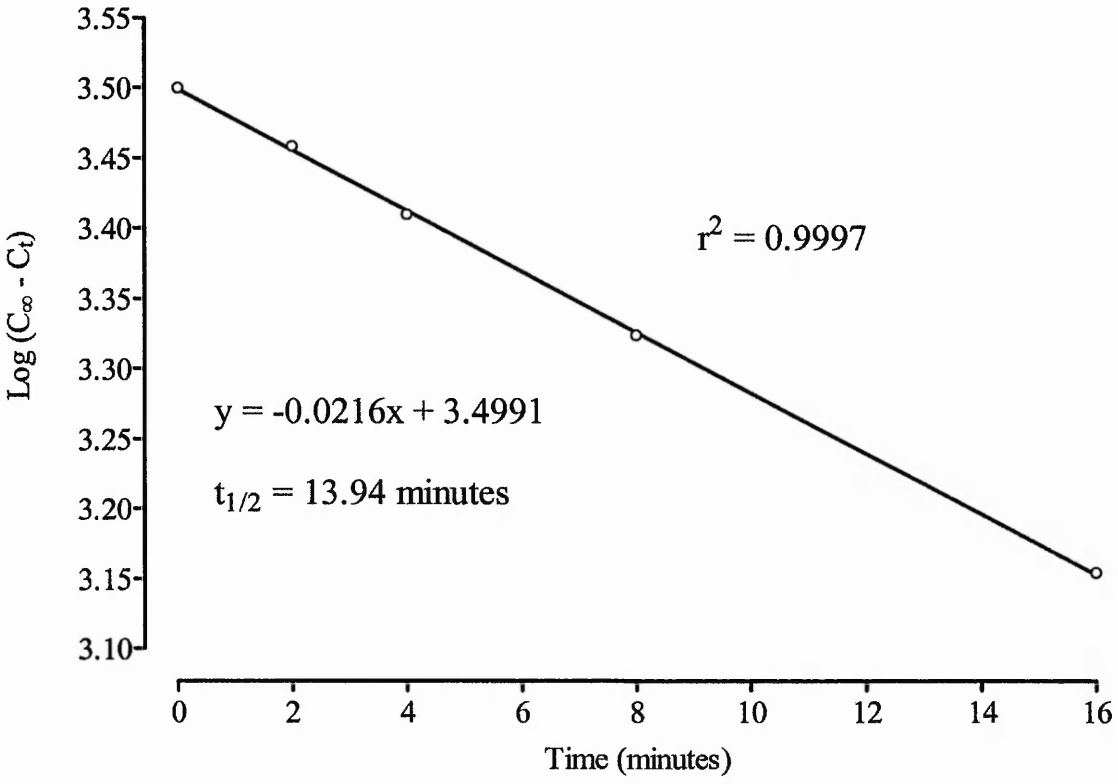


Figure 2. 4. 1. Semi log plot of THO loss rate over time for the calculation of the $t_{1/2}$. The $t_{1/2}$ was taken as the time at which y = c – log 2, where c was the value of the y intercept. The points denote the means of duplicate samples.

aliquots were added to separate scintillation vials containing 4ml of scintillation fluid (Ultima-gold, Packard). Activity was measured in 5 minute counts using a liquid scintillation counter (Tri-Carb 2100TR, Packard).

The temperature was maintained throughout the experiment at 15^0C, apart from cave populations where it was 8^oC. After the experiment the animals were transferred to a non-spiked media and left for several hours to unload before wet weight of the animals was determined.

Semi-log plots of THO loss rate over time demonstrated that water efflux was governed by a single rate constant (see figure 2. 4. 1.) The $t_{1/2}$ was determined from the regression line calculated from log (C_∞ - C_t) at each time t_n. The time at which $y = c - \log 2$ (i.e. the time taken for the count at time 0 to halve), where c was the value of the y-axis intercept, gave the $t_{1/2}$.

2. 4. 1. Validity of water permeability measurements using isotope-labelled water.

Water permeability measurements using isotope labelled water, mainly in the form deuterium (DHO) and tritium (THO), have been used extensively over the last 30 years (reviewed in Rasmussen & Andersen, 1996). Water permeability measurements are determined through the presence of either external or internal isotope labelled water, which passively diffuse between compartments across a membrane towards equilibrium. The speed of movement and consequently the time taken for equilibrium to be reached is the measure of the animals' permeability to water

The advantages of using the isotope labelled water technique in determining water permeability are as follows; (1) they are simple to perform on most aquatic organisms; (2)

have low financial cost; (3) experimental animals are not harmed during the experiment and therefore, can be used again as their own control; (4) changes in water permeability during the course of the experiment can be studied (Rasmussen & Andersen, 1996). These advantages have contributed to the widespread use of isotope labelled water to determine water flux and water permeability measurements.

Despite these advantages water permeability measurements using isotope labelled water have been criticised on the basis that they have not given values comparable with those obtained from other types of measurements, e.g. net water flux as determined by urinary excretion (Rasmussen & Andersen, 1996). The two main areas of concern are, (1) the possible isotope effects and, (2) the lack of representability of the internal compartment. For these reasons water permeability measurements are often referred to as 'apparent'.

Due to the differences in their molecular mass, the rate of movement of labelled (THO, DHO) and unlabelled water species has been found to vary. The diffusion rate of THO (molecular mass 20Da) and H_2O (molecular mass 18Da) differ by less than 10% (Rankin & Davenport, 1981). In the purple shore crab *Hemigrapsus nudus*, water fluxes measured using THO, DHO and H_2O were found to be not significantly different, at 91, 95 and 100% respectively (Smith & Rudy, 1972). Also no significant effects of hydrogen isotopes were found from measurements of water permeability in frog skin (King, 1969; Wang *et al.*, 1953). However, in the same study, Wang *et al.*, (1953) found that the diffusion rate of $H_2{}^{18}O$ was significantly slower (14%) than the diffusion rate of THO or DHO. Hence, if water permeability is to be measured with radiolabelled water, isotopes of hydrogen are advised rather than oxygen.

The second main area of criticism concerns the lack of information about the internal compartment and thus whether the measured value is a true measure of water permeability

or a possible artefact. The variation in the thickness of the unstirred layers on either side of the membrane would likely affect the diffusion gradient and potentially cause underestimation of water permeability measurements (Dainty & House, 1966). Alternatively the presence of small pores, such as aquaporins, in the membrane might be expected to over estimate the permeability measurements from one side of the membrane to the other (Lockwood *et al.* 1982).

Permeability based on flux measurements can substantially underestimate the net passage of water down an osmotic gradient, resulting in a marked difference in the ratio of the osmotic permeability (P_{os}, as determined from the net transfer of water) and the diffusional permeability (P_{diff}, determined from the flux of THO) especially in isolated tissues that may lack any irrigation (Bolt, 1989). The P_{os}: P_{diff} ratios of water permeability in amphibian skin can often exceed 10, reaching 27.2 in *Bufo regularis* (Maetz, 1968). However, the P_{os}: P_{diff} ratios of tissues that are well irrigated on both sides of the membrane such as gills are generally lower. For example, in the gills of the crabs *Libinia emarginata* and *Carcinus maenas* the P_{os}: P_{diff} ratios range between 1 and 2.5. These low P_{os}: P_{diff} ratios are due to good irrigation of the outside by respiratory currents and the inside by blood flow causing a reduction in the unstirred layers both sides of the membrane (Bolt, 1989). In support of this Bolt (1989) reported a sharp rise in the $t_{1/2}$ of *G. duebeni* after heart failure from 6 to 60min, emphasising the importance of the respiratory and circulatory currents in diminishing the unstirred layers.

Since heart rate can affect the irrigation of the internal membrane and thus potentially affect water permeability measurements, it is important to consider that heart rate can differ markedly in some species on acclimation to different salinities. In the crab *L. emarginata* the heart rate was found to correlate with the observed changes in water flux (Cornell, 1979). However, the heart rate of more euryhaline species such as *G. duebeni* does not

significantly alter when transferred between salinities and differences that do occur are such that heart rate is actually faster in dilute media (Bolt *et al.* 1980). Also Hume and Berlind (1976) found that the heart rate of *C. maenas* increased on acclimation to more dilute sea water media when the animal was found to be less permeable (higher $t_{1/2}$). Therefore, it is unlikely that the observed water fluxes after a change in the external salinity can be attributed to changes in heart rate. Any increase in heart rate with dilution of the external environment would tend to increase water permeability, rather than the reductions observed.

Attempts to further validate THO measurements of water permeability were carried out by Bolt (1985). He compared THO flux measurements with calculated permeabilities using urine flow rates measured with Cr EDTA as a marker. It was assumed that in *G. duebeni* urinary flow out of the animal matched the net flow into the animal. Thus, measurements of urine flow and osmotic gradient should permit calculations of the half time of exchange of water ($t_{1/2}$) for the animal (Bolt, 1985). From these results a good agreement was found between the measured $t_{1/2}$ using THO and the calculated $t_{1/2}$ using the urine measurements. Therefore, Bolt (1985) concluded that THO measurements of water permeability were real and unlikely to be artefacts of the experimental technique in crustaceans.

In conclusion, it seems highly unlikely for unstirred layers or other artefacts to be responsible for often large changes in water fluxes observed on transfer between different salinities in crustacean gills. Therefore, the observed changes in water flux must be either due to changes in the effective surface area or a real change in hydraulic permeability. Hence, it is presumed that the changes observed are real changes in hydraulic permeability.

2. 5. Measurements of sodium fluxes

2. 5. 1. Sodium influx

Animals were transferred from their acclimation media to a sealed vessel containing 50ml of 11mM Na solution spiked with 0.925MBq. ml^{-1} ^{22}Na. A sodium concentration of 11mM was used in the loading medium since the sodium uptake mechanism in the gammarids investigated is saturated at 11mM sodium or less (Sutcliffe, 1967a; Sutcliffe & Shaw, 1967). The animals were left to load in the sealed vessel for 30min at 15°C. Rapid transfer of *G. duebeni* from 2% to 100% sea water, was found to have no effect on sodium influx within 2 hours of transfer (Dawson, 1982). Therefore, it was presumed that the 30min loading period was time enough for sufficient sodium uptake to occur, allowing ^{22}Na activity to be measured without any change in sodium influx during loading.

After the 30min loading period, animals were taken out and rinsed thoroughly, first in non-spiked 11mM Na solution, and then in the acclimation media in order to increase survival and remove any superficial tracer. The animals were then placed in scintillation vials containing 1ml of acclimation media. Since ^{22}Na is a gamma emitter, sodium uptake was determined from 60 second counts with a gamma counter (Minaxi γ Auto-gamma 500 series, Packard). Tubes containing 1ml acclimation media without the animal were used as the blank, and the value obtained was subtracted from all sample counts measured. The animals were transferred to a non-spiked acclimation media and left for several hours before wet weight of the animals were determined. Animals were removed of superficial water with tissue paper, before wet weights were measured on a microbalance (Sartorius). The specific activity (counts. 60 s^{-1}. μmole Na^{-1}) of loading medium was calculated from its sodium concentration and the radioactive counts of a known volume (10μl). Sodium influx was expressed as μmoles Na. 100mg wet $weight^{-1}$. $hour^{-1}$, determined from the equation below.

$$\text{Sodium influx} = \frac{\text{counts. 60 s}^{-1}\text{. animal}^{-1}}{\text{specific activity}} \times \frac{100}{\text{wet weight}} \times 2$$

$$= \text{ μmoles Na. 100mg wet weight}^{-1}\text{. hour}^{-1}$$

2. 5. 2. Total body sodium

Animals were transferred from their acclimation media to a sealed vessel containing 50ml of 1.76mM ^{22}Na solution (sodium concentration of dechlorinated fresh water) spiked with approximately 0.148MBq. ml^{-1} ^{22}Na. Animals were allowed to load with ^{22}Na for two days, long enough for the specific activity of sodium in their bodies to reach equilibrium with that of the medium. After loading, the animals were rinsed three times with non-radioactive acclimation medium to remove any superficial tracer. They were then placed in 1ml of unlabelled acclimation medium and the radioactivity of the medium counted for 60 seconds in a gamma counter (Minaxi γ Auto-gamma 500 series, Packard). Animals were then dried with tissue paper and weighed. Tubes containing 1ml unlabelled acclimation media without animals were used as blanks and the resulting values subtracted from all sample counts measured.

As mentioned in the previous section (2. 5. 1.), the specific activity of loading medium was calculated from its sodium concentration and the radioactive counts of a known volume. The total body sodium concentration (μmole Na. 100mg wet weight^{-1}) was determined from the equation below.

$$\text{Total body sodium} = \frac{\text{counts. 60 s}^{-1}\text{. animal}^{-1}}{\text{specific activity}} \times \frac{100}{\text{wet weight}}$$

$$= \text{ μmoles Na/ 100mg wet weight}$$

It was assumed that the response of the radioactive sodium isotope, ^{22}Na was not significantly different from the sodium isotope ^{23}Na, normally present in the animal and medium.

2. 5. 3. Sodium efflux

Sodium efflux was determined in the same animals as those used to determine the total body sodium concentrations. Animals were therefore preloaded with ^{22}Na so that the specific activity of sodium in their bodies was equal to that of the medium. After counts were made to determine total body sodium concentrations, animals were immediately placed into 200ml of non-radioactive dechlorinated fresh water for 60min. The large volume (200ml) was assumed to be large enough to prevent the back-flux of tracer into the animal that may have caused an underestimate in sodium efflux calculations. The animals were then removed, rinsed and placed in vials containing 1ml of dechlorinated fresh water, and the vials counted. The sodium efflux was determined from the equation below.

$$\text{Sodium efflux} = \frac{(\text{counts. } 60\ s^{-1})_{0min} - (\text{counts. } 60\ s^{-1})_{60min}}{\text{specific activity}} \times \frac{100}{\text{wet.wt.}}$$

$$= \quad \mu\text{moles Na. } 100\text{mg wet weight}^{-1}\text{. hour}^{-1}$$

In addition, total body sodium concentrations and sodium efflux rates were determined in *G. pulex* when acclimated to 15% and 30% sea water. In these cases animals were transferred from their acclimation media to a sealed vessel containing 50ml of either 95mM ^{22}Na solution (sodium concentration of 15% sea water) or 190mM ^{22}Na solution (sodium concentration of 30% sea water), spiked with approximately 0.148MBq. ml^{-1} ^{22}Na.

2. 6. Determining blood ion concentrations

2. 6. 1. Extraction of gammarid blood

A small amount of methyl dichlorosilane was added to a clean watch glass. This was then allowed to evaporate in a fume cupboard before the watch glass was wiped clean with tissue paper. The dimethyl dichlorosilane coated the watch glass with a hydrophobic surface. This was to ensure haemolymph formed a discrete drop of liquid, rather than spread out over its surface. Approximately 5ml of mineral oil was added to the cleaned watched glass. Glass pipettes were then prepared using a Bunsen burner and forceps; the end of the glass pipette was melted and pulled in a way to create a fine point, designed for the extraction of blood.

Animals were taken out of their appropriate acclimation media, dried on tissue paper and held between first finger and thumb exposing the dorsal surface thereby enabling a fine pipette to be inserted between the 2^{nd} and 4^{th} dorsal plates under which the main blood vessel runs. Blood was extracted by capillary action and expelled under a layer of mineral oil in the watch glass. The amount of blood extracted from a single animal varied greatly with maximum volumes approaching 10µl. Where possible individual blood samples were analysed, although in most cases the blood samples were pooled from a number of animals. 1µl aliquots of blood from separate samples were placed in individual 0.5ml microcentrifuge tubes containing 99µl of distilled water, these were vortexed and stored at -80^{0}C for later analysis.

2. 6. 2. Ion Chromatography

The main cation concentrations of the blood were determined using ion chromatography (Dionex dx100). Blood cation analysis was carried out on the CS12 cation-exchange column (Dionex), using 20mM methanesulphonic acid (MSA) as the eluant.

The stored blood samples were taken out of the –80^{O}C freezer and allowed to thaw. All samples were vortexed before 30µl was diluted (10 fold) in 270µl of deionised water. The 300µl samples and ion standards were placed into separate 0.5ml PolyVials (Dionex) and the filter caps (Dionex) inserted. The vials were placed in the Dionex racks and positioned on the auto-sampler to await analysis. The samples were automatically run through the CS12 cation-exchange column and data analysed in Dionex computer software (Dionex AI-450). Concentrations of ions in the blood samples were calculated from the ion standard calibration curves. Standard curves were made from dilutions of a stock standard solution of 100ppm for all ions listed (Table 2. 6. 2. 1.). A typical standard curve for sodium can be seen in figure 2. 6. 2. 1.

Table 2. 6. 2. 1. List of ion standards for the determination of blood ion concentrations.

	Element	Atomic Mass	Compound	Molecular weight	Wt for 100ppm ($g.l^{-1}$)
Cations	Lithium	6.9	LiCl	42.39	0.614
	Sodium	23	NaCl	58.44	0.254
	Ammonium	17	NH_4Cl	53.49	0.315
	Potassium	39.1	KCl	74.55	0.19
	Magnesium	24.3	$MgSO_4.7H_2O$	246.48	1.01
	Calcium	40.1	$CaCl_2.2H_2O$	147.02	0.367

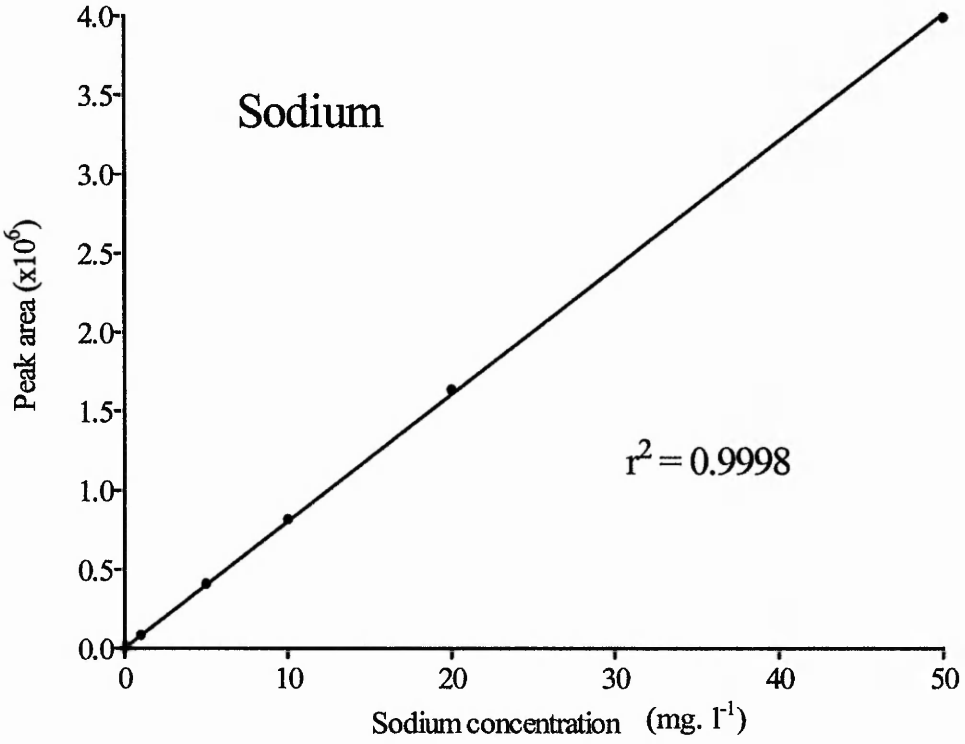


Figure 2. 6. 2. 1. A typical standard curve obtained from ion chromatography (Dionex) used for the calculation of blood ion concentrations.

2. 6. 3. Flame photometry

To prevent contamination of the ion chromatography column with copper, a Corning 410 flame photometer was used in copper experiments to measure haemolymph sodium concentrations. Haemolymph samples were extracted in the same way as that described previously (section 2. 6. 1). However, in this case 1µl of haemolymph was diluted in 5ml of deionised water. A range of NaCl standards were similarly diluted. The flame photometer was used to produce a standard curve from the diluted standards. This then allowed determination of haemolymph sodium concentrations.

2. 7. Oxygen measurements

Rates of oxygen consumption in *Gammarus pulex* were determined through the adaptation of an Oxygen Electrode Unit (Hansatech).

2. 7. 1. Electrode preparation

A few drops of electrolyte (50% saturated KCl) were placed on the central dome of the electrode disc, covering the platinum cathode and filling the surrounding well housing the silver anode. A 2cm square piece of cigarette paper ('a spacer') was placed over the central cathode and moistened by the electrolyte, providing electrical continuity between the two electrodes. A 2cm square of polytetrafluoroethylene (PTFE) membrane was placed on top of the paper spacer and a rubber O-ring applied, stretching the membrane over the surface of the electrode dome. A second, larger O-ring was fitted to the channel surrounding the electrode dome (figure 2. 7. 1. 1.)

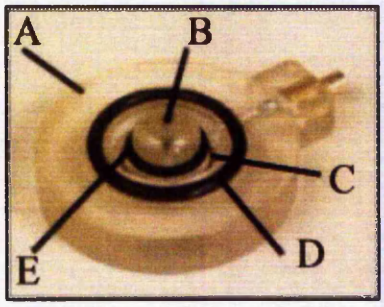


Figure 2. 7. 1. 1. An electrode disc (A) showing position of cathode (B), anode (C), and O-rings (D, E).
source: www.hansatech-instruments.com

The electrode disc was inserted at the bottom of the reaction chamber. Keeping the apparatus upright, avoiding spillage of the electrolyte, the base was threaded into position. The dome of the electrode disc protrudes into the lumen of the reaction vessel forming the floor of the reaction chamber. The electrode disc was connected to a control box (Hansatech CB1-D), converting the current generated by the electrode in response to oxygen into a voltage signal large enough for recording. The voltage signal was allowed 30min-1hour to stabilise before readings were taken. The control box was connected via an AD11/12 (Pico Technology Ltd) to a PC (Viglen, 488mHz processor) with data logging software (Picoscope, Pico Technology Ltd).

The reaction chamber was filled with 2ml of fully oxygenated dechlorinated fresh water, and a magnetic flea was inserted into the chamber. The speed of the stirring was controlled with a magnetic stirrer. Temperature was maintained in the reaction chamber by pumping temperature-controlled water into and out of the water jacket. The water movements through the water jacket were controlled by a water circulation unit (Grant). The temperature of the water was maintained with the combined effects of a water bath and cooling coil.

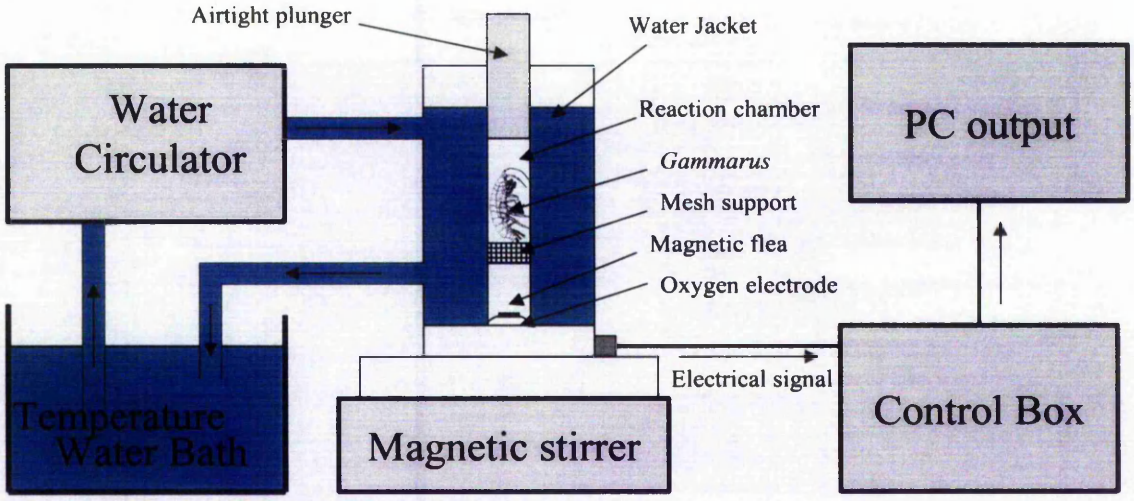


Figure 2. 7. 1. 2. Schematic representation of the oxygen electrode unit, used for the measurements of oxygen consumption in *G. pulex*. A prepared electrode disc was fixed at the base of the reaction chamber, which was connected via a control box to a Viglen 488mHz PC with Picoscope data logging software.

2. 7. 2. Calibration of the Oxygen electrode

The oxygen electrode unit was set up as described above. Sodium dithionite (40mg) was added to the water in the reaction vessel. The plunger was then placed in position at the top of the reaction chamber preventing contact with the air. The sodium dithionite reacts with the dissolved oxygen according to equation 1.

$$Na_2S_2O_4 + O_2 + H_2O \rightarrow NaHSO_4 + NaHSO_3 \qquad \text{(equation 1)}$$

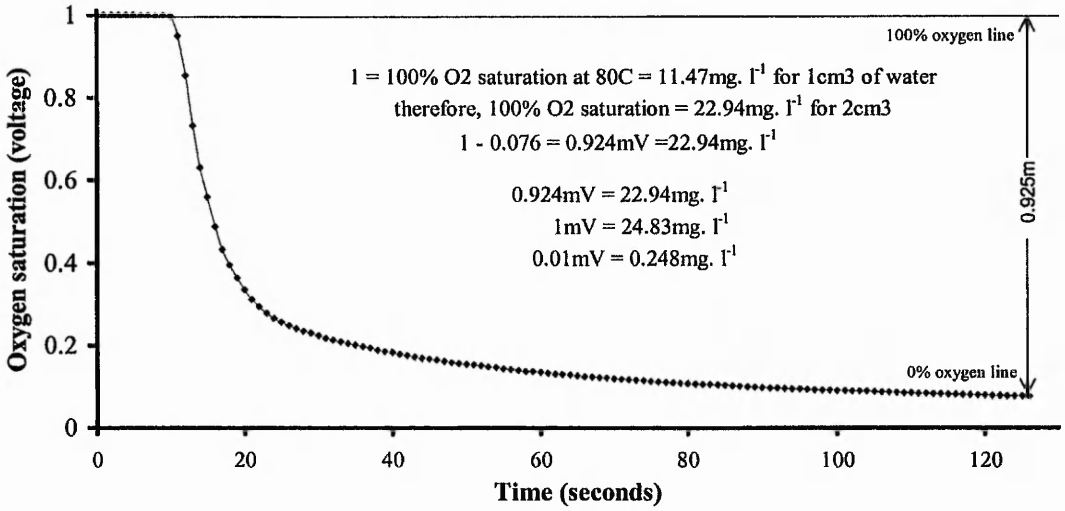


Figure 2. 7. 2. 1. Calibration of oxygen electrode. Addition of sodium dithionate reduces the dissolved oxygen content of the water, causing rapid drop in signal output.

The signal output drops reaching a new equilibrium ("0% oxygen line") after approximately two to three minutes. At this point the water is devoid of dissolved oxygen. The difference between the "100% oxygen line" and the "0% oxygen line" represents the oxygen concentration of the water contained in the reaction vessel (see figure 2. 7. 2. 1.). The values for the oxygen content were obtained from the empirical formula (Truesdale *et al.*, 1955, equation 2).

$$C_s = 14.16 - 0.3943T + 0.007714T^2 - 0.0000646T^3 \qquad \text{(equation 2)}$$

(C_s = saturation concentration (mg. l^{-1}) and T = temperature)

2. 7. 3. Measurements of oxygen consumption in *G. pulex*

Individual animals were placed in the enclosed reaction vessel containing 2ml of fully oxygenated dechlorinated fresh water. The unit was allowed to stabilise for one minute after the addition of the animal, before measurements were recorded for two hours. Oxygen consumption rates were expressed as μmoles oxygen. mg wet weight^{-1}. hour^{-1}. For the cave populations of *G. pulex,* oxygen consumption rates were measured at a temperature

representative of their natural environment (i.e. 8°C). The cave populations were compared to the river Wye population, also measured at 8°C. The Creswell population, containing parasite infected and uninfected *G. pulex* were measured at 15°C. The background rate of oxygen consumption was measured as described above, but with the absence of *G. pulex*. This accounted for any reduction in oxygen concentration not caused through the respiration of *G. pulex*, and was subtracted from the voltage readings as the blank.

2. 8. Statistical Analysis

All means were compared with the appropriate use of a One-way ANOVA and Duncan's multiple range test (SPSSWIN). A Student's paired t-test (Excel) was applied when only two data sets were compared.

CHAPTER THREE

Gill Na^+, K^+-ATPase

3. 1. Introduction

Sodium, potassium-dependent adenosine triphosphatase (Na^+, K^+-ATPase), otherwise known as the sodium pump or sodium-potassium pump is a primary active transport system, ubiquitous to all animals. Na^+, K^+-ATPase is a transmembrane protein composed of 3 subunits (α, β, γ) (Towle *et al.* 2001). The α-subunit provides the catalytic function, binding and hydrolysing ATP, the β-subunit is thought to anchor the complex in the basolateral membrane, whilst the γ-subunit, not always present, is thought to serve a regulatory role (Therien & Blostein, 2000). Located in the basolateral membrane of gill epithelium, Na^+, K^+-ATPase actively pumps 3 sodium ions outwards from the cytosol into the haemolymph and 2 potassium ions inwards from the haemolymph to the cytosol for every one molecule of ATP hydrolysed (reviewed in Glynn, 1985).

3. 1. 1. Activity and dynamics in Crustacean gills

The activity of gill Na^+, K^+-ATPase in crustaceans has gained considerable attention. Gill Na^+, K^+-ATPase has been previously characterised in a number of higher crustaceans including, the Purple shore crab *Hemigrapsus nudus* (Corotto & Holliday, 1996), the mud fiddler crab *Uca pugnax* (Holliday, 1985), the shore crab *Carcinus maenas* (Siebers *et al.*, 1983, 1985, 1992, Winkler, 1986), the blue crab *Callinectes sapidus* (Towle & Holleland, 1986), as well as the European lobster *Homarus gammarus* (Lucu & Devescovi, 1999). In all these animals significant changes in gill Na^+, K^+-ATPase activity were found to occur when animals were acclimated to varying concentrations of external salinity, with increased gill Na^+, K^+-ATPase activity in species acclimated to dilute sea water.

Evidence suggests the possibility that two levels of enzymatic control are involved in the acclimation to salinity change. During long term adaptation to dilute media the synthesis of new enzyme occurs. In contrast, short term changes are thought to be due to the increased catalytic activity of pre-existing enzyme triggered through low cellular sodium concentrations and the action of neuroendocrine factors (Lucu & Flik, 1999; Lucu, 1990, Siebers *et al.*, 1983).

From western blot analysis of gill Na^+, K^+-ATPase protein, a 2.1 fold increase was found in the shore crab *C. maenas* three weeks after transfer to dilute media (Lucu & Flik, 1999). Since no significant increases in protein levels were recorded after 4 hours, this supports the view that synthesis of new enzyme is a long term acclimation response. In the blue crab *Callinectes sapidus*, quantitative reverse transcription and polymerase chain reaction (RT-PCR), western blotting and immunocytochemistry were employed to investigate the correlation between α-subunit mRNA and protein abundance in relation to the osmoregulatory response (Towle *et al.*, 2001). Two weeks after transfer of crabs from 35 to 5 salinity, enhanced Na^+, K^+-ATPase was not accompanied by any notable difference in the relative proportions of protein in the posterior gills, suggesting that the enhanced Na^+, K^+-ATPase activity may result from the increased activity of existing enzyme rather than the synthesis of new enzyme.

Several studies have implicated the gills of isopods and amphipods as the sites of active ion transport in lower crustaceans (Mantel & Farmer, 1983). With the exception of the hypo-osmoregulating brine shrimp *Artemia* (reviewed by Conte, 1984) and the isopod *Idotea wosnesenskii* (Holliday, 1988), little work has been carried out on the active transport processes of lower crustaceans. This is particularly true for gammarid amphipods, where gill Na^+, K^+-ATPase has yet to be explored. The aim of this study is to characterise gill Na^+, K^+-ATPase in the freshwater amphipod *Gammarus pulex,* including partial

characterisation of the enzyme in several other gammarids. Following characterisation of this enzyme, the effects of external salinity acclimation on the activity of gill Na^+, K^+-ATPase, are determined. Gill Na^+, K^+-ATPase activity is compared between gammarids from fresh water, brackish water and sea water habitats.

3. 2. Results

3. 2. 1. Characterisation of gill Na^+, K^+-ATPase in *G. pulex*

The characterisation of gill Na^+, K^+-ATPase in *G. pulex* acclimated to dechlorinated fresh water can be seen in figures 3. 2. 1. to 3. 2. 6. The dose response curve illustrates the effects of ouabain on gill Na^+, K^+-ATPase activity (Fig. 3. 2. 1.). Ouabain a specific inhibitor of Na^+, K^+-ATPase showed a K_i value of 3.25 mM, which relates to the concentration of ouabain required to cause a 50% inhibition of the enzyme. Maximum enzyme inhibition was reached at 10 mM ouabain. Enzyme activity with optimum ion concentrations and 10mM ouabain was comparable with the activity found when Na^+ and K^+ were absent from the assay solution. Therefore, 10 mM ouabain was taken as the concentration causing 100% inhibition of gill Na^+, K^+-ATPase. In *G. duebeni,* 10mM ouabain was previously found to cause complete inhibition of gill Na^+, K^+-ATPase (Lloyd Mills, pers. com.).

The effect of sodium concentration in the assay solution on the activity of gill Na^+, K^+-ATPase for *G. pulex* can be seen in figure 3. 2. 2. Highest levels of gill Na^+, K^+-ATPase activity were found at 100 mM sodium. Enzyme activity decreased at sodium concentrations greater than 100mM sodium, approaching 50% maximum activity levels at sodium concentrations of 200 mM and greater. Very low levels of activity were recorded when sodium was absent from the assay solution.

Gill Na^+, K^+-ATPase showed typical Michaelis-Menten kinetics for the range of potassium concentrations in the assay solution (Fig. 3. 2. 3). The K_m value (the concentration of the external medium at which the enzyme is 50% saturated) was 3.85 mM, with 100% saturation of the enzyme at concentrations greater than 15 mM potassium. For magnesium (Fig. 3. 2. 4) the K_m value was 3.2 mM. Maximum enzyme activity was found at

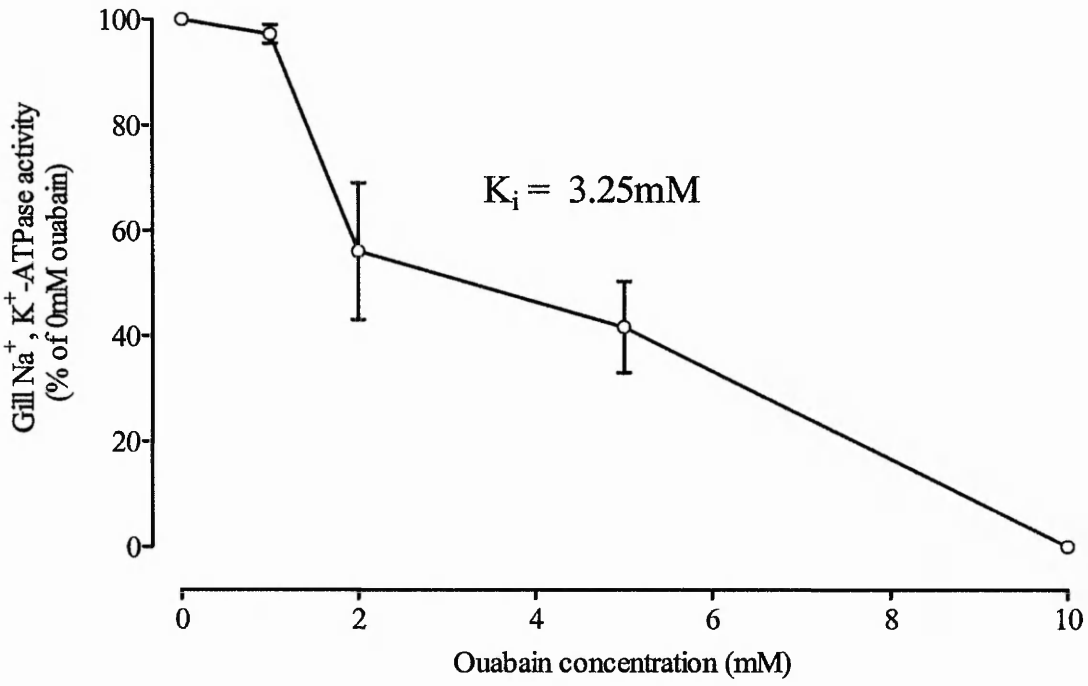


Figure 3. 2. 1. Effect of ouabain on gill Na^+, K^+-ATPase activity in *G. pulex* (mean ± SE, n=3). K_i - concentration causing 50% inhibition of the enzyme.

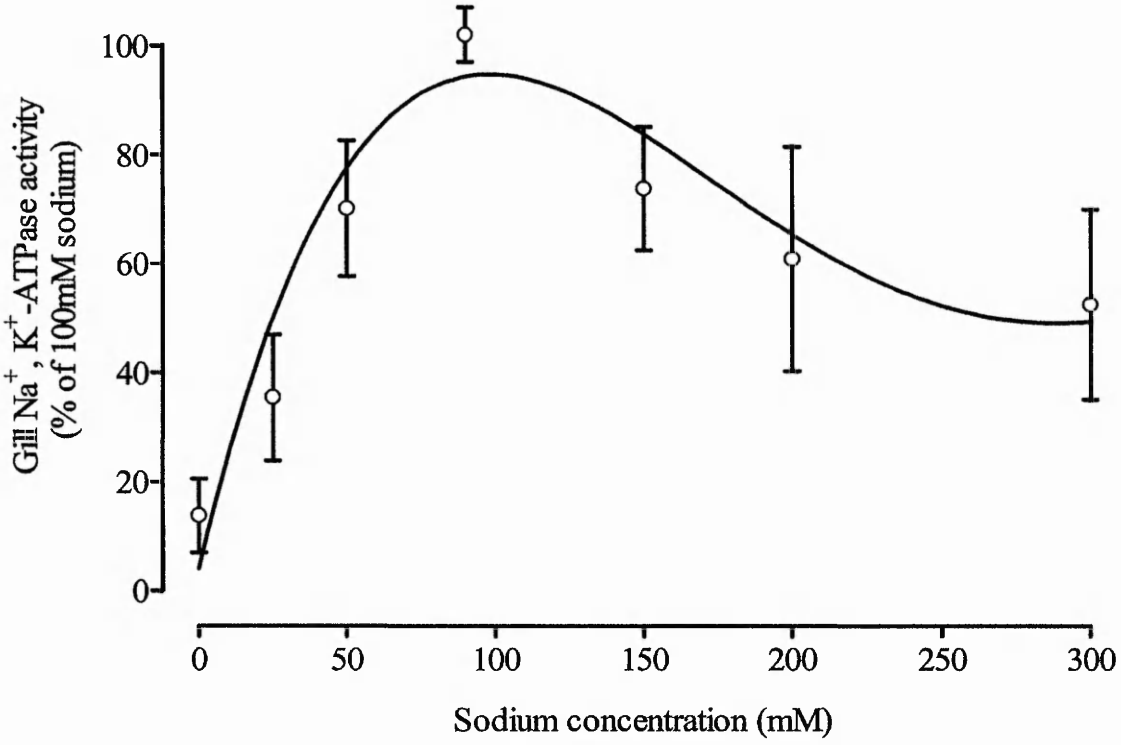


Figure 3. 2. 2. The effect of sodium on gill Na^+, K^+-ATPase activity in *G. pulex* (mean ± SE, n=3, for 50, 100 & 150mM Na n =5).

approximately 10 mM magnesium, with activity decreasing at concentrations greater than this. Gill Na^+, K^+-ATPase activity was undetected when magnesium was absent from the assay solution.

As observed for potassium, gill Na^+, K^+-ATPase showed typical Michaelis-Menten kinetics for the range of ATP concentrations in the assay solution (Fig. 3. 2. 5). A K_m value of 0.37 mM ATP was recorded, with gill Na^+, K^+-ATPase activity becoming saturated at concentrations approaching 1.75 mM ATP. Gill Na^+, K^+-ATPase had a pH optimum of 7.2 (Fig. 3. 2. 6.).

Characterisation of gill Na^+, K^+-ATPase activity with respect to sodium concentration was determined for *G. tigrinus*, *G. duebeni celticus* and the Northern Ireland population of *G. pulex* (Fig. 3. 2. 7.) Maximum gill Na^+, K^+-ATPase activity for all 3 gammarids was found at 100 mM sodium, identical to that found for *G. pulex* (Creswell population, Fig. 3. 2. 2), and therefore 100mM sodium was used in all subsequent gill Na^+, K^+-ATPase assays.

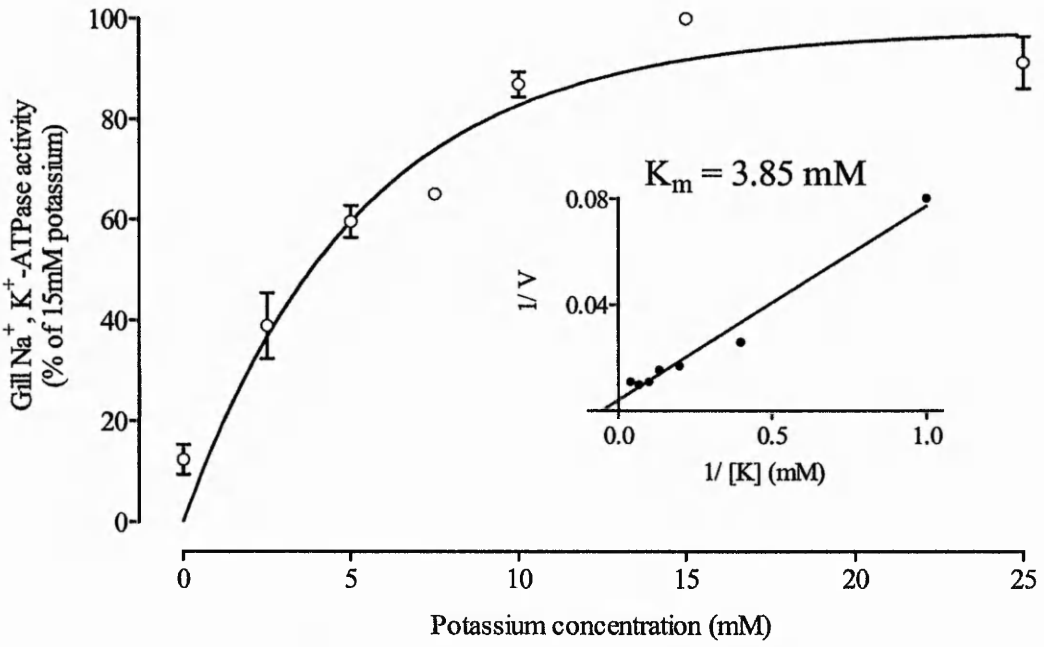


Figure 3. 2. 3. The effect of potassium on gill Na^+, K^+-ATPase activity in *G. pulex* (mean ± SE, n=3). K_m - concentration corresponding to 50% activity, determined from the Lineweaver-Burk plot (insert).

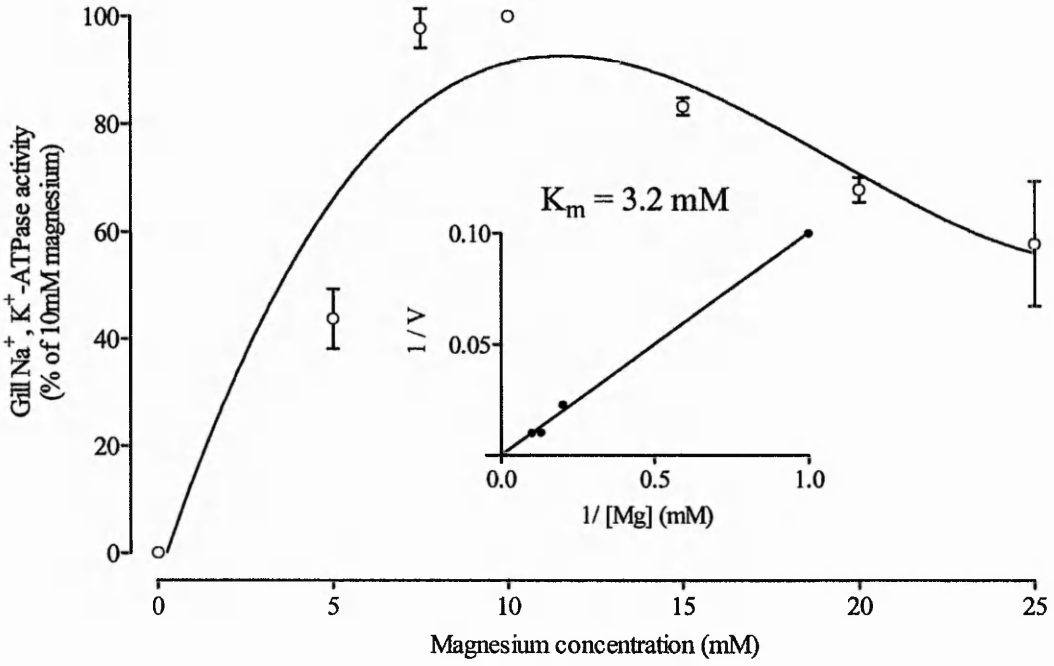


Figure 3. 2. 4. The effect of magnesium on gill Na^+, K^+-ATPase activity in *G. pulex* (mean ± SE, n=3). K_m - concentration corresponding to 50% activity, determined from the Lineweaver-Burk plot (insert).

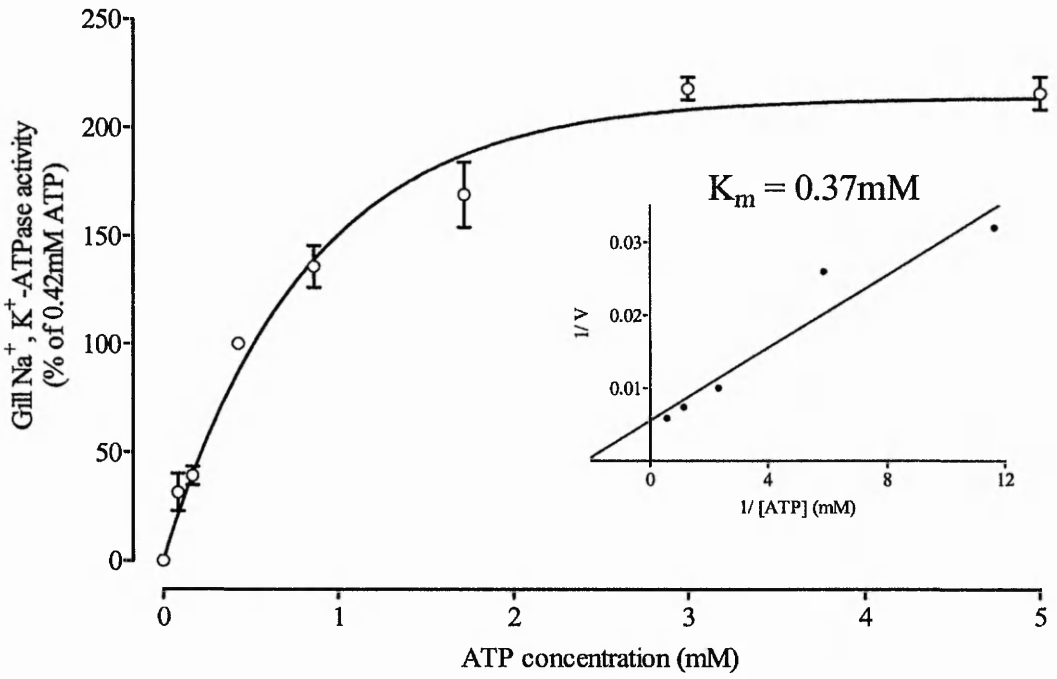


Figure 3. 2. 5. The effect of disodium ATP on gill Na^+, K^+-ATPase activity in *G. pulex* (mean ± SE, n=3). K_m - concentration corresponding to 50% activity, determined from the Lineweaver-Burk plot (insert).

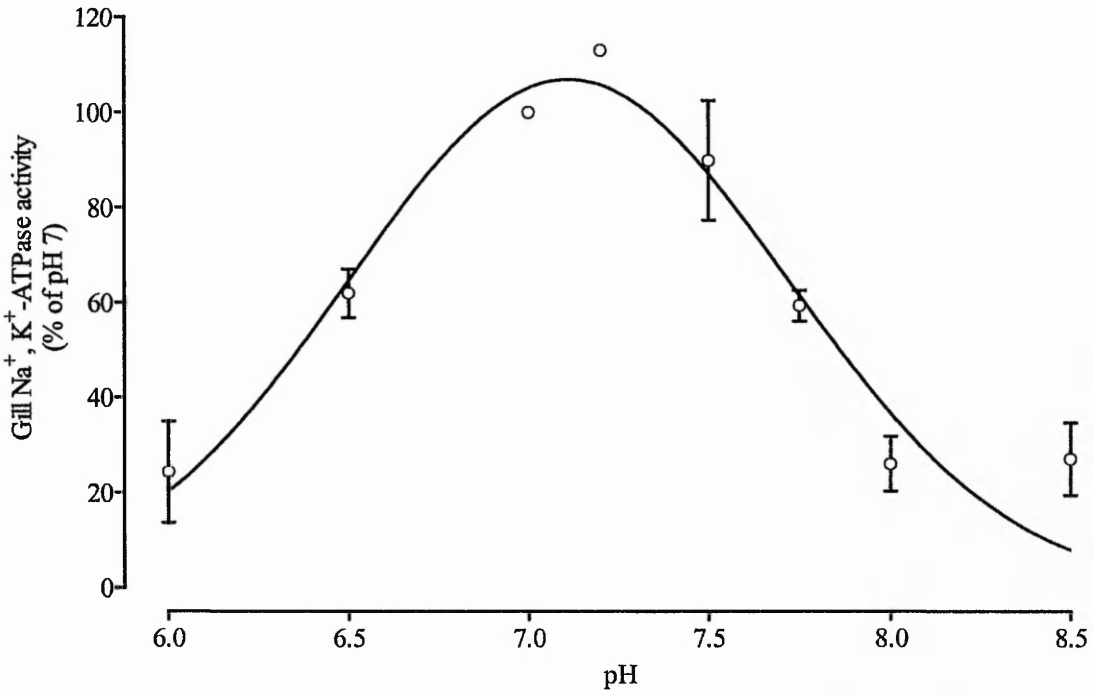


Figure 3. 2. 6. The effect of pH on gill Na^+, K^+-ATPase activity in *G. pulex* (mean ± SE, n=3).

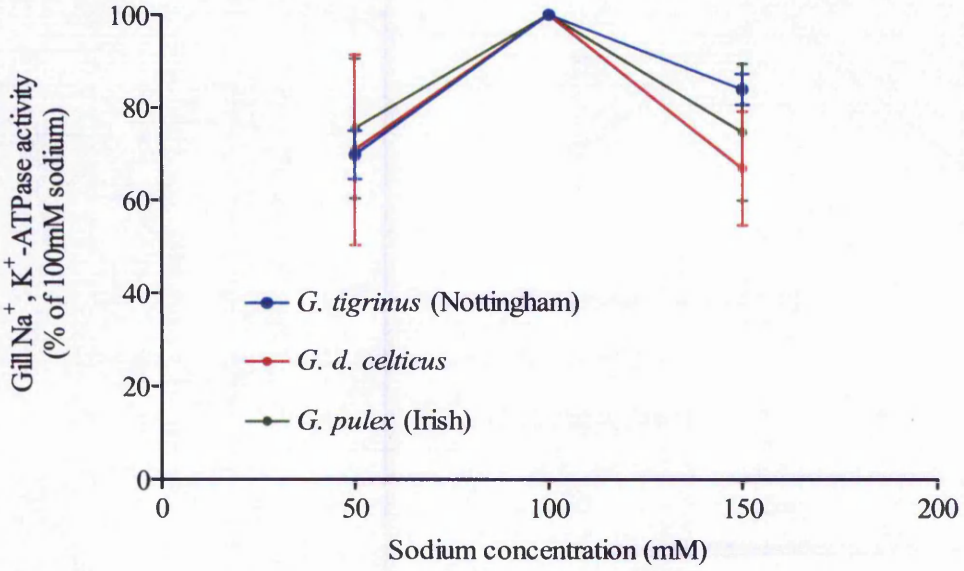


Figure 3. 2. 7. The effect of sodium on gill Na^+, K^+-ATPase activity in three species of gammarid (mean ± SE, n=3).

3. 2. 2. The effects of salinity on gill Na^+, K^+-ATPase activity

The effects of 5 day salinity acclimation on gill Na^+, K^+-ATPase activity in gammarids from fresh, brackish and sea water environments can be seen in figures 3. 2. 8. to 10. Highest levels of gill Na^+, K^+-ATPase activity were found for all freshwater gammarids when acclimated to fresh water (Fig. 3. 2. 8.). Gill Na^+, K^+-ATPase activity decreased with increased salinity in all gammarids. In *G. d. celticus*, a significant decrease in gill Na^+, K^+-ATPase activity, from fresh water levels, was found at 25% sea water ($p<0.05$). No further significant reduction in gill Na^+, K^+-ATPase activity was seen until *G. d. celticus* was acclimated to 100% sea water. In *D. villosus*, significant reduction in gill Na^+, K^+-ATPase activity, from fresh water levels, was found at 15% sea water ($p<0.05$), levelling out between 25% and 50% sea water. For the three *G. pulex* populations no significant difference in gill Na^+, K^+-ATPase activity was found between fresh water and 15% sea water. A Significant reduction in gill Na^+, K^+-ATPase activity was seen on acclimation to 30% sea water in all three *G. pulex* populations ($p<0.05$).

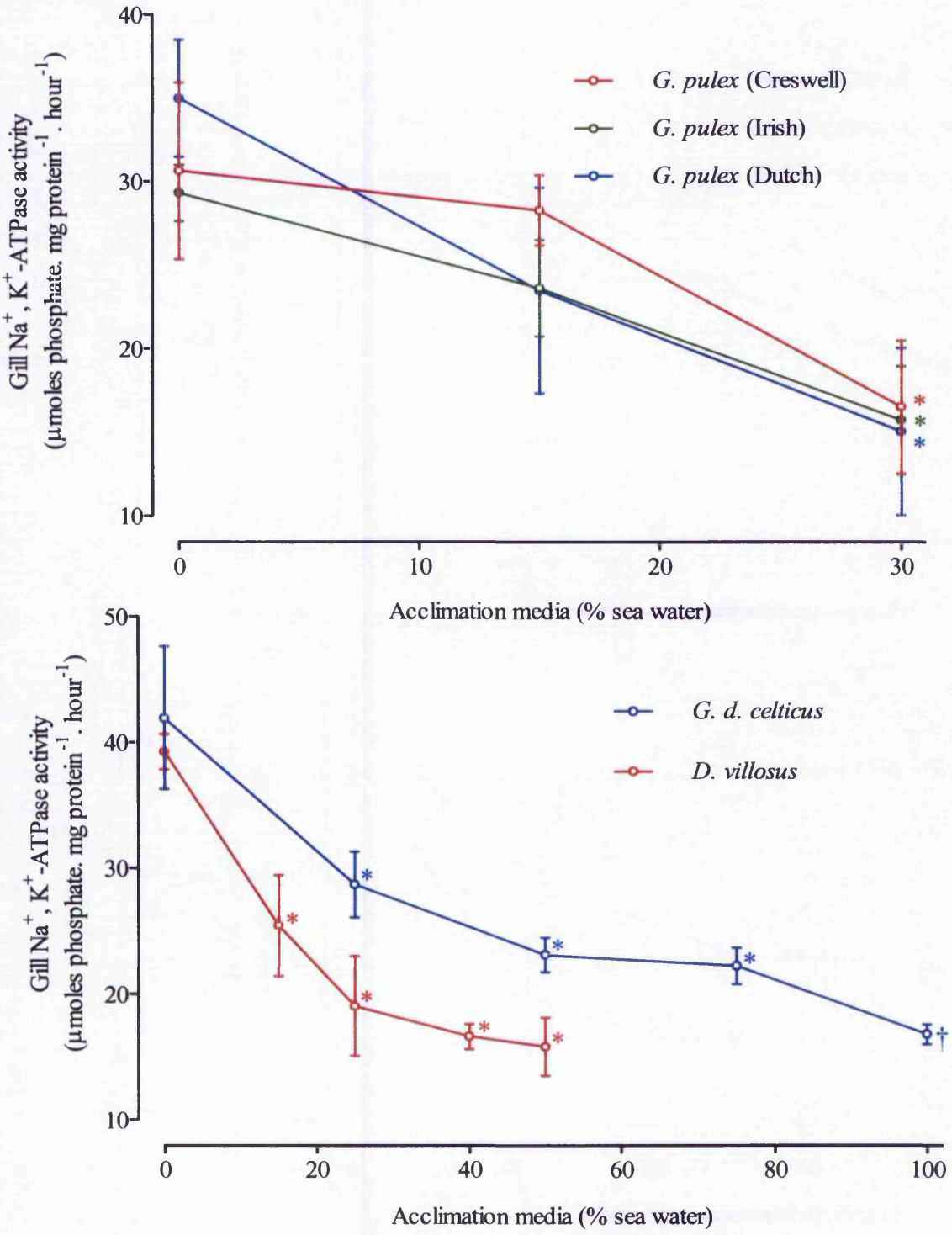
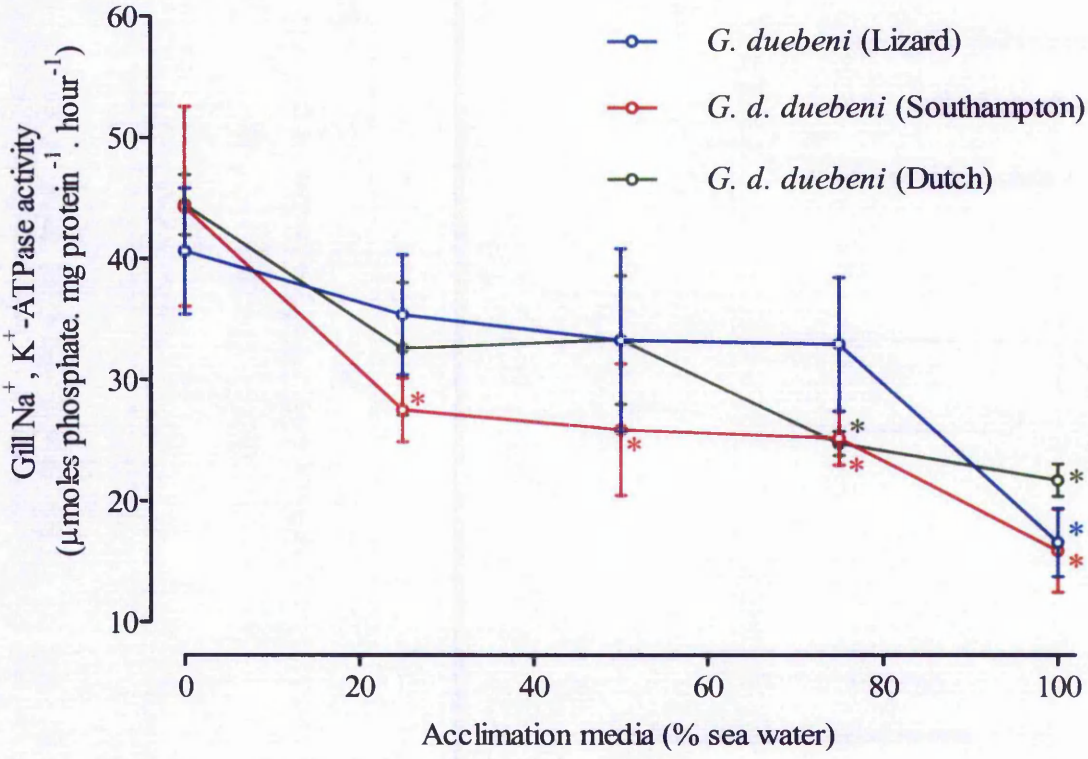


Figure 3. 2. 8. The effects of acclimation salinity on gill Na^+, K^+-ATPase activity in freshwater gammarids (mean ± SE, n=3). * significant difference from activity at 0% sea water, $p<0.05$. † significant difference from activity at 75% sea water and less. Symbol colour corresponds to line colour.

In the brackish water gammarids (Fig. 3. 2. 9.), gill Na^+, K^+-ATPase activity decreased with increased salinity with highest activity levels in fresh water. In *G. tigrinus* a significant reduction in gill Na^+, K^+-ATPase activity was found on acclimation to 50% sea water. Acclimation of *G. tigrinus* to 100% sea water was required to cause a further significant reduction in gill Na^+, K^+-ATPase activity. A significant reduction in gill Na^+, K^+-ATPase activity, from fresh water levels, was seen *G. zaddachi* and *G. d. duebeni* (Southampton population) when acclimated to 25% sea water. In contrast, no significant reduction in gill Na^+, K^+-ATPase activity was seen in *G. d. duebeni* (Dutch population) or *G. duebeni* (Lizard population) until acclimated to 75% and 100% sea water respectively.

In the marine gammarids (Fig. 3. 2. 10.), gill Na^+, K^+-ATPase activity decreased with increased salinity with highest activity levels at the most dilute sea water concentration (25%). There was no significant difference in gill Na^+, K^+-ATPase activity between *C. maenas* and *G. locusta* at 25% or 100% sea water ($p>0.05$).

Gill Na^+, K^+-ATPase activity in all fresh and brackish water gammarids following 5 day acclimation to dechlorinated fresh water was plotted together to enable easy comparison (Fig. 3. 2. 11). Highest gill Na^+, K^+-ATPase activity was found in the brackish water *G. tigrinus* (60.1 ± 4.8 μmoles phosphate. mg $protein^{-1}$. hr^{-1}), which with exception to *G. zaddachi* was significantly higher than all other gammarids ($p<0.05$). Gill Na^+, K^+- ATPase activity in fresh water acclimated *G. tigrinus* was approximately 50% higher than that of the *G. pulex* populations, and 25% higher than the *G. duebeni* populations. Furthermore, gill Na^+, K^+-ATPase activity for *G. zaddachi* was significantly higher than the three *G. pulex* populations ($p<0.05$). Gill Na^+, K^+-ATPase activities for the remaining brackish water gammarids (*G. d. duebeni* & *G. duebeni*) were not significantly different from the freshwater gammarids when acclimated to fresh water.



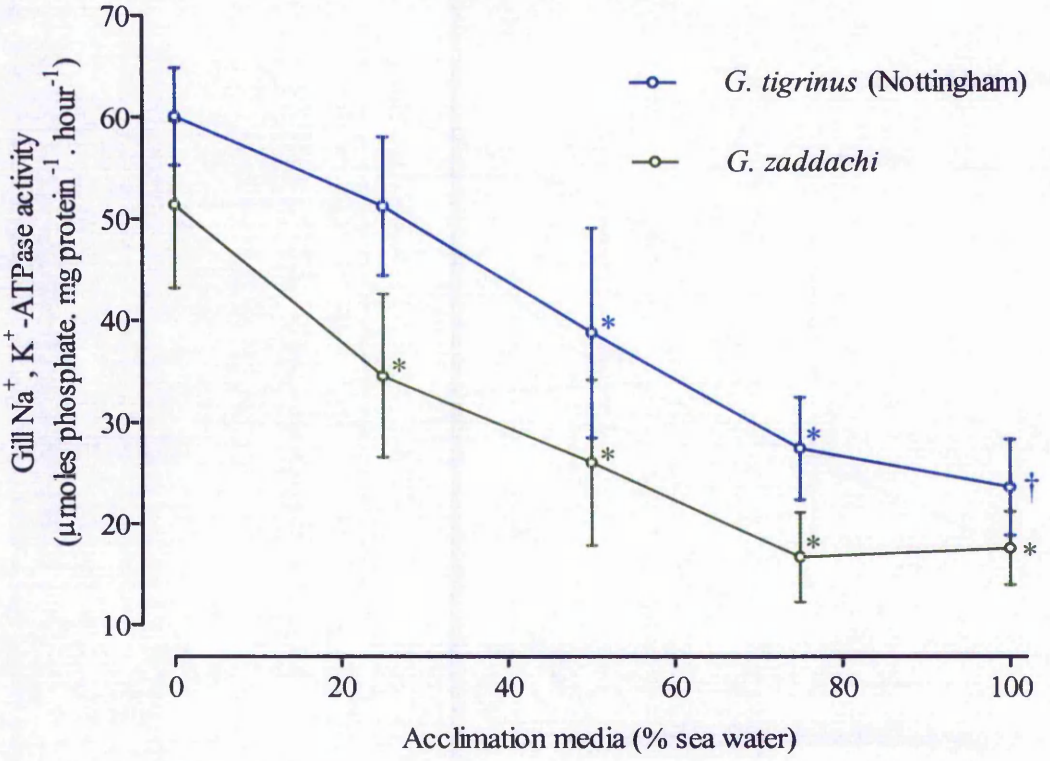


Figure 3. 2. 9. The effects of acclimation salinity on gill Na^+, K^+-ATPase activity in brackish water gammarids (mean ± SE, n=3).* significant difference from activity at 0% sea water, $p<0.05$. † significant difference from 50% sea water and less. Symbol colour corresponds to line colour.

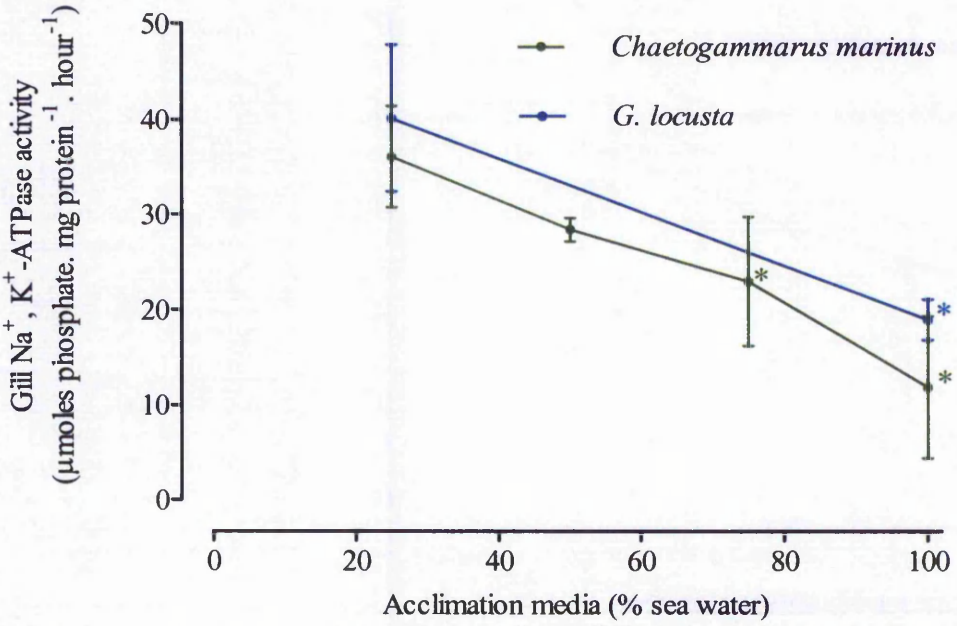


Figure 3. 2. 10. The effects of acclimation salinity on gill Na^+, K^+-ATPase activity in two seawater gammarids (mean ± SE, n=3). * significant difference from activity at 25% sea water, $p<0.05$. Star colour corresponds to line colour.

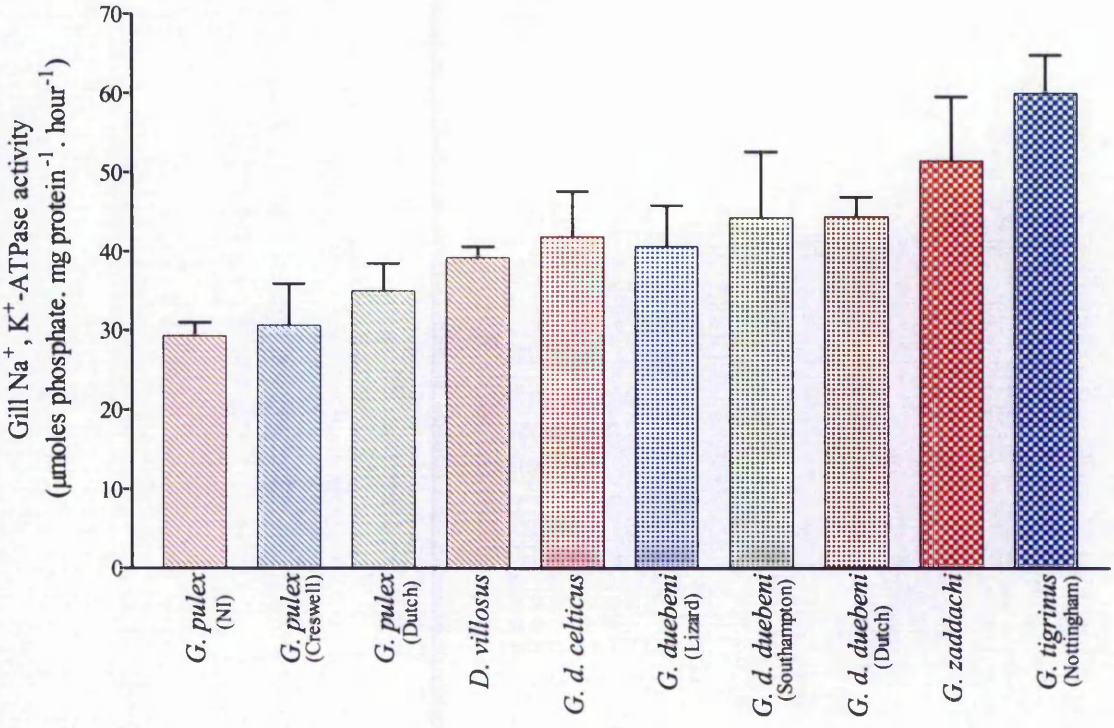


Figure 3. 2. 11. Gill Na^+, K^+-ATPase activity in gammarid species from fresh, brackish and sea water habitats after acclimated for 5 days in dechlorinated fresh water (mean ± SE, n=3).

3. 2. 3. Salinity effects on haemolymph sodium concentration

The regulation of haemolymph sodium concentration with respect to external salinity in all gammarids investigated can be seen in figure 3. 2. 12. All gammarids maintain haemolymph sodium concentrations hyperosmotic when in dilute media and approach isosmoticity between 25 and 50% sea water. The hyperosmotic gradient between the haemolymph and the external media was highest in fresh water media for the fresh and brackish water gammarids and at 25% sea water in the marine gammarids, as they were unable to survive acclimation to more dilute media.

3. 2. 4. Salinity effects on gill Mg^{2+} ATPase activity

The effects of 5 day salinity acclimation on gill Mg^{2+} ATPase activity in gammarids from fresh, brackish and sea water environments can be seen in figure 3. 2. 13. In contrast to Na^+, K^+-ATPase, salinity had no general effect on gill Mg^{2+} ATPase activity in gammarids, but remained relatively constant throughout the salinity acclimation range.

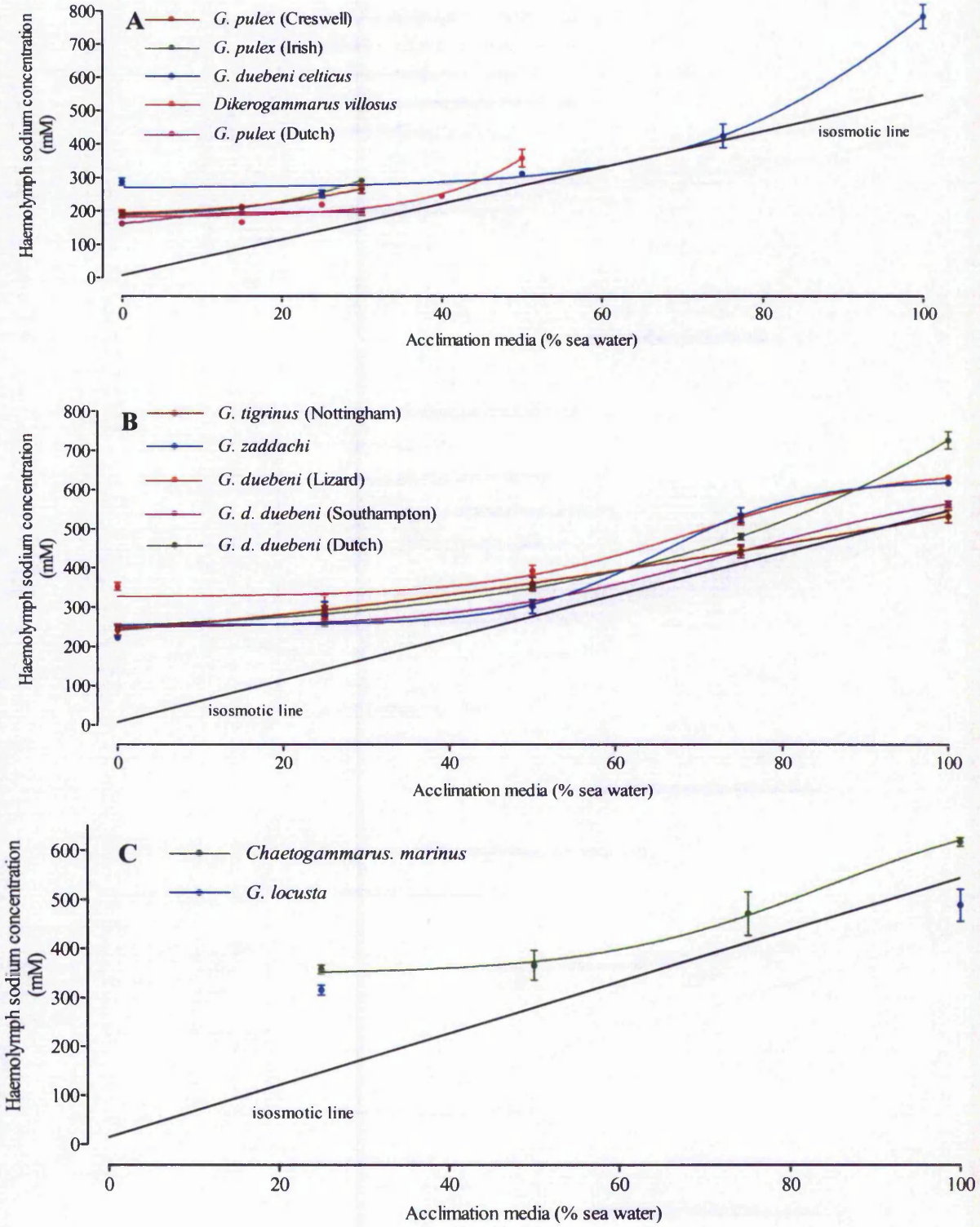


Figure 3. 2. 12. The effects of acclimation salinity on haemolyph sodium concentration in gammarids from freshwater (A), brackish water (B) and seawater (C) habitats (mean ± SE, n=5).

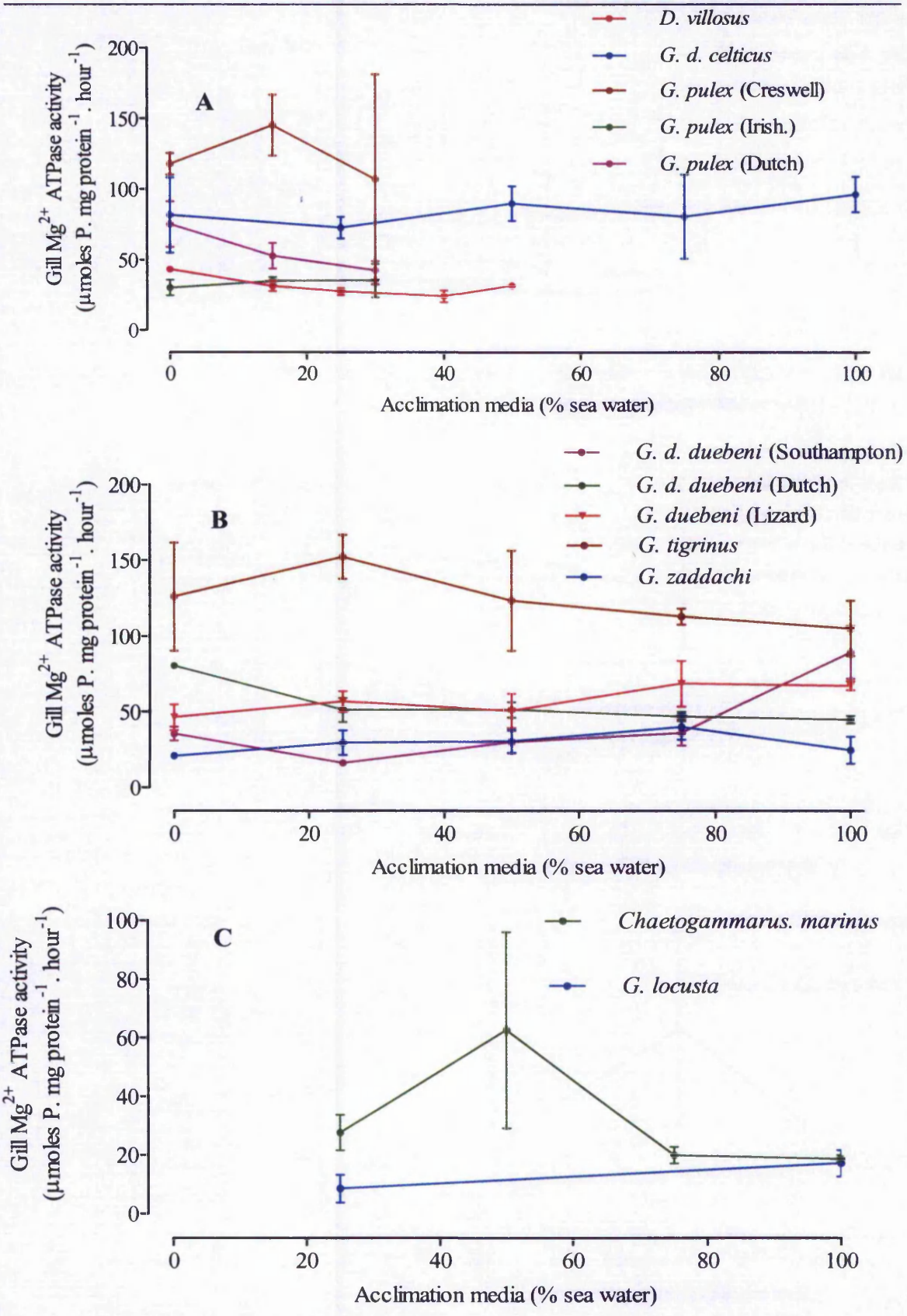


Figure 3. 2. 13. The effects of acclimation salinity on gill Mg^{2+} ATPase activity in fresh water (A), brackish water (B) and sea water (C) species of gammarid (mean ± SE, n=3).

3. 3. Discussion

3. 3. 1. Characterisation of gill Na^+, K^+-ATPase in *G. pulex*

Despite the increasing number of studies regarding the changes in gill Na^+, K^+-ATPase activity with variation in external salinity in crustaceans, few papers present data on the ion and cofactor requirements of this enzyme. Crustacea gill Na^+, K^+-ATPase whose ion and cofactor requirements have been published include, the purple shore crab *Hemigrapsus nudus* (Corotto & Holliday, 1996), the mud fiddler crab *Uca pugnax* (Holliday, 1985) the blue crab *Callinectes sapidus* (Neufeld *et al.,* 1980) and the brine shrimp *Artemia salina* (Holliday *et al.,* 1990). Characterisation of gill Na^+, K^+-ATPase in *G. pulex* will be discussed in relation to these previous characterisation studies.

Ouabain was found to have a half-inhibition value (K_i) of 3.25 mM. This K_i value was much larger than those previously obtained in other crustaceans, such as *Hemigrapsus nudus* (95 μM, Corotto & Holliday, 1996), *Callinectes sapidus* (0.5 mM, Neufeld *et al.,* 1980) and *Uca pugnax* (0.08 mM, Holliday 1985). The high K_i value denotes the lack of binding affinity and thus reduced inhibition of gill Na^+, K^+-ATPase by ouabain, the reasons for which are not fully understood. Ouabain is thought to bind to the extracellular side of the system with binding and inhibition dependent on the ligands present (Glynn 1985). The compositions of ligands in gill membranes do vary to some extent between species, resulting in a difference in binding affinity. These potential differences in binding affinity may contribute to the reduced inhibition of gill Na^+, K^+-ATPase by ouabain. Furthermore, increased potassium concentrations in the incubation media have been thought to partly reduce inhibition of gill Na^+, K^+-ATPase by ouabain in animal cells (Albers, 1976). However, the amount of potassium used in the assay solutions corresponds to values previously used in gill Na^+, K^+-ATPase assays of other crustaceans and is unlikely to have caused the differences found in this study.

One ouabain molecule binds per enzyme molecule (Glynn, 1985), consequently a greater number of enzyme molecules present in the gills of *G. pulex* may be responsible for the increased K_i value. Rather than speculate further, investigations to locate and quantify Na^+, K^+-ATPase in gammarid gills would prove useful.

Gill Na^+, K^+-ATPase activity was maximal at 100 mM sodium, with substrate inhibition occurring at sodium concentrations greater than this. The optimal sodium concentration for gill Na^+, K^+-ATPase activity in *G. pulex* was similar to that previously found in the hyperregulating shore crab *Carcinus maenas* (Siebers *et al.*, 1985). As external salinities increase, the haemolymph-external medium ionic gradient is reduced. In this case passive sodium efflux is minimal, hence the need for active sodium uptake to replace these lost ions is reduced. The strong substrate inhibition may represent the basic mechanism to provide an activity response of the enzyme to sudden environmental salinity changes (i.e. enzyme activity is reduced as the external environment and the haemolymph approach equilibrium).

Typical saturation kinetics were observed for potassium and substrate ATP. The K_m value for potassium (3.85 mM), was similar to that found in *Idotea wosnesenskii* (4 mM, Holliday, 1988) and *Uca pugnax* (2 mM, Holliday, 1985). For substrate ATP, the K_m value (0.37 mM) was also in a similar range to those observed in gill Na^+, K^+-ATPase of other crustaceans, such as *Hemigrapsus nudus* (0.6 mM, Corotto & Holliday, 1996), *Callinectes sapidus* (0.19mM Neufeld *et al.*, 1980), *Idotea wosnesenskii* (0.9mM, Holliday, 1988) and *Uca pugnax* (0.56 mM, Holliday, 1988).

The K_m value of magnesium (3.2 mM) was found to correspond to the values found in other crustaceans, such as *Hemigrapsus nudus,* (2.6mM, Corotto & Holliday, 1996) and *Uca pugnax* (3.33mM, Holliday, 1985). When the magnesium concentration was altered gill Na^+, K^+-ATPase of *G. pulex* did not show saturation kinetics, but instead increased

magnesium concentrations caused a reduction in enzyme activity. This substrate inhibition by magnesium, although not found in all crustacean gills, has been reported in the gills of *Uca pugnax* (Holliday, 1985) as well as the brine shrimp *Artemia salina* (Holliday *et al.,* 1990). The reasons why higher magnesium levels cause enzyme inhibition are unclear, particularly since enzyme inhibition only occurs in certain crustacean species and not others. The optimum Mg^{2+}/ ATP ratio for *G. pulex* gill Na^+, K^+-ATPase activity was approximately 2:1 at an ATP concentration of 5 mM. This ratio was also reported in other crustaceans, such as *U. pugnax* (Holliday, 1985) and *A. salina* (Holliday *et al.,* 1990; Ewing *et al.*, 1974).

The optimum pH for Na^+, K^+-ATPase activity was found at 7.2. This value is similar to that found in other crustaceans such as *Hemigrapsus nudus* (Corotto & Holliday, 1996) and identical to that of *Idotea wosnesenskii* (Holliday, 1988) and *Artemia salina* (Holliday *et al.,* 1990). It would be expected therefore, that gill Na^+, K^+-ATPase functions at a pH value close to 7.2 in the epithelial cells of *G. pulex* gills.

3. 3. 2. Gill Na^+, K^+-ATPase characterisation in other gammarids

The sodium activity profiles of gill Na^+, K^+-ATPase for *G. d. celticus*, *G. tigrinus* and *G. pulex* (Northern Ireland population) were identical to that found in *G. pulex* (Creswell population), with maximum enzyme activity at 100 mM sodium. Sodium is considered the most important ion of Na^+, K^+-ATPase (Albers, 1976). It was assumed that gill Na^+, K^+-ATPase activity of the other gammarids had similar activity profiles for the additional ion and cofactor requirements. This assumption allowed gill Na^+, K^+-ATPase activity to be compared between different gammarids.

3. 3. 3. The effects of salinity on gill Na^+, K^+-ATPase activity: comparison between gammarids from fresh, brackish and sea water habitats

It has been well established that gill Na^+, K^+-ATPase is the main driving force for active ion uptake in crustacean gills, with activity governed by the external sea water concentration (reviewed by Péqueux, 1995). In this study, gill Na^+, K^+-ATPase activity of all gammarids investigated was dependent upon the acclimation salinity. Maximum gill Na^+, K^+-ATPase activity occurred in all gammarids when acclimated to their most dilute sea water media. This maximum enzyme activity coincided with the maximum osmotic gradient maintained by all gammarids in the most dilute acclimation media, and implicates gill Na^+, K^+-ATPase involvement in the active uptake of sodium from dilute media in hyperregulating gammarids.

The significant reductions in gill Na^+, K^+-ATPase activity found in all gammarids investigated, coincided with their haemolymph sodium concentrations approaching isosmoticity with the external salinity. The smaller osmotic gradient found with increased salinity, decreases ion loss from the haemolymph. Such a decrease in ion loss reduces the need for the active uptake of ions facilitated by gill Na^+, K^+-ATPase.

When acclimated to fresh water, gill Na^+, K^+-ATPase activity in the freshwater species was generally lower than the brackish water gammarids. This was particularly the case in the freshwater *G. pulex* populations, which were significantly lower than the two euryhaline gammarids *G. tigrinus* and *G. zaddachi*. Although gill Na^+, K^+-ATPase activity in the *G. pulex* populations was approximately 25% lower than the populations and subspecies of *G. duebeni*, they were not found to be significantly different ($p>0.05$). This lack of statistical significant was attributed to the limited number of replicates used, due to the lack of availability of samples.

The lower gill Na^+, K^+-ATPase activity in the *G. pulex* populations corresponds with the maintenance of a lower osmotic gradient between the haemolymph and the external medium. Haemolymph sodium concentrations of the *G. pulex* populations were significantly lower than those of the brackish water gammarids. This significantly lower haemolymph sodium concentration exhibited by *G. pulex* populations indicates a lower osmotic gradient between the haemolymph and external medium. The maintenance of a lower osmotic gradient by *G. pulex* populations would reduce the energy required for active ion uptake. This may be an adaptation to fresh water, reducing energy expenditure on osmoregulation, and enabling *G. pulex* to expend more energy on growth and reproduction.

With the exception of *G. zaddachi*, the euryhaline amphipod *G. tigrinus* was found to exhibit significantly higher gill Na^+, K^+-ATPase activity than all the other gammarids studied when acclimated to fresh water. It was unclear why *G. tigrinus* exhibited such high enzyme activity, particularly over other brackish water species such as *G. d. duebeni*, since haemolymph sodium concentrations in *G. tigrinus* were not higher than those of the other brackish water gammarids. Replacement of sodium ions due to increased sodium loss from the haemolymph to the external media, when acclimated to fresh water, may provide a partial explanation. Since *G. tigrinus* are on average significantly smaller than the other brackish water gammarids studied, their relatively larger surface area: volume ratio may contribute to an increased sodium loss. This may result in an increase in the active sodium uptake of *G. tigrinus* despite similar haemolymph sodium concentrations to other brackish water gammarid species.

Gill Na^+, K^+-ATPase activity exhibited by the two marine amphipods *Chaetogammarus marinus* and *G. locusta* was highest in the most dilute salinity (25% sea water). Activity levels were comparable with those found for the brackish water species. Marine gammarids inhabit waters of almost constant salinity and are therefore likely to be poorly adapted to

dilute media. Investigations in to the structural integrity of gammarid gills have shown poorly developed microtubule systems, which are associated with the septate junctions in gammarid gills (Shires *et al.*, 1995). The microtubule system plays an important role in the mechanical buttressing of the septate junctions against disruption by osmotic stress. Consequently the poorly developed microtubule system found in marine gammarids, would lead to increased ion loss in hypo-osmotic media, which is responsible for the poor survival rate of marine crustacea in dilute media. The high rate of gill Na^+, K^+-ATPase activity exhibited by these marine gammarids in dilute media may be an attempt to balance the potentially higher ion loss.

3. 3. 4. The effects of salinity on gill Mg^{2+} ATPase activity

Overall, gill Mg^{2+} ATPase activity was not affected by acclimation salinity and remained relatively constant throughout the salinity acclimation range. This agrees with similar work carried out on the shore crab *C. maenas* (Siebers *et al.*, 1983). In the Southampton population of *G. d. duebeni* however, gill Mg^{2+} ATPase activity increased significantly when acclimated to 100% sea water. In contrast, gill Mg^{2+} ATPase activity in the Dutch populations of *G. d. duebeni* and *G. pulex* decreased with increased salinity. The reasons for these unexpected salinity effects on gill Mg^{2+} ATPase activity are unknown. The "gill Mg^{2+} ATPase" represents all gill ATPase activity except Na^+, K^+-ATPase. The majority of this ATPase is Mg^{2+} ATPase (Sola *et al.*, 1995), although other complexes such as Ca^{2+} ATPase may also be present. Differences in the relative proportions of these ATPases in the gill homogenates may influence the rate of phosphate liberation in the assay solutions.

In the gill homogenates of *C. maenas*, Mg^{2+} ATPase accounted for approximately 20% of the total ATPase activity (Siebers *et al.*, 1983). The proportions of Mg^{2+} and Na^+, K^+-ATPase activity differed markedly between the gammarids in this study. With the

exception of *G. locusta*, where Mg^{2+} ATPase was approximately half the activity of Na^+, K^+-ATPase, Mg^{2+} ATPase was equal to, or twice the activity of Na^+, K^+-ATPase in gill homogenates. The Mg^{2+} ATPase/ Na^+, K^+-ATPase ratio tended to increase with increased sea water acclimation in all gammarids studied. This was due to the reduction in Na^+, K^+-ATPase activity with increased salinity.

CHAPTER FOUR

Osmoregulation of Invasive species

Amphipod crustaceans have featured as invaders throughout the world, aided by human intervention through shipping, deliberate introductions to increase fish stocks and fish farming practices, and even during ecological experiments (Jazdzewski, 1980; Pinkster *et al.*, 1992; Dick *et al.*, 1997; Dick *et al.*, 1999). Such invasions have often been to the detriment of the native amphipod populations, with radical alterations in aquatic community structures (Conlan, 1994).

This chapter investigates osmoregulatory aspects of two separate amphipod invasions; 1) the relatively recent (since mid 1990s, Dick & Platvoet, 2000) invasion of the Ponto-Caspian amphipod *Dikerogammarus villosus* in the freshwater bodies of the Netherlands, and 2) the displacement of the native freshwater amphipod *G. d. celticus* by the invading freshwater species *G. pulex* in Northern Ireland. Comparisons of the osmoregulatory physiology of both native and invading amphipod species have been carried out to provide further understanding of the species interactions that take place.

4. 1. Osmoregulation of the invasive amphipod *Dikerogammarus villosus* in The Netherlands. Comparisons with endogenous species

4. 1. 1. Introduction

The rapid invasion of *Dikerogammarus villosus* from the Ponto – Caspian region into Western Europe has occured over the last ten years following the opening of the Main – Danube Canal in 1992 (van der Velde *et al.*, 2000). The construction of the Main - Danube Canal allowed important industrial links to be made between the waterways of Eastern and Western Europe. However, in so doing, links were also established between the aquatic ecosystems of these two areas. This has aided the geographical distribution of the amphipod *D. villosus* to the detriment of many native species (Nesemann *et al.*, 1995). In 1994-5, *D. villosus* was found in the River Rhine at the German/ Dutch border (Bij de Vaate & Kinke, 1995) and is currently migrating thorough the waters of The Netherlands (Dick & Platvoet, 2000) and France (Devlin *et al.*, 2001). It is predicted that *D. villosus* will invade further reaches of continental Europe, the British Isles, and even the Great Lakes of North America, *via* the ballast water of transoceanic ships (Dick & Platvoet, 2001).

The presence of *D. villosus* in the water bodies of The Netherlands has coincided with the disappearance and/ or reduction of the native *G. duebeni duebeni* and the invading North American amphipod *G. tigrinus* (Dick & Platvoet, 2000; 2001; van der Velde *et al.*, 2000). The successful establishment of *D. villosus* in the waters of Western Europe has been attributed to its predatory appetite (Dick & Platvoet, 2000), as well as its dispersal behaviour and high fecundity (van der Velde *et* al., 2000). Such attributes have allowed *D. villosus* to overcome any biotic resistance of new host communities (Dick & Platvoet, 2001).

4. 1. 1. 1. Predatory interactions

Laboratory microcosm experiments have been used to assess the predatory interactions between the invading *D. villosus* (Fig. 1. 5. 2.) and two previously dominant amphipods *G. d. duebeni* (Fig. 1. 5. 4.) and *G. tigrinus* (Fig. 1. 5. 3.) (Dick & Platvoet, 2000; Dick *et al.*, 2002). Through these experiments high frequencies of predation by male *D. villosus* on female *G. d. duebeni* were found. In general, most predatory interactions between crustaceans occur during the approximately 12 hour post-moult period (Dick, 1989), when the exoskeleton is shed exposing the soft flesh (Dick, 1996a; MacNeil *et al.*, 1997). However, due to its predatory appetite and larger mouthparts, *D. villosus* was able to break through the harder exoskeleton of intermoult female *G. d. duebeni*. Consequently, predation of female *G. d. duebeni* by male *D. villosus* was not restricted to animals in post moult. Similar interactions were also found between male *D. villosus* and female *G. tigrinus* (Dick & Platvoet, 2000). These strong predatory interactions in favour of *D. villosus*, coupled with its high fecundity, appear to explain the replacement of both *G. d. duebeni* and *G. tigrinus* with this invading amphipod.

In The Netherlands, the physical size of *D. villosus* (up to 30 mm, Dick & Platvoet, 2000; personal observations) provides it with a predatory advantage over the other Dutch gammarids. However, in the French waters of the Moselle River, *D. villosus* are approximately half the size of the Dutch population, with a maximum size of 15mm in length. In the Moselle River, *D. villosus* were found to co-occur with *G. pulex* and *G. tigrinus* (Devlin *et al.*, 2001). No investigation, has so far, been reported on the interactions between these French populations. Whilst it might be expected that *D. villosus* would replace these two populations, it would be interesting to determine whether the reduced size of *D. villous* reduces its predatory advantage.

4. 1. 1. 2. Physiology of *Dikerogammarus villosus*

The possibility of global dispersal of *D. villosus, via* ballast water of ocean going vessels, has encouraged investigations of the physiological tolerance of this species to a variety of environmental variables, including salinity, temperature and oxygen levels (van der Velde *et al.*, 2000; Brujis *et al.*, 2001). With regard to salinity tolerance, *D. villosus* occupies habitats ranging between fresh water and 10 salinity (Jazdzewski & Konopacka, 1988). However, through gradual acclimation *D. villosus* can tolerate up to 20 salinity, although high mortalities occur at 25 salinity and greater (Brujis *et al.*, 2001). It was assumed that *D. villosus* could survive sudden increases in external salinity provided the salinity does not exceed 20 (Brujis *et al.*, 2001). This led the authors to suggest that *D. villosus* may be able to survive incomplete ballast water exchange and subsequently be dispersed over large distances.

Apart from the observation of the salinity tolerance range of *D. villosus*, no information is yet available on the osmoregulation of this invasive amphipod. Information on the osmoregulatory abilities of *D. villosus* would therefore be useful to further the understanding of this important invasive species. Since osmoregulation in dilute media is an energy demanding process, differences in the osmoregulatory capabilities between gammarid species may provide one with an energetic advantage over another. Such an energetic advantage might influence the competitive interactions that take place between animals that occupy similar habitats. The aim of this study is to provide information on the osmoregulation of the invasive species *D. villosus*. In addition, information on the osmoregulation of three competing amphipods, *G. duebeni duebeni*, *G. tigrinus* and *G. pulex* from The Netherlands is shown.

4. 1. 2. Results

Only limited numbers of *G. tigrinus* were collected from the Netherlands. Consequently, information was not obtained for *G. tigrinus* acclimated to 100% sea water. In addition, measurements of gill enzyme activity and rapid transfer experiments were not possible in this species. Furthermore, rapid transfer experiments were not carried out for the Dutch population of *G. pulex* due to the limited numbers available.

4. 1. 2. 1. Haemolymph ion concentrations

Sodium

Haemolymph sodium concentrations were maintained hyperosmotic in all species when acclimated to fresh and oligohaline waters (Fig. 4. 1. 2. 1.). Isosmoticity was approached at approximately 30% and 40% sea water in *G. pulex* and *D. villosus* respectively, and approximately 50% sea water in the more euryhaline amphipods *G. tigrinus* and *G. d. duebeni*. In fresh water, the haemolymph sodium concentrations of the four species were significantly different from each other (*G. d. duebeni* > *G. tigrinus* > *D. villosus* > *G. pulex*, $p<0.05$).

Potassium

Haemolymph potassium concentrations were maintained strongly hyperosmotic in all four species throughout their salinity acclimation ranges (Fig. 4. 1. 2. 2.). No significant difference was found between the species when acclimated to fresh water. Similar haemolymph potassium profiles were displayed by *G. tigrinus* and *G. d. duebeni*, haemolymph concentration gradually increased with increased salinity. In contrast, *G. pulex* displayed a sharp increase in haemolymph potassium concentration when acclimated to 15% and 30% sea water. Unlike these other gammarids, *D. villosus* maintained relatively stable haemolymph potassium concentrations up to 40% sea water. However, in 50% sea

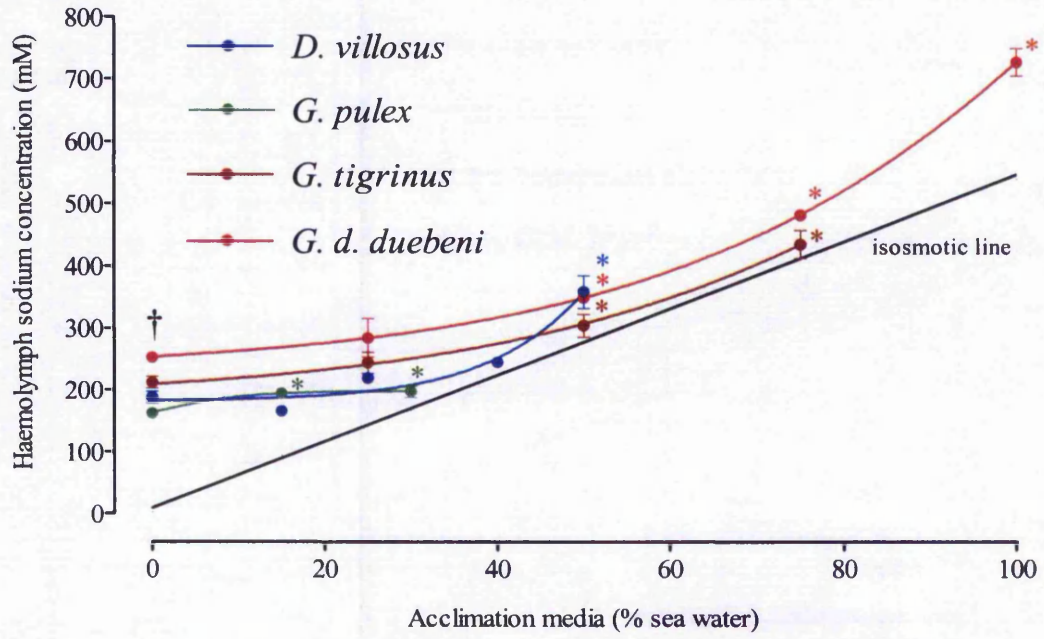


Figure 4. 1. 2. 1. Haemolymph sodium concentration in four species of gammarid collected from the Netherlands, following a five day acclimation to media of increasing sea water concentration. (mean ± SE, n =5). * Significant difference from freshwater values, $p<0.05$ (star colour corresponds to line colour); † significant difference between species in fresh water, $p<0.05$.

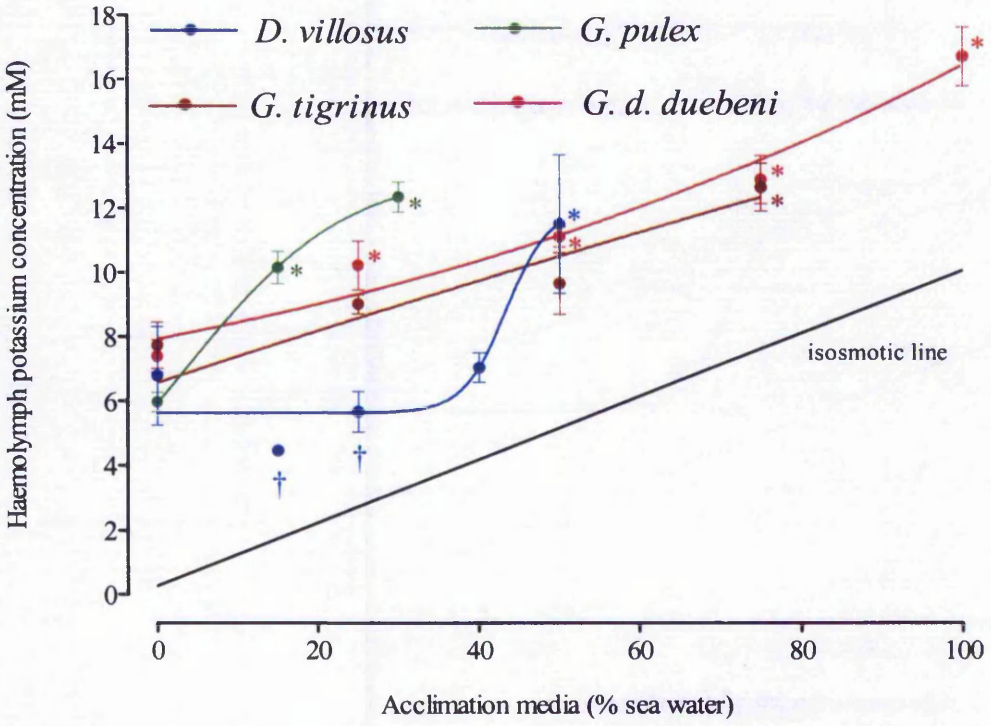


Figure 4. 1. 2. 2. Haemolymph potassium concentration in four species of gammarid collected from the Netherlands, following a five day acclimation to media of increasing sea water concentration. (mean ± SE, n =5). * Significant difference from freshwater values, $p<0.05$; † significant difference between species, $p<0.05$ (star and cross colours correspond to line colours).

water, a sharp increase in haemolymph concentration was shown, giving haemolymph potassium concentrations similar to those of *G. tigrinus* and *G. d. duebeni*.

Magnesium

Haemolymph magnesium concentrations were maintained hyperosmotic in fresh water and hypo-osmotic in concentrations greater than 15% sea water (Fig. 4. 1. 2. 3). In fresh water, haemolymph magnesium concentrations were maintained significantly higher in *D. villosus* (9.1 ± 0.9 mM) and significantly lower in *G. d. duebeni* (5.1 ± 0.5 mM, $p<0.05$) than the other two species. With exception to *G. pulex*, haemolymph magnesium was maintained at relatively constant levels in all gammarids despite increases in external salinity, until significant increases in haemolymph magnesium were shown at 50%, 75% and 100% sea water in *D. villosus*, *G. tigrinus* and *G. d. duebeni* respectively.

Calcium

Haemolymph calcium was maintained hyperosmotic in all species throughout the salinity acclimation range (Fig. 4. 1. 2. 4.). In fresh water, haemolymph calcium concentrations were maintained significantly higher in *G. d. duebeni* (20.9 ± 0.9 mM) and significantly lower in *D. villosus* (10.9 ± 1.6 mM, $p<0.05$) than the other two species. In *D. villosus*, haemolymph calcium was maintained relatively stable, significantly lower than the other three gammarids between fresh water and 40% sea water. A rapid increase in haemolymph calcium was shown when *D. villosus* was acclimated to 50% sea water, corresponding with similar increases in the other haemolymph cation concentrations at this sea water concentration. A sharp increase in haemolymph calcium was shown between fresh water and 15% sea water in *G. pulex*. Similar increases by *G. pulex* were shown for the other haemolymph cations between fresh water and 15% sea water.

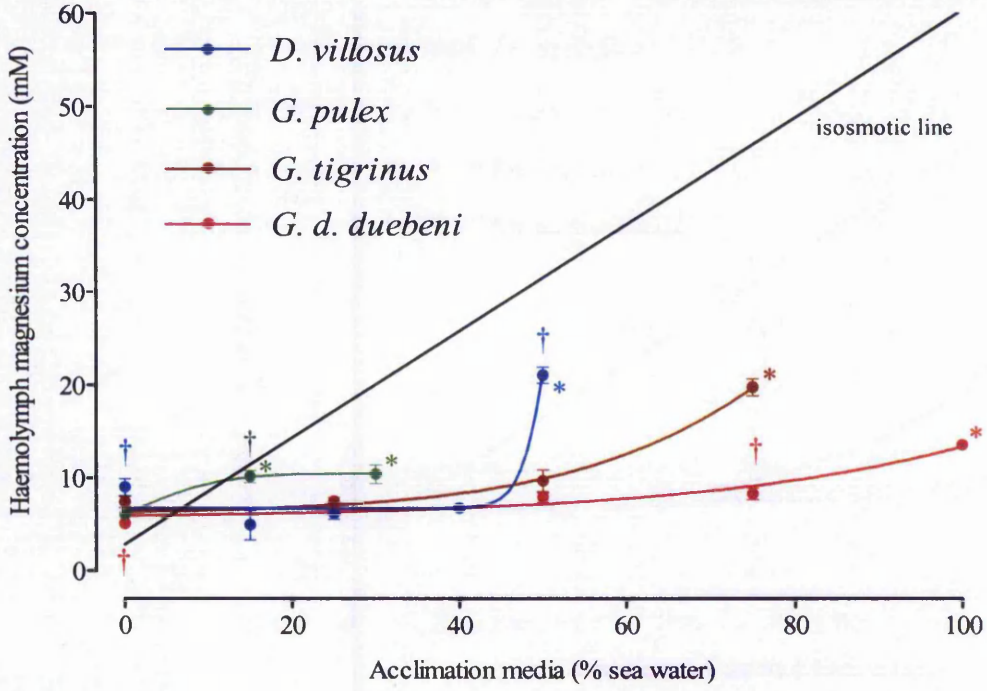


Figure 4. 1. 2. 3. Haemolymph magnesium concentration in four species of gammarid collected from the Netherlands, following a five day acclimation to media of increasing sea water concentration. (mean ± SE, n =5). * Significant difference from freshwater values, $p<0.05$; † significant difference between species, $p<0.05$ (star and cross colours correspond to line colours).

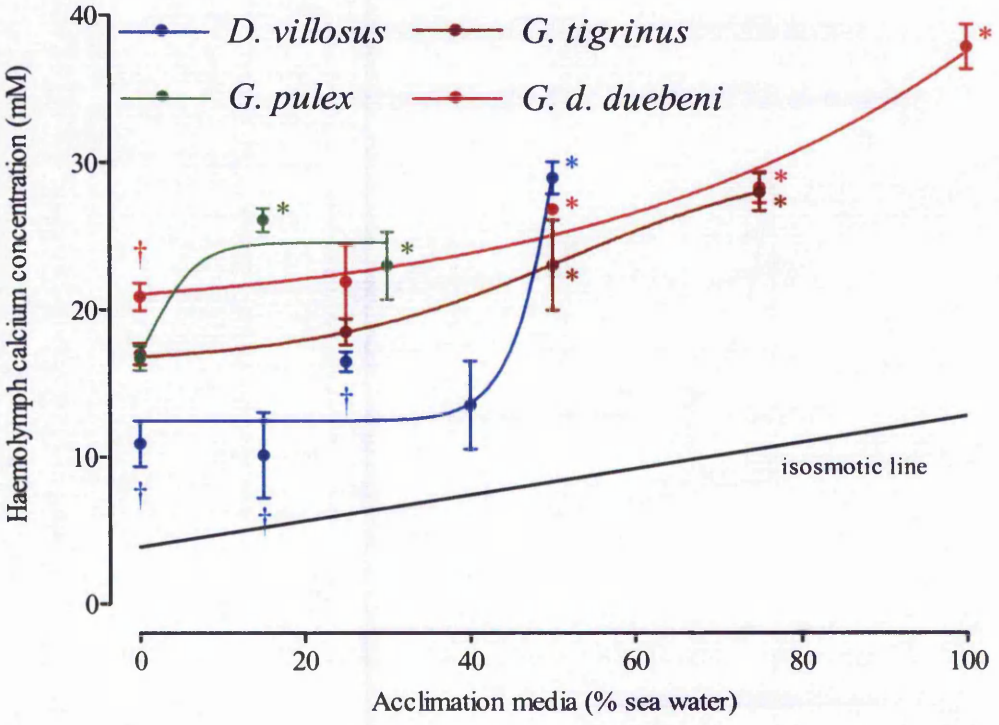


Figure 4. 1. 2. 4. Haemolymph calcium concentration in four species of gammarid collected from the Netherlands, following a five day acclimation to media of increasing sea water concentration. (mean ± SE, n =5). * Significant difference from freshwater values, $p<0.05$; † significant difference between species, $p<0.05$ (star and cross colours correspond to line colours).

4. 1. 2. 2. Water permeability and sodium flux

In fresh water, *G. pulex* displayed a significantly lower $t_{1/2}$ than the other three gammarids (Fig. 4. 1. 2. 5.). No significant difference was found in $t_{1/2}$ with increased sea water acclimation in *G.* pulex. In contrast, significant differences were found between the $t_{1/2}$ of the other three gammarids with increased salinity ($p<0.05$). A rapid decrease in $t_{1/2}$ was found to occur between 50% (15.2 ± 1.3 min) and 75% sea water (6.5 ± 0.6 min) in *G. d. duebeni*. In *D. villosus* a significant decrease in $t_{1/2}$ was found between fresh water (14.8 ± 0.5 min) and 15% sea water (11.6 ± 1.3 min) acclimation. No further reduction in $t_{1/2}$ was found in *D. villosus* with increased salinity up to 50% sea water acclimation. In *G. tigrinus*, $t_{1/2}$ decreased significantly from fresh water (12.9 ± 1.1 min) to 25% sea water (8.4 ± 0.9 min, $p<0.05$), a further significant reduction was found when acclimation to 75% sea water (5.9 ± 0.4 min, $p<0.05$) compared to values at 25% & 50% sea water ($p<0.05$).

The mechanisms of sodium influx in the brackish water *G. d. duebeni* become saturated at 11mM sodium (Sutcliffe, 1971a), whereas in the more freshwater *G. pulex* sodium influx is saturated at 1mM external sodium (Shaw & Sutcliffe, 1961). In the present study, no significant difference in sodium influx rate was found when 5.5 mM, 11 mM or 16.5 mM sodium was present in the loading medium (Table 4. 1. 2. 1.). Therefore, it was assumed that the sodium influx mechanism in *D. villosus* becomes saturated at less than 5.5 mM sodium. Consequently, the ^{22}Na loading solution containing nominal concentrations of 11 mM sodium was deemed suitable for the measurement of sodium influx in *D. villosus*. Sodium influx measurements for all gammarids investigated were determined with 11 mM sodium in the loading medium.

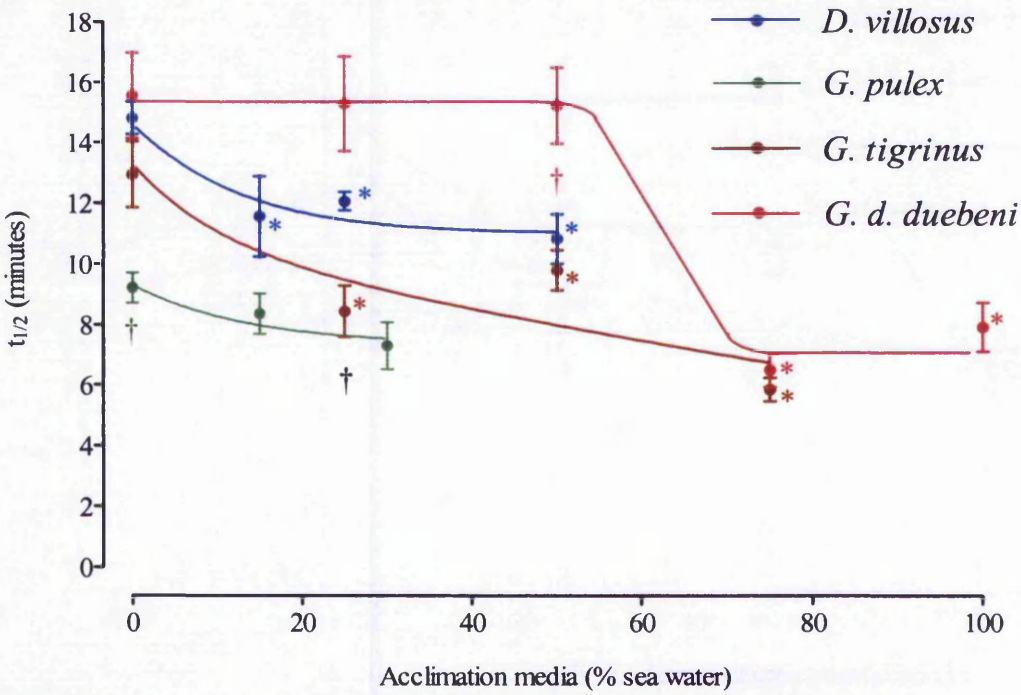


Figure 4. 1. 2. 5. Half-time of exchange of body water ($t_{1/2}$) in four species of gammarid collected from the Netherlands, following a five day acclimation to media of increasing sea water concentration. (mean ± SE, n =5). * Significant difference from freshwater values, $p<0.05$; † significant difference between species, $p<0.05$ (star and cross colours correspond to line colours).

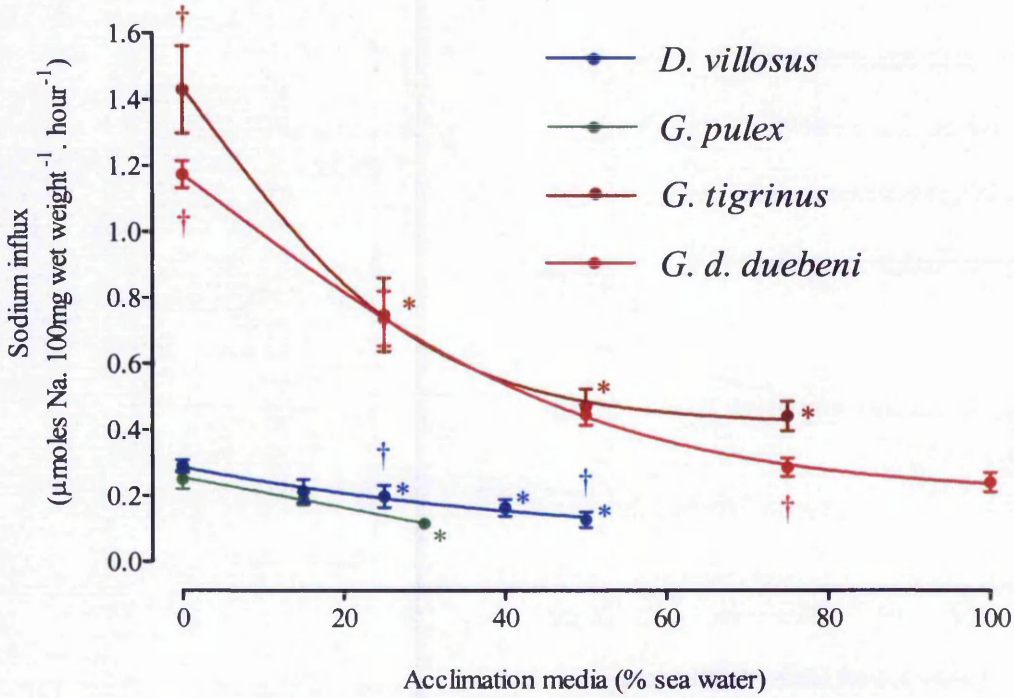


Figure 4. 1. 2. 6. Sodium influx in four species of gammarid from the Netherlands, following a five day acclimation to media of increasing sea water concentration. (mean ± SE, n =5). * Significant difference from freshwater values, $p<0.05$; † significant difference between species, $p<0.05$ (star and cross colours correspond to line colours).

Table 4. 1. 2. 1. Saturation of sodium uptake mechanisms in fresh water acclimated *Dikerogammarus villosus*.

Sodium concentration of loading solution (mM)	Sodium Influx (mean ± SE) (μmoles sodium. 100 mg wet weight^{-1}. hour^{-1})
5.5	0.35 ± 0.02
11	0.33 ± 0.04
16.5	0.34 ± 0.03

Sodium influx decreased with increased sea water acclimation in all four gammarids (Fig. 4. 1. 2. 6.). In fresh water, sodium influx was significantly higher in *G. tigrinus* (1.4 ± 0.13 μmoles Na. 100 mg wet weight^{-1}. hour^{-1}) than *G. d. duebeni* (1.2 ± 0.04 μmoles Na. 100 mg wet weight^{-1}. hour^{-1}, $p<0.05$). These values were both approximately six times the sodium influx of the more freshwater species *G. pulex* (0.25 ± 0.03 μmoles Na. 100mg wet weight^{-1} .hour^{-1}) and *D. villosus* (0.29 ± 0.02 μmoles Na. 100 mg wet weight^{-1}. hour^{-1}, $p<0.05$). In the two more euryhaline species *G. tigrinus* and *G. d. duebeni* sodium influx was found to reduce at a similar rate with increased sea water acclimation, with almost identical values of sodium influx at 25% and 50% sea water. Sodium influx appeared to level off at 75% sea water in *G. tigrinus*, whilst further reductions in *G. d. duebeni* at 75% sea water resulted in a significant difference between these two species at this sea water concentration ($p<0.05$). In the freshwater species *G. pulex and D. villosus*, sodium influx was essentially similar, decreasing gradually with increased sea water concentrations. Concentrations of 25% and 30% sea water were required to cause a significant reduction in sodium influx from the freshwater value in *D. villosus* and *G. pulex* respectively ($p<0.05$).

Further comparison between the sodium fluxes of *D. villosus* and *G. d. duebeni* acclimated to fresh water can be seen in figure 4. 1. 2. 7. Although sodium influx was found to differ significantly between the two species ($p<0.05$), no significant difference was found

between the sodium efflux rates. In addition, the total body sodium of *G. d. duebeni* was significantly higher than that of *D. villosus* ($p<0.05$).

4. 1. 2. 3. Rapid salinity transfer

Experiments were carried out to determine the ability of *G. d. duebeni* and *D. villosus* to alter their sodium influx in response to large instantaneous changes in external salinity. Instant transfer of freshwater acclimated *G. d. duebeni* to media of 100% sea water resulted in a rapid decrease in sodium influx, reaching new stable levels approximately 2 to 4 hours after transfer (Fig. 4. 1. 2. 8.). New stable levels of sodium influx were also reached 2 to 4 hours after transfer of sea water acclimated *G. d. duebeni* to fresh water. Due to the limited salinity tolerance of *D. villosus*, freshwater acclimated animals were transferred to 50% sea water, whilst the reverse transfer was not made (Fig. 4. 1. 2. 9.). Significant differences in sodium influx were shown 3 hours after transfer ($p<0.05$), reaching new levels 3 to 4 hours after transfer of freshwater acclimated *D. villosus* to 50% sea water.

Rapid transfer of freshwater acclimated *G. d. duebeni* to 100% sea water resulted in a gradual decrease in $t_{1/2}$, reaching new stable levels approximately 18 hours after transfer (Fig. 4. 1. 2. 10.). In contrast, instant transfer of 100% sea water acclimated *G. d. duebeni* to fresh water, resulted in a rapid increase in $t_{1/2}$, reaching equilibrium approximately 4 hours after transfer.

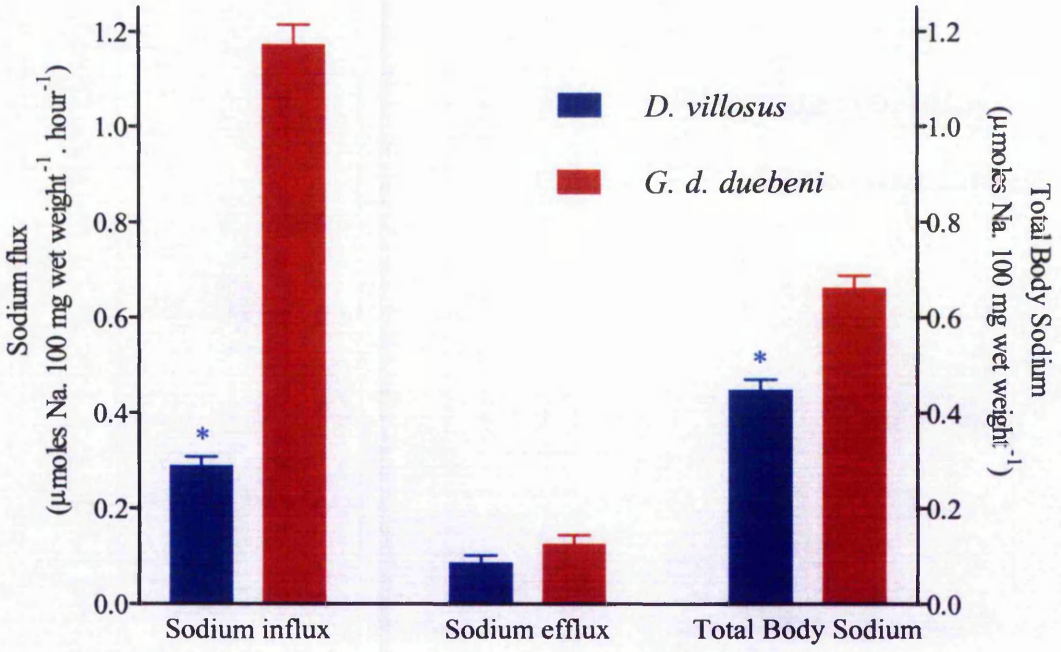


Figure 4. 1. 2. 7. Comparison of sodium influx, efflux and total body sodium between the freshwater *Dikerogammarus villosus* the brackish water *G. d. duebeni* from the Netherlands, acclimated for five days in fresh water (mean ± SE, n =5). * Significant difference between species, $p<0.05$.

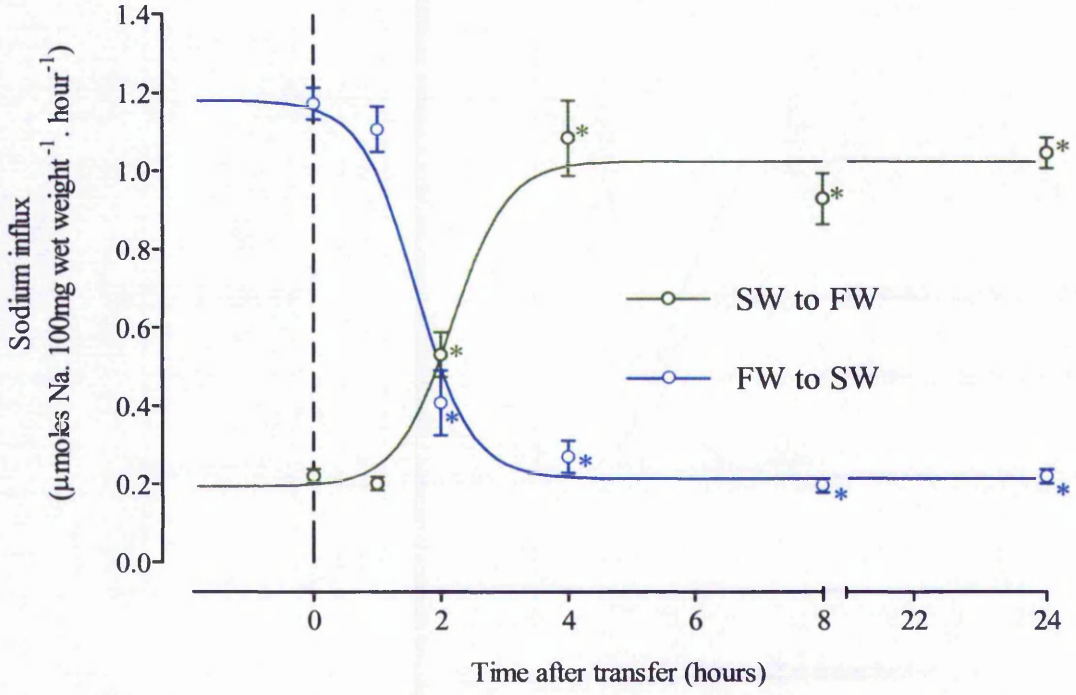


Figure 4. 1. 2. 8. Change in sodium influx following transfer of *G. d. duebeni* from fresh water (FW) to sea water (SW) (blue line) and from SW to FW (green line) (mean ± SE, n =5). * Significant difference from control (0 hours), $p<0.05$.

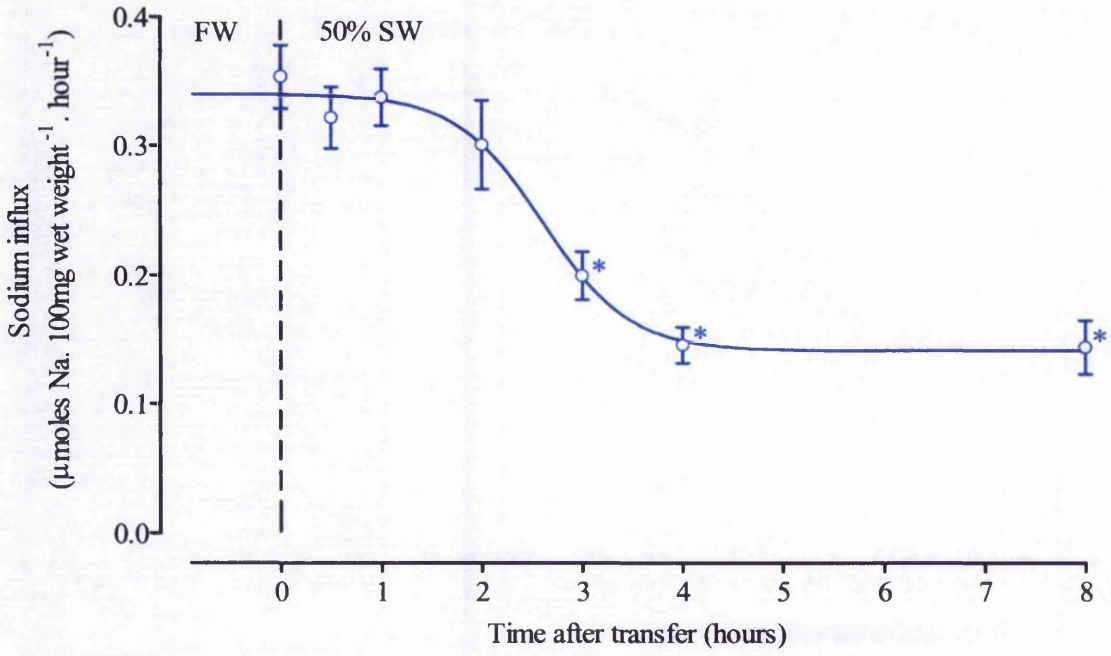


Figure 4. 1. 2. 9. Change in sodium influx following transfer of *D. villosus* from fresh water (FW) to 50% sea water (SW) (mean ± SE, n =5). * Significant difference from control (0 hours), $p<0.05$.

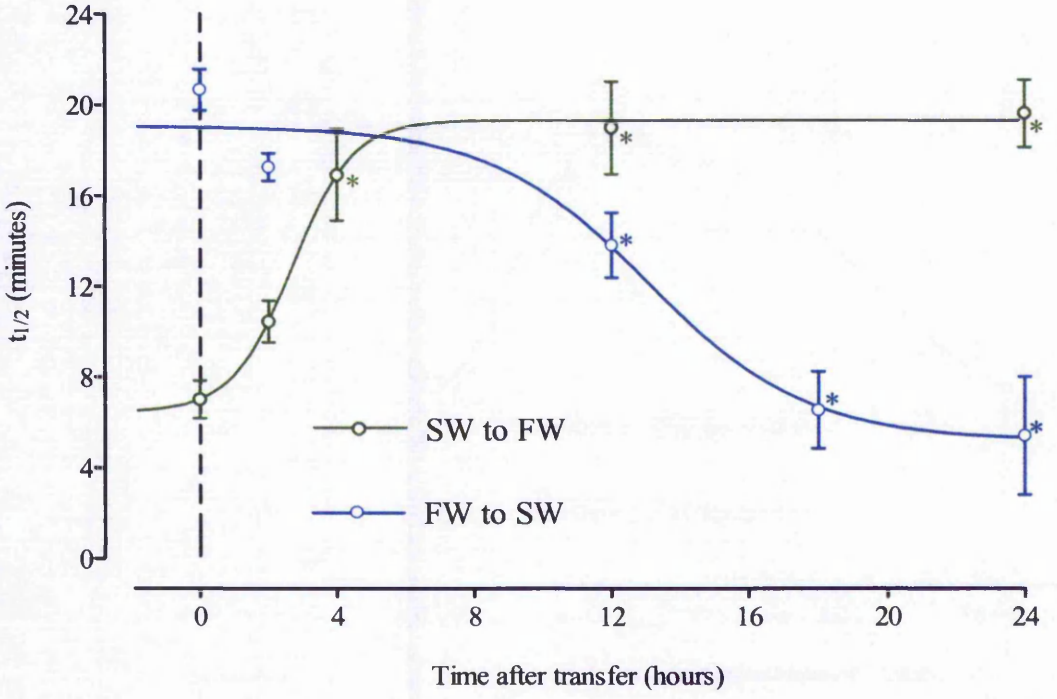


Figure 4. 1. 2. 10. Change in the half-time of exchange of body water ($t_{1/2}$) following transfer of *G. d. duebeni* from fresh water (FW) to sea water (SW) (blue line) and from SW to FW (green line) (mean ± SE, n =5). * Significant difference from control (0 hours), $p<0.05$.

4. 1. 3. Discussion

4. 1. 3. 1. Haemolymph ion concentration

The ability of aquatic organisms to maintain haemolymph ion concentrations within cell tolerance limits with respect to the external salinity is vital for their survival. For all the haemolymph ion concentrations measured, significant increases were seen when *G. pulex* was acclimated to 15% sea water. The inability of *G. pulex* to maintain stable haemolymph ion concentrations with only relatively small changes in external salinity reflects the poor osmoregulatory powers of this freshwater amphipod. Increases in haemolymph ion concentrations are accompanied by similar increases in tissue ion concentration (Beadle & Cragg, 1940). The increase of intracellular ions is believed to be responsible for the poor survival rates of *G. pulex* when acclimated to increased salinity media (Lockwood, 1961). In contrast, *D. villosus* was able to maintain relatively constant haemolymph ion concentrations between fresh water and 40% sea water, although at 50% sea water acclimation, all ion concentrations rose sharply to significantly higher levels. The inability of *D. villosus* to maintain haemolymph ion concentrations, particularly sodium, and consequently intracellular sodium concentrations when acclimated to 50% sea water, may explain the poor survival rate of *D. villosus* at the limit of its salinity tolerance range.

Haemolymph sodium concentrations in freshwater acclimated Dutch gammarids were maintained at significantly higher concentrations in the brackish water species *G. d. duebeni* and *G. tigrinus*. The lowest haemolymph sodium concentration was maintained by the most freshwater adapted species *G. pulex*. The invading amphipod *D. villosus* was also found to have a significantly lower haemolymph sodium concentration in fresh water when compared to the two brackish water species. The maintenance of a higher hyperosmotic sodium gradient requires increased levels of active sodium uptake (Péqueux, 1995). Consequently, the lower sodium gradient maintained by *G. pulex* and *D. villosus*, reflects increased freshwater adaptation, which could potentially provide these species with an

energetic advantage over the brackish water forms in the fresh waters of The Netherlands. Further discussion of haemolymph sodium regulation will continue with regard to sodium flux and water permeability in the following section (4. 1. 3. 2 - 3.).

Magnesium has anaesthetic properties in marine invertebrates (Pantin, 1946), and has been found to exert significant physiological effects on the neuromuscular junctions of crustaceans (Morritt & Spicer, 1993). High haemolymph magnesium concentrations have been correlated with low levels of locomotory activity in crustaceans (Morritt & Spicer, 1993; Frederich *et al.*, 2000). In fresh water, significantly higher haemolymph magnesium concentrations were found in *D. villosus* when compared to that measured in the other Dutch amphipods. In the laboratory aquarium, *D. villosus* appeared less active than other gammarids, frequently attaching to stones and moving only as a result of physical disturbance (personal observation). The low activity of *D. villosus* may be a reflection of the comparatively high haemolymph magnesium concentrations in this species in fresh water. In addition, haemolymph magnesium concentrations were significantly higher in *D. villosus* acclimated to 50% sea water than when acclimated to more dilute media. This seawater concentration is the upper limit of the salinity tolerance range of this species (Brujis *et al.*, 2001). The reduced fitness of *D. villosus* at 50% sea water can result in sluggish movement and inactivity possibly highlighted by the significantly high haemolymph magnesium concentrations at this salinity.

4. 1. 3. 2. Water permeability

The ability of aquatic organisms to alter their half time of exchange of body water ($t_{1/2}$) is of particular physiological importance to animals that occupy water bodies of fluctuating salinity (Bolt *et al.*, 1980). The decrease in $t_{1/2}$ in sea water media aids the uptake of salts *via* passive forces and thus potentially reduces the energy requirements of active salt uptake. It is well established that the brackish water *G. d. duebeni* can alter its $t_{1/2}$ with

increasing seawater acclimation (Lockwood *et al.*, 1973; Bolt *et al.*, 1980; Lockwood & Bolt, 1989). The rapid decrease in $t_{1/2}$ between 50% and 75% sea water agrees with previous reports (Lockwood *et al.*, 1973; Bolt *et al.*, 1980), coinciding with haemolymph approaching isosmoticity with the external media.

Changes in $t_{1/2}$ with increasing salinity were also found in the brackish water species *G. tigrinus*. Unlike that found for *G. d. duebeni*, where rapid changes in $t_{1/2}$ occured between 50% and 75% sea water, the $t_{1/2}$ of *G. tigrinus* decreased gradually with increasing salinity. This is the first report of *G. tigrinus* altering its $t_{1/2}$ with changing salinity. Outflux experiments with tritiated water failed to show any change in $t_{1/2}$ with increased seawater concentration in two other *G. tigrinus* populations from Northern Ireland (Chapter 4. 2) and England (data not shown). Comparisons with these two populations revealed a similar $t_{1/2}$ in all *G. tigrinus* populations between 25% and 75% sea water. However, the Dutch *G. tigrinus* population had a significantly higher $t_{1/2}$ in fresh water. This increase in $t_{1/2}$ would be an advantage to animals inhabiting fresh water, limiting ion loss to the hypo-osmotic external environment. Therefore, the Dutch *G. tigrinus* population appears to demonstrate increased adaptation to fresh water when compared to the *G. tigrinus* populations from Ireland and England.

The inability to alter $t_{1/2}$ with changing salinity is common to stenohaline as well as some euryhaline crustaceans. Such inability to alter $t_{1/2}$ with changing salinity was demonstrated in this study by the freshwater amphipod *G. pulex*. This supports previous reports on the $t_{1/2}$ of other *G. pulex* populations (Sutcliffe, 1971d).

The invading amphipod *D. villosus* was found to significantly decrease its $t_{1/2}$ between fresh water and 15% sea water. Decreasing $t_{1/2}$ is an advantage when haemolymph is isosmotic with the external media, allowing increased passive uptake of ions saving energy

by reducing active ion uptake. However, decreasing $t_{1/2}$ whilst still maintaining hyperosmoticity would increase ion loss. This may account for the slight reductions in some haemolymph ion concentrations (Na, K, Mg) at 15% sea water in *D. villosus*.

4. 1. 3. 3. Sodium flux

Significantly higher sodium influxes were found in the two brackish water species *G. d. duebeni* and *G. tigrinus*, when acclimated to fresh water. Both *G. d. duebeni* and *G. tigrinus* had significantly higher haemolymph sodium concentrations than the more freshwater adapted species (*G. pulex* and *D. villosus*). Therefore, it seems likely that the higher sodium influx rates of the brackish water species were to maintain these higher haemolymph sodium concentrations. In fresh water, sodium influx of *D. villosus* was similar to that of the highly freshwater adapted species *G. pulex*, suggesting that *D. villosus* is equally well adapted to fresh waters.

Sodium influx was found to decrease with increasing salinity in all Dutch gammarids. In *G. d. duebeni* and *G. tigrinus* sodium influx decreased significantly between fresh water and 50% sea water, reaching relatively stable levels at salinities greater than 50% sea water. The partial levelling out of the sodium influx at concentrations greater than 50% sea water in *G. d. duebeni* and *G. tigrinus* corresponds with the haemolymph sodium concentrations and external media approaching isosmotic.

The $t_{1/2}$ of *G. d. duebeni* and *D. villosus* were very similar when acclimated to fresh water. Considering this similarity as well as the maintenance of a significantly higher osmotic gradient in *G. d. duebeni* than *D. villosus* when acclimated to fresh water, it would be expected that passive sodium loss to the relatively low sodium environment would be greater in the former than the latter. Despite this, no significant difference between the sodium efflux rates of *D. villosus* and *G. d. duebeni* were found, when acclimated to fresh

water. Reclaiming sodium from the urine through active uptake in the antennal glands reduces sodium loss to the external environment and is a technique exhibited by *G. d. duebeni* (Lockwood, 1961). The production of hypotonic urine relative to the haemolymph ion concentration may explain the unexpectedly low sodium efflux seen in *G. d. duebeni*. Whether *D. villosus* has the ability to produce hypotonic urine is not currently known. The low sodium efflux found by *D. villosus* in this study may suggest it has similar capabilities, but may simply reflect the lower haemolymph-external medium ionic gradient maintained by this species in fresh water compared to that of *G. d. duebeni*.

The high sodium influx rates of the brackish water species when acclimated to fresh water would suggest that significantly higher rates of active sodium uptake are taking place in the brackish water species. Measurements of gill Na^+, K^+-ATPase activity showed greater activity in *G. d. duebeni* than either *G. pulex* or *D. villosus* when in fresh water (results displayed in Chapter 3. 2.). However, despite these apparent differences no significant difference in gill Na^+, K^+-ATPase activity between these species was found in fresh water. This lack of statistical significance was attributed to the limited number of replicates used ($n = 3$). It is likely that increasing sample size would statistically confirm these apparent differences, particularly between gill Na^+, K^+-ATPase activity of *G. pulex* and *G. d. duebeni* when in fresh water.

4. 1. 3. 4. Rapid transfer experiments

The ability to rapidly reduce sodium influx after instant transfer to increased salinity might enable *D. villosus* to maintain its haemolymph sodium concentration and subsequent intracellular ions to within cell tolerance limits. Such ability would have great benefits for invading amphipods such as *D. villosus*, enabling them to cross waters of raised salinity, as well as to potentially survive sudden partial ballast water exchange.

Despite being highly adapted to fresh waters, *D. villosus* was able to rapidly alter its sodium influx, reaching new stable levels 4 hours after transfer of fresh water acclimated animals to 50% sea water. However, it must be noted that all *D. villosus* that were rapidly transferred showed strong signs of reduced fitness, with most dying if left for 24 hours in 50% sea water. The inability of *D. villosus* to maintain extracellular ion concentrations within limits tolerable to cells was probably responsible for the 100% mortality of *D. villosus* 24 hours after transfer from fresh water to 50% sea water.

In contrast to *D. villosus*, the highly euryhaline *G. d. duebeni* was able to survive rapid transfer from either fresh water to 100% sea water or vice versa. Transfer to hyper- or hypo- osmotic media brought about large changes in sodium influx after 4 hours, a similar response time as *D. villosus*. The rapid change in sodium influx by *G. d. duebeni* enabled it to maintain intracellular ions within cell tolerance limits, and confirms previous reports on rapid transfer experiments with *G. d. duebeni* populations (Bolt *et al.*, 1980).

4. 1. 3. 5. Ecological significance with regard to *D. villosus* invasion

High predation pressures exerted by *D. villosus* on the amphipods *G. d. duebeni* and *G. tigrinus* have resulted in the latter two species being replaced by the former in the Dutch waters (Dick & Platvoet, 2000). The present study has found the osmoregulation of *D. villosus* to be more adapted to fresh and oligohaline waters than that of the two more brackish water forms *G. d. duebeni* and *G. tigrinus*. Due to the maintenance of a significantly lower haemolymph sodium concentration and lower sodium influx by *D. villosus*, less energy would be used on osmoregulation. The reduction of energy required for osmoregulation could provide *D. villosus* with a significant energetic advantage over the other two species in the oligohaline waters of The Netherlands. It is unlikely however, for this energetic advantage in oligohaline waters exhibited by *D. villosus* over *G. d. duebeni* and *G. tigrinus* to be solely responsible for the replacement of latter two species by

the former. Rather it should be considered, as a contributory factor that may allow more energy to be used in growth and reproduction. The aggressive predatory behaviour would have a more direct effect on displacement.

Table 4. 1. 3. 1. The main ion concentrations of water samples taken from the sampling sites of the Dutch amphipod species (mean ± SD).

Ion concentration (mM)	Dutch amphipod sampling site			
	D. villosus	*G. tigrinus*	*G. pulex*	*G. d. duebeni*
Sodium	4.4 ± 0.3	4.31 ± 0.31	2.1 ± 0.32	84.53 ± 0.25
Potassium	0.37 ± 0.08	0.3 ± 0.1	0.4 ± 0.13	1.46 ± 0.12
Magnesium	1.15 ± 0.16	1.2 ± 0.14	0.8 ± 0.23	9.19 ± 0.84
Calcium	4.1 ± 0.24	4.7 ± 0.24	2.06 ± 0.1	7.69 ± 1.3

From this study the osmoregulation of *D. villosus* and *G. pulex* were very similar, both maintaining low haemolymph ion concentrations and low levels of sodium influx whilst in fresh water. Due to these similarities, it is unlikely that *D. villosus* would have any osmoregulatory advantage over *G. pulex* in fresh water. Indeed, it was previously reported that the survival rate of *D. villosus* decreases slightly when acclimated to fresh water as apposed to its ideal salinity of 0.3 (Brujis *et al.*, 2001). It has been previously found that *G. pulex* is extremely well adapted to fresh water and has ion uptake mechanisms with high sodium affinities, allowing uptake of sodium and chloride from fresh waters with very low ion content (Sutcliffe, 1967b; 1971c). The ion content of the water samples where each gammarid was collected shows that *G. pulex* is located in fresh waters with lower ion concentrations than *G. tigrinus* or *D. villosus*, with *G. d. duebeni* in water of approximately 10% sea water (4 salinity, Table 4. 1. 3. 1.). It is suggested that the replacement of *D. villosus* by *G. pulex* may not occur in fresh waters that contain low ion content, since *D. villosus* are unable to maintain stable haemolymph ion concentrations in such media. In the present study, *D. villosus* were only acclimated to fresh water (1.76 mM Na) between 5 and 10 days and during this time haemolymph ion concentrations were maintained within stable limits. It may be informative to monitor the haemolymph ion concentration in fresh waters

with low ion content (<1mM Na) over a period of a few months to determine whether stable haemolymph ion concentrations can be maintained. There have been no reports, as yet, on the displacement of *G. pulex* by *D. villosus* from freshwater bodies with low ion concentrations.

In the Moselle River in France, both *G. pulex* and *D. villosus* were found to co-exist (Devlin *et al.*, 2001). Individual *D. villosus* from the Moselle River were significantly smaller in size (15mm length) than those of the Dutch population (30mm length). It could be suggested that the reduced size of *D. villosus* may lower the predatory advantage it has been found to hold over the Dutch amphipod communities (Dick & Platvoet, 2000). In the Moselle River, differences in osmoregulation between the competing amphipods may be more influential in determining species interactions.

Survival of rapid salinity change

It has previously been suggested that *D. villosus* can survive incomplete ballast water exchange if a large enough population was present and the salinity does not exceed 20, enabling the potential for the global distribution of *D. villosus* (Brujis *et al.*, 2001). The present study fails to support this claim, since rapid transfer experiments revealed 100% mortality in *D. villosus* within 24 hours after transfer from fresh water to 50% sea water (20 salinity, since 100% sea water is equal to 40 salinity throughout this thesis).

4. 2. Osmoregulation of the indigenous amphipod *Gammarus duebeni celticus* in fresh waters of Northern Ireland; comparisons with the introduced amphipods *G. pulex* and *G. tigrinus*.

4. 2. 1. Introduction

Ireland's freshwater invertebrate communities have been identified as having low species diversity, when compared to Britain and Continental Europe (Dick, 1996a). However, through intended and accidental anthropogenic inputs, species numbers have increased in many Irish freshwater communities. One such example is Lough Neagh where numbers of fish and macro-crustacean species have doubled since the 17th century (Dick, 1996b).

The most widespread freshwater crustacean in Northern Ireland is the indigenous amphipod *Gammarus duebeni celticus*. This species is the freshwater form of the brackish amphipod *G. duebeni* (Stock & Pinkster, 1970). The freshwater *G. d. celticus* has been found to be morphologically as well as physiologically distinct from the brackish water form, as a result of long term adaptation to its fresh water environment (Pinkster *et* al., 1970; Stock & Pinkster, 1970; Lockwood *et al.*, 1973; Bolt *et al.*, 1980; Lockwood & Bolt, 1989; Chapter 5, present study).

The freshwater amphipod *G. d. celticus* was once the only freshwater amphipod present in Ireland (Hynes, 1954; Strange & Glass, 1979). However, the euryhaline amphipod *G. tigrinus*, which is native to North America (Reynolds, 2001), was introduced to Ireland during the First World War. This probably occurred *via* bilge water or ballast tanks of Naval vessels following transatlantic crossing (Costello, 1993). *G. tigrinus* has often been found to occupy brackish water habitats or fresh waters in which the ion content has been raised due to pollution (Bird, 1989; Terrell-Nield, 1995). *G. tigrinus* has been reported to be spreading in England and Ireland (Gledhill *et al.*, 1993). It has also increased its range in

continental Europe, after it was introduced into the polluted Rivers Werra and Weser to boost invertebrate stocks (Bulnheim, 1985).

Following the accidental introduction of *G. tigrinus*, the freshwater amphipod *G. pulex* was deliberately introduced into Ireland in the 1950s in order to increase prey items for fish in angling waters (Strange & Glass, 1979). The interactions of the three species (*G. d. celticus*, *G. pulex* and *G. tigrinus*), and their comparative abundance in the fresh waters of Northern Ireland have been well documented (Hynes, 1955b; Pinkster, 1975; Pinkster *et al.,* 1977; Dick *et al.*, 1993; Dick, 1996a; Dick *et al.*, 1999; MacNeil *et al.*, 1997). These reports have highlighted the elimination of the native *G. d. celticus* by the invasive *G. pulex* from many freshwater rivers and lakes in Northern Ireland, and the exclusion of *G. tigrinus* by both *G. d. celticus* and *G. pulex*.

4. 2. 1. 1. Interactions between *G. d. celticus*, *G. pulex* and *G. tigrinus*

There have been a variety of ideas concerning the mechanism(s) underlying the exclusion of *G. d. celticus* by *G. pulex* from freshwater bodies. Differences in reproductive output were suggested, since the reproductive output of *G. pulex* was found to be 2 to 3 times greater than that of *G. d. celticus* (Hynes, 1955a; Sutcliffe, 1993). The reproductive output of *G. tigrinus* is 2 to 3 times that of *G. pulex* (Bird, 1989) although *G. tigrinus* was still out competed by *G. pulex* in the River Poulter, Nottinghamshire (Terrell-Nield, 1995). However, this phenomena only occured when salinity in the River Poulter declined after cessation of mine pumping effluent into this water system. In laboratory experiments high levels of predatory interaction were found to exist between gammarids, which led to the suggestion that differences in reproductive potential alone were insufficient to provide one species with an advantage over another (Dick 1996a).

Mutual but differential predation interactions have been documented between Irish freshwater amphipods (Dick *et al.*, 1990a; Dick *et al.*, 1990b; Dick *et al.*, 1994, Dick, 1996a). Within this process males of both species kill and eat single, recently moulted female congenerics. However, male *G. pulex* were found to kill significantly more female congenerics than do male *G. d. celticus*. Furthermore, *G. d. celticus* were found to be more cannibalistic than *G. pulex* (Dick *et al.*, 1997). The physiology of the moulting process in *G. d. celticus* results in individuals of this species remaining vulnerable to both predation and cannibalism for longer than *G. pulex* (Dick, 1992, 1995). It is this mutual but differential predation between *G. pulex* and *G. d. celticus*, which is believed to account for the elimination and replacement of the latter by the former in many Irish rivers (Dick *et al.*, 1990a; Dick *et al.*, 1990b; Dick *et al.*, 1994, Dick, 1996a).

Mutual but differential predation between freshwater populations of *G. d. celticus* and *G. pulex* has been unable to entirely explain all field observations. Hynes (1954) conducted transplantation experiments with *G. pulex* into several freshwater streams in the Isle of Man, originally containing only *G. d. celticus* (later published by Dick *et al.*, 1997). In these experiments *G. pulex* was unsuccessful in displacing *G. d. celticus* from the freshwater streams. These results disagree with those found in the Irish freshwater populations where *G. d. celticus* is displaced by *G. pulex* (Dick *et al.*, 1990a; Dick *et al.*, 1990b; Dick *et al.*, 1994, Dick, 1996a). Similar resistance by *G. d. celticus* to *G. pulex* invasion has been found to occur in freshwater streams in Brittany (Stock, 1993; Dunn, 1995). Also, the exclusion of *G. pulex* has been reported from a freshwater stream in Ireland that was previously found to contain almost equal proportions of *G. pulex* and *G. d. celticus* (Dick *et al.*, 1994). The reasons for the resistance of *G. d. celticus* to *G. pulex* invasion in these instances were not known. Differences in environmental factors, such as the chemical/ ionic composition of the water acting upon the physiological capabilities of the two amphipods may provide partial explanation.

The amphipod *G. tigrinus* is often found in oligohaline and brackish waters in North America and Western Europe (Reynolds, 2001). In Northern Ireland they occur in fresh water, although exclusion of *G. tigrinus* by both *G. pulex* and *G. d. celticus* through predatory interactions from many of these fresh waters in Northern Ireland has been reported (Dick, 1996a). Predatory interactions between *G. pulex* and *G. tigrinus* were strongly influenced by the ionic concentration of the selected water bodies (Dick & Platvoet, 1996). Predation frequencies were in favour of *G. pulex* in waters of low conductivity, whereas no differential predation occurred in waters of higher conductivity. Consequently, *G. pulex* excluded *G. tigrinus* in fresh waters, whilst the reverse was true in oligohaline waters (Dick & Platvoet, 1996). In waters of raised salinity, ecological adaptation exhibited by *G. tigrinus* has led to higher fecundity and shorter generation time (Bird, 1989). This adaptation was believed to be responsible for the exclusion of *G. pulex* by *G. tigrinus* in oligohaline waters (Bird, 1989).

The effects of ionic concentration on the interactions between competing species, highlights the importance of osmoregulation in aquatic gammarid amphipods. In fresh water, the active uptake of salts is an energy demanding process (Sutcliffe, 1993). The ability of a freshwater species to reduce its energy expenditure would likely provide this species with an advantage over its competitors. The differences in energy expenditure could potentially influence competitive interactions, such as intraguild predation, in favour of the species most adapted to the environment. The aim of this study is to compare the osmoregulatory physiology of the three Irish freshwater amphipods *G. d. celticus, G. pulex* and *G. tigrinus*. Differences in osmoregulatory physiology are discussed with regard to the competitive interactions between these three amphipods, and to provide a physiological explanation to contribute to the current understanding of intraguild predation for the exclusion of *G. d. celticus* by *G. pulex* from freshwater systems in Northern Ireland.

4. 2. 2. Results

4. 2. 2. 1. Haemolymph ion concentration

Due to the limiting numbers of *G. tigrinus* collected from Northern Ireland, it was only possible to determine the main haemolymph ion concentrations in the two Irish freshwater species *G. duebeni celticus* and *G. pulex* (Figs 4. 2. 2. 1 to 5).

Sodium

Haemolymph sodium concentrations were maintained significantly higher in *G. d. celticus* (288 ± 10.8 mM) than *G. pulex* (189 ± 6.1 mM) when acclimated to fresh water ($p<0.05$, Fig 4. 2. 2. 1.). Haemolymph sodium concentrations in *G. d. celticus* were hyperosmotic in fresh water approaching isosmoticity when acclimation to seawater concentrations greater than 50% sea water. Due to high mortalities in *G. pulex* they were not acclimated to salinities greater than 30% sea water, although Sutcliffe (1967b) found that isosmoticity is reached in *G. pulex* around 40-50% sea water.

Potassium

Haemolymph potassium concentration was hyperosmotic in both freshwater species over their range of seawater concentrations (Fig. 4. 2. 2. 2.). In fresh water, haemolymph potassium was maintained at significantly higher concentrations in *G. d. celticus* (12.3 ± 0.2 mM) than *G. pulex* (8.6 ± 0.5 mM, $p<0.05$). Haemolymph potassium rose sharply with increased salinity in *G. pulex*. In contrast, haemolymph potassium concentrations in *G. d. celticus* were maintained relatively stable between 0 and 75% sea water, and only significantly increased at 100% sea water.

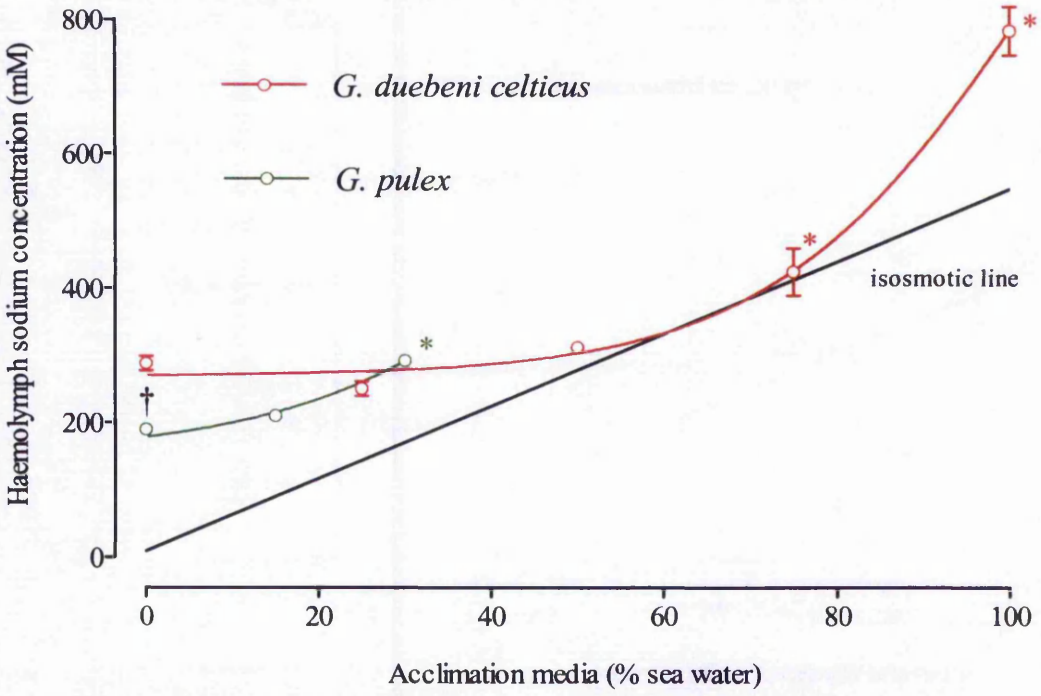


Figure 4. 2. 2. 1. Haemolymph sodium concentration in *Gammarus duebeni celticus* and *G. pulex* from Northern Ireland, following a five day acclimation to media of increasing seawater concentration. (mean ± SE, n =5).* Significant difference from fresh water, $p<0.05$; † significant difference between species, $p<0.05$).

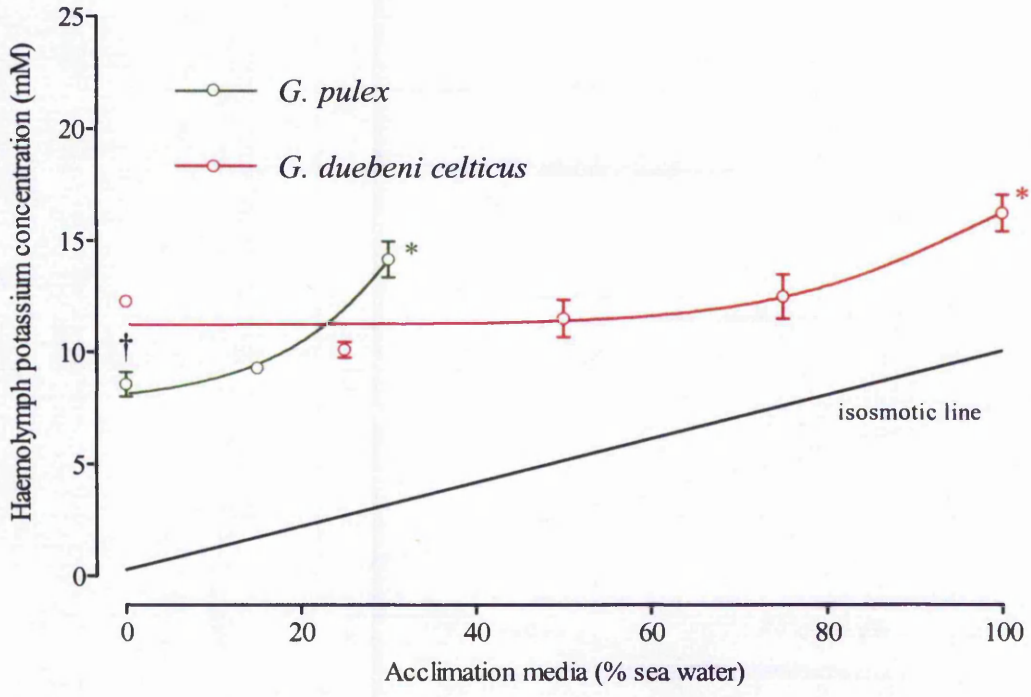


Figure 4. 2. 2. 2. Haemolymph potassium concentration in *Gammarus duebeni celticus* and *G. pulex* from Northern Ireland, following a five day acclimation to media of increasing seawater concentration. (mean ± SE, n =5). * Significant difference from fresh water, $p<0.05$; † significant difference between species, $p<0.05$).

Magnesium

Haemolymph magnesium concentrations were maintained hyperosmotic to fresh water in both *G. pulex* and *G. d. celticus* (Fig. 4. 2. 2. 3.). Haemolymph magnesium concentrations were maintained hypo-osmotic in both species at acclimation salinities greater than approximately 15% sea water. Higher haemolymph magnesium concentrations were maintained in freshwater acclimated *G. d. celticus* (17.3 ± 1.7 mM) than *G. pulex* (6.3 ± 0.5 mM, $p < 0.05$). Haemolymph magnesium concentrations in *G. d. celticus* were maintained relatively stable between fresh water and 75% sea water, increasing significantly at 100% sea water. Haemolymph magnesium concentrations increased significantly in *G. pulex* at 15% and 30% sea water acclimation.

Calcium

Similar haemolymph calcium concentrations were found in *G. d. celticus* and *G. pulex* acclimated to fresh water (Fig. 4. 2. 2. 4.). However, with increased seawater concentrations, haemolymph calcium concentrations were found to rise sharply in *G. pulex*, with significantly higher calcium levels at 30% sea water. In *G. d. celticus*, no significant increase in haemolymph calcium was found with increased salinity.

4. 2. 2. 2. Water permeability and sodium flux

Half time of exchange of tritiated water ($t_{1/2}$) and sodium influx were compared between the three freshwater amphipods *G. d. celticus*, *G. pulex* and *G. tigrinus*. No significant difference in $t_{1/2}$ was found in all three Irish freshwater species with increasing salinity acclimation (Fig. 4. 2. 2. 5.). In fresh water the $t_{1/2}$ of *G. d. celticus* (17.2 ± 0.96 min) was significantly higher than both *G. pulex* (10.8 ± 1.6 min) and *G. tigrinus* (9.6 ± 1.9 min). This comparatively higher $t_{1/2}$ in *G. d. celticus* was maintained throughout the salinity acclimation range, being significantly higher than *G. tigrinus* at 50%, 75% and 100% sea water.

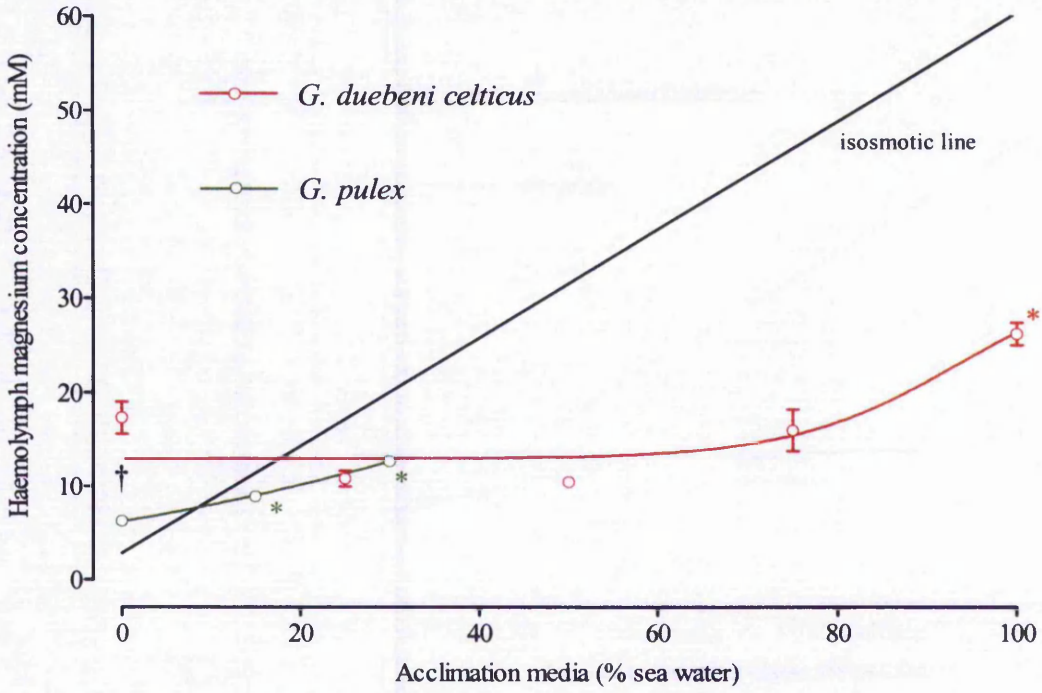


Figure 4. 2. 2. 3. Haemolymph magnesium concentration in *Gammarus duebeni celticus* and *G. pulex* from Northern Ireland, following a five day acclimation to media of increasing seawater concentration. (mean ± SE, n =5). * Significant difference from fresh water, $p<0.05$; † significant difference between species, $p<0.05$.

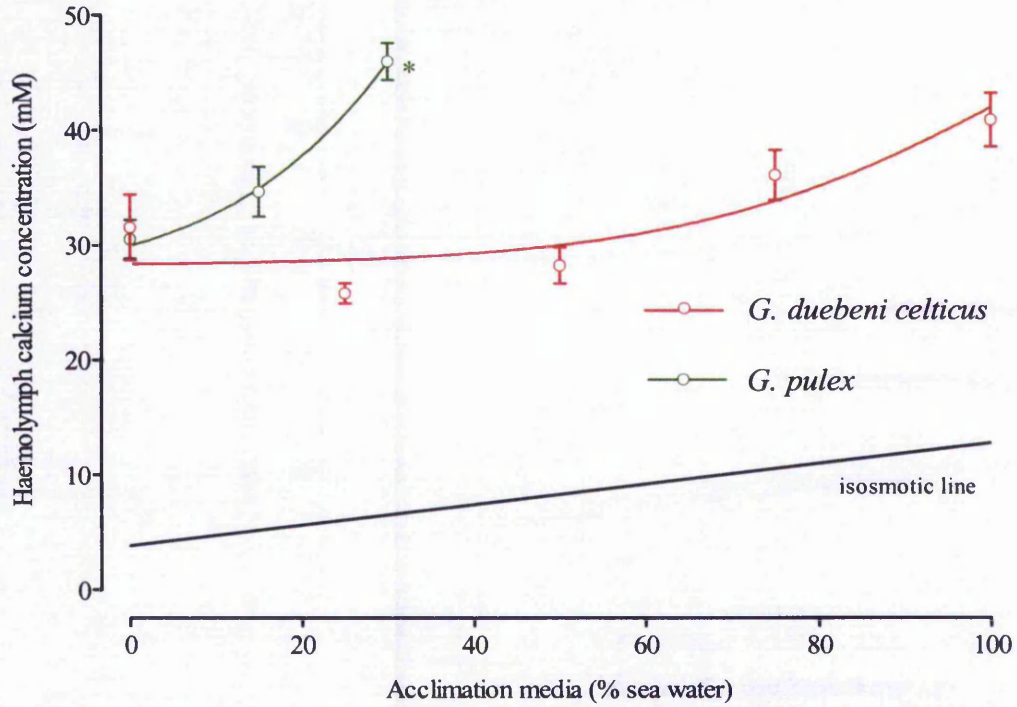


Figure 4. 2. 2. 4. Haemolymph calcium concentration in *Gammarus duebeni celticus* and *G. pulex* from Northern Ireland, following a five day acclimation to media of increasing seawater concentration. (mean ± SE, n =5). * Significant difference from fresh water, $p<0.05$.

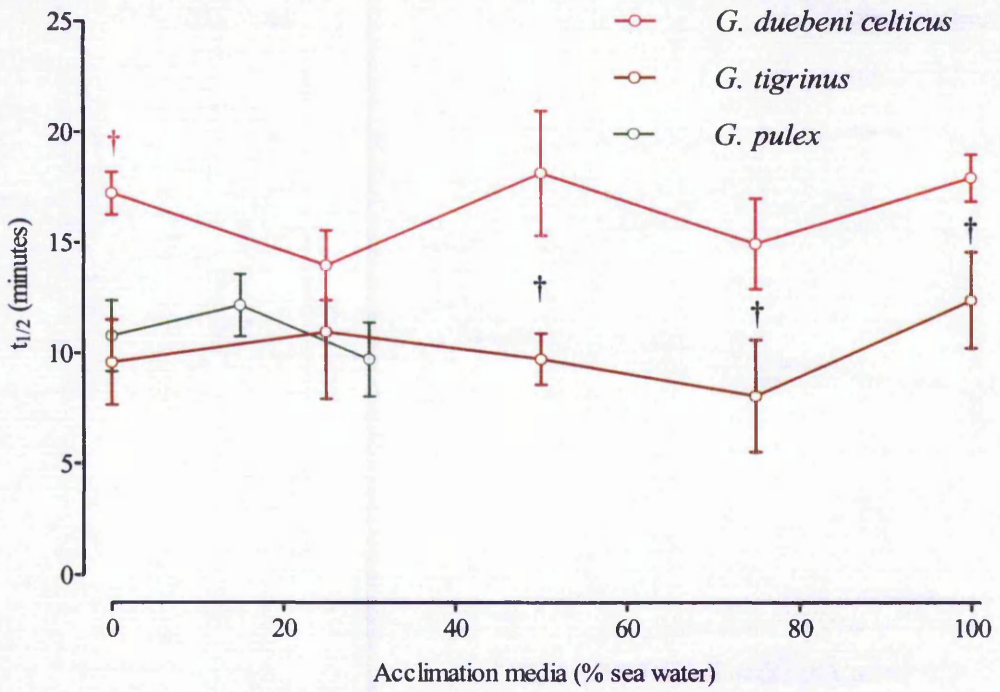


Figure 4. 2. 2. 5. Half-time of exchange of body water ($t_{1/2}$) in *Gammarus duebeni celticus, G. tigrinus* and *G. pulex* from Northern Ireland, following a five day acclimation to media of increasing seawater concentration. (mean ± SE, n =5). † Significant difference between species, $p<0.05$.

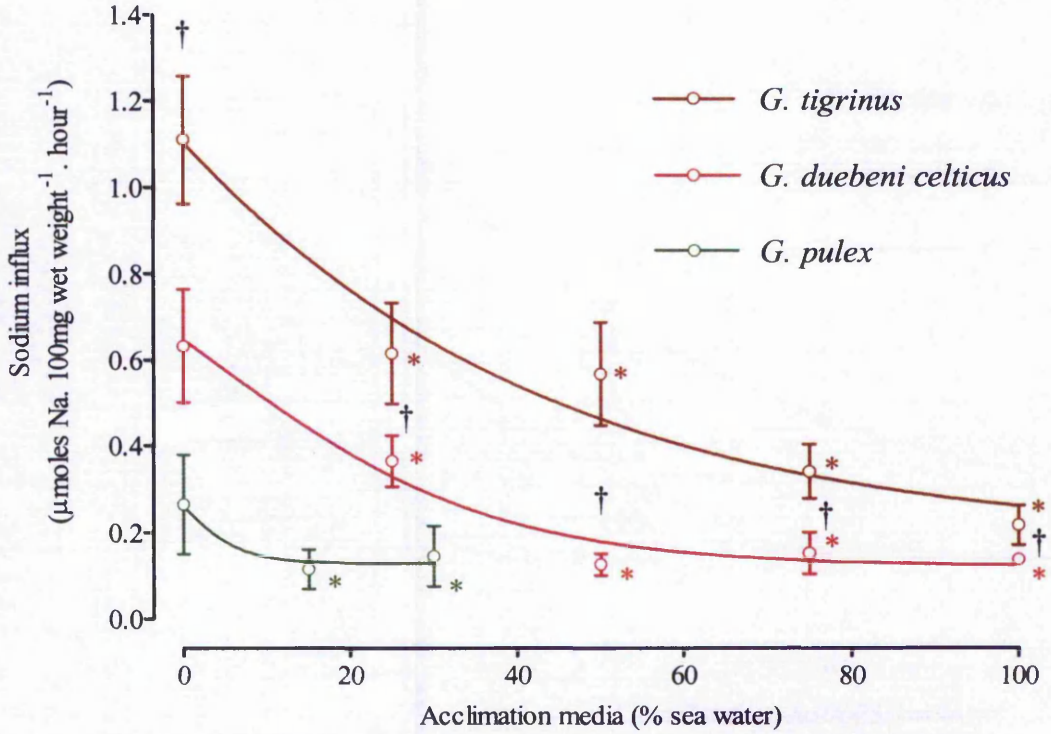


Figure 4. 2. 2. 6. Sodium influx in *Gammarus duebeni celticus, G. tigrinus* and *G. pulex* from Northern Ireland, following a five day acclimation to media of increasing seawater concentration. (mean ± SE, n =5). * Significant difference from fresh water, $p<0.05$; † significant difference between species, $p<0.05$.

Sodium influx was found to decrease significantly with increased seawater concentration in all species (Fig. 4. 2. 2. 6.). In fresh water, the sodium influxes for the three species were significantly different from each other, with significantly higher levels in *G. tigrinus* (1.1 ± 0.15 μmoles Na. 100mg wet weight^{-1}. hour^{-1}) and significantly lower levels in *G. pulex* (0.26 ± 0.12 μmoles Na. 100mg wet weight^{-1}. hour^{-1}). Comparison of sodium influx between *G. tigrinus* and *G. d. celticus* up to 100% sea water revealed significantly higher levels in *G. tigrinus* at all acclimation salinities.

Sodium flux and total body sodium concentrations were compared between *G. d. celticus* and *G. pulex* when acclimated to fresh water (Fig. 4. 2. 2. 7.). It was not possible to include *G. tigrinus* in these studies due to the low numbers collected. Significant differences in sodium influx and total body sodium were found between *G. d. celticus* and *G. pulex* ($p<0.05$). However, no significant difference in sodium efflux was found between these two species.

4. 2. 2. 3. Rapid salinity transfer

Experiments were carried out to determine the ability of *G. d. celticus* and *G. pulex* to alter their sodium influx in response to large instantaneous changes in external salinity. Instant transfer of freshwater acclimated *G. d. celticus* to media of 100% sea water resulted in a rapid decrease in sodium influx, reaching new stable levels approximately 8 hours after transfer (Fig. 4. 2. 2. 8.). A similar response time (8 hours) for an increase in sodium influx was observed when 100% sea water acclimated *G. d. celticus* were transferred to fresh water. In comparison *G. pulex* were unable to significantly alter their sodium influx within 24 hours after transfer from either fresh water to 30% sea water or from 30% sea water to fresh water (Fig. 4. 2. 2. 9.).

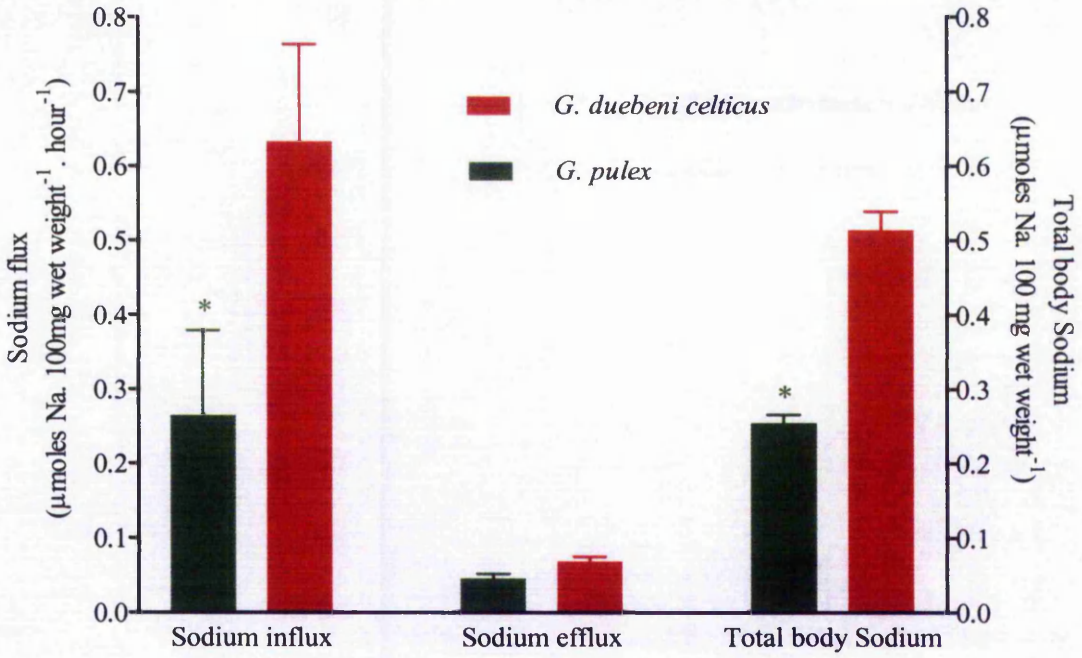


Figure 4. 2. 2. 7. Comparison of sodium influx, efflux and total body sodium between *Gammarus duebeni celticus* and *G. pulex* from Northern Ireland, acclimated for five days in fresh water (mean ± SE, n =5). * Significant difference between species, $p<0.05$.

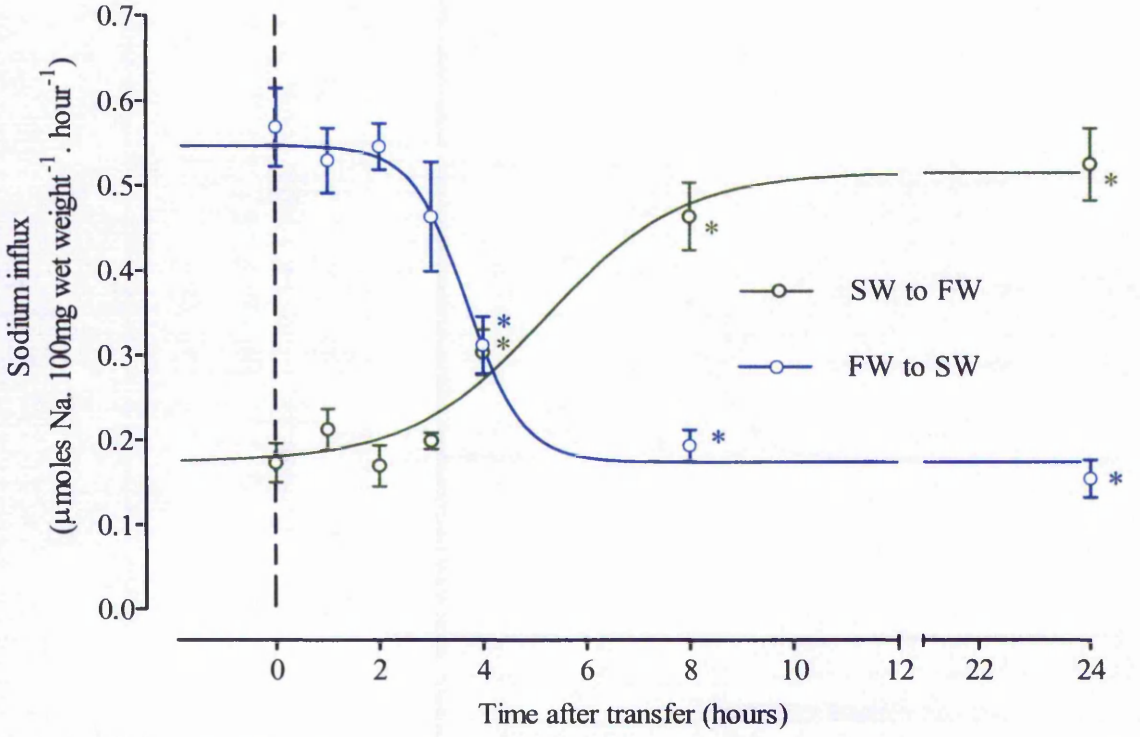


Figure 4. 2. 2. 8. Change in sodium influx following transfer of *G. d. celticus* from fresh water (FW) to sea water (SW, blue line) and from SW to FW (green line) (mean ± SE, n =5). * Significant difference from control (0 hours), $p<0.05$.

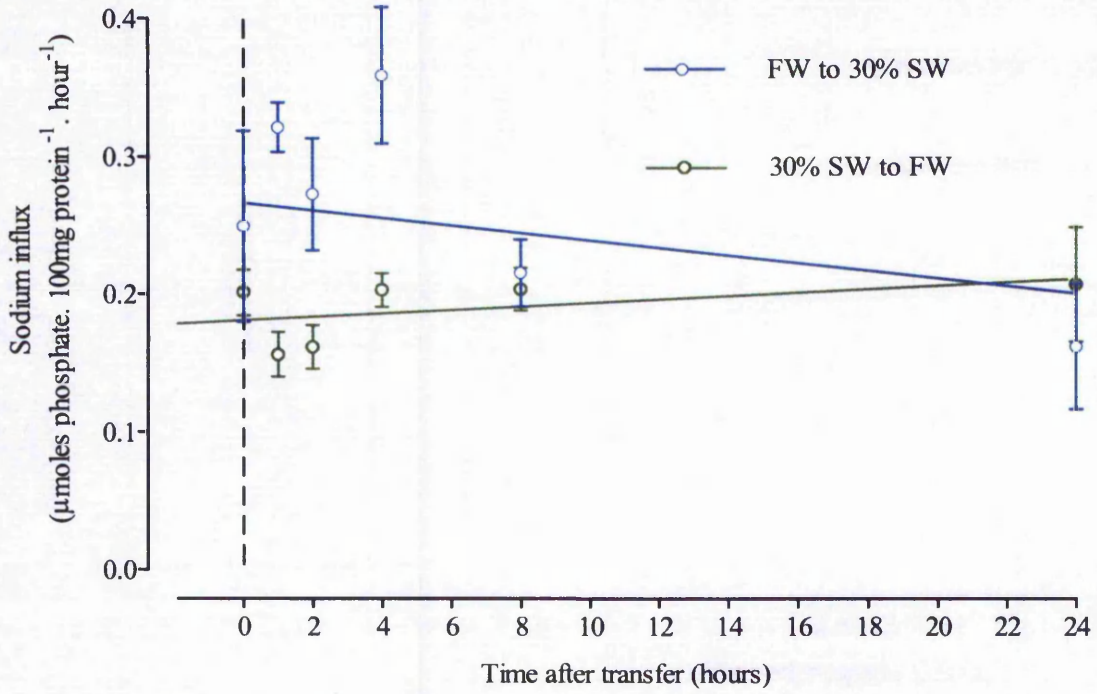


Figure 4. 2. 2. 9. Change in sodium influx following transfer of *G. pulex* from fresh water (FW) to 30% sea water (SW, blue line) and from 30% SW to FW (green line) (mean ± SE, n =5). No significant difference from control value (at 0 hours).

4. 2. 3. Discussion

4. 2. 3. 1. Haemolymph ion concentration

The maintenance of haemolymph sodium concentrations hyperosmotic to fresh water is an energy demanding process, which requires the active uptake of sodium against the outwardly directed concentration gradient. Freshwater species have adapted to such hypo-osmotic media by reducing their haemolymph-external medium ionic gradient, thereby reducing the energy requirements needed to maintain haemolymph ion concentrations. Comparison between the two Irish freshwater amphipods *G. d. celticus* and *G. pulex*, when acclimated to fresh water, revealed significantly lower haemolymph sodium concentration in *G. pulex*. The lower haemolymph sodium concentration exhibited by *G. pulex* may suggest an adaptation to fresh water, which could provide *G. pulex* with an energetic advantage over *G. d. celticus*. Further discussion of sodium regulation will continue with regard to sodium influx in the later section (4. 2. 3. 3.).

As described for haemolymph sodium concentrations, haemolymph potassium, magnesium and chloride concentrations were maintained at significantly lower levels in *G. pulex* than *G. d. celticus*. Collectively, these differences equate to relatively large differences in haemolymph osmolality between the two species. Haemolymph osmolality was not measured in this study, since a micro-litre osmometer was not available to measure the limited gammarid blood samples. Previous measurements of haemolymph osmolality in *G. pulex* have been recorded as 300 mOsmol. kg^{-1} to fresh water (Shires *et al.*, 1995). From comparisons between the blood ion concentrations of these two species, haemolymph osmolality in *G. d. celticus* might be expected to be in the region of 400 -500 mOsmol. kg^{-1} in fresh water. The higher osmolality in fresh water is more typical of a brackish water species, such as *G. d. duebeni* (Shaw & Sutcliffe, 1961; Sutcliffe, 1967a) or *G. tigrinus* (Koop & Grieshaber, 2000).

Potassium is used as a counter-ion for the movement of sodium by the transport enzyme Na^+, K^+-ATPase (Péqueux, 1995). Consequently effects on sodium regulation might be expected to cause corresponding effects on haemolymph potassium concentration. However, the potassium channels on the basolateral membrane of the gill epithelium are also involved in potassium homeostasis (Onken & Riestenpatt, 1998). The basolateral potassium channel allows potassium, pumped into the cell from the haemolymph by Na^+, K^+-ATPase, to leak back out into the haemolymph. Consequently, the changes in haemolymph potassium concentration with respect to salinity are not controlled by the same process responsible for sodium movement. This may explain why potassium fails to become isotonic at about 50% sea water, unlike that seen for sodium.

In freshwater acclimated animals, haemolymph magnesium concentrations were significantly higher in *G. d. celticus* than *G. pulex*. Pantin, (1946) suggested that magnesium has anaesthetic properties in marine invertebrates. Magnesium ions can exert profound physiological effects on the neuromuscular junctions of crustaceans (Morritt & Spicer, 1993), with haemolymph magnesium concentrations correlated with low levels of activity (Morritt & Spicer, 1993; Frederich *et al.*, 2000). Therefore, the higher haemolymph magnesium concentrations found in *G. d. celticus* compared to *G. pulex*, may reflect lower levels of activity exhibited by the former over the latter. Higher activity levels of *G. pulex* over *G. d. celticus* may influence the competitive interactions between these two species, which have resulted in the replacement of the latter by the former (Dick, 1996a; Dick *et al.*, 1993; Dick *et al.*, 1994). However, rather than further speculate on the possible implications of the higher activities of *G. pulex*, the relationship between haemolymph magnesium concentration and activity levels in these amphipods needs to be established.

All haemolymph ion concentrations in *G. pulex* increased sharply to significantly higher levels at 30% sea water acclimation. The inability of *G. pulex* to maintain constant ion

concentrations with relatively small changes in external salinity may reflect the poor osmoregulatory powers of *G. pulex* with increased salinity. It has been previously found that a rise in blood chloride concentrations is accompanied by a marked increase in tissue chloride concentration (Beadle & Cragg, 1940). The inability of *G. pulex* to maintain low intracellular concentrations of ions such as chloride was believed to be responsible for the poor survival rates of this species in increased salinity media (Lockwood, 1961). There was also no attempt by *G. pulex* to reduce the rise in blood ion concentration by producing a more concentrated urine when acclimated to increased salinities (Lockwood, 1961). In contrast, *G. d celticus* were able to maintain relatively constant haemolymph ion levels between fresh water and at least 50% sea water for the main Na ion, and even up to 75% sea water for other ions (Mg, Ca, K). The differences in haemolymph ion concentrations with increasing salinity reflect variations in osmoregulatory abilities, with *G. d. celticus* capable of dealing with higher salinities than *G. pulex*.

4. 2. 3. 2. Water permeability

In all three of the Irish amphipods, *G. pulex*, *G. tigrinus* and *G. duebeni celticus,* the half time of exchange of body water ($t_{1/2}$) was not affected by alterations in the external salinity, but remained relatively constant throughout the salinity range. Animals that are unable to alter their $t_{1/2}$ with respect to the external salinity are believed to be those that inhabit water bodies with little or no fluctuations in salinity (Lockwood, 1986). Therefore, since these freshwater gammarids do not experience salinity fluctuations in their natural environment, it may not be surprising that they were unable to alter their $t_{1/2}$.

The $t_{1/2}$ for *G. d. celticus* was in agreement with previous work on this species (Bolt, 1986; Lockwood & Bolt, 1989), and was much higher than that found in either *G. pulex* or *G. tigrinus*. In general, freshwater adapted crustaceans often maintain a lower osmotic gradient and have a higher $t_{1/2}$ than those living in brackish or fully saline environments

(Rasmussen & Andersen, 1996). For example, a high $t_{1/2}$ was found in the freshwater crabs *Uca minax, Uca pugilator* and *Uca rapax* due to the increased freshwater adaptation (Hannan & Evans, 1973). The higher $t_{1/2}$ in *G. d. celticus* would reduce ion loss from the gammarid, when acclimated to fresh water. Increasing $t_{1/2}$ and subsequent ion loss would contribute to the maintenance of the higher haemolymph ion concentrations found in *G. d. celticus*.

4. 2. 3. 3. Sodium flux

Sodium influx was found to decrease with increasing salinity in all Irish gammarids. In *G. d. celticus*, sodium influx decreased significantly between fresh water and 50% sea water. At acclimation salinities of 50% sea water and higher, sodium influx was maintained at minimum equilibrium levels. The levelling out of the sodium influx at 50% sea water in *G. d. celticus* corresponds with the haemolymph sodium concentration approaching isosmotic with the external media at this seawater concentration.

Haemolymph sodium concentrations were markedly hyperosmotic when *G. d. celticus* was acclimated to 100% sea water. This high haemolymph sodium concentration in 100% sea water acclimated *G. d. celticus* could not be attributed to increased levels of sodium influx, since minimum sodium influx was seen at this seawater concentration. Furthermore, since sodium loss would be expected to be at a minimum in 100% sea water acclimated animals, it is unlikely that lower sodium loss rates in *G. d. celticus* could account for such high haemolymph sodium concentrations. A reduction in haemolymph volume with increased salinity may account for such increases in sodium concentration. However, there is no physiological evidence to suggest that *G. d. celticus* can or would alter its haemolymph volume upon seawater acclimation. Rather than further speculate upon this point, confirmation of this unusually high haemolymph sodium concentration is required. Unfortunately due to limited samples collected this was not possible in this study.

Comparison of the rate of sodium influx with respect to salinity between the three Irish gammarids, revealed significant differences that may be related to their haemolymph ion concentration and the degree of adaptiveness to fresh water. The most striking difference was observed in fresh water, with *G. tigrinus* having a sodium influx rate almost double that of *G. d. celticus* and almost five times that of *G. pulex.* The significantly higher sodium influx in freshwater acclimated *G. tigrinus* would suggest the maintenance of a significantly higher sodium concentration in this species, though this awaits confirmation.

Sodium efflux between *G. pulex* and *G. d. celticus* was not found to be significantly different when these species were acclimated to fresh water. Reclaiming sodium ions from the urine enables *G. pulex* to produce hypotonic urine with respect to the ionic concentration of their body fluids, thereby reducing sodium efflux (Sutcliffe & Shaw, 1967). The antennal glands in crustaceans are thought to be the organs responsible for claiming sodium from the urine, through the action of Na^+, K^+-ATPase (Weihrauch *et al.*, 1998). The production of hypotonic urine in *G. d. celticus* has not been investigated although the low sodium efflux found by this species may suggest it has the same capacity as *G. pulex* to reclaim sodium from the urine. In addition, part of the reduction in the rate of sodium loss may also be due to a reduction in the diffusion across the body surface (Sutcliffe, 1967a). The comparatively higher $t_{1/2}$ of *G. d. celticus* than *G. pulex* may suggest that sodium loss is reduced in the former species through this pathway.

The sodium influx rates in *G. d. celticus* were double that of *G. pulex* when acclimated to fresh water. These higher rates of sodium influx were responsible for the maintenance of a significantly higher haemolymph sodium concentration found in *G. d. celticus* than *G. pulex*. Higher haemolymph sodium concentrations of *G. d. celticus* than *G. pulex* was supported by the higher total body sodium values found in the former than the latter. The maintenance of haemolymph sodium hyperosmotic to fresh water is an energy demanding

process, requiring the active uptake of sodium against the ionic concentration gradient (Péqueux, 1995). The increased levels of sodium influx in freshwater acclimated *G. d. celticus* and *G. pulex* corresponded with the increased activity of the active transport enzyme Na^+, K^+-ATPase in both these species. Consequently, larger amounts of energy are required to maintain stable haemolymph sodium concentrations in *G. d. celticus* than *G. pulex*. Such differences in the energy requirements of osmoregulation, are likely to provide *G. pulex* with a competitive advantage over *G. d. celticus* in fresh water.

Gill Na^+, K^+-ATPase activity and sodium influx in *G. d. celticus* and *G. pulex* showed similar profiles with increasing salinity. In *G. d. celticus*, gill Na^+, K^+-ATPase activity reached basal equilibrium levels at 50% sea water (from Chapter 3) corresponding with the point at which haemolymph sodium approaches isosmotic with the external media.

4. 2. 3. 4. Rapid transfer experiments

The ability to rapidly alter sodium influx and $t_{1/2}$ mechanisms following instant transfer to either hyper- or hypo-osmotic media is of great advantage to aquatic animals that occupy waters of cyclic and/or fluctuating salinities (Bolt, 1983). Hence it is not unexpected that no significant change in sodium influx or $t_{1/2}$ was seen in rapid transfer experiments with *G. pulex*. In *G. d. celticus* no change in $t_{1/2}$ was seen 2 hours after transfer to either hyper- or hypo-osmotic media (data not shown). However, differences in sodium influx rates were seen approximately 8 hours after transfer from fresh water to sea water and vice versa. The ability of *G. d. celticus* to alter its sodium influx with rapid changes in external salinity is unlikely to be an advantage in its natural fresh water habitats of Ireland. However, despite generations of freshwater adaptation in *G. d. celticus*, it seems that some osmoregulatory mechanisms, such as rapid alteration in sodium influx, exhibited by *G. d. duebeni* have been retained. Comparison between the osmoregulatory abilities of the freshwater *G. d. celticus* and the brackish water *G. d. duebeni* can be seen in Chapter 5.

4. 2. 3. 5. Ecological significance of osmoregulatory differences

The replacement of *G. d. celticus* with *G. pulex* has been suggested to be due to a process of differential but mutual predation (Dick, 1996a; Dick *et al.*, 1999; Dick *et al.*, 1993). This study has clearly demonstrated that the Irish freshwater amphipod *G. pulex* is more adapted to fresh water than *G. d. celticus*. In these hyperosmotic regulators, the ability of *G. pulex* to maintain a lower haemolymph sodium concentration reduces the requirements for active sodium uptake and thus reduces the energy demands on this species. The lower energetic cost of osmoregulation in freshwater acclimated *G. pulex* could provide *G. pulex* with a competitive advantage over *G. d. celticus* in fresh water, which may act as a contributory factor influencing displacement of the latter by the former.

Furthermore, both *G. pulex* and *G. d. celticus* have been found to exclude *G., tigrinus* from many freshwater bodies in Northern Ireland (Dick, 1996a). The high sodium influx measurements of freshwater acclimated *G. tigrinus*, suggest that more energy was required to maintain stable haemolymph sodium concentrations. This added energy expenditure of *G. tigrinus* in fresh water, may contribute to its exclusion by *G. pulex* and *G. d. celticus* from many freshwater systems in Northern Ireland. Although, Dick (1996a) suggested differential predation as a likely explanation for *G. tigrinus* exclusion, these differences in their osmoregulatory adaptation to fresh water cannot be ignored.

Previous interactions between *G. pulex* and *G. tigrinus* have been influenced by salinity (Dick & Platvoet, 1996). The displacement of *G. tigrinus* by *G. pulex* occurred in waters of low conductivity whilst the reverse was true in waters of high conductivity. This was attributed to the fact that *G. pulex* was more physiologically adapted to fresh waters, whilst *G. tigrinus* prefers more oligohaline waters. Similar salinity adaptation may be effecting the competitive interactions between *G. pulex* and *G. d. celticus*, since the former is more adapted to fresh waters than the latter.

With increased salinity, the competitive advantage gained by *G. pulex* in fresh water, may be altered by the inability of this highly adapted freshwater species to maintain constant haemolymph ion concentrations. Therefore, slight increases in salinity may reduce fitness in *G. pulex* and affect the outcome of competitive interactions between itself and *G. d. celticus*. Consequently, in waters of increased ionic concentration, *G. d. celticus* may have the capacity to resist replacement by *G. pulex*, as reported in certain freshwater bodies of the Isle of Man and Ireland (Dick *et al.*, 1997). Although, in these waters, the ionic compositions were not measured, the differences in osmoregulatory capacities between these two freshwater species could prove crucial in influencing the competitive interactions between these two species.

CHAPTER FIVE

Geographically isolated populations of *Gammarus duebeni*: differences in their osmoregulatory physiology

5. 1. Introduction

The amphipod *Gammarus duebeni* is an extremely euryhaline species, able to tolerate large fluctuations in external salinity. They are typically found in brackish water environments including littoral zone rock pools, estuarine creeks and pans of salt marshes, where they are subject to wide salinity variations of a gradual or sudden nature (Lockwood, 1992; Sheader, 1983). Furthermore, they can also be found in fresh water habitats of Britain and Ireland (Hynes, 1954), as well as in extremely hypersaline environments, up to 73 salinity (Forsman, 1951). Investigation into the adaptation of isolated populations of *G. duebeni* to these different habitats, has uncovered marked morphological and physiological differences between the brackish water and fresh water forms (Stock & Pinkster, 1970; Pinkster *et al.*, 1970; Sutcliffe, 1972, 1978, 2000).

5. 1. 1. Morphological differences

Reid (1939) was first to identify physiological distinctions between the fresh and brackish water forms of *G. duebeni*. This led authors to search for morphological differences between the two forms. Differences in morphology were found based on the relative dimensions of the length and width of the merus (meropodite), the 4th segment of pereopod 7 (the 5th walking leg, see Fig. 5. 1. 1. 1.) in populations of *G. duebeni* (Stock & Pinkster, 1970). These morphological differences led to the proposal of two subspecies. The subspecies *G. duebeni celticus* was applied to animals from fresh water habitats where the length of the merus was greater than twice the width (ratio > 2). In the brackish water populations from Pas de Calais northwards to Sweden, the merus length was less than twice the width (ratio < 2), for these populations the subspecies *G. duebeni duebeni* was formed

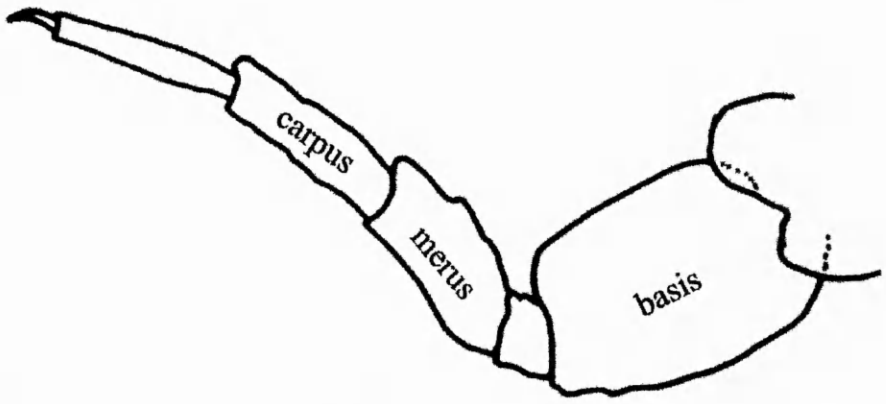


Figure 5. 1. 1. 1. The pereopod 7 of a male *G. duebeni* from fresh water in Ireland (taken from Sutcliffe, 2000).

(Stock & Pinkster, 1970). It was pointed out by Sutcliffe (1972) that due to allometric growth patterns in gammarids, the relative dimensions of certain parts of the body, including the pereopods, change in proportion to other parts of the body as the animal grows. Therefore, to minimise the effects of natural variation between individuals, Sutcliffe (1972) measured only fully adult males and converted the width and length measurements into logarithms (Sutcliffe, 2000). From these measurements two morphological distinct subspecies, *G. duebeni celticus* (freshwater form) and *G. duebeni duebeni* (brackish water form) were confirmed. In addition, an intermediate form was identified, comprising of animals from fresh water habitats close to the western and northern coastlines of the British Isles, where sodium and chloride concentrations of the fresh water media can become periodically raised due to sea spray (Sutcliffe, 1972).

There is some concern as to whether a single morphological feature (merus ratio) is sufficient for the construction of two subspecies of *G. duebeni*, particularly since the merus width measurements have been found to narrow in brackish water *G. duebeni* after long term (4 year) acclimation to fresh water (Sutcliffe, 2000). However, for the purpose of this study the nomenclature for two subspecies *G. duebeni celticus*, and *G. duebeni duebeni* was used. In addition, the 'Lizard' population was classified as belonging to the intermediate group *G. duebeni*.

5. 1. 2. Differences in osmoregulatory physiology

The differences in osmoregulatory physiology between the freshwater *G. d. celticus* and brackish water *G. d. duebeni* have been related to the higher degree of adaptiveness of the former to fresh water (Lockwood, 1992). In dilute media, animals have the tendency to lose salts and gain water, due to the high osmotic and ionic gradients between the body fluids and the external medium. *G. d. celticus* has been previously found to have a higher affinity for sodium ions than *G. d. duebeni*. This increases the uptake rate of sodium in fresh waters with low sodium concentrations over that achieved by *G. d. duebeni* (Sutcliffe, 1967a, 1971a). Hence *G. d. celticus* is more able to maintain its haemolymph sodium concentrations in media with low sodium content (Sutcliffe, 1971a).

An important physiological distinction between brackish water and freshwater stocks of *G. duebeni* is the ability of the former to alter their apparent permeability to water, as the osmotic gradient between the haemolymph and the external medium varies (Lockwood *et al.,* 1973; Lockwood & Inman, 1973, Bolt *et al.*, 1980; Bolt, 1983). In the brackish water *G. d. duebeni,* apparent permeability to water is greatest in more saline media, where the haemolymph ionic concentration becomes almost isosmotic with the external media. In contrast, the freshwater *G. d. celticus* has lost the ability to alter water permeability, and permeability has been found to remain relatively low irrespective of external salinity (Lockwood & Bolt, 1989).

The aim of this chapter is to compare the osmoregulatory physiology of four entirely isolated populations of *G. duebeni* from North West Europe. These four isolated populations of *G. duebeni* include; 1) a single population of the freshwater subspecies *G. d. celticus* (Lough Neigh, Northern Ireland), 2) two populations of the brackish subspecies *G. d. duebeni* (Totton Marsh, Southampton and Petten, The Netherlands), and 3) a single *G. duebeni* population from a fresh water stream near the coast with raised sodium content due

to sea spray (Lizard, Cornwall population). Haemolymph ion concentration, sodium flux, water permeability, and gill Na^+, K^+-ATPase activity are compared with respect to salinity acclimation. In addition, the effects of rapid salinity transfer on sodium influx and water permeability is investigated. The results of these experiments are discussed with respect to the natural habitat of each population.

5. 2. Results

5. 2. 1. Haemolymph ion concentration

The comparison of the main haemolymph ion concentrations between four stock populations of *G. duebeni* can be seen in figures 5. 2. 1 to 4. The isosmotic line in all graphs corresponds to what the haemolymph ion concentrations would be if the values simply mirrored that of the acclimation media.

Sodium

Haemolymph sodium concentration was found to increase with increasing salinity in all four populations of *G. duebeni* (Fig. 5. 2. 1.). Haemolymph sodium was maintained at hyperosmotic levels in fresh water and 25% sea water, approaching isosmoticity at 50% sea water and above. However, significant differences were found between populations at fresh water and 100% sea water concentrations ($p<0.05$). In fresh water acclimated animals, haemolymph sodium concentrations were maintained at a significantly higher level in the Lizard population (352.7 ± 9.7 mM) than in the three other populations ($p<0.05$). Furthermore, in 100% sea water acclimated animals, whilst both the Southampton and Lizard populations remained closely isosmotic with the external media; the haemolymph sodium was maintained at significantly higher concentration in the Dutch population (726 ± 22 mM, $p<0.05$). In the Irish freshwater population, haemolymph sodium was maintained at even higher concentrations (781 ± 36 mM) than that recorded for the other three populations at 100% sea water acclimation ($p<0.05$).

Potassium

Haemolymph potassium concentrations were found to increase with increased salinity acclimation, and remained hyperosmotic to the external media in all four populations of *G. duebeni* (Fig. 5. 2. 2.). The haemolymph potassium profiles for the two brackish

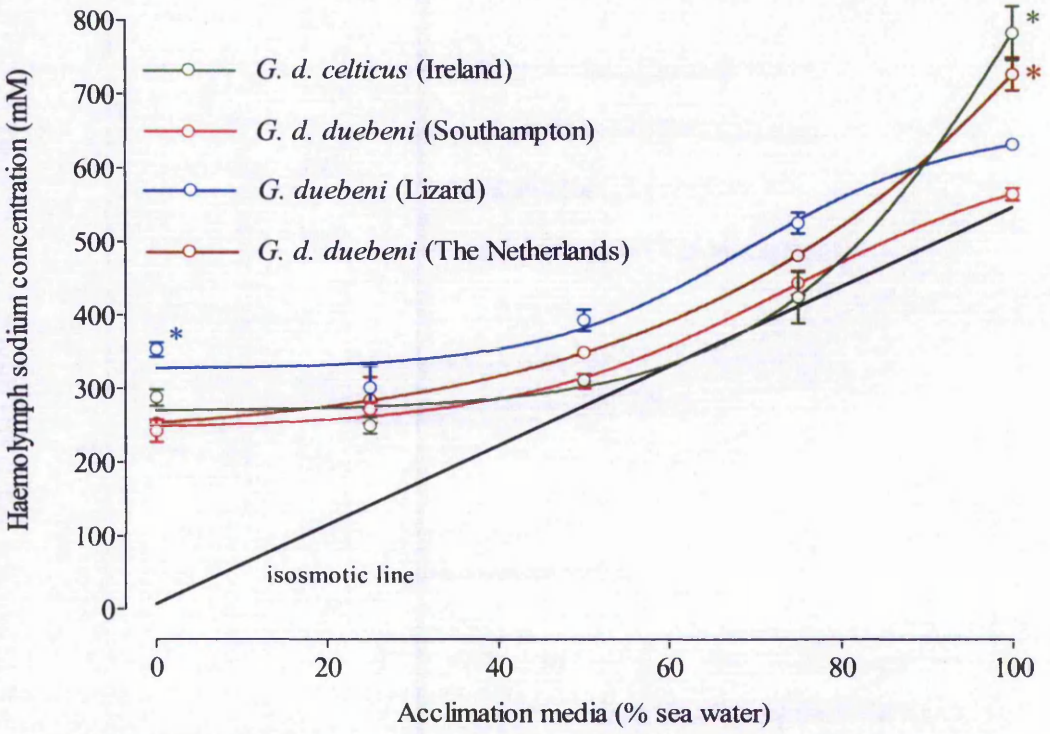


Figure 5. 2. 1. Haemolymph sodium concentration in four stock populations of *G. duebeni*, following a five day acclimation to media of increasing sea water concentration. (mean ± SE, n =5). * significant difference from the other 3 populations, $p<0.05$.

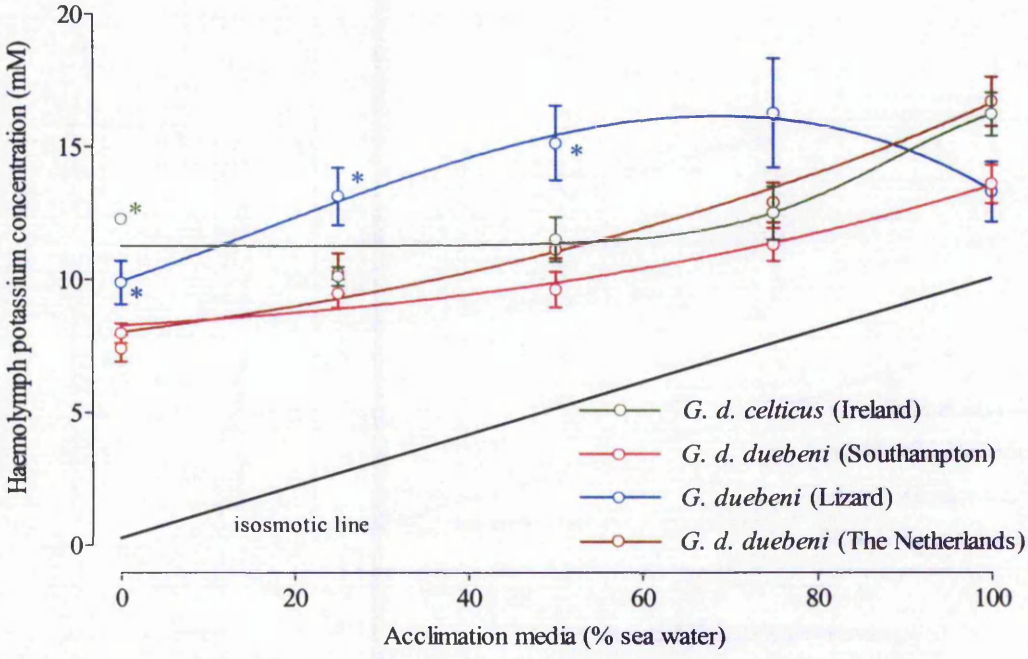


Figure 5. 2. 2. Haemolymph potassium concentration in four stock populations of *G. duebeni*, following a five day acclimation to media of increasing sea water concentration. (mean ± SE, n =5). * significant difference from the other 3 populations, $p<0.05$.

populations (*G. d. duebeni*) from The Netherlands and Southampton were similar throughout the salinity acclimation range. This was also apparent for the fresh water Irish species (*G. d. celticus*), except for differences in haemolymph potassium concentrations in fresh water acclimation media. In fresh water, haemolymph potassium concentrations in the Irish freshwater *G. d. celticus* were significantly higher (12.3 ± 0.2 mM) than that found in the other three populations ($p<0.05$). In fresh water, the value for the Lizard population was intermediate between the higher value of *G. d. celticus* and the lower values of the two brackish water populations. The haemolymph potassium profile with increasing salinity acclimation in the Lizard populations differed from that of the other three populations, resulting in significantly higher concentrations at 25% and 50% sea water ($p<0.05$).

Magnesium

In the Lizard population, haemolymph magnesium concentration was not significantly different with increased sea water concentration. Haemolymph magnesium concentration remained relatively stable in the other three populations, except for significant increases at 100% sea water for the Dutch and Irish populations and at 75% and 100% for the Southampton population. In fresh water, the haemolymph magnesium concentrations were maintained hyper-osmotic in all four populations (Fig. 5. 2. 3). The magnesium concentrations of both brackish water *G. d. duebeni* populations were significantly lower than that recorded for both the Lizard population (over the entire salinity acclimation range) and Irish population (at 0%, 75% & 100% sea water, $p<0.05$). The haemolymph magnesium concentrations were hypo-osmotic at 25% sea water for all populations except the Lizard population, which remained hyper-osmotic, becoming hypo-osmotic at sea water concentrations greater than approximately 40% sea water. The haemolymph magnesium concentrations for the Lizard population remained significantly greater than that of the two brackish water species throughout the entire salinity acclimation range ($p<0.05$).

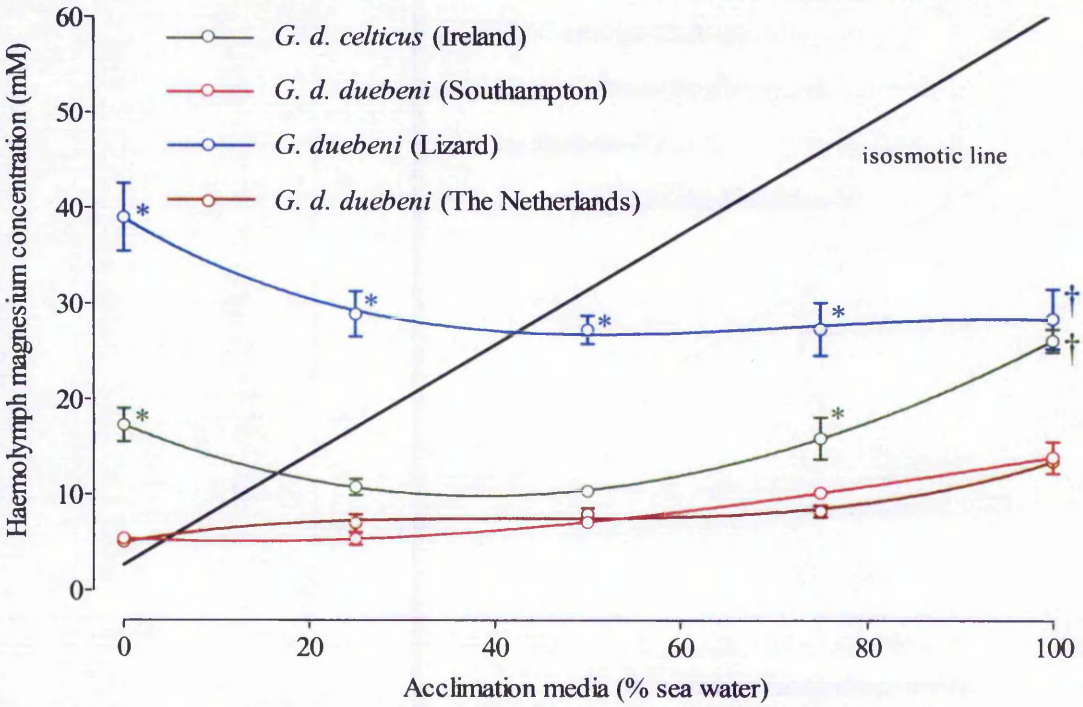


Figure 5. 2. 3. Haemolymph magnesium concentration in four stock populations of *G. duebeni*, following a five day acclimation to media of increasing sea water concentration. (mean ± SE, n =5). * significant difference from the other 3 populations, $p<0.05$; † significant difference from Dutch and Southampton populations, $p<0.05$.

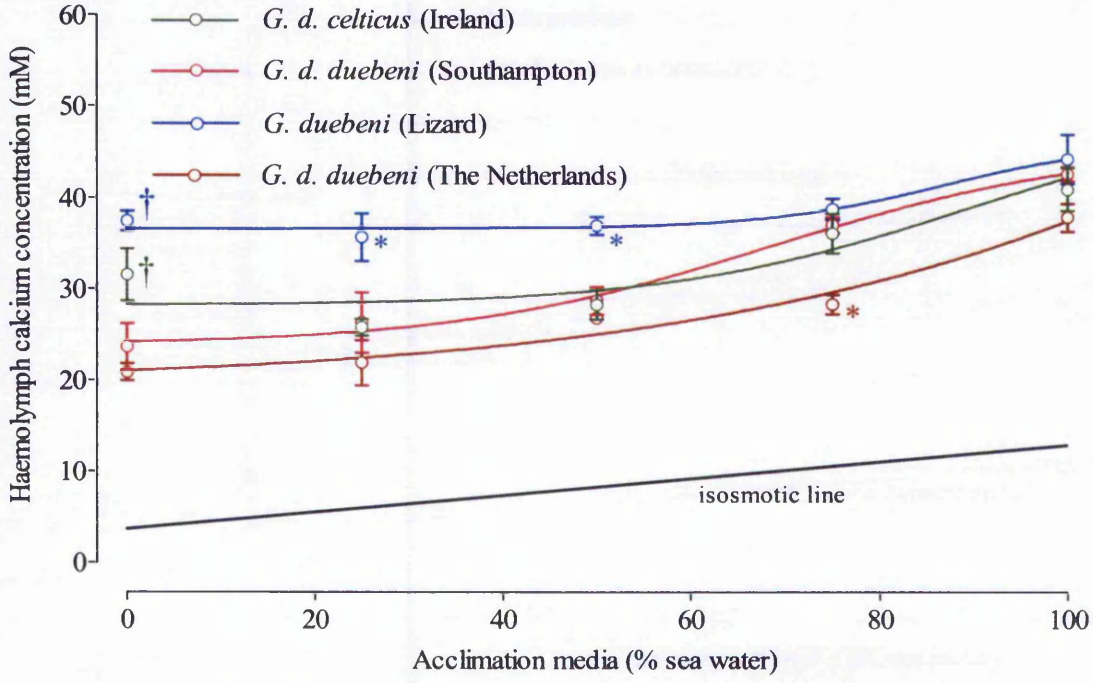


Figure 5. 2. 4. Haemolymph calcium concentration in four stock populations of *G. duebeni*, following a five day acclimation to media of increasing sea water concentration. (mean ± SE, n =5). * significant difference from the other 3 populations, $p<0.05$; † significant difference from Dutch and Southampton populations, $p<0.05$).

The haemolymph magnesium concentration of the Irish fresh water population were maintained at a level intermediate between the lower brackish water and higher fresh water Lizard populations throughout the salinity range.

Calcium

Haemolymph calcium concentrations were maintained strongly hyperosmotic to the external medium in all populations and at all acclimation salinities (Fig. 5. 2. 4.). In all populations, the haemolymph calcium concentration was maintained relatively constant at all salinities, increasing slightly at 100% sea water. Haemolymph calcium concentrations at all salinities were higher in the Lizard population, significantly higher than all other populations at 25% and 50% sea water ($p<0.05$). The haemolymph calcium concentrations in both the freshwater Irish and Lizard populations were significantly greater than the two brackish water populations during fresh water acclimation ($p<0.05$).

5. 2. 2. Water permeability

The half-time of exchange of body water ($t_{1/2}$) did not significantly differ with increasing seawater acclimation in the Irish freshwater *G. d. celticus* population (Fig. 5. 2. 5.). No significant difference was found between the $t_{1/2}$ measurements of the four populations at either fresh water, 25% or 50% sea water. There was a rapid reduction in $t_{1/2}$ between 50 and 75% sea water in the two brackish water populations (Southampton & Dutch) as well as the fresh water Lizard population. This reduction in $t_{1/2}$ was responsible for the significant difference between these populations and the Irish freshwater population at 75% and 100% sea water ($p<0.05$).

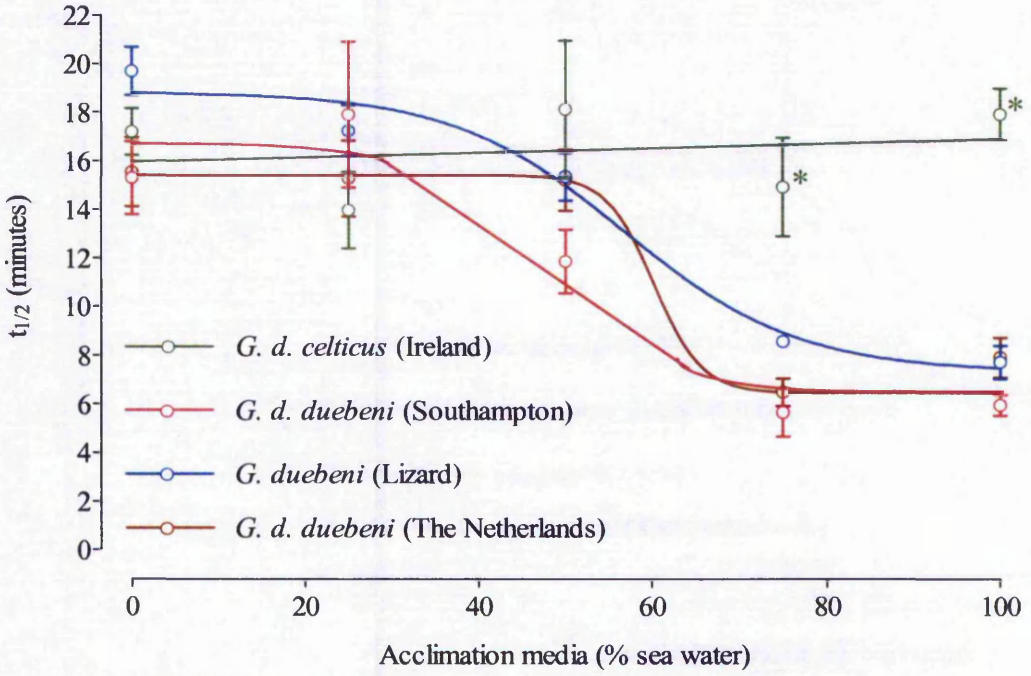


Figure 5. 2. 5. Half-time for the exchange of body water ($t_{1/2}$) in four stock populations of *G. duebeni*, following a five day acclimation to media of increasing sea water concentration. (mean ± SE, n =5). * significant difference from the other 3 populations, $p<0.05$.

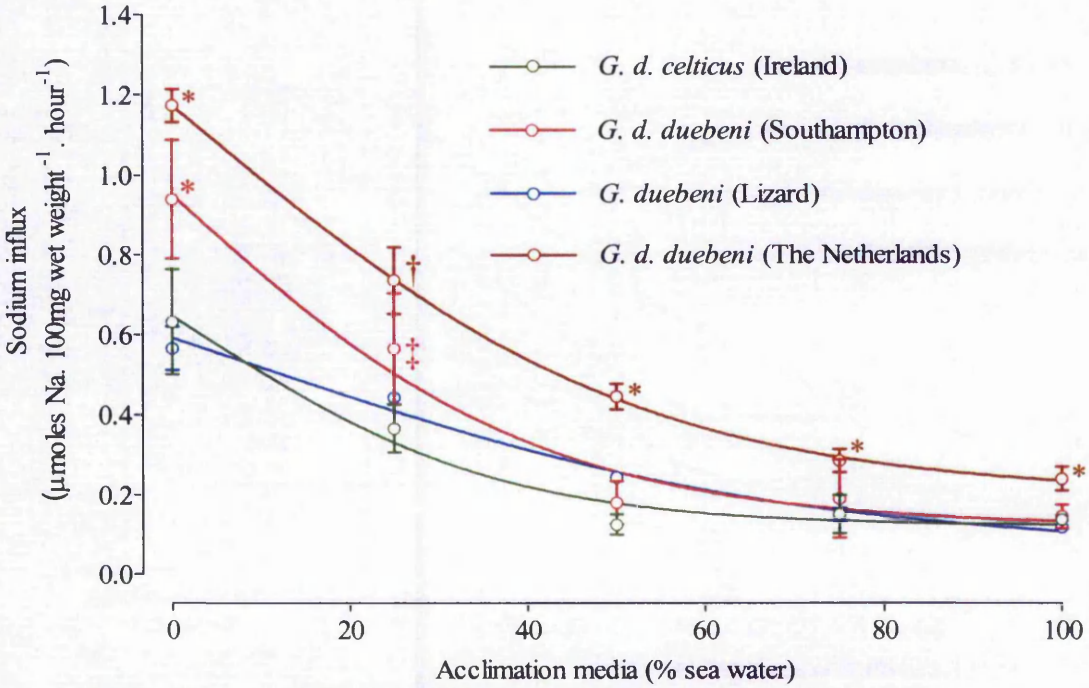


Figure 5. 2. 6. Sodium influx in four stock populations of *G. duebeni*, following a five day acclimation to media of increasing sea water concentration. (mean ± SE, n =5). Significant difference from: 1) the other 3 populations * $p<0.05$, 2) Irish and Lizard populations † $p<0.05$, 3) Irish population ‡ $p<0.05$).

5. 2. 3. Sodium flux

Sodium influx decreased with increased salinity acclimation in all populations of *G. duebeni* (Fig. 5. 2. 6.). In fresh water, the sodium influxes of the two brackish water populations (Dutch and Southampton) were significantly higher than the fresh water Irish and Lizard populations. At 25% sea water acclimation, the sodium influx of the Dutch population was significantly higher than the two freshwater populations (Irish and Lizard, $p<0.05$), whilst the Southampton population was significantly different from the freshwater Irish population ($p<0.05$). The sodium influx of the Dutch brackish water population was significantly higher than all other populations at 50%, 75% and 100% sea water ($p<0.05$).

The sodium influx, efflux and total body sodium was compared between the Irish freshwater *G. d. celticus* and the Dutch brackish water *G. d. duebeni* (Fig. 5. 2. 7.). The sodium influx in the Dutch population was almost twice that measured in the Irish population. There was a significant difference in sodium efflux between *G. d. celticus* and *G. d. duebeni* ($p<0.05$), the sodium efflux in *G. d. celticus* was approximately half that of *G. d. duebeni*. There was also a significant difference ($p<0.05$) in total body sodium between the two populations, with lower total body sodium in *G. d. celticus* than *G. d. duebeni*.

5. 2. 4. Rapid salinity transfer experiments

Sodium influx

Experiments were carried out to determine the speed of response of sodium influx to large instantaneous changes in external salinity. Instant transfer of freshwater acclimated *G. d. duebeni* (Southampton) to media of 100% sea water, resulted in a rapid decrease in sodium influx, reaching new stable levels 2 hours after transfer (Fig. 5. 2. 8.). A similar response time (2 hours) for an increase in sodium influx was observed when 100% sea water acclimated *G. d. duebeni* (Southampton) were transferred to fresh water. In comparison to

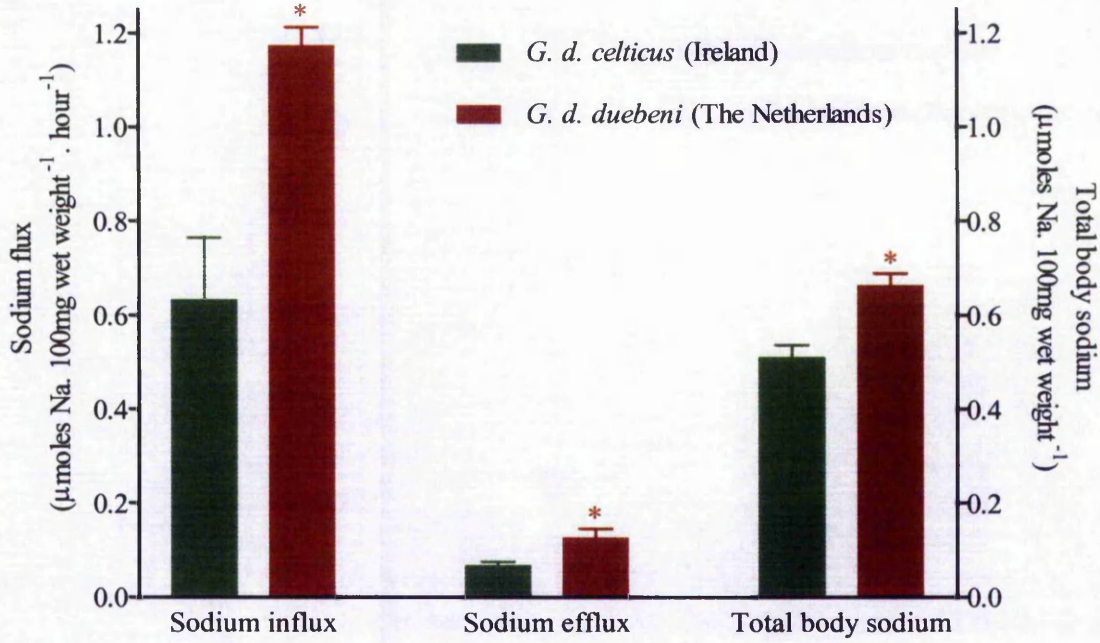


Figure 5. 2. 7. Comparison of sodium influx, efflux and total body sodium between the Irish freshwater subspecies *G. d. celticus* and the Dutch brackish water subspecies *G. d. duebeni*, acclimated for five days in fresh water (mean ± SE, n =5). * significant difference between subspecies, $p<0.05$.

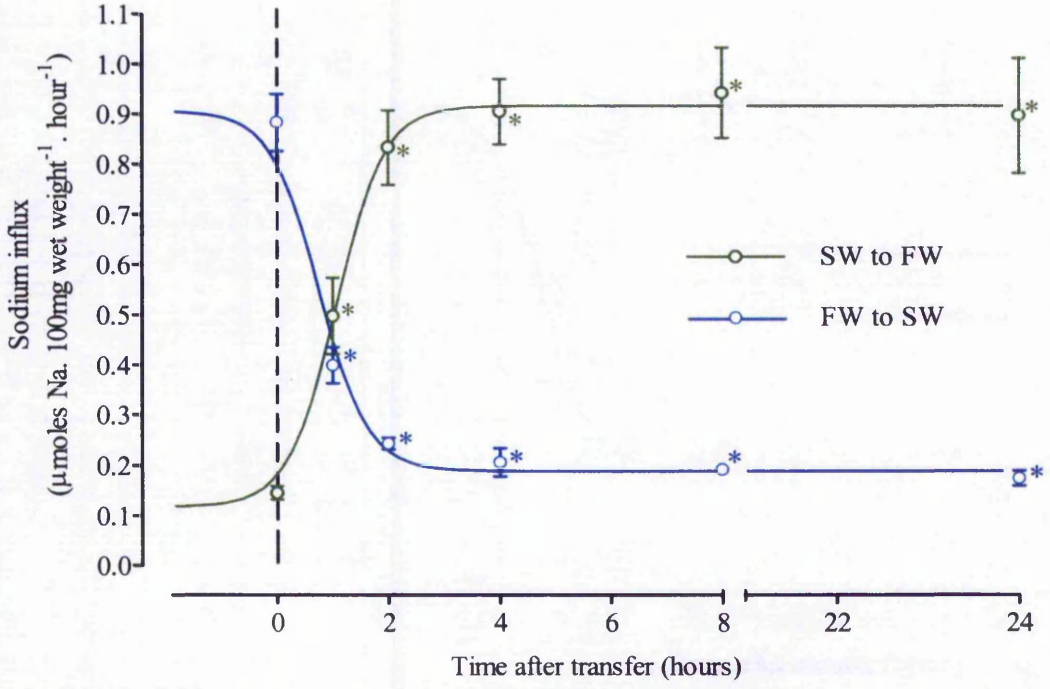


Figure 5. 2. 8. Change in sodium influx following transfer of *G. d. duebeni* (Southampton population) from fresh water (FW) to sea water (SW, blue line) and from SW to FW (green line) (mean ± SE, n =5). * significant difference from control (0 hours), $p<0.05$.

the Southampton population of *G. d. duebeni*, instant transfer of freshwater acclimated *G. d. duebeni* (Dutch) to media of 100% sea water also resulted in a rapid decrease in sodium influx (Fig. 4. 1. 2. 8.). However, new sodium influx levels were reached approximately 4 hours after transfer. *G. d. duebeni* (Dutch) also took 4 hours to reach new increased levels of sodium influx following transfer of seawater acclimated animals to fresh water.

Instant transfer of freshwater acclimated *G. d. celticus* to media of 100% sea water resulted in a rapid decrease in sodium influx, reaching new stable levels by 8 hours after transfer (Fig. 4. 2. 2. 8.). A similar response time (8 hours) for an increase in sodium influx was observed when 100% seawater acclimated *G. d. celticus* were transferred to fresh water. The 4-8 hour response time required for the change in sodium influx following transfer from fresh water to sea water and vice versa, was markedly slower than that observed for either of the brackish water populations (Figs 5. 2. 8. & 4. 1. 2. 8.).

Half time of exchange of body water ($t_{1/2}$)

The change in the half time of exchange of body water ($t_{1/2}$) following instantaneous transfer from fresh water to sea water and vice versa was examined in *G. d. duebeni* collected from The Netherlands (Fig. 4. 1. 2. 10.). Rapid transfer of freshwater acclimated *G. d. duebeni* to 100% sea water resulted in a decrease in $t_{1/2}$, reaching new stable levels approximately 18 hours after transfer. In contrast, instant transfer of 100% seawater acclimated *G. d. duebeni* to fresh water, resulted in a rapid increase in $t_{1/2}$, reaching equilibrium approximately 4 hours after transfer.

5. 3. Discussion

5. 3. 1. Haemolymph ion concentration

A typical steady state haemolymph sodium concentration curve of a hyper-osmotic- iso-osmotic regulator was exhibited for all four populations of *G. duebeni*. This agrees with previous reports on the brackish water *G. d. duebeni* (Beadle & Cragg, 1940; Lockwood, 1961; 1964; Sutcliffe, 1967a, 1971a, 1971b). Whilst the haemolymph sodium profiles of the four populations were very similar, differences between certain populations did occur in fresh water and 100% sea water acclimation. Haemolymph sodium regulation is discussed in relation to sodium flux dynamics in the following section (section 5. 3. 2.).

In fresh water media, all cations measured were maintained at a higher level in the freshwater populations (Irish and Lizard) than the brackish water populations (Southampton and Dutch). Haemolymph sodium concentrations have been found to fall further in brackish water populations than freshwater populations when exposed to media containing less than 10mM sodium (<2% sea water, Sutcliffe, 1967a). The ability of freshwater populations of *G. duebeni* to maintain a higher haemolymph sodium concentration when in fresh water, was found to be due to the higher affinity of the sodium transport system, and reduced sodium loss in the urine in freshwater populations (Sutcliffe, 1971a). Recovery of potassium and calcium ions from the urine may provide an explanation for the higher haemolymph concentrations of these ions found in the freshwater populations when acclimated to fresh water.

Haemolymph magnesium concentrations were significantly higher in the two freshwater populations than in the two brackish water populations throughout the salinity acclimation range. High haemolymph magnesium concentrations have been suggested to act as an effective anaesthetic in marine invertebrates (Pantin, 1946). High levels of magnesium in

the haemolymph have been correlated to low activity levels in decapod crustaceans. This has led to the suggestion that high haemolymph magnesium levels could effect their geographical distribution (Morritt & Spicer, 1993; Frederich *et al.*, 2000). The high haemolymph magnesium concentrations and consequently low activity levels may be related to the relative competition the animals experience in their natural environments. The absence of competitors, experienced by the 'Lizard' population, may reduce the need for high activity levels; hence the high haemolymph magnesium contributes more to the ionic strength of the haemolymph. In waters of high competition, there may be a need to reduce haemolymph magnesium concentration, increasing activity and thereby becoming a stronger competitor. The high levels of competition experienced by the brackish water *G. d. duebeni* populations may be responsible for the maintenance of low haemolymph magnesium concentrations. In addition, the relatively recent invasion of *G. pulex* into the Irish waters has increased competition for *G. d. celticus* (Dick, 1996a). This may have resulted in the lowering of haemolymph magnesium concentrations allowing for higher activity levels. This however, is pure speculation and a relationship between activity levels and haemolymph magnesium concentration in *G. duebeni* populations needs to be established.

5. 3. 2. Water permeability

The ability of aquatic organisms to alter their $t_{1/2}$ is of particular physiological importance in animals that occupy water bodies of fluctuating salinity. The two brackish water populations of *G. d. duebeni* from The Netherlands and Southampton, and the freshwater 'Lizard' population *G. duebeni* were found to lower their $t_{1/2}$ with increasing seawater acclimation. The change in $t_{1/2}$ by these three populations was not linear, but a rapid decrease in $t_{1/2}$ occurred between 50 and 75% sea water. This rapid change in $t_{1/2}$ at 50% to 75% sea water coincides with haemolymph approaching isosmoticity with the external media. In contrast, the Irish freshwater *G. duebeni celticus* was unable to alter its $t_{1/2}$ with

respect to increasing salinity. These findings confirm those found by previous authors (Lockwood *et al.*, 1973; Bolt *et al.*, 1980; Bolt, 1989; Lockwood & Bolt, 1989). The Irish freshwater subspecies *G. d. celticus* has been isolated from the sea for hundreds of generations (Hynes, 1954). It has been suggested that due to this isolation from saline influences, *G. d. celticus* has lost the ability to alter its $t_{1/2}$ with respect to external salinity (Lockwood & Bolt, 1989). Consequently, this inability to alter its $t_{1/2}$ may partially explain why *G. d. celticus* cannot survive sudden increases in external salinity, although it can survive a stepped salinity increase up to 100% sea water over a period of days (Bolt, 1986).

Morphological changes in the gill epithelia have been found to occur with changing salinity acclimation. In the gills of gammarids, junctional microtubules have been found to play a key role in the mechanical buttressing of the septate junctions against osmotic stress (Shires *et al.*, 1994, 1995). In gammarids that experienced low osmotic stress, such as the marine amphipod *G. locusta*, only short septate junctions accompanied by a small number of 'junctional' microtubules were observed (Shires *et al.*, 1995). In contrast, the septate junctions of *G. d. duebeni* were significantly longer than those of *G. locusta*. In the gill epithelium of *G. d. duebeni*, the length of the septate regions were found to be slightly greater when acclimated to sea water than to 2% sea water. In *G. d. celticus*, the most complex junctional microtubular systems were seen, which were found to alter dramatically with acclimation salinity. In fresh water, the septate junctions were accompanied by a single row of junctional microtubules in neighbouring cells. However, in sea water doublets of the junctional microtubules were seen, with some microtubules linked to the plasma membrane by dense strands (Shires *et al.*, 1994, 1995).

These differences in gill epithelia could potentially contribute to the observed flux differences of *G. duebeni* when acclimated to fresh water and sea water, by varying the extent of the unstirred layers beneath the cuticle (Lockwood *et al.*, 1973). However,

measurements of urine flow rates in *G. duebeni* were found to closely match the theoretical urine volume expected on the basis of the water flux and fraction difference between the blood and media (Lockwood & Inman, 1973). Consequently, the flux data was believed not to be affected by interferences with diffusional exchange and to reflect actual changes in water permeability (Lockwood *et al.*, 1973). There has been much concern as to whether these changes in $t_{1/2}$, determined from flux measurements using radioisotope labelled water, reflect true changes in hydraulic permeability or are merely artefacts of the experimental design (reviewed by Lockwood *et al.*, 1982; Rasmussen & Andersen, 1996). A more detailed evaluation of the use of radioisotope labelled water to determine permeability measurements can be found in chapter 2. 5. 1.

5. 3. 3. Changes in sodium influx

Sodium influx was found to decrease with increasing salinity in all four populations of *G. duebeni*. At acclimation salinities greater than or equal to 50% sea water, little change in sodium influx was found with increased salinity, maintaining low levels in all *G. duebeni* populations. This corresponds to the haemolymph sodium concentration approaching isosmoticity with external media at 50% sea water (Lockwood, 1992).

In fresh water, the sodium influxes of the two brackish water populations of *G. d. duebeni* were significantly higher than that recorded for either of the two freshwater forms. However, haemolymph sodium concentrations in fresh water were not found to be significantly higher in brackish water populations despite this increase in sodium influx. One possible explanation for this discrepancy may be the higher sodium losses in brackish water populations when acclimated to fresh water. It has been previously found that the freshwater populations of *G. duebeni* are able to reduce sodium loss from the urine by producing hypotonic urine in relation to their body ion concentration (Sutcliffe, 1971a). The sodium efflux was compared between the Irish freshwater *G. d. celticus* and the

brackish water *G. d. duebeni* collected from The Netherlands. The brackish water populations were found to have a significantly higher rate of sodium efflux than the freshwater population when acclimated to fresh water. Consequently, the higher sodium influx rates experienced by the brackish water populations could be due to the balancing out of the higher sodium loss when acclimated to fresh water.

The lower sodium influx in freshwater populations (Irish and Lizard) compared to the brackish water populations (Southampton and Dutch) when acclimated to fresh water, suggests an adaptation to life in fresh water. Active uptake of sodium and other ions from fresh water requires energy to pump ions into the animal against an outwardly directed ionic gradient. To reduce this energy expenditure in dilute media, freshwater animals often lower their haemolymph sodium concentrations. In addition, changes in relative permeability will reduce sodium loss. In both these cases active sodium uptake is reduced. In the present study, the freshwater populations of *G. d. celticus* and *G. duebeni* did not demonstrate lower haemolymph sodium concentrations than the brackish water populations. In fact, the haemolymph sodium concentrations for the 'Lizard' population were significantly higher than the other three gammarids. Lower body permeability in this freshwater population may allow for the higher haemolymph sodium concentrations exhibited.

The sodium influx rates in the brackish water *G. d. duebeni* population from The Netherlands were maintained at significantly higher levels throughout the salinity acclimation range. These high sodium influx rates of *G. d. duebeni* in fresh water are needed to offset the relatively high sodium loss rates when acclimated to fresh water. However, when acclimated to salinities greater than 50% sea water the haemolymph sodium concentration approaches isosmotic with the external media. In such isosmotic conditions the sodium loss rates are expected to be at a minimum. Consequently, the

significantly higher sodium influx rates of the brackish water *G. d. duebeni* from The Netherlands when acclimated to 100% sea water, together with the lower sodium loss rates in this media, may partially explain the significantly higher haemolymph sodium concentrations found at this salinity. This explanation however, does not account for the extremely high haemolymph sodium concentrations found in *G. d. celticus* when acclimated to sea water. The inability of *G. d. celticus* to alter its $t_{1/2}$ may be responsible for the high haemolymph sodium concentrations. Such a low $t_{1/2}$ will produce an extremely low sodium efflux potentially leading to a high sodium concentration when acclimated to 100% sea water. Rather than speculate further, confirmation of this high haemolymph sodium concentration is required.

Despite differences in sodium influx between the brackish and freshwater populations of *G. duebeni* acclimated to fresh water, no significant difference in gill Na^+, K^+-ATPase activity was found between these populations (Chapter 3. 2.). This lack of statistical significance was partially attributed to the limited number of replicates used (n = 3). It is believed that increasing sample size might highlight the apparent differences that seem to exist from sodium influx data.

5. 3. 4. Rapid transfer experiments: effects on water permeability and sodium influx

The ability to rapidly alter sodium influx and $t_{1/2}$ following instant transfer to either hyper- or hypo-osmotic media is of great advantage to aquatic animals that occupy waters of cyclic and/or fluctuating salinities. Such rapid alteration in either of these osmoregulatory mechanisms could potentially aid in the maintenance of haemolymph sodium concentration despite salinity changes in the external environment (Bolt, 1983; Lockwood, 1992).

Sodium influx was found to decrease after instant transfer of freshwater acclimated animals to 100% sea water, and rapidly increase after transfer of 100% seawater acclimated animals to fresh water in both *G. d. duebeni* and *G. d. celticus* populations. However, the speed of change in sodium influx after transfer to either hyper- or hypo-osmotic media was found to differ between all three populations of *G. duebeni*. These differences in the rate of change in sodium influx can be related to the individual environments which each of these populations inhabits.

The fastest rate of change in sodium influx was shown by the brackish water *G. d. duebeni* collected from Totton Marsh, Southampton. Here they are found in small shallow water filled depressions. In this population new levels of sodium influx were reached approximately 2 hours after transfer to either hyper- or hypo- osmotic media. This Southampton population is often exposed to fluctuations in external salinity in its natural environment, ranging from 1-22 (Lockwood & Inman, 1973). In the brackish water *G. d. duebeni* collected from The Netherlands, new levels of sodium influx were obtained between 2 and 4 hours after transfer to either hyper- or hypo-osmotic media. This population occupies large bodies of low saline waters (85 ± 0.25 mM Na, approx. 4-6 salinity), which remain relatively constant all year round except during extremely high tides and storms when seawater incursion results in raised salinities (Platvoet, pers. com.). It could be argued therefore that this *G. d. duebeni* population has become less adapted to rapid salinity fluctuations since it is less likely to experience such salinity fluctuations in its natural environment.

In comparison to these two populations of *G. d. duebeni*, the response in the exclusively freshwater *G. d. celticus* population was significantly slower following transfer to either hyper- or hypo- osmotic media. New levels of sodium influx were reached 8 hours after transfer, 2 to 4 times slower than that seen in the *G. d. duebeni* populations. This

experimental population of *G. d. celticus* was collected from Lough Neigh, Northern Ireland, which maintains constant fresh water conditions and has been isolated from sea water influences for many generations (Dick, 1996a). Consequently, adaptation to this constant fresh water environment has resulted in the inability of *G. d. celticus* to rapidly respond to sudden changes in external salinity. The inability of *G. d. celticus* to rapidly alter its sodium influx, combined with its failure to change $t_{1/2}$ with changes in external salinity, contributes to this freshwater population being unable to withstand sudden salinity shock (Lockwood & Bolt, 1989).

The ability of brackish water species such as *G. d. duebeni* to alter their $t_{1/2}$ and sodium influx with fluctuating salinities, aids in the maintenance of haemolymph ion concentrations by allowing uptake of water and salts whilst in almost isosmotic saline media and restricting such movements in hypo-osmotic fresh water (Bolt, 1983). Instantaneous changes in $t_{1/2}$ in response to rapid fluctuations in external salinity would have clear advantages in animals exposed to such rapid fluctuations in their natural environment.

The speed of change in $t_{1/2}$ upon transfer to hypo- and hyper- osmotic media in *G. d. duebeni* collected from Totton Marsh, Southampton has been previously investigated (Lockwood *et al.*, 1973; Bolt, 1983). From influx measurements using tritiated water, an increase in $t_{1/2}$ occurred almost instantaneously (<5min) after transfer of 2% seawater acclimated *G. d. duebeni* (Southampton) to 100% sea water media. A decrease in $t_{1/2}$ was then observed approximately 16-18 hours after transfer, coinciding with the haemolymph sodium concentration approaching isosmotic with the external media (Lockwood *et al.*, 1973; Bolt, 1983). In the present study when freshwater acclimated *G. d. duebeni,* collected from The Netherlands, were transferred to 100% sea water, no significant increase in $t_{1/2}$ was detected immediately after transfer. However, a decrease in $t_{1/2}$ was seen in the Dutch

population approximately 18 hours after transfer, similar to that reported in the Southampton population (Lockwood *et al.*, 1973; Bolt, 1983).

The speed of change in $t_{1/2}$ was found to differ between the two populations of *G. d. duebeni* (Southampton & Dutch) after transfer of 100% seawater acclimated animals to dilute media. Changes in $t_{1/2}$ were found to occur almost instantaneously (< 5min) after transfer of 100% seawater acclimated *G. d. duebeni* (Southampton) to dilute media (Dawson, 1982; Lockwood *et al.*, 1973). In comparison, the Dutch population of *G. d. duebeni* used in this study showed no significant difference in $t_{1/2}$ 2 hours after transfer to hypertonic fresh water. The inability of the Dutch population of *G. d. duebeni* to instantaneously alter its $t_{1/2}$, different from that reported for the Southampton population (Dawson, 1982; Lockwood *et al.*, 1973), may lie in the adaptation of these two populations to their contrasting natural habitats.

The factors that control $t_{1/2}$ in gammarids are not understood. A variety of neuroendocrine factors have been suggested as controlling osmoregulatory processes in crustaceans. These include biogenic amines such as dopamine, 5-hydroxytryptamine (5-HT) and octopamine (reviewed in Kamemoto, 1991). More recently the neuropeptide crustacean hypoglycemic hormone (CHH) (Spaning-Pierrot *et al.*, 2000), and a sequiterpene compound methyl farnesoate (Lovett *et al.*, 2001) have also been advanced as possible controlling influences.

These hormones have been found to increase sodium uptake through the up regulation of gill Na^+, K^+-ATPase and/ or H^+-ATPase as well as the increase in the number of apical sodium channels in the gills of a variety of crustaceans (Sommer & Mantel, 1988; Trausch *et al.* 1989; Bianchini & Gilles, 1990; Ahl & Brown, 1991; Eckhardt *et al.*, 1995). It is likely that similar hormonal control of osmoregulation occurs in gammarids. However, the instantaneous changes in $t_{1/2}$ and sodium influx following transfer of *G. d. duebeni* to

hyper- or hypo-osmotic media, appears to preclude the action of a hormonal system (Lockwood *et* al., 1973; Bolt *et* al., 1983). In such situations, rapid changes in osmoregulation may be influenced by differences in transepithelial potential across the body surface, since changes in the osmotic gradient were ruled out (Lockwood *et al.*, 1973).

CHAPTER SIX

The effect of copper on osmoregulation in *G. pulex*

6. 1. Introduction

Copper is an essential trace element in biological systems, used in the correct functioning of a number of proteins, including carbonic anhydrase (Henry, 1996), cytochrome oxidase (Hassall and Dangerfield, 1990) and the crustacean respiratory protein haemocyanin (Taylor & Anstiss, 1999). However, copper is toxic if internal concentrations exceed the capacity of physiological and biochemical detoxification processes (Rainbow, 1992). Excessive build up of copper in the natural environment often occurs through increases in anthropogenic inputs from mining, agrochemical and manufacturing plants (Taylor *et al.*, 1994). Due to the position of the sources of these inputs, rivers are often burdened with relatively high copper concentrations (e.g. Carnon River, Cornwall 600 μg. l^{-1} copper). This can result in aquatic organisms experiencing direct waterborne contact with high copper concentrations, and consequential detrimental effects on the local fresh water communities (Bryan & Langston, 1992).

The bioavailability and subsequent toxicity of copper to aquatic organisms increases with decreasing salinity (McLusky *et al.*, 1986). The increased bioavailability in dilute media is due to salinity effects on metal speciation (increase in free Cu^{2+} ion concentration) and/ or the reduction in competitive interactions with other major ions (i.e. Mg^{2+} and Ca^{2+}) for sites on the ion transporters (Taylor & Anstiss, 1999). It has also been suggested that exposure to high copper concentrations when simultaneously burdened with increased osmoregulatory demands at low salinity enhances vulnerability to trace metal toxicity (McLusky *et al.*, 1986). Consequently, hyper-regulating freshwater crustaceans, such as *G. pulex*, may be expected to be particularly sensitive to elevated environmental copper concentrations.

In crustaceans, the gills are a major site for the uptake of waterborne copper. Hence, gills are often exposed to relatively high environmental copper concentrations. This has resulted in gill damage in several crustaceans, such as the isopod *Jaera nordmanni* (Bubel, 1976), the shrimp *Penaeus japonicus* (Soegianto *et al.*, 1999), and the crab *Carcinus maenas* (Nonnotte *et al.*, 1993). Exposure to waterborne copper in crustaceans may result in the impairment of osmoregulatory as well as respiratory ability. Such osmoregulatory and respiratory impairment has been shown in *C. maenas* (Hansen *et al.*, 1992; Nonnotte *et al.*, 1993; Lawson *et al.*, 1995), and *P. japonicus* (Bambang *et al.*, 1995; Soegianto *et al.*, 1999).

In the freshwater amphipod *G. pulex*, elevated environmental copper concentrations have been found to have a variety of physiological effects. These include: increased locomotion (Taylor *et al.*, 1994), raised respiratory currents (Gerhardt, 1995), enhanced oxygen consumption (Kedwards *et al.*, 1996), reduced feeding rate (Blockwell *et al.*, 1998), reduced growth rate (Maund *et al.*, 1992) and ultimately death (Taylor *et al.*, 1991). These parameters are effectively integrated whole animal responses to the physiological effects of copper toxicity.

The amphipod *G. pulex* actively maintains hyper-osmotic haemolymph concentrations in its natural fresh water environment (Sutcliffe, 1967). Since the active maintenance of haemolymph ion concentrations within cell tolerance limits is essential for survival, the impairment of osmoregulation by copper is likely to result in reduced fitness and subsequent death. So far, the effects of copper on osmoregulation in gammarids have not been explored. Therefore, the aim of this study is to investigate the effects of copper toxicity on sodium regulation in *G. pulex*. The effects of copper on sodium regulation are determined from measurements of haemolymph sodium concentration, sodium influx and water permeability. Copper toxicity to gill Na^+, K^+-ATPase activity is also assessed. In

addition, the mechanisms of copper toxicity to this important active ion transporter are investigated.

6. 2. Results

6. 2. 1. Haemolymph sodium concentration

The effects of 24 hour *in vivo* copper exposure on haemolymph sodium concentration in *G. pulex* can be seen in figure 6. 2. 1. Nominal copper concentrations of 25 μg. l^{-1} caused a significant reduction in haemolymph sodium concentration compared to control (no copper added dechlorinated tap water, $p<0.05$). A copper concentration of 100 μg. l^{-1} caused haemolymph sodium concentrations to fall to approximately 65% of the control (no copper added) value. Increasing copper concentration up to 1000μg. l^{-1} failed to significantly reduce haemolymph sodium concentration from that achieved with 100 μg. l^{-1} copper exposure.

The time course for the effects of *in vivo* copper exposure of 100 and 1000 μg. l^{-1} copper on haemolymph sodium concentration was investigated (Fig. 6. 2. 2.). Nominal concentrations of 100 and 1000 μg. l^{-1} copper had almost identical effects on reducing haemolymph sodium concentration. By 4 hours, both 100 and 1000 μg. l^{-1} copper had significantly reduced haemolymph sodium concentration to less than 72% of control values ($p<0.05$). Increasing the duration of *in vivo* copper exposure up to 1 day did not significantly reduce haemolymph sodium concentration below that achieved after 4 hours.

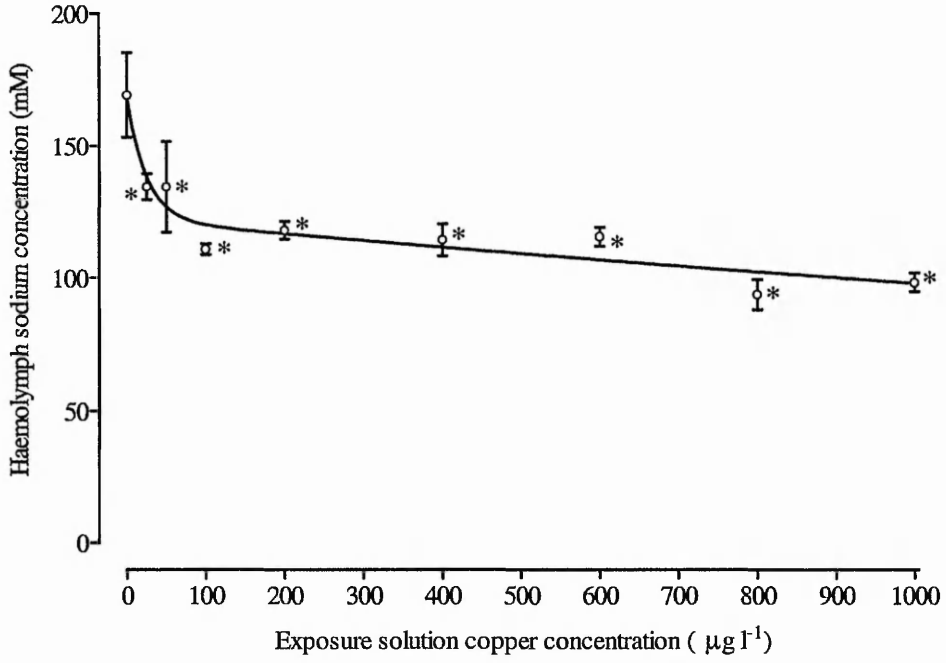


Figure 6. 2. 1. The effect of short term copper exposure (24 hours) on haemolymph sodium concentration in *G. pulex* (mean ± SE, n=5). * Significant difference from control, $p<0.05$.

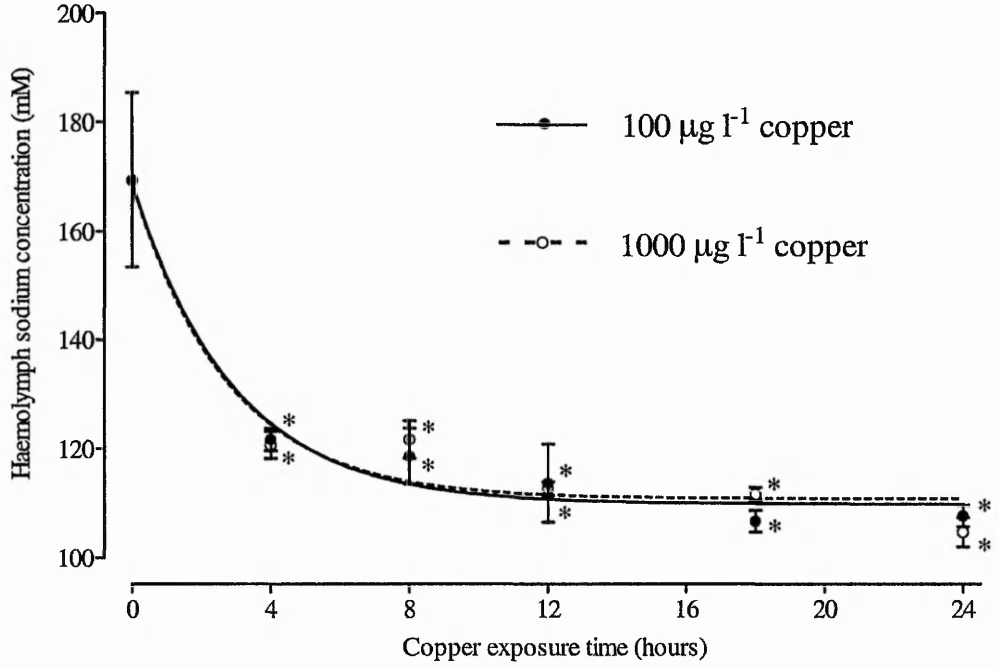


Figure 6. 2. 2. Time course for the effect of 100 and 1000µg. l^{-1} copper on haemolymph sodium concentration in *G. pulex* (mean ± SE, n=5). * Significant difference from control, $p<0.05$.

6. 2. 2. Water permeability and sodium flux

The effects of *in vivo* copper exposure on the half-time of exchange of body water ($t_{1/2}$) were investigated (Figs 6. 2. 3 & 4.). Exposure to 100 μg. l^{-1} copper failed to significantly alter $t_{1/2}$ within 1 day, or even by 102 hours. Increasing the exposure concentration to 1000 μg. l^{-1} copper also had no significant effect on $t_{1/2}$ up to 1 day exposure.

Sodium influx was significantly inhibited by 100 and 1000 μg. l^{-1} copper within 2 hours of *in vivo* exposure (Fig. 6. 2. 5.). No further significant reduction in sodium influx was seen when exposure duration was increased up to 1 day. After 1 hour exposure to 100 and 1000 μg. l^{-1} copper, sodium influx decreased to approximately 77% and 85% of control levels respectively. However, only the decrease in sodium influx caused by 100 μg. l^{-1} copper after 1 hour exposure was significantly different from the control value.

6. 2. 3. Gill enzyme activity

The effects of *in vitro* copper exposure on gill Na^+, K^+-ATPase activity revealed significant inhibition by 10 μg. l^{-1} copper or greater in the assay solution (Fig. 6. 2. 6.). Gill Na^+, K^+-ATPase activity significantly decreased with increasing copper concentrations in the assay solution ($p<0.05$). Maximum inhibition of gill Na^+, K^+-ATPase activity, to approximately 10% of control values, was achieved at 1000 μg. l^{-1} copper. In contrast, no significant inhibition of gill Mg^{2+} ATPase activity was found when exposed to *in vitro* copper concentrations up to 300 μg. l^{-1} copper (Fig. 6. 2. 7.). A copper concentration of 1000μg. l^{-1} was required to significant reduce gill Mg^{2+} ATPase to approximately 30% of control levels ($p<0.05$).

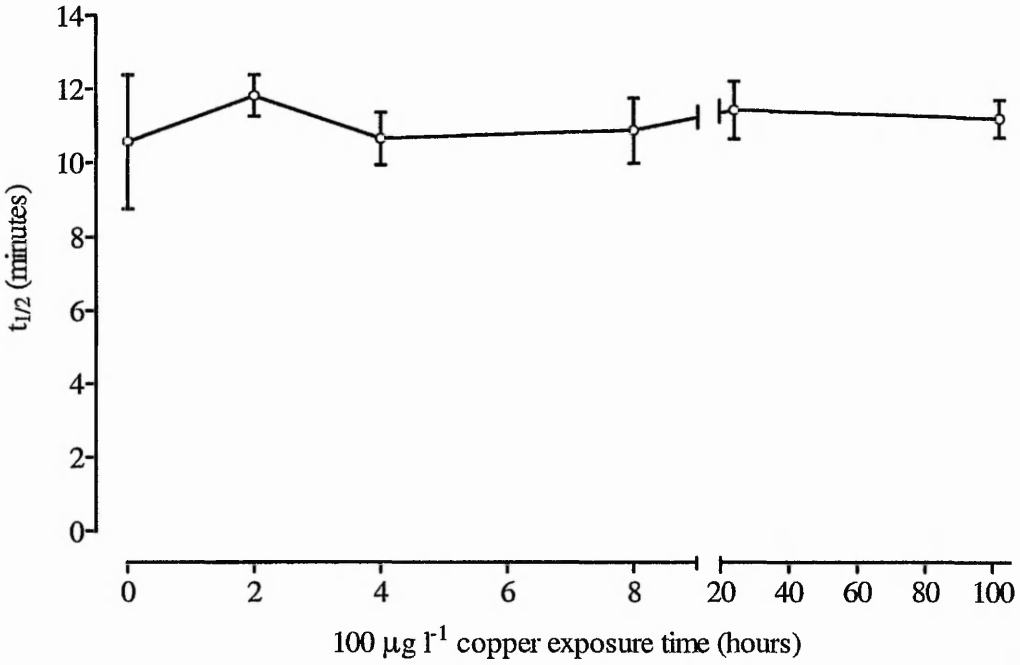


Figure 6. 2. 3. Time course for the effects of 100µg l^{-1} copper on the half-time of exchange of body water ($t_{1/2}$) in *G. pulex.* (mean ± SE, n =5).

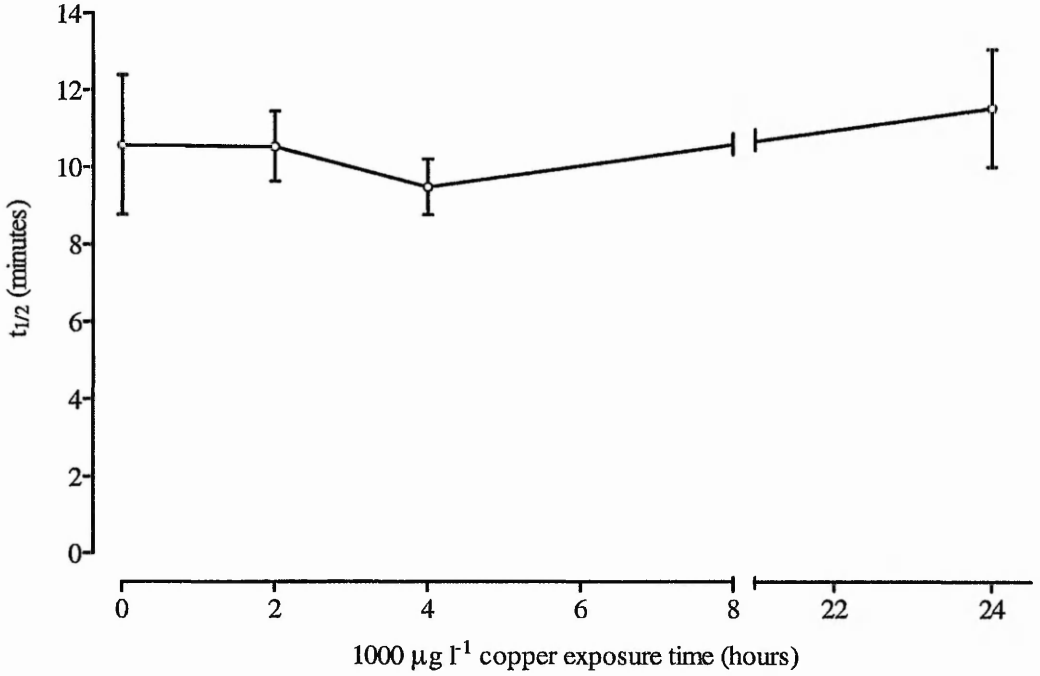


Figure 6. 2. 4. Time course for the effects of 1000µg l^{-1} copper on the half-time of exchange of body water ($t_{1/2}$) in *G. pulex.* (mean ± SE, n=5).

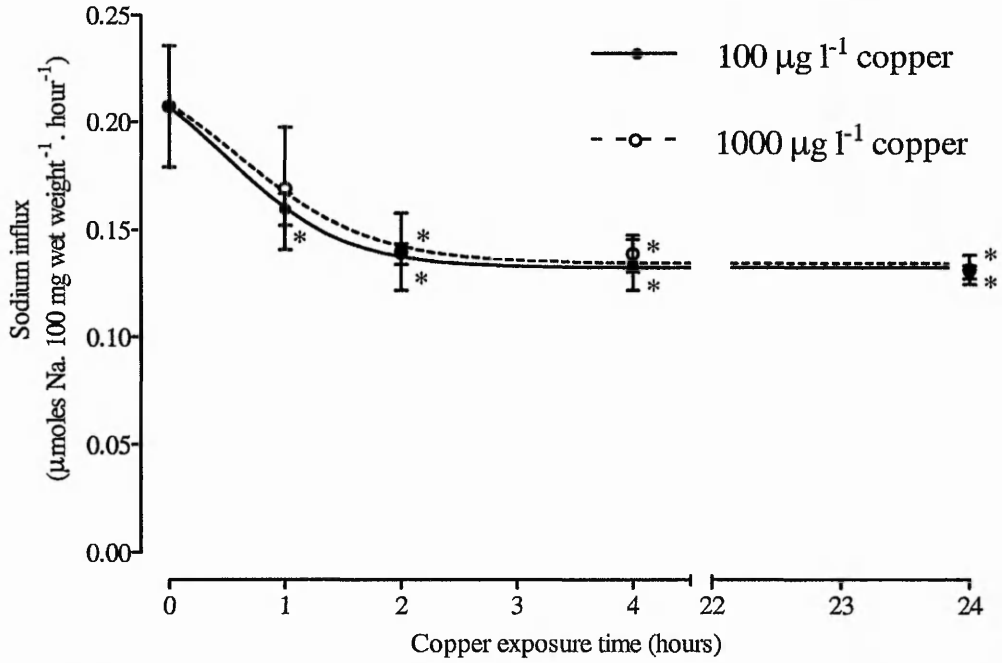


Figure 6. 2. 5. The effect of 100 and 1000μg l^{-1} copper on the sodium influx rate in *G. pulex* (mean ± SE, n=5). * Significant difference from control, $p<0.05$.

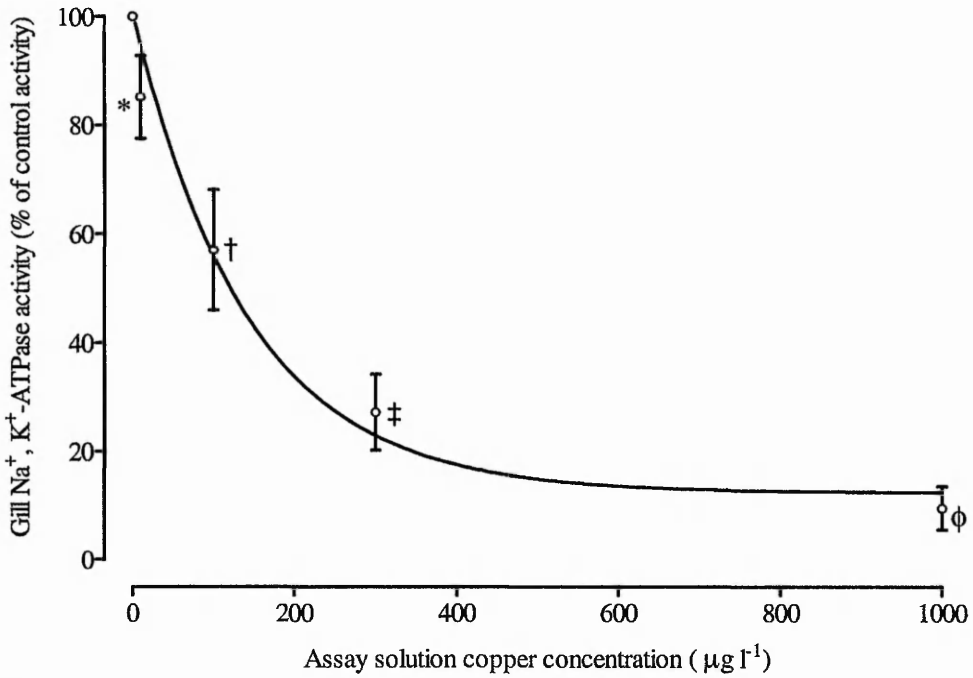


Figure 6. 2. 6. The effect of copper on gill Na^+, K^+-ATPase activity in *G. pulex* (mean ± SE, n=3). * Significant difference from control, $p<0.05$; † significant difference from 10μg. l^{-1} copper or less, $p<0.05$; ‡ significant difference from 100μg. l^{-1} copper or less, $p<0.05$; ϕ significant difference from 300μg. l^{-1} copper or less, $p<0.05$.

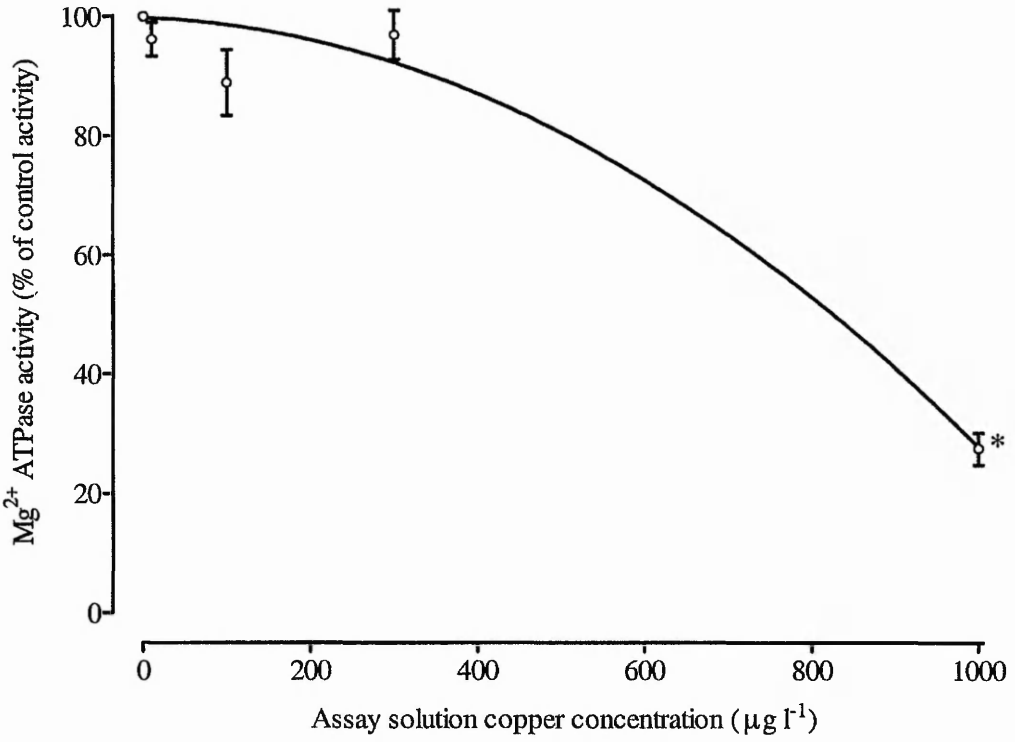


Figure 6. 2. 7. The effect of copper on gill Mg^{2+} ATPase activity in *G. pulex* (mean ± SE, n=3).* Significant difference from control, $p<0.05$.

The mechanism of copper inhibition to gill Na^+, K^+-ATPase activity was also investigated (Fig. 6. 2. 8.). Gill Na^+, K^+-ATPase activity was significantly inhibited by 1000 μg. l^{-1} copper to 10% of control values ($p<0.05$). By adding the metal chelator (1mM DTPA), gill Na^+, K^+-ATPase activity was significantly increased to 24% of control levels ($p<0.05$). Gill Na^+, K^+-ATPase activity increased to 55% of control values when 1mM DTT (metal chelator and sulphydryl reagent) was added to the assay solution. This gill Na^+, K^+-ATPase activity was significantly higher than that achieved through metal chelation alone (i. e. addition of 1mM DTPA, $p<0.05$).

In contrast, gill Mg^{2+} ATPase responded differently to copper and chelator treatment (Fig. 6. 2. 9.). *In vitro* exposure to 1000 μg. l^{-1} copper significantly inhibited gill Mg^{2+} ATPase activity to 33% of control levels. The metal chelator (1 mM DTPA) and the metal chelator plus sulphydryl reagent (1mM DTT) increased gill Mg^{2+} ATPase activity to 92% and 86%

of control levels respectively. The improvement in gill Mg^{2+} ATPase activity achieved through the addition of 1mM DTPA was significantly better than that achieved by adding 1mM DTT to the assay solution. This was opposite to that observed for gill of Na^+, K^+-ATPase activity.

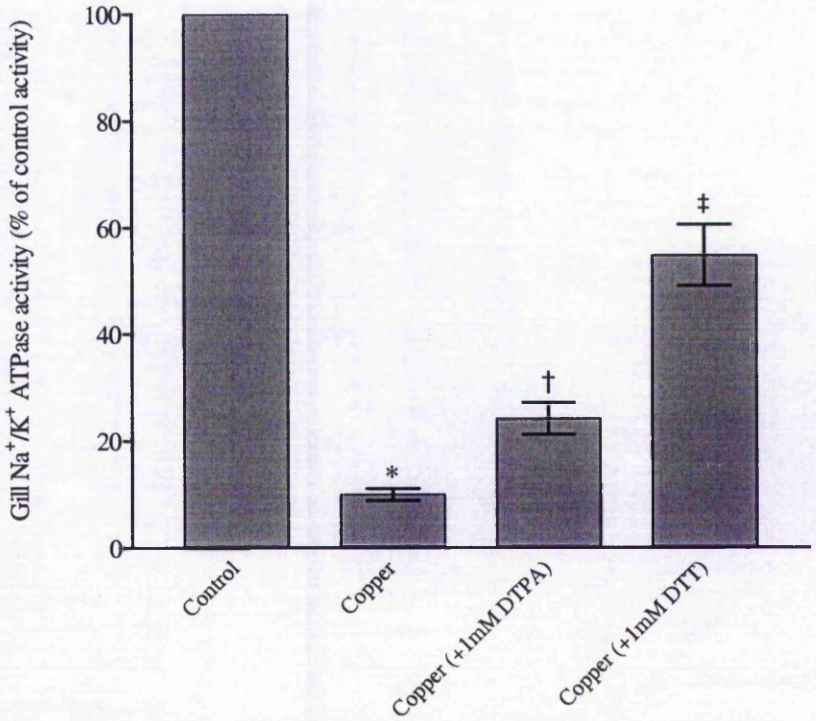


Figure 6. 2. 8. Diethylenetriaminepentaacetic acid (DTPA) and dithiothreitol (DTT) reverse the inhibitory effects of copper on gill Na^+, K^+-ATPase in *G. pulex* (mean ± SE, n=3).* Significant difference from control, $p<0.05$; † significant difference from control or 1000μg. l^{-1} copper treated, $p<0.05$; and ‡ significant difference from control, 1000μg. l^{-1} copper treated, or copper and DTPA treated, $p<0.05$.

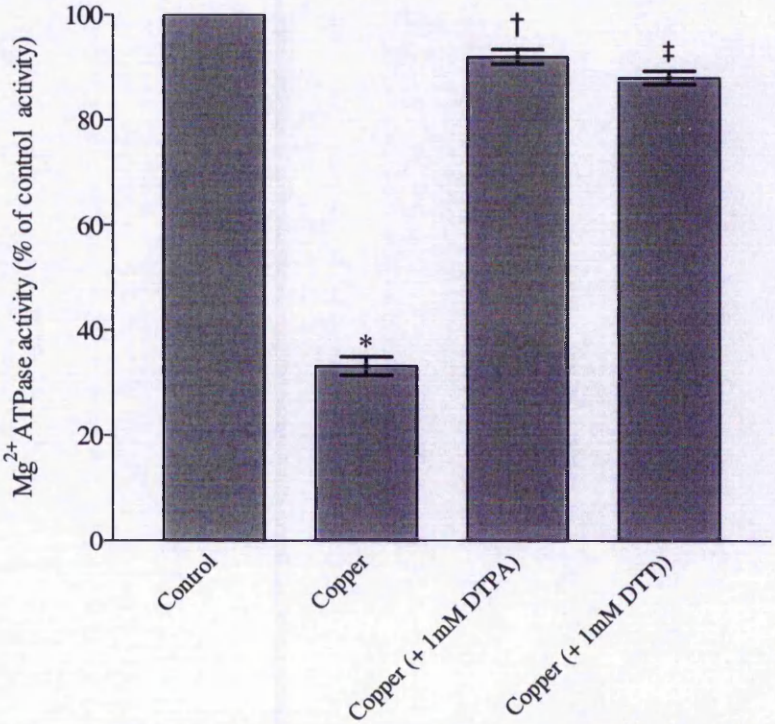


Figure 6. 2. 9. Diethylenetriaminepentaacetic acid (DTPA) and dithiothreitol (DTT) reverse the inhibitory effects of copper on gill Mg^{2+} ATPase activity in *G. pulex* (mean ± SE, n=3). * Significant difference from control, $p<0.05$; † significant difference from control or 1000μg. l^{-1} copper treated, $p<0.05$; and ‡ significant difference from control, 1000μg. l^{-1} copper treated, or copper and DTPA treated, $p<0.05$.

6. 2. 4. Copper pre-exposure on gill enzyme activity

Five day exposure to 100 and 1000μg l^{-1} copper caused significant reductions ($p<0.05$) in haemolymph sodium concentrations, to 76% and 60% of the control value respectively (Fig. 6. 2. 10.). These reductions in haemolymph sodium concentration as a result of 5 days copper exposure were almost identical to the reductions found after only 1 day (Fig 6. 2. 1.).

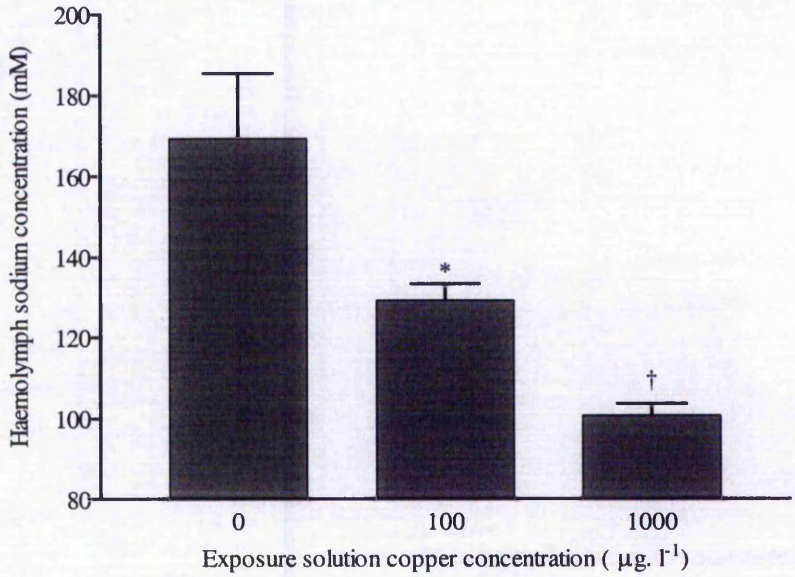


Figure 6. 2. 10. The effects of 5 day copper exposure at 100 and 1000μg l^{-1} copper on haemolymph sodium concentration in *G. pulex* (mean ± SE, n=5).* significant difference from control (no copper), $p<0.05$; † significant difference from control and 100μg. l^{-1} copper, $p<0.05$).

Gill Na^+, K^+-ATPase activity

Experiments were conducted to determine if pre-exposure of whole animals to either 100 or 1000 μg l^{-1} copper for 1 and/ or 5 days would have any effect on the copper toxicity to gill Na^+, K^+-ATPase activity in the assay solution. In control animals with no pre-exposure, gill Na^+, K^+-ATPase activity was significantly inhibited by 100 and 1000 μg l^{-1} copper in the assay solution, reducing enzyme activity to 53% and 5% of the control value respectively (Fig. 6. 2. 11.). The effects of 24 hour pre-exposure to 100 μg l^{-1} copper showed no effect on gill Na^+, K^+-ATPase activity when copper was absent from the assay solution. Pre-exposure to 100 μg l^{-1} copper for 1 day also made no significant difference to the toxic effects of 1000 μg l^{-1} copper in the assay solution on gill Na^+, K^+-ATPase activity. However, pre-exposure to 100 μg l^{-1} copper for 1 day had a significant effect on reducing 100 μg l^{-1} copper *in vitro* toxicity to gill Na^+, K^+-ATPase, resulting in an increase in gill Na^+, K^+-ATPase activity, to 90% of the control levels. Increasing the pre-exposure duration of 100 μg l^{-1} copper to 5 days caused further reductions in 100 μg l^{-1} copper *in vitro*

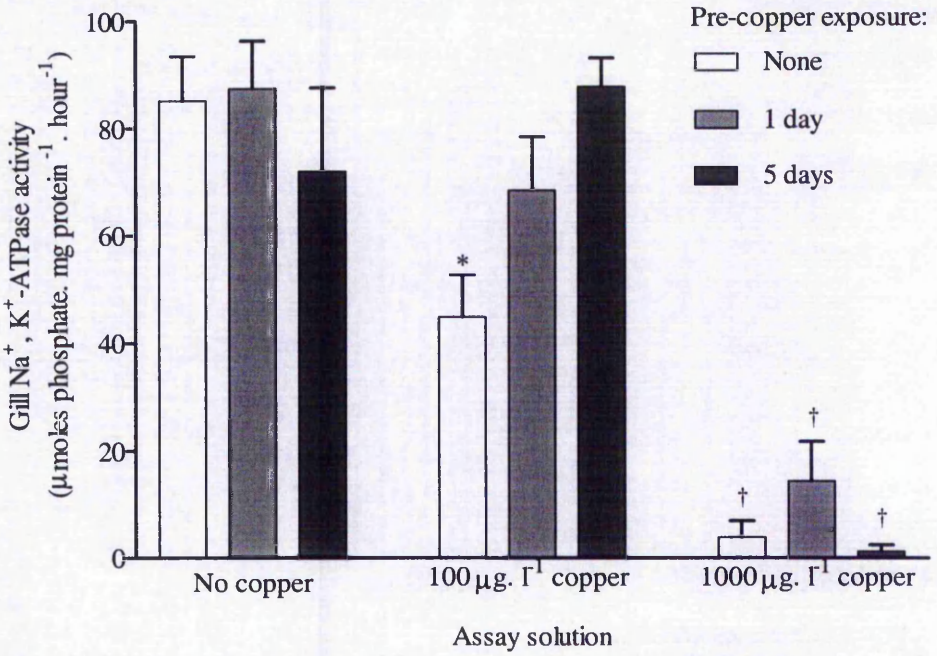


Figure 6. 2. 11. The effects of 1 and 5 day pre-exposure to 100μg l^{-1} copper on *in vitro* copper toxicity to gill Na^+, K^+-ATPase in *G. pulex* (mean ± SE, n=3). * significant difference from control (no copper), $p<0.05$; † significant difference from control and 100μg l^{-1} copper, $p<0.05$.

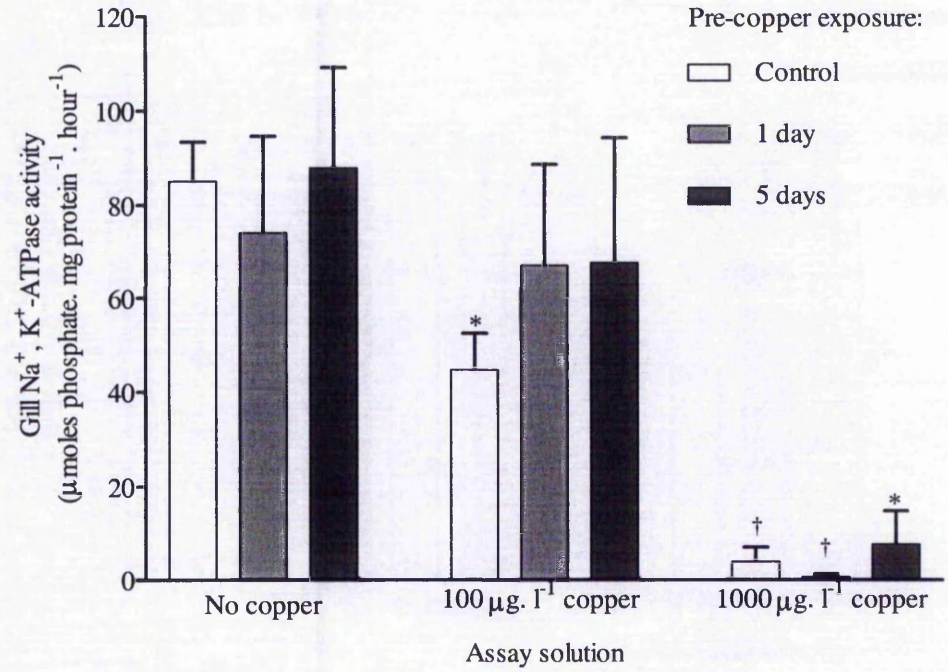


Figure 6. 2. 12. The effects of 1 and 5 day pre-exposure to 1000μg l^{-1} copper on *in vitro* copper toxicity to gill Na^+, K^+-ATPase in *G. pulex* (mean ± SE, n=3). * significant difference from control (no copper), $p<0.05$; † significant difference from control and 100μg l^{-1} copper, $p<0.05$.

toxicity to gill Na^+, K^+-ATPase. This resulted in further increases in gill Na^+, K^+-ATPase activity to approximately 120% of the control levels.

Similar reduction in 100 $\mu g\ l^{-1}$ copper toxicity to gill Na^+, K^+-ATPase were found when *G. pulex* were pre-exposed to 1000 $\mu g\ l^{-1}$ copper for 1 and 5 days (Fig. 6. 2. 12.). Increasing the pre-exposure duration of 1000$\mu g\ l^{-1}$ copper from 1 to 5 days caused no further increase in gill Na^+, K^+-ATPase over that achieved by 1 day exposure when 100 $\mu g\ l^{-1}$ copper was in the assay solution.

Note: The specific activity of gill Na^+, K^+-ATPase activity in *G. pulex* using hepes buffer (present chapter) was 2 to 3 times greater than when imidazole was used in the assay solution (Chapter 3). Possible reasons for this will be addressed in the general discussion (Chapter 9).

Gill Mg^{2+} ATPase activity

Gill Mg^{2+} ATPase activity without copper exposure was approximately 5 times greater than that of gill Na^+, K^+-ATPase activity. Copper was less toxic to gill Mg^{2+} ATPase than to Na^+, K^+-ATPase, resulting in a significant reduction in gill Mg^{2+} ATPase at 1000 but not at 100 $\mu g\ l^{-1}$ copper in the assay solution (Fig. 6. 2. 13.). Pre-exposure to 100 $\mu g\ l^{-1}$ copper for 1 day reduced gill Mg^{2+} ATPase activity in all assay solutions by approximately half of the activities recorded with no pre copper exposure. Pre-exposure to 100 $\mu g\ l^{-1}$ copper for 1 day did not alter copper toxicity to gill Mg^{2+} ATPase in the assay solution. Increasing the duration of 100 $\mu g\ l^{-1}$ copper pre-exposure to 5 days also reduced the overall gill Mg^{2+} ATPase activity in all the assay solution. However, this overall reduction in gill Mg^{2+} ATPase activity after 5 days was only two-thirds of the activities recorded with no pre-copper exposure, and significantly greater than the gill Mg^{2+} ATPase activities recorded

after 1 day pre-copper exposure. Pre-exposure to 100 $\mu g\ l^{-1}$ copper for 5 days did not alter copper toxicity to gill Mg^{2+} ATPase in the assay solution.

The overall gill Mg^{2+} ATPase activity after pre-exposure to 1000 $\mu g\ l^{-1}$ copper for 1 and 5 days (Fig. 6. 2. 14.) showed similar levels to those activities found after 5 days exposure to 100 $\mu g\ l^{-1}$ copper (Fig. 6. 2. 13.). There was no significant difference in overall Mg^{2+} ATPase activity between 1 and 5 day pre-exposure to 1000 $\mu g\ l^{-1}$ copper. Pre-exposure to 1000 $\mu g\ l^{-1}$ copper for 1day or even after 5 days, did not alter copper toxicity to gill Mg^{2+} ATPase in the assay solution.

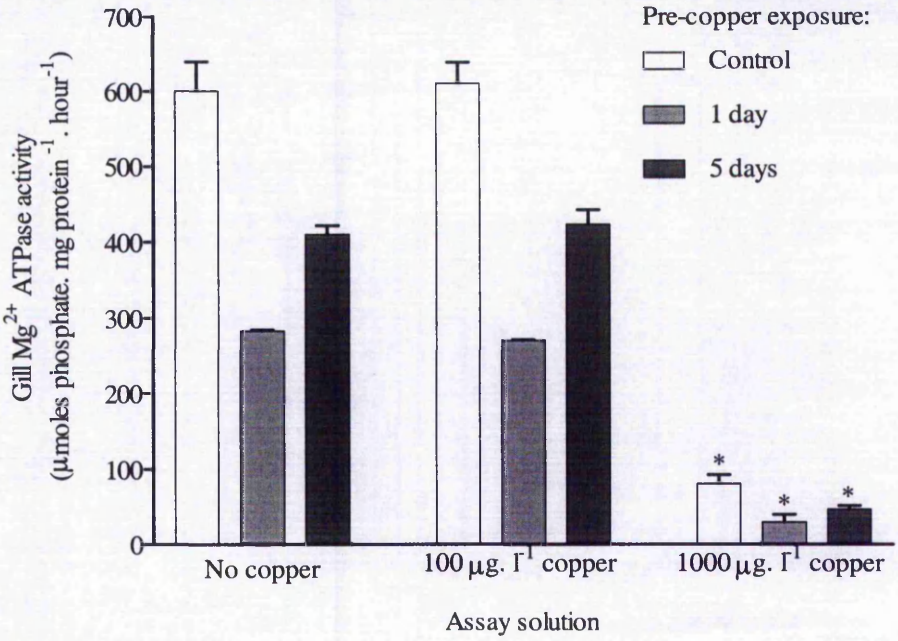


Figure 6. 2. 13. The effects of 1 and 5 day pre-exposure to 100μg l^{-1} copper on *in vitro* copper toxicity to gill Mg^{2+} ATPase in *G. pulex* (mean ± SE, n=3). * significant difference from control (no copper) and 100μg l^{-1} copper, $p<0.05$.

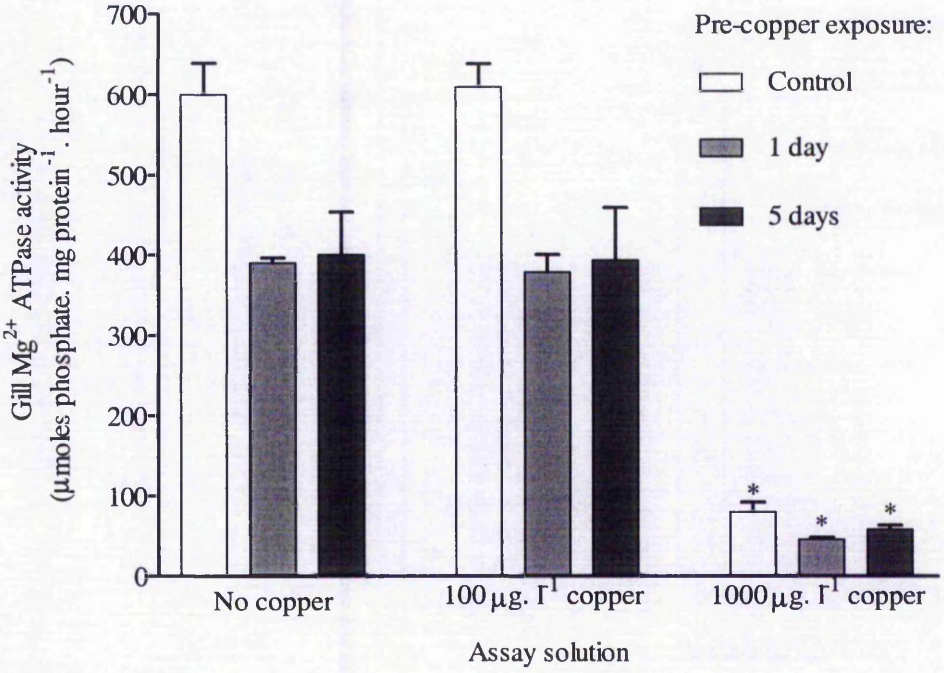


Figure 6. 2. 14. The effects of 1 and 5 day pre-exposure to 1000μg l^{-1} copper on *in vitro* copper toxicity to gill Mg^{2+} ATPase in *G. pulex* (mean ± SE, n=3). * significant difference from control (no copper) and 100μg l^{-1} copper, $p<0.05$.

6. 3. Discussion

6. 3. 1. Effects of copper on sodium regulation

The maintenance of haemolymph sodium concentration within cell tolerance limits is essential for survival. In freshwater animals, haemolymph sodium concentrations are maintained hyperosmotic to the external medium. In these circumstances animals have a tendency to gain water and lose salts. Crustaceans are able reduce ion loss and water access by restricting the overall body permeability to specialised structures, i.e. the gills. The gills, as well as providing a respiratory function, are the main organs involved in the active uptake of ions. The main pathway of copper uptake is also across the gill epithelium. Consequently copper toxicity to gill epithelial cells is likely to occur upon exposure to increased environmental copper concentrations.

Significant reductions in the haemolymph sodium concentration were found when *G. pulex* was exposed to sublethal copper concentrations. A possible explanation for the fall in haemolymph sodium concentration caused by copper exposure may be due to copper damage to the structural integrity of the gill epithelium. Structural gill damage by copper has been previously reported in the rainbow trout, *Oncorhyncus mykiss*, (Sola *et al.*, 1995), the shrimp *Penaeus japonicus* (Soegianto *et al.*, 1999) and the shore crab *Carcinus maenas* (Nonnotte *et al.*, 1993; Lawson *et al.*, 1995).

The gill epithelium of gammarids is composed of septate junctions with associated 'junctional' microtubules (Shires *et al.*, 1995). These structures are better developed in freshwater gammarids, such as *G. pulex*, thereby enabling their gills to stand up to the hypo-osmotic stress imposed by fresh water. Copper exposure could potentially lead to the partial separation of these septate junctions creating more leaky epithelium. This would result in a loss of sodium down the concentration gradient to the hypo-osmotic external

environment. In this case, sodium uptake may only be able to match sodium losses at a lower haemolymph sodium concentration. In this scenario, *G. pulex* would be expected to increase its permeability to both ions and water. Copper concentrations of 100 and 1000 $\mu g.\ l^{-1}$ failed to show any significant effect on the water permeability of *G. pulex*, despite the significant fall in haemolymph sodium concentration. This result suggests that even if structural gill damage did occur, it cannot account for the fall in haemolymph sodium concentration observed in *G. pulex*. Copper damage to the gill epithelium may cause an increase in sodium loss rate and/ or a reduction in the sodium uptake rate, which could explain the observed fall in haemolymph sodium concentration.

The absence of change in the gill permeability after copper exposure, rules out a non-specific increase in sodium loss across the gills. However, sodium loss in *G. pulex* occurs primarily through the urine (Lockwood, 1961). The reclamation of sodium ions from the urine occurs in the antennal glands of crustaceans (Horiuchi, 1980; Sarver *et al.*, 1994; Lin *et al.*, 2000). Antennal gland Na^+, K^+-ATPase was responsible for renal salt reabsorption in the freshwater crayfish *Procambarus clarkii*, enabling it to produce dilute urine (Sarver *et al.*, 1994). Copper effects on sodium loss in the urine were not examined in this study. However, since copper inhibited gill Na^+, K^+-ATPase, it may be expected that similar inhibition of Na^+, K^+-ATPase in the antennal gland could occur. This would result in a more concentrated urine, thereby increasing sodium loss, which would potentially contribute to the observed fall in haemolymph sodium concentration.

Copper toxicity to sodium uptake mechanisms may contribute to the lower haemolymph sodium concentration observed in *G. pulex*. The current model for sodium uptake in freshwater crustaceans involves an apical proton pump (V type H^+-ATPase), which actively pumps H^+ out of the cytoplasm. This creates an electrochemical gradient for the inward movement of sodium *via* sodium channels (Riestenpatt *et al.*, 1996; Onken & Riestenpatt,

1998). The driving force for sodium uptake across the gill epithelium is believed to be the activity of a basolateral enzyme Na^+, K^+-ATPase (Péqueux, 1995).

In the present study the sodium influx rate was significantly reduced 4 hours after *in vivo* exposure to both 100 and 1000μg. l^{-1} copper. This significant drop in the sodium influx rate to 67% of control values corresponds with the significant fall (to 72% of control) in haemolymph sodium concentration, due to copper exposure. Since gill Na^+, K^+-ATPase is the main driving force for the uptake of sodium (Péqueux, 1995), investigations were conducted to measure the effects of copper on gill Na^+, K^+-ATPase. A reduction in gill Na^+, K^+-ATPase could explain the significant fall in sodium influx rate.

Entry of heavy metals into aquatic crustaceans occurs primarily across the gills. It was shown in the Chinese mitten crab, *Eriocheir sinensis*, that heavy metals rapidly cross the gill cuticle and underlying epithelial layer before passing into the haemolymph (Barradas & Péqueux, 1996). The speed at which heavy metals can enter the gills after first exposure has been found to be rapid. Cadmium was detected in the gills of *C. maenas* within 15 minutes (Pedersen & Bjerregaard, 2000), whilst copper was detected in the gills of the Rainbow Trout, *Salmo gaidneri* after only 5 minutes of exposure (Taylor *et al.*, 2002). Similarly, it would be expected that copper rapidly enters the gills of *G. pulex*. Hence, copper would come into contacted with Na^+, K^+-ATPase located on the basolateral membrane of the gill epithelium before entering the haemolymph. Subsequent inhibition of gill Na^+, K^+-ATPase would affect sodium uptake, which may be responsible for the fall in haemolymph sodium concentration.

Previous studies on crustacean gill Na^+, K^+-ATPase, have found that its activity is severely inhibited by heavy metals (Boitel & Truchot, 1989; Hansen *et al.*, 1992; Weeks *et al.*, 1993; Postel *et al.*, 1998). In the present study gill Na^+, K^+-ATPase activity in *G. pulex* was

significantly inhibited by copper concentrations of 10μg. l^{-1} and greater. The fall in haemolymph sodium concentration after copper exposure was due to a reduction in the sodium influx rate caused, at least in part, by the inhibition of gill Na^+, K^+-ATPase.

Copper toxicity to Mg^{2+} ATPase activity was also demonstrated. Previous studies have disregarded the effects of heavy metals on Mg^{2+} ATPase activity, partly due to its lower sensitivity to heavy metal toxicity than Na^+, K^+-ATPase (Sola *et al.*, 1995; Watson & Beamish, 1981). In this study Mg^{2+} ATPase in *G. pulex* was significantly inhibited by copper, although as expected Mg^{2+} ATPase was far less sensitive to copper exposure than Na^+, K^+-ATPase. A copper concentration of 1000μg. l^{-1} was required to significantly inhibit Mg^{2+} ATPase activity. The effects of copper on both these enzymes were considered further, enabling the mechanisms of copper toxicity to be investigated.

Heavy metal toxicity to proteins occurs *via* a number of different mechanisms. Sulphydryl (-SH) groups on proteins are one target of heavy metal interaction. Metals are known to oxidise these –SH groups on proteins, affecting hydrogen bonding and subsequently altering the three dimensional structure of the protein (Dawson, 1982). The position and number of these –SH groups varies with different proteins, influencing the degree of heavy metal toxicity to the individual protein (Zichittella *et al.*, 2000). Furthermore, metals such as zinc can be vital for the functioning of certain enzymes, such as carbonic anhydrase (Henry, 1996). In these cases raised concentrations of heavy metals might displace the resident metal on the protein, resulting in alteration of the protein structure with subsequent effects on functional activity. Additionally, heavy metals can potentially combine with proteins *via* chelation and salt formation, resulting in reduction in functional activity (Dawson, 1982).

Experiments with the chelating agents (DTT & DTPA) were carried out to investigate the importance of heavy metal interactions with the functional –SH groups on Na^+, K^+-ATPase and Mg^{2+} ATPase activity. The addition of the heavy metal chelator DTPA after *in vitro* copper exposure was designed to remove copper bound to the enzyme. Copper bound to enzymes can potentially distort their structure, thereby inhibiting activity. Consequently, the reduction in enzyme inhibition after the addition of DTPA can be attributed to the removal of the copper from the enzyme. In the present study, the addition of DTPA was found to significantly reduce inhibition of Na^+, K^+-ATPase and Mg^{2+} ATPase activity. Mg^{2+} ATPase activity was restored to a significantly greater extent than was Na^+, K^+-ATPase by the addition of DTPA.

The heavy metal chelator DTT can reduce oxidised sulphydryl groups on proteins (Carmack & Kelly, 1968). Although DTT chelates metals, its chelating properties are much lower than those of DTPA. Therefore, by comparing the effects of DTPA and DTT on Na^+, K^+-ATPase and Mg^{2+} ATPase activity, copper toxicity through either its physical presence on the enzyme or through the oxidation of the –SH groups can be assessed. In the present study, the addition of DTT following *in vitro* copper exposure was found to reverse the inhibitory effects of copper on gill Na^+, K^+-ATPase to a significantly greater extent than DTPA. In contrast, the addition of DTT did not increase Mg^{2+} ATPase activity above that observed through the addition of DTPA. These results suggest that the –SH groups are less vital for the functioning of Mg^{2+} ATPase than they are for Na^+, K^+-ATPase. The location of –SH groups are important regarding effects on functional activity (Zichittella *et al.*, 2000). This study suggests that the location of the –SH groups in Na^+, K^+-ATPase and Mg^{2+} ATPase of *G. pulex* differs, resulting in the increased copper toxicity to the former than the latter. In conclusion, the fall in haemolymph sodium concentration was brought about by the reduction in sodium influx through, at least in part, by the inhibition of gill Na^+, K^+-

ATPase. The oxidation of the –SH groups on Na^+, K^+-ATPase was at least partly responsible for the reduction in its activity.

6. 3. 2. The effects of *in vivo* copper pre-exposure on *in vitro* copper toxicity to gill enzymes

In experiments where sub-lethal concentrations of copper (100μg. l^{-1}) were used in the assay medium, pre-exposure of whole *G. pulex* to water borne copper caused a significant increase in gill Na^+, K^+-ATPase activity. Factors that may be responsible for such an increase in gill Na^+, K^+-ATPase activity include: (1) an increase in the intrinsic activity of pre-existing enzyme units, (2) translation of new enzyme, and/ or, (3) increased expression of detoxifying proteins (e.g. metallothionein, glutathione).

Alteration in the catalytic rate of the enzyme has been found to occur in crustaceans following sudden exposure to hypo-osmotic stress (Towle & Taylor, 1976; Towle *et al.*, 1977). An increase in the expression of the existing enzyme in animals pre-exposed to sub-lethal concentrations of copper, could potentially disguise the inhibiting effects of the copper on the enzyme. Such an alteration in enzyme activity in response to sub-lethal metal stress may provide a possible explanation for the observed increases in gill Na^+, K^+-ATPase activity during *in vitro* 100μg. l^{-1} copper exposure.

Animals pre-exposed to sub-lethal copper concentrations may respond to enzyme inhibition with increased translation of new enzyme. Pre-exposure to sub-lethal copper concentrations for one and five days could potentially allow time enough for increased synthesis of new enzyme to occur. Sub-lethal copper exposure was responsible for the up-regulation of gill Na^+, K^+-ATPase activity in the rainbow trout, *Salmo gairdneri* (McGeer *et al.*, 2000). Furthermore, gradual proliferation of the ion regulatory chloride cells in the gills of the tilapia *Oreochromis mossambicus* were found to occur during waterborne copper exposure

(Li *et al.*, 1998). It has been suggested that the general stress hormone, cortisol may be partly responsible for the increased activity of gill Na^+, K^+-ATPase, through the increased synthesis of new enzyme in fish (Smith *et al.*, 2001). It may be reasonable to assume that sub-lethal copper exposure may result in a similar increase in enzyme expression in crustaceans. Such an increase in gill Na^+, K^+-ATPase expression could at least partly explain the increased activity levels of this enzyme following sublethal copper exposure.

Aquatic crustaceans have mechanisms that are able to counter the damaging effects of copper and other metals (Taylor & Anstiss. 1999). These aquatic animals, including crustaceans, have cells that possess a variety of antioxidant defences, which can protect against copper induced oxidative damage. Copper chelators such as metallothioneins (MTs) and glutathione (GSH) may remove excess copper. Anti-oxidant defences also include the enzymes superoxide dismutase (SOD), catalase and GSH peroxidase, which limit the formation of free radicals (Taylor & Anstiss. 1999). The synthesis of MTs are induced by the presence of heavy metals (Cherian & Goyer, 1978). It is believed that antioxidants such as MTs regulate the toxic effects of reactive metal ions by sequestering them, thus preventing the metals from interacting with essential structures within cells (Roesijadi, 1996; Brouwer & Brouwer, 1998). The crustacean midgut glands have been found to contain high levels of copper-metallothioneins as well as glutathione (Taylor & Anstiss. 1999). Therefore, pre-exposure to water borne copper may have triggered increased expression of these anti-oxidant defences such as MTs. These would subsequently sequester any excess copper; thereby reducing copper interactions with the enzyme and restoring gill Na^+, K^+-ATPase activity.

However, due to the nature of the Na^+, K^+-ATPase assay in this study, dilution of these anti-oxidant defences by the relatively large quantities of homogenate would have occurred. Such dilution would reduce the effectiveness of the anti-oxidants and is unlikely

to explain the observed reduction in copper toxicity. It is suggested that synthesis of new enzyme is more likely to be responsible for the increased activity of Na^+, K^+-ATPase enzyme in this study.

Metal exposure results in individuals that have acquired resistance to metal toxicity (Roesijadi, 1996). Metals, including copper, can displace zinc present in many proteins including carbonic anhydrase and MT. The displacement of zinc from these proteins and other zinc binding sites, releases the inhibition on a metal transcription factor. Consequently, animals previously exposed to metals may acquire resistance to metal toxicity (Roesijadi, 1996). High mortality and sensitivity to a metal-polluted stream was seen in a reference population of *G. pulex* when compared with a population previously exposed to metal pollution (Crane & Maltby, 1991; Maltby & Crane, 1994). Therefore, these studies suggest that *G. pulex* populations previously exposed to sub-lethal metal concentrations have an increased resistance to copper toxicity.

CHAPTER SEVEN

Effect of acanthocephalan parasite infection on osmoregulation in *G. pulex*

7. 1. Introduction

7. 1. 1. Life cycle of the Acanthocephala

The acanthocephala is a phylum of approximately 500 species of spiney worm-like parasites. All are endoparasitic and require two hosts to complete their life cycle: an intermediate host (e.g. gammarids), and a definitive host (e.g. fish or bird) (Crompton & Joyner, 1980). The adult attaches itself *via* a protrusible proboscis to the intestinal wall of the definitive host. The parasite does not possess a gut and nutrients are absorbed through the body wall. After mating, females retain the fertilised eggs in their body until a larval stage is reached. Infective larvae are released from the adult stage of the parasite and pass out in the faeces of the definitive host. When the intermediate host then swallows these eggs, the spindle shaped acanthor, usually armed with rostellar hooks and small body spines, hatches and bores into the intestinal wall of the host, eventually reaching the haemocoel. Within the haemocoel the acanthor grows and transforms into the infective cystacanth, which is enclosed in a delicate hyaline sheath produced by the larva. It remains in the haemocoel stage until the intermediate host is eaten by the definitive host (Chandler & Read, 1961).

7. 1. 2. Effects of acanthocephalan parasite infection in *G. pulex*

The two genera of acanthocephala important to *G. pulex* are *Polymorphus* and *Pomphorhynchus*. *Polymorphus minutus* and *Pomphorhynchus laevis* are two species that use *G. pulex* as their intermediate host. Although both these parasite species use the same intermediate host, the definitive host differs: *Polymorphus minutus* uses water birds, particularly ducks, whilst *Pomphorhynchus laevis* uses a few fish species (Barbel *Barbus*

barbus, Chub *Leuciscus cephalus*, Rainbow Trout *Salmo gairdneri* and Brown Trout *Salmo trutta*). It has been reported that adult *P. minutus* were found in natural infections in 86 avian spp (Crompton & Harrison, 1964). Infection of *G. pulex* by either *P. minutus* or *P. laevis* can be easily identified due to the presence of either a bright orange-red or an orange-yellow cystacanth, which can be seen through the cuticle of *G. pulex*. In the field, the frequency of acanthocephala parasite infection in *G. pulex* has been reported to be as much as 36% of the population (Kennedy & Rumpus, 1977), although in most cases, if present, approximately 5 to 10% of the population become infected.

7. 1. 2. 1. Phototaxis

The effects of the acanthocephalan parasites on *G. pulex* are widespread, including behavioural, morphological, physical and physiological changes (Marriott *et al.*, 1989; Bentley & Hurd, 1993; Bakker *et al.*, 1997; Bollache *et al.*, 2001; Plaistow *et al.*, 2001). These changes are believed to be evolved strategies by the parasites through which infected intermediate hosts are made more readily available to predation, thereby enhancing transmission to the predatory definitive hosts (Marriott *et al.*, 1989). Furthermore, the invading parasites are believed to alter the regulatory processes of the host improving the parasites' environment for enhanced growth and reproduction (Plaistow *et al.*, 2001).

Behavioural effects on *G. pulex* caused by the two acanthocephalan parasites *P. minutus* and *P. laevis* include differences in swimming activity, photo-reactivity, feeding rates, reproductive behaviour and behavioural drift (Poulton & Thompson, 1987; Marriot *et al.*, 1989; Pascoe *et al.*, 1995; Bakker *et al.*, 1997). Uninfected *G. pulex* are photophobic and negatively phototactic, causing the animal to seek the cover of darkness under rocks and boulders within the substrate, an avoidance strategy from potential predators. However, *G. pulex* infected with *P. laevis* were found to have a reduced tendency to seek darkness compared with non-infected animals (Marriott *et al.*, 1989), and were also more likely to be

seen swimming in open water than non-infected animals (Brown & Thompson, 1986). In contrast, no significant change in response to light was found in *G. pulex* infected with *P. minutus* compared to non-infected animals (Marriott *et al.*, 1989). In addition, *G. pulex* infected with *P. minutus* did not increase the time spent swimming when compared to non-infected animals. However, when they did swim, these *G. pulex* were 2 to 4 times more likely to swim closer to the surface water than non-infected animals (Marriott *et al.*, 1989). The increased exposure of infected *G. pulex* in surface waters has lead to higher densities of infected animals found in the drift population compared to the benthic population (McCahon *et al.*, 1991). These changes in behaviour in response to light and gravity brought about by parasite infection cause *G. pulex* to spend longer periods of time in open water, increasing the risk from predation.

7. 1. 2. 2. Cystacanth colouration

Other than the behavioural effects as outlined above, the actual physical appearance of the orange spot seen through the cuticle of *G. pulex* infected with either *P. minutus* or *P. laevis*, would be likely to increase its visibility to potential predators. Consequently, this would increase the parasites' chances of being passed on to the definitive host. Investigations into the effects of the orange spot on increasing predation of *G. pulex* have been carried out (Bakker *et al.*, 1997). Coloured paint was used to either mimic or hide the visible appearance of the orange spots in *G. pulex*. In laboratory controlled experiments, the presence of the orange spot was found to increase predation on *G. pulex* by the stickleback *Gasterosteus aculeatus*. Furthermore, in experiments where infected *G. pulex* had their orange spots hidden with body coloured paint, infected animals still suffered higher predation than uninfected animals, believed to be related to the difference in behaviour caused by the parasite.

7. 1. 2. 3. Physiology

The effects of the acanthocephalan parasite infection on the physiology of gammarids have received little attention. Research to date has involved the effects on (1) oxygen consumption rates (Rumpus & Kennedy, 1974), (2) haemolymph protein concentrations (Bentley & Hurd, 1993), and (3) the reallocation of energy resources (Plaistow *et al.*, 2001). Differences in oxygen consumption rates were found to occur in *G. pulex* infected with *P. laevis* (Rumpus & Kennedy, 1974). The oxygen consumption rates of *G. pulex* at 20^0C were found to decrease by 19.3% with the presence of cystacanths. Multiple infections failed to show any further reduction in oxygen consumption. In non-infected *G. pulex,* 30% of their haemolymph protein was haemocyanin, this rose to 45% in animals infected with cystacanths of *P. laevis* (Bentley & Hurd, 1993). They suggested that the increase in haemocyanin could provide infected gammarids with increased oxygen carrying capacity.

The acanthocephalan parasites are able to alter the physiological aspects of their hosts' internal environment in order to make them more favourable for the parasites' own growth and development. The acanthocephalan parasite *P. laevis* was found to alter the lipid and glycogen content of their host *G. pulex* (Plaistow *et al.*, 2001). Lipids are an important resource in invertebrates, providing twice as much energy per unit weight as carbohydrates and proteins (Hadley, 1985). In contrast, glycogen is used as the immediate energy source (Sparkies *et al.*, 1996). Consequently, effects on host glycogen levels are likely to indicate short-term physiological costs, whilst alteration in lipid content may signify more prolonged physiological effects, such as those linked to the re-allocation of energy (Plaistow *et al.*, 2001).

Although there has been a wide range of studies, which have found clear behavioural, physical and physiological effects of acanthocephalan parasites on *G. pulex*, as outlined

above, searches through the literature have failed to find any reports of parasite effects on osmoregulation in gammarids. To date, parasite effects on osmoregulation have been investigated in the estuarine crab *Rithropanopeus harrisii* (Reisser & Forward, 1991) and the juvenile chum salmon, *Oncorhynchus keta* (Urawa, 1993). The rhizocephalan parasite *Loxothylacus panopaei* was found not to alter the osmoregulatory ability of the hyper-regulating crab *R. harrisii* (Reisser & Forward, 1991). However, juvenile salmon, infected with the ectoparasitic flagellate *Ichthyobodo necator* were found to have a marked effect on osmoregulation through the epidermal destruction of the skin (Urawa, 1993).

Previous research has shown that acanthocephalan parasite infection can affect the mating success of *G. pulex* through changes in neuroendocrine activity (Bollache *et al.*, 2001). There has been increasing evidence of neuroendocrine factors controlling osmoregulatory processes in crustaceans (reviewed in Kamemoto, 1991). These neuroendocrine factors include amines (e.g. dopamine, 5-hydroxytryptamine (5-HT, serotonin), octopamine), neuropeptides (crustacean hyperglycemic hormone (CHH) Spanings-Pierrot *et al.*, 2000) and a newly discovered sequiterpene compound methyl farnesoate (Lovett *et al.*, 2001). It would seem possible therefore, that parasite changes in neuroendocrine modulation may affect osmoregulation in *G. pulex*. The aim of this chapter is to determine the effects of the acanthocephalan endoparasite *Polymorphus minutus* on the osmoregulation of *G. pulex*. The osmoregulatory effects of *P. minutus* are determined from measurements of haemolymph ion concentrations, water permeability, and sodium flux recorded over the salinity acclimation range of *G. pulex*. In addition, the effects of *P. minutus* on the oxygen consumption rates of *G. pulex* are determined.

7. 2. Results

7. 2. 1. Parasite effects on oxygen consumption in *G. pulex*

In addition to the effects on osmoregulation, investigations were carried out to determine the effects of *P. minutus* infection on the oxygen consumption in *G. pulex* (Fig. 7. 2. 1.). The rate of oxygen consumption decreased with decreasing oxygen concentration in the media. In both infected and uninfected groups, oxygen consumption was relatively stable between approximately 150 to 250 μmoles. l^{-1} oxygen. There was no significant difference in the rate of oxygen consumption between infected and uninfected animals throughout the range of oxygen concentrations measured.

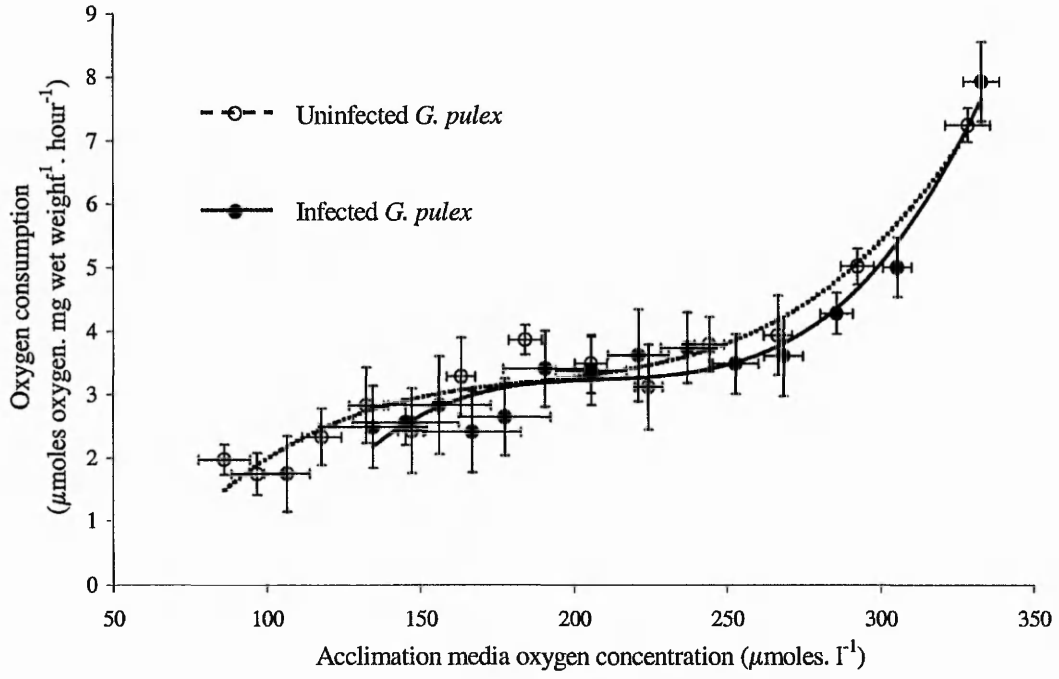


Figure 7. 2. 1. The effects of *P. minutus* on oxygen consumption rates in *G. pulex*, measured at 15 ± 0.1^{0}C (mean ± SE, n=5).

7. 2. 2. Parasite effects on haemolymph ion concentration

Sodium

The effects of the acanthocephalan endoparasite *P. minutus* on haemolymph ion concentrations in *G. pulex* can be seen in figures 7. 2. 2. to 5. Parasite infection had no significant effect on haemolymph sodium concentration in *G. pulex*, when acclimated to fresh water or 30% sea water (Fig. 7. 2. 2A.). However, at 15% sea water, haemolymph sodium concentrations were significantly higher in infected than in uninfected animals ($p<0.05$). The change in haemolymph sodium concentration with respect to the sodium gradient between the haemolymph and the external medium was calculated (Fig. 7. 2. 2B.). In uninfected animals, a significant increase in haemolymph sodium was seen at a sodium gradient between 120 and 100 mM sodium. In contrast, a significant increase in haemolymph sodium in infected animals, was seen at a sodium gradient between 190 and 170 mM sodium.

Potassium

In uninfected *G. pulex*, acclimation salinity had very little influence on haemolymph potassium concentration (Fig. 7. 2. 3.). The slight rise in haemolymph potassium concentration measured in 30% sea water was not significant. In contrast, haemolymph potassium concentrations significantly increased in infected animals acclimated to 15% and 30% sea water when compared to fresh water ($p<0.05$). Infected animals had significantly higher haemolymph potassium concentrations than uninfected animals when acclimated to 15% sea water ($p<0.05$).

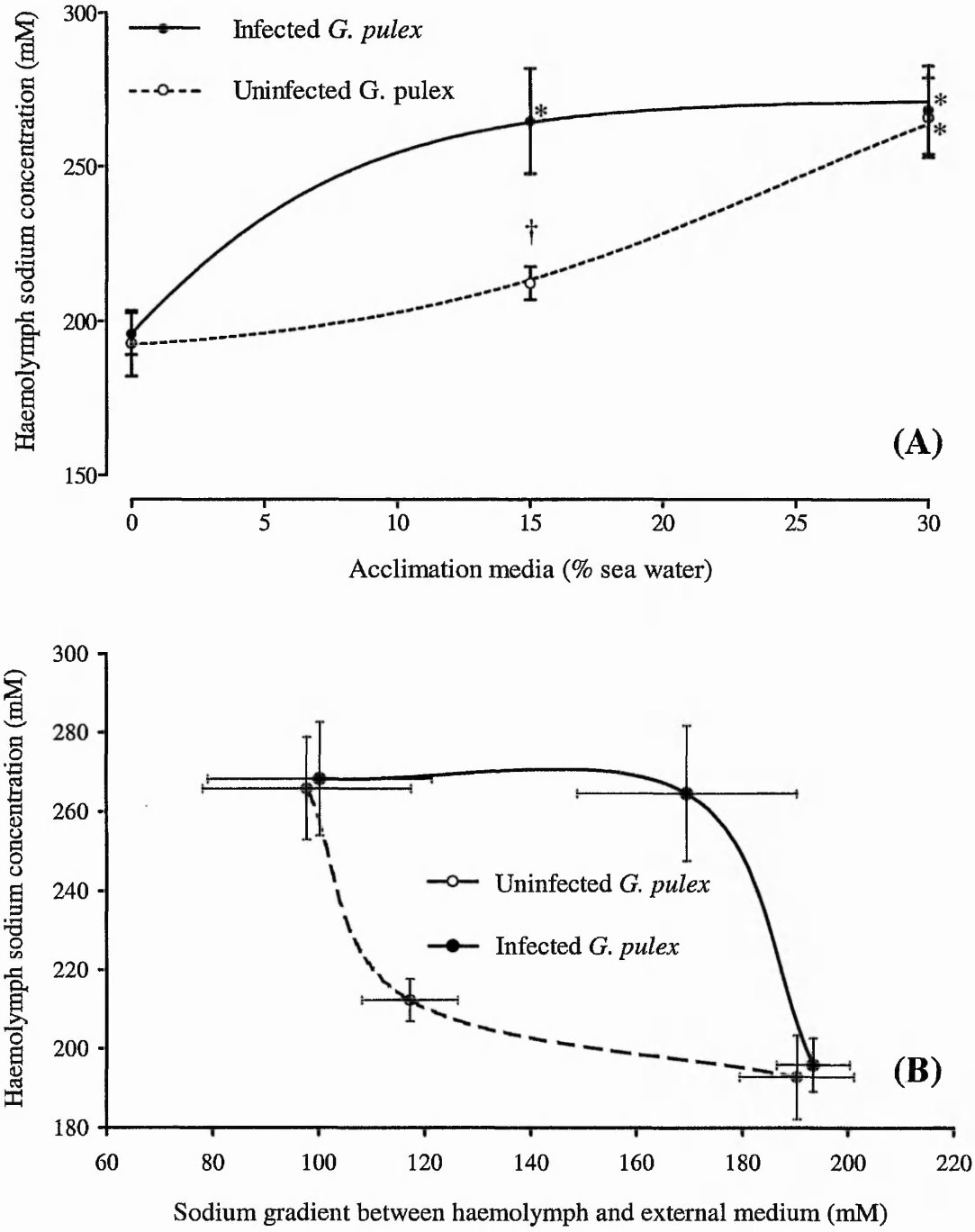


Figure 7. 2. 2. (A) The effects of *P. minutus* infection on haemolymph sodium concentration in *G. pulex*, acclimated to increasing sea water concentration (mean ± SE, n=5). * Significant difference from 0% sea water, $p < 0.05$; † significant difference between infected and uninfected animals, $p < 0.05$. (B) Differences in sodium gradient between infected and uninfected *G. pulex*, with respect to the haemolymph sodium concentrations from graph A.

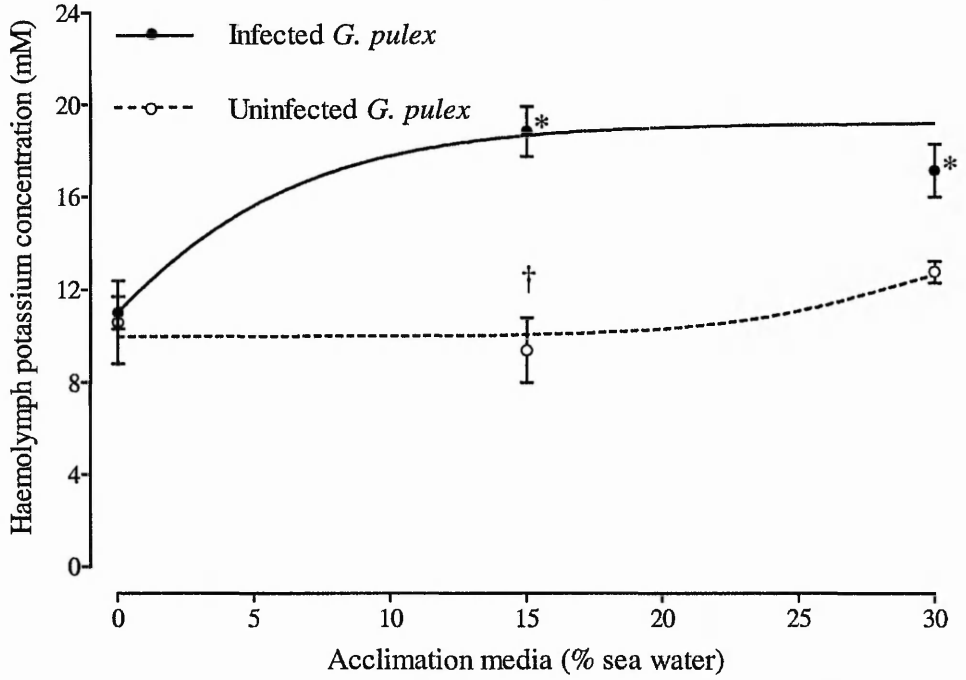


Figure 7. 2. 3. The effects of *P. minutus* infection on haemolymph potassium concentration in *G. pulex* acclimated to increased sea water concentration (mean ± SE, n=5). * significant difference from 0% sea water, $p<0.05$.† significant difference between infected and uninfected animals, $p<0.05$.

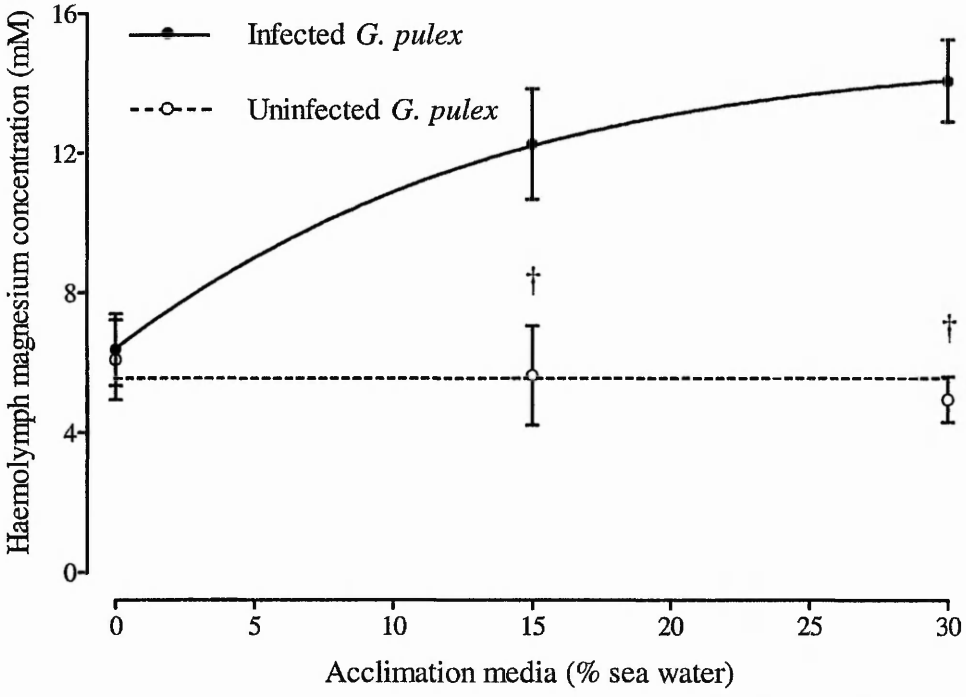


Figure 7. 2. 4. The effects of *P. minutus* infection on haemolymph magnesium concentration in *G. pulex* acclimated to increased sea water concentration (mean ± SE, n=5). † significant difference between infected and uninfected animals, $p<0.05$.

Magnesium

In uninfected *G. pulex*, acclimation salinity had no significant effect on haemolymph magnesium concentration (Fig. 7. 2. 4.). In contrast, haemolymph magnesium concentrations significantly increased in infected animals acclimated to 15% and 30% sea water when compared to fresh water ($p<0.05$). Infected animals had significantly higher haemolymph magnesium concentrations than uninfected animals when acclimated to 15% and 30% sea water ($p<0.05$).

Calcium

Significant differences in haemolymph calcium concentration between infected and uninfected animals were seen throughout the salinity acclimation range ($p<0.05$, Fig. 7. 2. 5.). Haemolymph calcium concentrations in infected animals were maintained approximately three times that of uninfected animals throughout the salinity acclimation range. Haemolymph calcium concentration was independent of the external salinity for both infected and uninfected animals.

7. 2. 3. Parasite effects on water permeability and sodium flux

The half time of exchange of body water ($t_{1/2}$) showed no significant difference between infected and uninfected animals at each of the acclimation salinities (Fig. 7. 2. 6.). In addition, the $t_{1/2}$ for both groups did not significantly differ with increased seawater acclimation.

The effects of *P. minutus* infection on the sodium influx rate of *G. pulex*, after acclimation to increasing sea water concentrations were investigated (Fig. 7. 2. 7A.). Parasite infection had no significant effect on sodium influx in *G. pulex*, when acclimated to fresh water or 30% sea water. However, at 15% sea water acclimation, the sodium influx for the uninfected animals was significantly lower than that of the infected animals ($p<0.05$). The

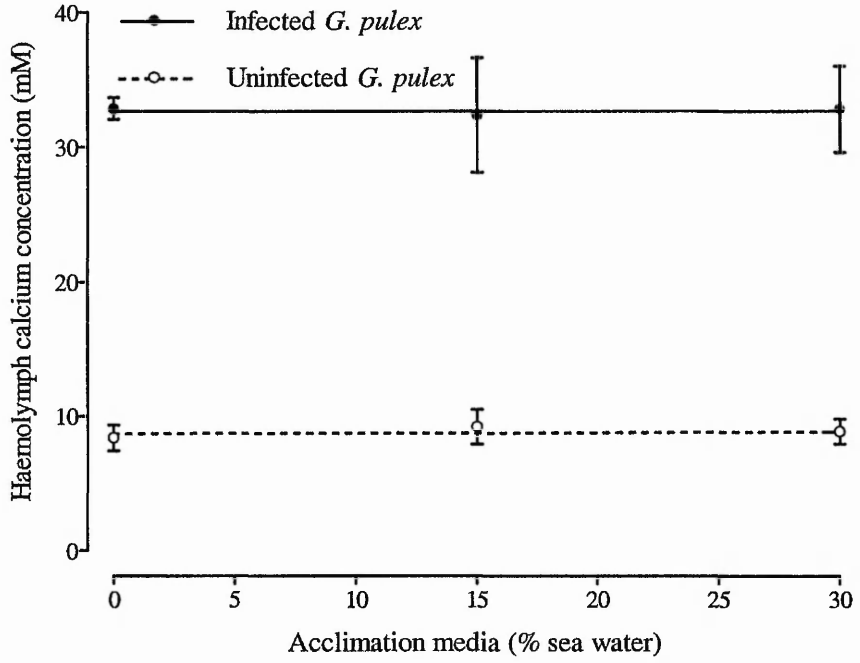


Figure 7. 2. 5. The effects of *P. minutus* infection on haemolymph calcium concentration in *G. pulex* acclimated to increased sea water concentration (mean ± SE, n=5). Infected animals were significant difference from uninfected animals at all salinities, $p<0.05$.

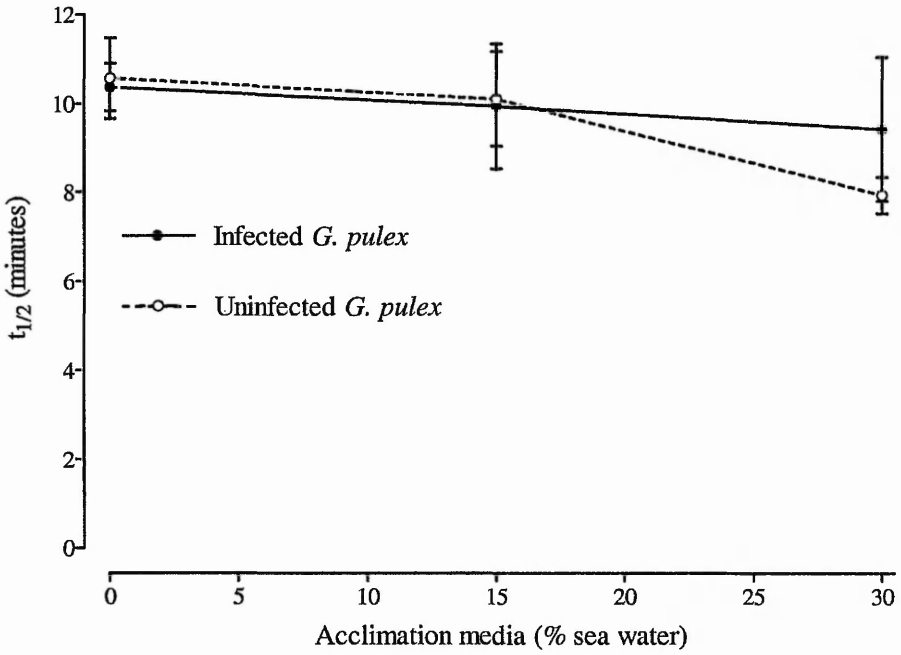


Figure 7. 2. 6. The effects of *P. minutus* infection on the half time of exchange of body water ($t_{1/2}$) in *G. pulex* acclimated to increasing sea water concentrations (mean ± SE, n=5). No significant difference between $t_{1/2}$ values.

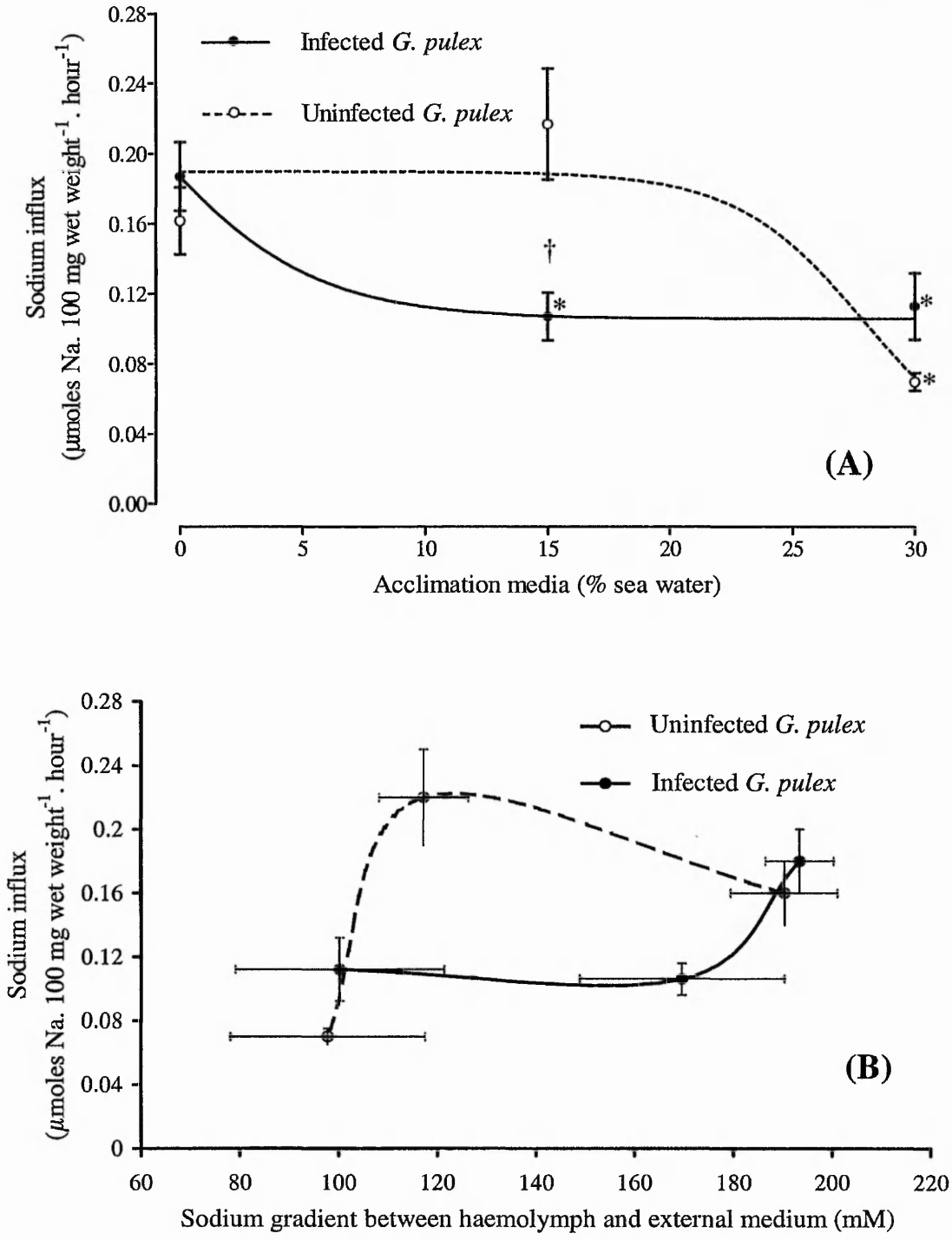


Figure 7. 2. 7. (A) The effects of *P. minutus* infection on sodium influx in *G. pulex*, acclimated to increasing sea water concentration (mean ± SE, n=5). * Significant difference from 0% sea water, $p<0.05$; † significant difference between infected and uninfected animals, $p<0.05$. (B) Differences in sodium gradient between infected and uninfected *G. pulex*, with respect to the sodium influx values from graph A.

change in the rate of sodium influx with respect to the sodium gradient between the haemolymph and the external medium was calculated (Fig. 7. 2. 7B.). In uninfected animals, a significant decrease in the rate of sodium influx was seen at a sodium gradient between 120 and 100 mM sodium. In contrast, a significant decrease in the rate of sodium influx in infected animals, was seen at a sodium gradient between 190 and 170 mM sodium.

As found for sodium influx, parasite infection had no significant effect on sodium efflux in *G. pulex*, when acclimated to fresh water or 30% sea water (Fig. 7. 2. 8A.). However, at 15% sea water acclimation, the sodium efflux for the uninfected animals was significantly greater than that of the infected animals ($p<0.05$). The change in the rate of sodium efflux with respect to the sodium gradient between the haemolymph and the external medium was calculated (Fig. 7. 2. 8B). The rate of sodium efflux decreased as the sodium gradient increased in both infected and uninfected animals. The lower rate of sodium efflux in infected than uninfected animals at 15% sea water coincides with the higher sodium gradient found in the former than the latter at this salinity.

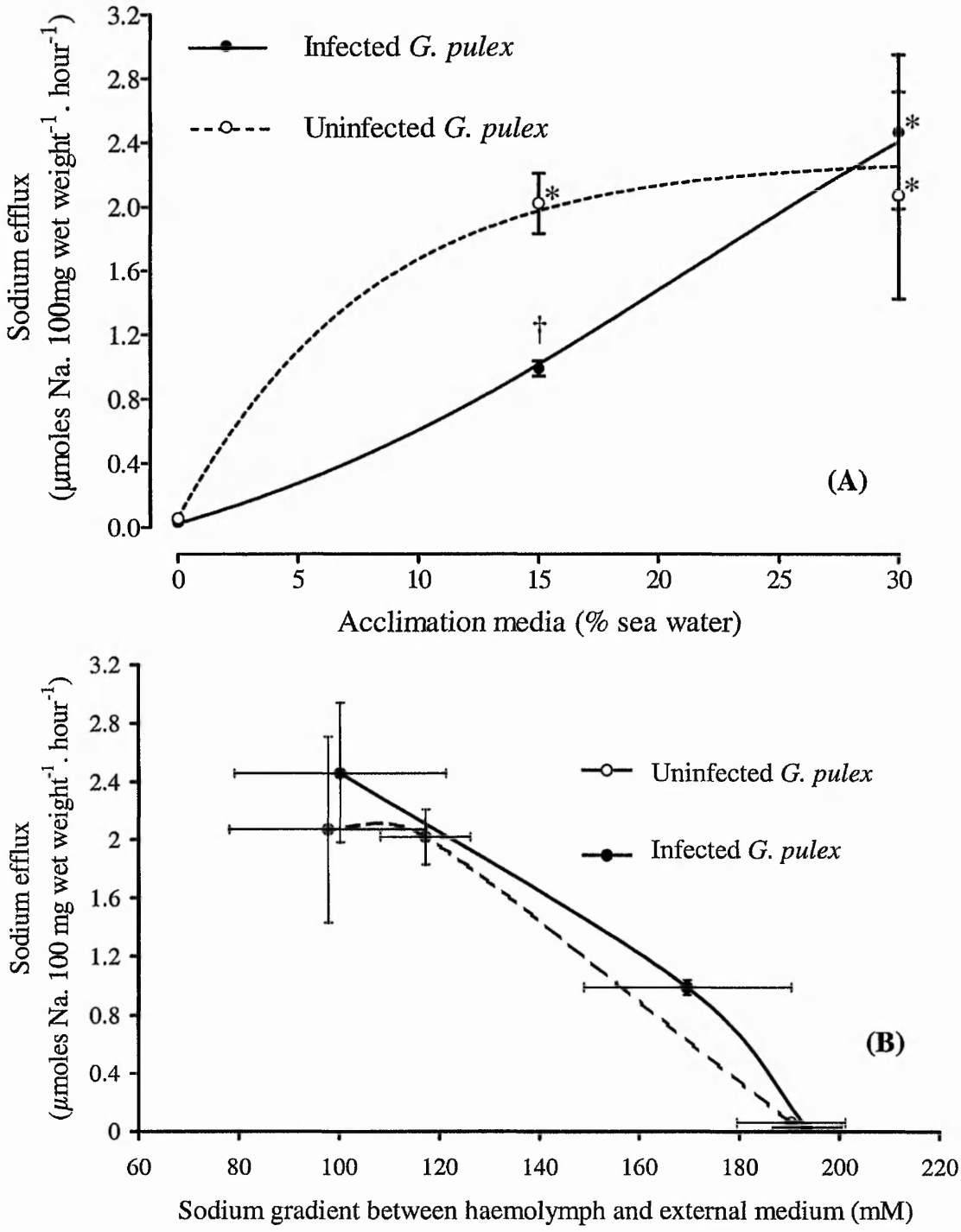


Figure 7. 2. 9. (A) The effects of *P. minutus* infection on sodium efflux in *G. pulex*, acclimated to increasing seawater concentration (mean ± SE, n=5). * Significant difference from 0% seawater, $p < 0.05$; † significant difference between infected and uninfected animals, $p < 0.05$. (B) Differences in sodium gradient between infected and uninfected *G. pulex*, with respect to the sodium efflux values from graph A.

7. 3. Discussion

7. 3. 1. Rates of oxygen consumption in *G. pulex*

The physical presence of mature cystacanths of *P. minutus* in the haemocoel of the intermediate host *G. pulex* may be expected to displace the internal organs including the heart thereby effecting the circulatory efficiency. In addition, the increased body burden of infected animals may impose increased demands on the infected animal, which as a result may require an increased oxygen consumption rate. However, despite these potential parasite effects, no difference was found in the oxygen consumption rates of *G. pulex* infected with cystacanths of *P. minutus* when compared to uninfected animals. This differs from the effects of *Polymorphus laevis* on the rate of oxygen consumption in *G. pulex* (Rumpus & Kennedy, 1974). In this case, *P. laevis* infection was found to cause a significant reduction in the rate of oxygen consumption in *G. pulex*.

The body burden of parasite infection in *G. pulex* may have more of an effect when the host becomes exposed to high stress environments, such as waters lacking in dissolved oxygen content. Therefore, in future studies measurements of oxygen consumption in *G. pulex* infected with *P. minutus* should be carried out over a wider range of dissolved oxygen concentrations, particularly in waters approaching anoxia.

7. 3. 2. Haemolymph ion concentration

Cystacanths of *P. minutus* present in the haemocoel of *G. pulex* were found to have significant effects on a number of haemolymph ion concentrations. Haemolymph sodium concentration between infected and uninfected *G. pulex* was significantly different when acclimated to 15% sea water. These differences in haemolymph sodium concentration were found to be due to differences in the sodium flux mechanisms influenced by parasite

infection. This will be discussed in relation to sodium flux mechanism in the following section (7. 3. 3.).

Although, no significant difference was seen in freshwater acclimated animals, at 15% and 30% sea water, infected *G. pulex* had significantly higher haemolymph magnesium concentrations than uninfected animals. Haemolymph magnesium is believed to have anaesthetic properties in marine invertebrates, with high haemolymph magnesium correlated with low levels of activity in crustaceans (Morritt & Spicer, 1993; Frederich *et al.*, 2000). Consequently, the relatively high haemolymph magnesium concentrations exhibited by infected *G. pulex* in dilute sea water may have subsequent effects on host behaviour through increased lethargy. Reduction in activity of infected *G. pulex* could potentially influence competitive interactions in oligohaline waters. However, rather than speculate further upon the possible implications of the reduced activity in infected *G. pulex*, the relationship between haemolymph magnesium concentration and activity levels in these amphipods needs to be established.

Marked differences in haemolymph calcium concentration were found between infected and uninfected *G. pulex*. Infected *G. pulex* had approximately three times higher calcium concentrations than uninfected animals in all acclimation salinities. The maintenance of a constant haemolymph calcium concentration with increasing salinity in both infected and uninfected *G. pulex* suggests that the differences in calcium levels do not reflect changes in osmoregulation. In Crustacea, including *G. pulex*, haemolymph calcium concentrations are strongly influenced by the developmental stage of the animal (Wright, 1980). Although recently moulted animals were not used in these experiments, animals two to three days prior to moulting can lose more than 40% of their body calcium (Wright, 1980). Therefore, animals in pre-moult may have significantly lower haemolymph calcium concentration than those in the intermoult stage. Consequently, the differences in haemolymph calcium

concentrations observed between infected and uninfected *G. pulex* may simply reflect the differences in the developmental stages of the two groups.

Acanthocephalan parasites have been found to accumulate calcium from their host, such as the fish intestinal parasite *Acanthocephalus lucii*, where tissue calcium concentrations were higher than those of its host tissues (Sures *et al.*, 1999). Such accumulation of calcium by *A. lucii*, was responsible for skeletal deformations of the host fish through deprivation of essential calcium (Taraschewski, 1989). Such strong ion absorption is typical of intestinal parasites (Taraschewski, 2000). Although calcium absorption is unlikely to occur to the same extent by *P. minutus* from the haemocoel of *G. pulex*, it is likely that at least some accumulation of calcium will take place. However, such accumulation by *P. minutus* is likely to result in significantly lower haemolymph calcium in *G. pulex* rather than the high calcium concentrations found in this study. One suggestion is that higher haemolymph calcium concentrations may arise from the release of calcium from the hosts intracellular stores. Such high haemolymph calcium concentrations would aid in its uptake by *P. minutus*. The accumulation of calcium from *G. pulex*, could potentially effect host growth and development by increasing the duration of the intermoult period. However, this is speculation and further work to investigate ion uptake in *P. minutus* from the its intermediate host *G. pulex* is required, before effects on host growth and development can be assessed.

7. 3. 3. Water permeability and sodium flux

Infection of *G. pulex* with one or more cystacanths of *P. minutus* was found to have a significant effect on the sodium regulation in animals acclimated to 15% sea water when compared to uninfected animals. However, the haemolymph sodium concentrations of infected and uninfected *G. pulex* were almost identical in fresh water and 30% sea water.

The difference in 15% sea water acclimation suggests an effect of parasite infection on sodium regulation in *G. pulex*. The increase in haemolymph sodium concentration at 15% sea water between infected and uninfected animals could be simply due to a reduction in water influx by the parasitised animals. However, $t_{1/2}$ measurements failed to show any significant effect of *P. minutus* infection. Therefore, the increase in haemolymph sodium concentration at 15% sea water was possibly due to either an increase in sodium uptake or a reduction in sodium loss caused by *P. minutus* infection.

An increase in sodium influx could help produce a higher haemolymph sodium concentration if there is no change in sodium efflux. However, experimental determination of the sodium influx in infected and uninfected animals over the salinity range revealed apparently contradictory findings. Whilst in fresh water and 30% sea water, the sodium influx rates did not differ between infected and uninfected animals, at 15% sea water sodium influx was found to be significantly lower in infected animals, despite the increase in haemolymph sodium concentration compared to uninfected *G. pulex*. However, investigation of sodium efflux revealed a significant reduction in sodium efflux in infected animals compared to uninfected at 15% sea water acclimation. This reduction in sodium loss at 15% sea water is therefore consistent with the increase in haemolymph sodium concentration found at this salinity, despite the fall in sodium influx.

A whole host of biological, physical and behavioural effects have been found to be elicited by *P. minutus* on its intermediate host *G. pulex*. These changes have clear advantages for the parasite, either by aiding in its development within the intermediate host (Plaistow *et al.*, 2001) or by increasing its chances of transmission to the definitive host (Marriott *et al.*, 1989), thereby completing its life cycle. The present study has demonstrated that osmoregulatory changes in parasite infected *G. pulex* do occur in oligohaline waters. However, the benefits to the manipulative parasite in terms of development and/ or

increased transmission to its definitive host as a result of such changes are difficult to see. Due to the lack of benefits to the parasite, it is unlikely that the changes in the osmoregulation of parasite infected *G. pulex* are as a result of direct manipulation by the parasite. Osmoregulatory changes by *P. minutus* infection are more likely to be due to indirect effects caused by changes in related physiological parameters. These indirect effects could potentially arise from alterations in energy stores primarily used to enhance parasite growth (Plaistow *et al.*, 2001) and/ or the release of neurotransmitters aimed at causing behavioural modifications of the host (Maynard *et al.*, 1996).

In hyper-osmotic regulators, such as *G. pulex*, the maintenance of the osmotic gradient between the internal fluids and the external medium requires energy. In fresh water, *G. pulex* has been estimated to spend approximately 11% of its daily energy budget on active ion uptake to replace those lost to the environment (Sutcliffe, 1984). Consequently, parasite effects on energy resources could potentially affect osmoregulatory capabilities of the host. Infection of *G. pulex* with cystacanths of *P. laevis* were found to have significant effects on the host's lipid and glycogen contents, although only females infected with *P. laevis* were reported to have significant reductions in lipid content (Plaistow *et al.*, 2001). This reduction in lipid content in gravid females was thought to be due to a partial castration of the females by *P. laevis* (Poulton & Thompson, 1987), since females accumulate a large resource store prior to egg laying (Sutcliffe, 1993). This large lipid store was utilised by the developing parasite. There have been no investigations into the effects of *P. minutus* on the resource content of *G. pulex*. However, it would be expected that the effects would be more magnified than that exhibited by *P. laevis*, since *P. minutus* has been found to cause complete castration of female *G. pulex* (Ward, 1986). Consequently, osmoregulation in *G. pulex* infected by *P. minutus* may be affected by the reduced energy available for active ion uptake in gravid females. However, in the present study no differentiation was made

between male and female *G. pulex*, therefore the reduction in lipid resources is unlikely to be responsible for the differences in sodium regulation.

Increased glycogen levels were found in *G. pulex* infected with *P. laevis* irrespective of sex or reproductive stage (Plaistow *et al.*, 2001). Similar increases have been reported in parasitised brine shrimps *Artemia spp.* (Amat *et al.*, 1991). This increase in glycogen concentration was suggested to be either an indirect consequence of the increased energy demands experienced by infected *G. pulex*, or the result of adaptive manipulation of the hosts energy reserves (Plaistow *et al.*, 2001). The increased glycogen concentration may supply *G. pulex* with the energy required for active ion uptake as well as the active reclamation of sodium from the urine. The ability to reclaim sodium from the urine enables *G. pulex* to produce a hypotonic urine, thereby reducing sodium loss (Lockwood, 1961). The antennal gland of crustaceans is believed to play an important role in the reabsorption of sodium from the urine through the action of Na^+, K^+-ATPase (Sarver *et al.*, 1994; Horiuchi, 1980). Since this is an energy demanding process, higher glycogen levels reported in parasitised *G. pulex*, may provide more energy for the increased active ion uptake, reducing sodium loss and subsequent lowering of haemolymph sodium concentrations.

Increased swimming activity was observed in *G. pulex* infected with cystacanths of *P. laevis*. Theoretically, increased swimming activity might have an indirect effect on osmoregulation by increasing haemolymph circulation through the gills. This increased circulation may reduce the thickness of unstirred layers on the internal side of the membrane. Furthermore, increased swimming activity may increase water currents and thus reduce the thickness of the unstirred layers on the external side of the membrane. Both these events may subsequently increase the diffusion gradients between haemolymph and external medium, altering ion and water fluxes. However, gammarid gills are often well

ventilated, consequently one would expect the thickness of the unstirred layers to be at a minimum. Therefore, it would be unlikely for increased swimming activity to have any significant effect on osmoregulation.

The behavioural modifications, which have been found to occur in a whole host of gammarids infected with acanthocephalan parasites, are thought to be driven by changes in the neuroendocrine modulation, elicited by acanthocephalan parasites. Cystacanths of *Polymorphus paradoxus* have been suggested to affect the behaviour of their intermediate host *G. lacustris* through alterations in serotonergic modulatory activity (Helluy & Holmes, 1990). Injection of serotonin into uninfected *G. lacustris* was found to cause the same alterations in the hosts behaviour as observed in infected *G. lacustris.* Octopamine was also found to suppress these serotonin effects.

In a more recent study, infection of *G. lacustris* by cystacanths of *P. paradoxus* was correlated with changes in the anatomy of the serotonergic neurons of the amphipod's central nervous system (Maynard *et al.*, 1996). Infected *G. lacustris* had an increased number of varicosities (swollen sites of neurotransmitter serotonin release along neural processes) than uninfected animals. This increase in serotonin in infected *G. lacustris* was linked to altered intermediate host behaviour through changes in central nervous system function.

Osmoregulation in crustaceans is controlled by neuroendocrine factors, which are released from a variety of sources into the haemolymph, from where they are transported to the target organs to induce the appropriate physiological effects. These factors include biogenic amines such as dopamine, serotonin and octopamine (reviewed in Kamemoto, 1991). In the shore crab *Carcinus maenas* (Sommer & Mantel, 1988) and the Chinese spider crab *Eriocheir sinensis* (Trausch *et al.* 1989; Bianchini & Gilles, 1990) the release of

neuroamines, such as dopamine and serotonin have been shown to increase sodium uptake through the increased activity of Na^+, K^+-ATPase in the gills. Therefore, the changes in sodium regulation exhibited by *G. pulex* infected with cystacanths of *P. minutus* in this study, may reflect alterations in the serotonergic modulation. These changes in serotonin levels, besides causing alterations in the host behaviour, are believed to indirectly influence mechanisms responsible for haemolymph sodium regulation.

CHAPTER EIGHT

Physiology of *G. pulex* cave populations

8. 1. Introduction

Cave environments are devoid of light and often characterised as stable environments with low food and oxygen levels (Malard & Hervant, 1999; Culver, 1985). As a consequence, many aquatic cave environments are typically low in species abundance and diversity when compared to surface water habitats (Holsinger, 1988). Investigations have found some caves to have relatively larger fluctuations in their physiochemical regimes than the corresponding surface waters (Culver *et al.*, 1995). Hence, in order to live in cave environments it is likely that specialised morphological, physiological and behavioural adaptations should be evident.

8. 1. 1. Morphological differences in cave animals

Populations of the freshwater amphipod *Gammarus minus*, have been found to occur in both isolated subterranean environments and surface environments. Comparison of the morphological characteristics of the two populations has found some marked differences between the two groups, which have been suggested to be due to evolutionary adaptations to their contrasting environments. The morphological characters of hypogean (underground) *G. minus* that differ from epigean (surface) populations include, 1) reduction in the size of the eyes, 2) increase in length of the antennae, 3) increase in overall body length, 4) increase in apparent fragility due to lengthening of pereopods and uropods, and 5) change in colour from brown to blue and even white (Hüppop, 1985; Culver *et al.*, 1995).

The reduction in the size of the eye in hypogean animals relates to changes in the central nervous system (CNS). The optic lobe of hypogean *G. minus* was approximately half the

size of the optic lobe of the epigean population (Culver *et al.*, 1995). This morphological reduction in the eye was reflected in the animals' response to light. Epigean *G. minus* were found to be much more photophobic than their hypogean counterparts, this was due to the reduction of an integrated visual system with which hypogean *G. minus* could respond to light (Vawter *et al.*, 1987).

In addition to the reduction of the optic lobe, further changes in the CNS involve the increased dominance of the olfactory lobe. The olfactory lobe of hypogean *G. minus* was found to be on average 25% larger than that of the epigean population (Culver *et al.*, 1995). The increase in size of the olfactory lobe would likely cause an increase in the animals' sensitivity to chemosensory stimuli, which would have great benefits to animals living in environments of complete darkness. Further to heightened chemosensory function in hypogean animals, an increased tactile sense would also seem to benefit animals living in complete darkness. Hypogean populations of *G. minus* have been found to have enlarged antennae, the first antenna was found to be 25 to 45% larger than that of the epigean *G. minus* (Holsinger & Culver, 1970). This increased radius of tactile sense would likely aid in the animals movement in waters without light, and appears to be a selective adaptation to life in a cave.

In surface water environments, the brown colouration of gammarid amphipods is an adaptation to their benthic existence. The brown colour aids in the camouflage of the animal against the brown substrate background, and is likely to aid in keeping them undetected from prowling predators, who use visual awareness to track down their prey. In a cave environment however, colour has no importance since waters are in darkness. Consequently, lack of body pigmentation will not confer a selective disadvantage and may cause a reduction in the overall metabolic cost *via* a cessation of pigment synthesis. The *G.*

pulex populations from the Speedwell cavern system were transparent (Personal observation).

8. 1. 2. Physiological and behavioural differences in cave animals

Due to the absence of light in caves, most if not all autotrophic production is absent. Consequently cave waters are often characterised by very low levels of food. Therefore, the capability to withstand such low levels of food is crucial to the survival of cave species. Two main mechanisms have been highlighted in the hypogean invertebrates *Stenasellus virei, Niphargus virei* and *N. rhenorhodanensis* (Hervant, 1996; Malard & Hervant, 1999). These include increased levels of energy storage during times of food abundance and metabolic saving adjustments stimulated by food limitations. Such strategies would allow a hypogean organism to withstand long periods of starvation. For example, the hypogean invertebrates *S. virei, N. virei* and *N. rhenorhodanensis* can survive for longer than a year without feeding (Gibert & Mathieu, 1980).

Hypogean organisms generally have a reduced metabolic rate compared to closely related epigean species, due to adaptation to the low nutrient environment (Culver *et al.*, 1995). However, experiments carried out on both hypogean and epigean populations of *G. minus* revealed a higher metabolic rate for the hypogean population (Culver & Poulson, 1971). Culver *et al.*, (1995), suggested that the lack of a reduced metabolic rate found in cave populations was due to the relatively rich detrital food base in their natural habitat. In this case the cave habitat was rich in food, which is unlike that found in most isolated ground water cave systems (Malard & Hervant, 1999).

Differences in the behavioural responses of hypogean and epigean crustaceans have also been found to occur with respect to starvation. The epigean *Asellus aquaticus* and *Gammarus fossarum* responded to food deprivation with marked increases in locomotory

activity (Hervant *et al.*, 1997b). These authors suggested that this was an attempt to search for food at the beginning of the starvation period. In contrast, the hypogean species *S. virei, N. virei* and *N. rhenorhodanensis* reduce their energy expenditure by reducing both movement and ventilation during long term starvation. This is referred to as a 'sit and wait' strategy, conserving energy until food becomes available (Hervant *et al.*, 1997a, 1997b). Such reductions in energy expenditure could be critical to survival in nutrient deficient environments.

The effects of low food supply in cave environments have at least in part been overcome in hypogean species *via* a reduced metabolic rate during periods of low food availability; an increased storage capacity during periods of adequate food; and restricted energy expenditure from a lessening in locomotory activity. However, despite these measures to cope with such food limiting environments, the lack of food is likely to have an impact upon the fitness of the organism. Poor nutrition may lead to a reduction in reproductive potential, particularly for females, as they tend to invest a large proportion of energy on egg production. A fall in fecundity will have subsequent effects on the population. Poor nutrition, resulting in lower energy levels also has the potential to deleteriously influence other physiological mechanisms such as osmoregulation.

The low metabolic rate among hypogean animals has been considered mainly as an adaptation to a decreased food supply. However, low metabolic rates in hypogean populations have been linked to an adaptation to low oxygen concentrations present in cave waters (Hüppop, 1985; Danielopol *et al.*, 1994; Hervant *et al.*, 1997a, 1997b, 1998). Due to the absence of light and autotrophic production in cave waters, oxygen is not produced. Here the oxygen concentration is determined by the rate of oxygen transport from the atmosphere and by the rate of oxygen consumption (Malard & Hervant, 1999).

The low oxygen content of many cave waters requires hypogean species to tolerate periods of dysoxic (0.3 – 3.0 mg. l^{-1}. O_2), suboxic (< 0.3 mg. l^{-1}. O_2), and even anoxic conditions. The hypogean species *S. virei, N. virei* and *N. rhenorhodanensis* survived significantly longer under severe hypoxia than any other epigean crustaceans previously studied, with LT_{50} (lethal time causing 50% mortality) values of 61.7, 52.1 and 46.7 hours respectively (Hervant *et al.*, 1995, 1996). In contrast, the epigean crustaceans *G. fossarum* and *A. aquaticus* had much lower LT_{50} values of 6.3 and 19.7 hours respectively indicating reduced survival when exposed to severe hypoxia.

The long survival times of many hypogean animals have been suggested to be due to the combination of four mechanisms: 1) high energy storage for anaerobic metabolism, 2) low metabolic rate in normoxia, 3) high recovery by re-synthesising energy stores during post-hypoxic stress, and 4) reduction in locomotion and ventilation (Hervant *et al.*, 1996; Malard & Hervant, 1999; Hervant *et al.*, 1999).

In contrast to many hypogean crustaceans, cave populations of *G. minus*, were not found to have increased tolerance to severe hypoxia (Hervant *et al.*, 1999). The lack of tolerance to low oxygen levels was considered to be due to the observation that these hypogean populations of *G. minus* did not experience long-term environmental hypoxia (Culver *et al.*, 1995). Thus it displays morphological but not physiological adaptations to the subterranean habitat (Hervant *et al.*, 1999). Consequently, high resistance to hypoxia is not universally found in hypogean organisms, but is more related to oxygen availability and/ or to the energetic state of each cave ecosystem (Hervant *et al.*, 1999).

The 'sit and wait' strategy adopted by hypogean animals in response to food shortages has also been linked to waters of severe hypoxia. The hypogean crustaceans *S. virei, N. virei* and *N. rhenorhodanensis* exhibited no escape response and weaker hyperventilation at the

onset of severe hypoxia, when compared to the epigean crustaceans *G. fossarum* and *A. aquaticus* (Hervant *et al.*, 1997a, 1997b). The hypogean isopod *Proasellus slavus* also reduced its exploratory movements under severe hypoxia (Danielopol *et al.*, 1992, 1994).

The transparent appearance of hypogean *G. pulex* suggests that at least some morphological adaptation to cave habitats has occured. As yet, no investigations of physiological significance have been carried out on cave populations of *G. pulex*. Since the osmoregulatory activity of freshwater amphipods is substantial (around 11% of total energy expenditure in *G. pulex*, Sutcliffe, 1984), any adaptation to reduce their energetic cost in the hypogean low energy environment would be beneficial.

The osmoregulation of cave dwelling organisms has received very little attention. In the current literature only investigations on calcium regulation in hypogean organisms are reported (Culver *et al.*, 1995). Therefore, this study aims to provide preliminary information on the osmoregulatory physiology of an epigean population and two isolated hypogean populations of *G. pulex*. Osmoregulatory physiology is determined through measurements of haemolymph ion concentrations, water flux and sodium regulation. In addition the oxygen consumption rates of hypogean and epigean *G. pulex* populations are compared.

8. 2. Results

8. 2. 1. Study site

The Peak-Speedwell Cavern system is located in the Peak District National Park (Ordnance Survey grid reference, SK139 827) and is designated as a Site of Special Scientific Interest (SSSI). It was developed at least one million years ago and extends approximately 20km long and 290 metres deep (Waltham *et al.*, 1997). The water source for the Peak Cavern is largely by autogenic recharge (water that has only had contact with limestone bedrock and overlying soil) from rainwater. In contrast, the Speedwell Cavern is largely fed by allogenic recharge (water that originates on non-limestone) from nearby streams (Wood *et al.*, 2002). The River Wye population was located near to the cave water system (Ordnance Survey grid reference, SK164 732).

8. 2. 2. Oxygen consumption

The oxygen consumption profiles for both River Wye and Speedwell cave populations of *G. pulex* were very similar throughout the range of oxygen concentrations in the acclimation media (Fig. 8. 2. 1.). However, the oxygen consumption rate of the Peak cave population differs markedly at higher levels of oxygen concentration (>300μmoles. l^{-1}). In all populations, the maximum rate of oxygen consumption was achieved at the highest measured oxygen concentrations. The maximum oxygen consumption of the Peak cave population (7.8 ± 0.9 μmoles O_2. mg wet $weight^{-1}$. $hour^{-1}$ at 358 ± 6.2μmoles O_2. l^{-1}) was significantly greater ($p<0.05$) than that recorded for either the River Wye (5.75 ± 0.24 μmoles O_2. mg wet $weight^{-1}$. $hour^{-1}$ at 375 ± 4.23 μmoles O_2. l^{-1}) or the Speedwell cave (4.1 ± 0.44 μmoles O_2. mg wet $weight^{-1}$. $hour^{-1}$ at 363 ± 3.79 μmoles O_2. l^{-1}) populations. At lower oxygen concentrations (<300μmoles. l^{-1}) the rates of oxygen consumption in all three populations were very similar ranging between approximately 1- 2 μmoles O_2. mg wet $weight^{-1}$. $hour^{-1}$).

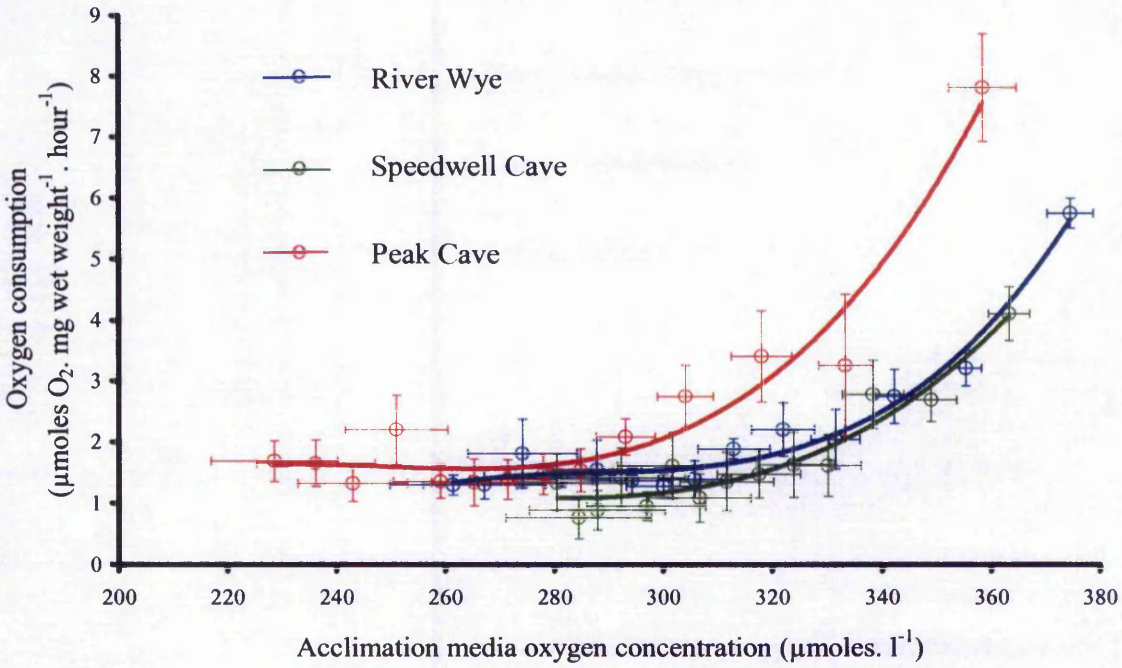


Figure 8. 2. 1. Rate of oxygen consumption in *G. pulex* acclimated to fresh water; comparisons with two isolated cave populations, at 8 ± 0.1^{0}C (mean ± SE, n=5).

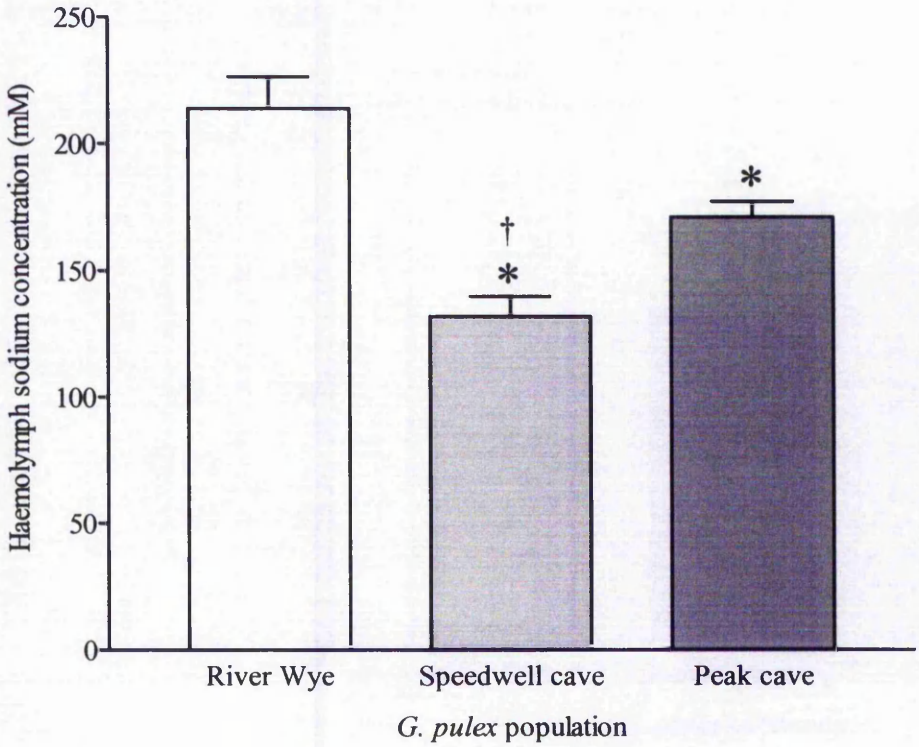


Figure 8. 2. 2. Haemolymph sodium concentration in *G. pulex*, comparisons with two isolated cave populations (mean ± SE, n=5). * Significant difference from River Wye population, $p<0.05$; † significant difference between cave populations, $p<0.05$.

8. 2. 3. Haemolymph ion concentrations

Sodium

The differences in haemolymph ion concentrations between a River Wye population and two entirely isolated cave populations of *G. pulex* after fresh water acclimation can be seen in figures 8. 2. 2 to 6. Significant differences ($p<0.05$) in haemolymph sodium concentration were found between the River Wye population and both Speedwell cave and Peak cave populations of *G. pulex* (Fig. 8. 2. 2.). The haemolymph sodium concentrations of the Speedwell cave and Peak cave populations were 61% and 80% of the River Wye population respectively. In addition, the haemolymph sodium concentration of the Speedwell cave population was significantly lower ($p<0.05$) than that of the Peak cave population.

Potassium

Haemolymph potassium concentrations (Fig. 8. 2. 3.) for the three populations of *G. pulex* exhibited a similar trend to that previously described for sodium. Haemolymph potassium concentrations of the Speedwell cave and Peak cave were 56% and 78% of the River Wye population respectively. However, despite this a significant difference was found only between the River Wye and Speedwell cave populations ($p<0.05$).

Magnesium

The haemolymph magnesium concentration in the Speedwell cave population was found to be significantly different ($p<0.05$) from both the River Wye and the Peak cave populations (Fig. 8. 2. 4.). No significant difference in haemolymph magnesium concentration was found between the River Wye and the Peak cave populations.

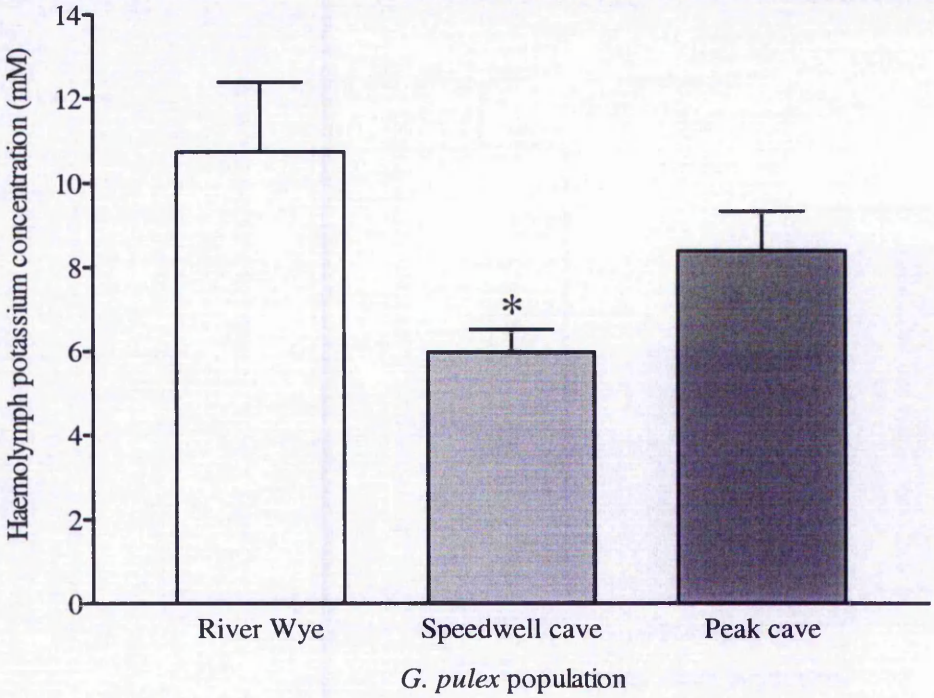


Figure 8. 2. 3. Haemolymph potassium concentration in *G. pulex*, comparisons with two isolated cave populations (mean ± SE, n=5). * Significant difference from River Wye population, $p<0.05$.

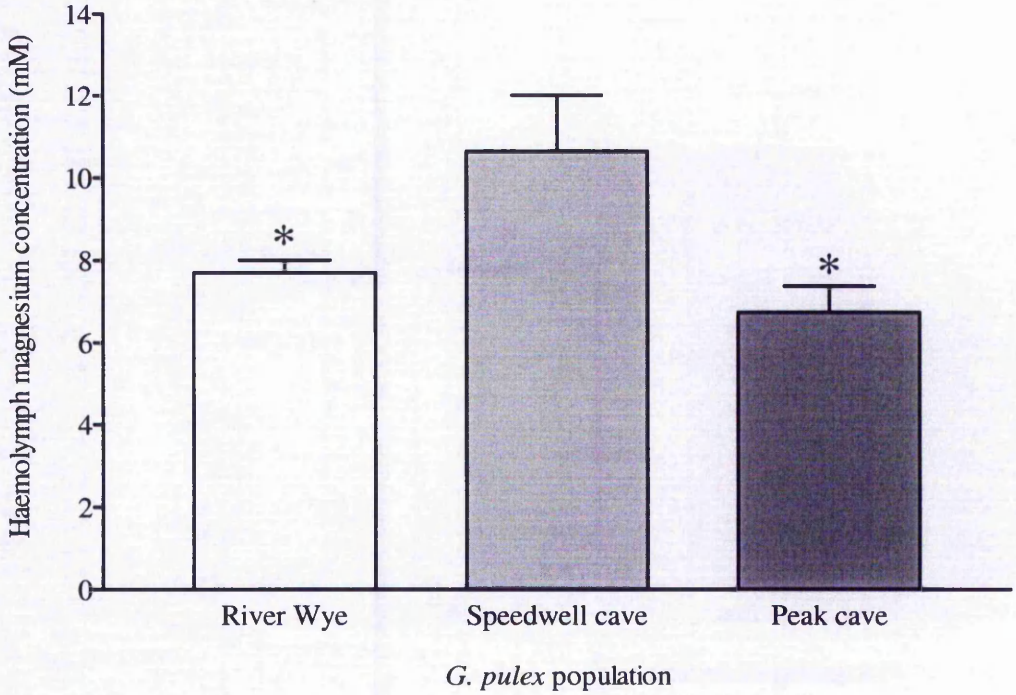


Figure 8. 2. 4. Haemolymph magnesium concentration in *G. pulex*, comparisons with two isolated cave populations (mean ± SE, n=5). * Significant difference from Speedwell population, $p<0.05$.

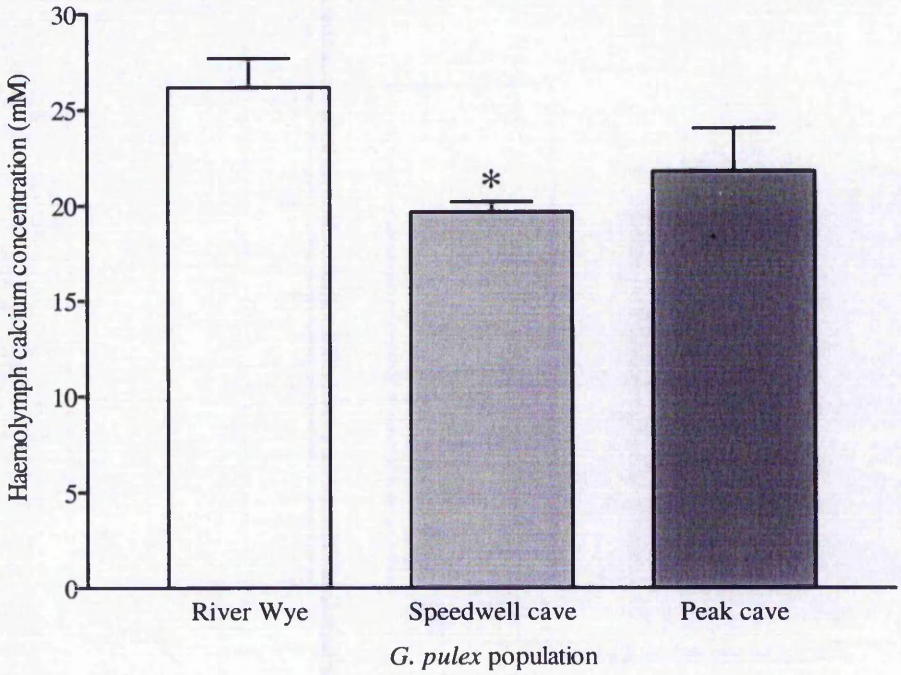


Figure 8. 2. 5. Haemolymph calcium concentration in *G. pulex*, comparisons with two isolated cave populations (mean ± SE, n=5). * Significant difference from River Wye population, $p<0.05$.

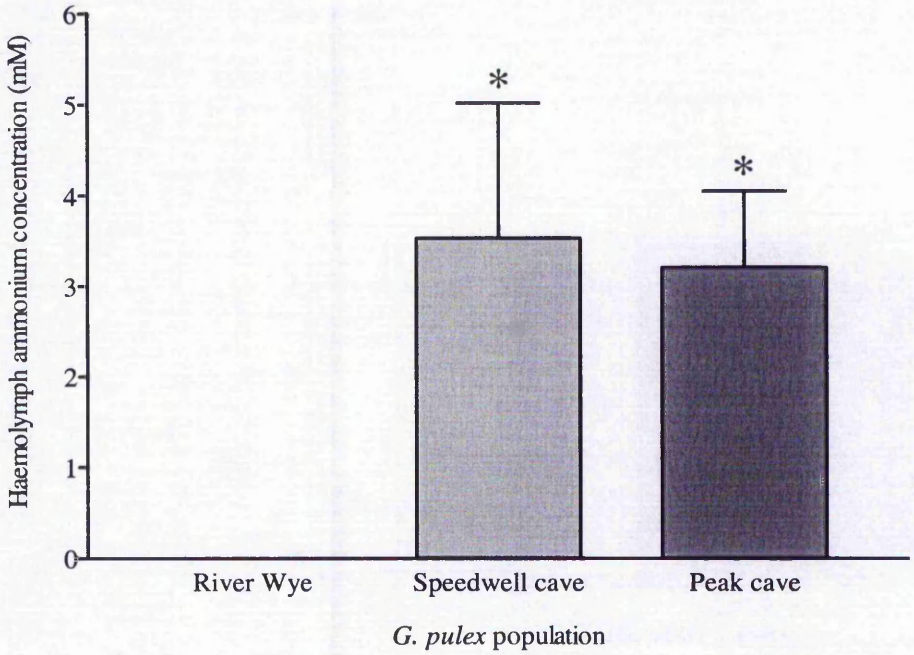


Figure 8. 2. 6. Haemolymph ammonium concentration in *G. pulex*, comparisons with two isolated cave populations (mean ± SE, n=5). * Significant difference from River Wye population, $p<0.05$.

Calcium

Both Speedwell cave and Peak cave populations were found to have lower haemolymph calcium concentrations than the River Wye population (Fig. 8. 2. 5.). For the haemolymph calcium concentration only the Speedwell cave population was significantly different from the River Wye population ($p<0.05$).

Ammonium

Ammonium was not detected in the haemolymph of the River Wye population (Fig. 8. 2. 6.). In contrast, haemolymph ammonium was in Speedwell cave and Peak cave populations, which were significantly greater than the River Wye population ($p<0.05$).

8. 2. 4. Water permeability and sodium flux

There was no significant difference between the half time of exchange of tritiated water ($t_{1/2}$) between the three populations of *G. pulex* (Fig. 8. 2. 7.). Furthermore, sodium influx measurements were not significantly different between the three populations (Fig. 8. 2. 8.).

The specific activity of gill Na^+, K^+-ATPase and Mg^{2+} ATPase was higher when hepes buffer was used in the assay solution (Chapter 6) rather than imidazole (Chapter 3). Therefore, hepes was used as the enzyme buffer in experiments to determine the specific enzyme activity of epigean and hypogean populations of *G. pulex*.

Although comparison of the gill Na^+, K^+-ATPase activity in all three populations of *G. pulex*, revealed marked differences between the River Wye and the two cave populations (Fig. 8. 2. 9.), large standard deviations resulted in the finding that there were no significant differences between the populations ($p>0.05$). Also, no significant differences were found between Mg^{2+} ATPase activities of the three *G. pulex* populations (Fig. 8. 2. 10.). Gill Mg^{2+} ATPase activity was however, three to five times greater than the gill Na^+, K^+- ATPase activity for all three *G. pulex* populations.

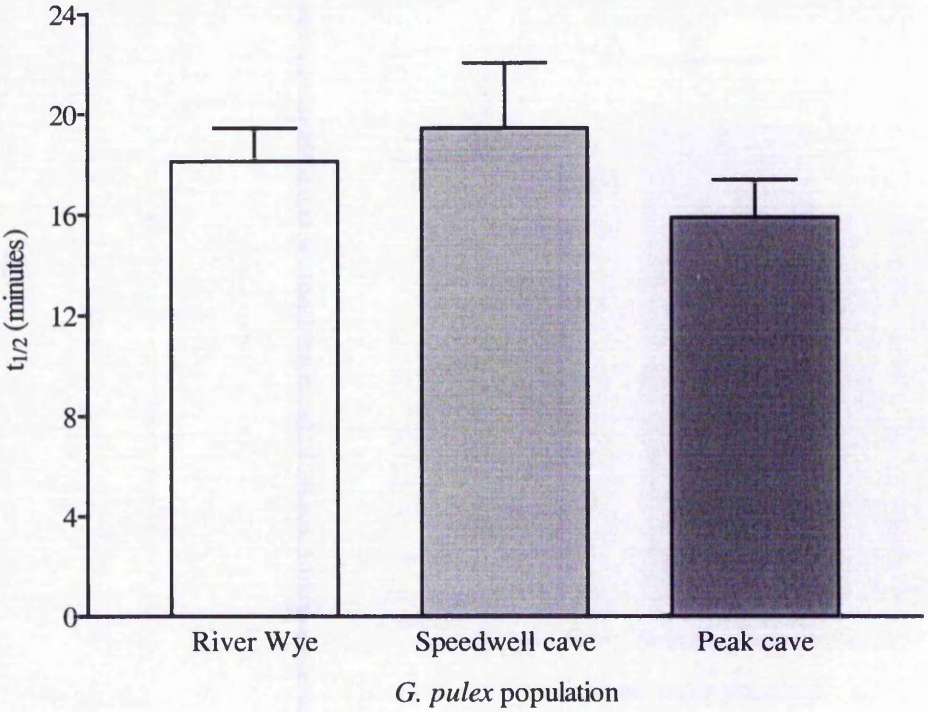


Figure 8. 2. 7. Half time of exchange of body water ($t_{1/2}$) in *G. pulex*, comparisons with two isolated cave populations (mean ± SE, n=5). No significant differences between mean $t_{1/2}$ values.

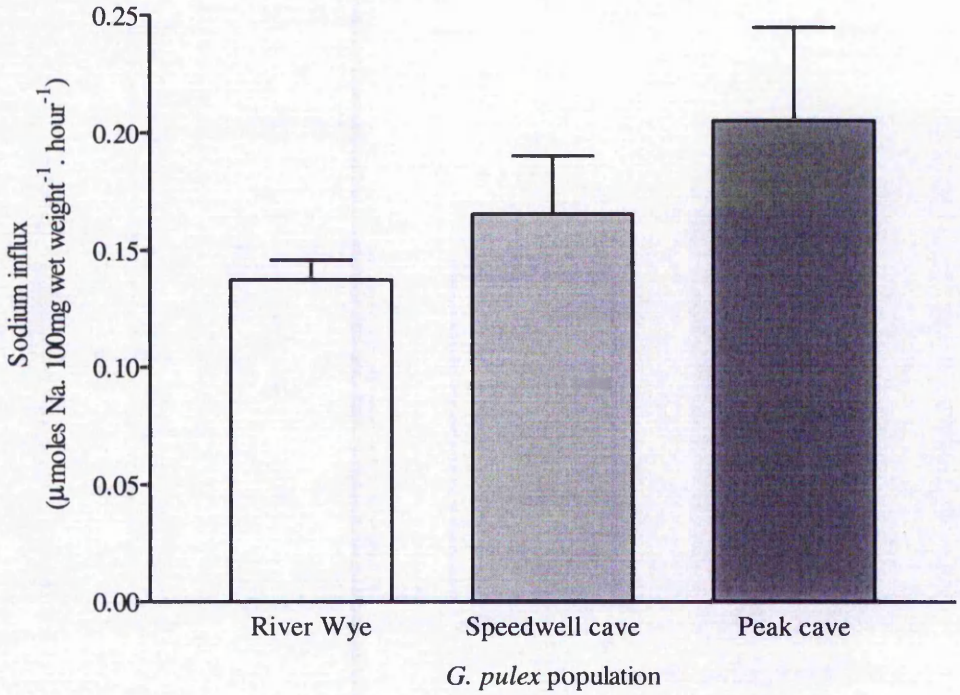


Figure 8. 2. 8. Sodium influx in *G. pulex*, comparisons with two isolated cave populations (mean ± SE, n=5). No significant difference between population means.

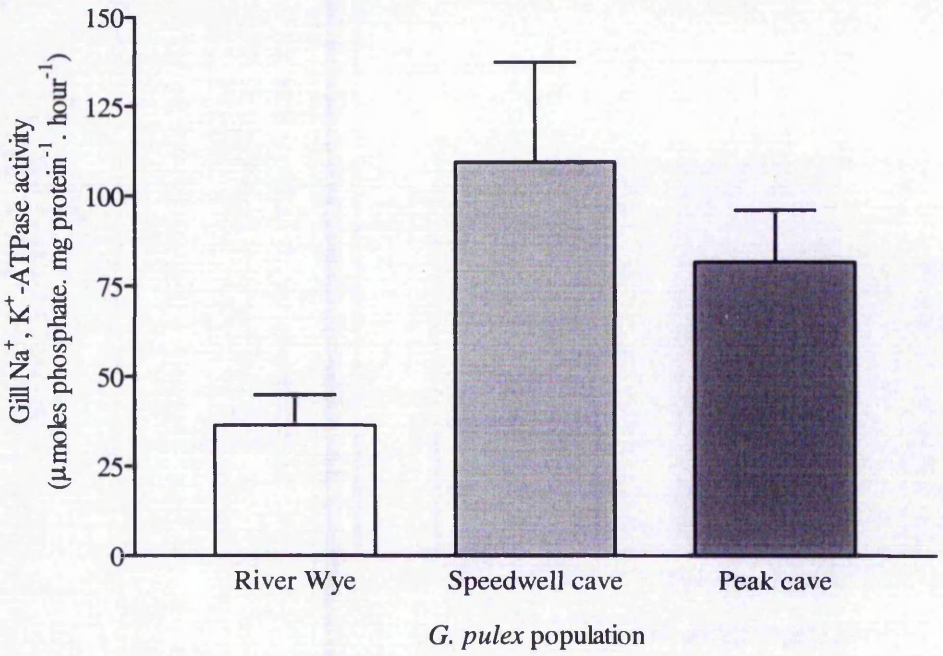


Figure 8. 2. 9. Gill Na^+, K^+-ATPase activity in *G. pulex* acclimated to fresh water; comparisons with two isolated cave populations (mean ± SE, n=3). No significant difference between population means. Hepes buffer used in assay solution.

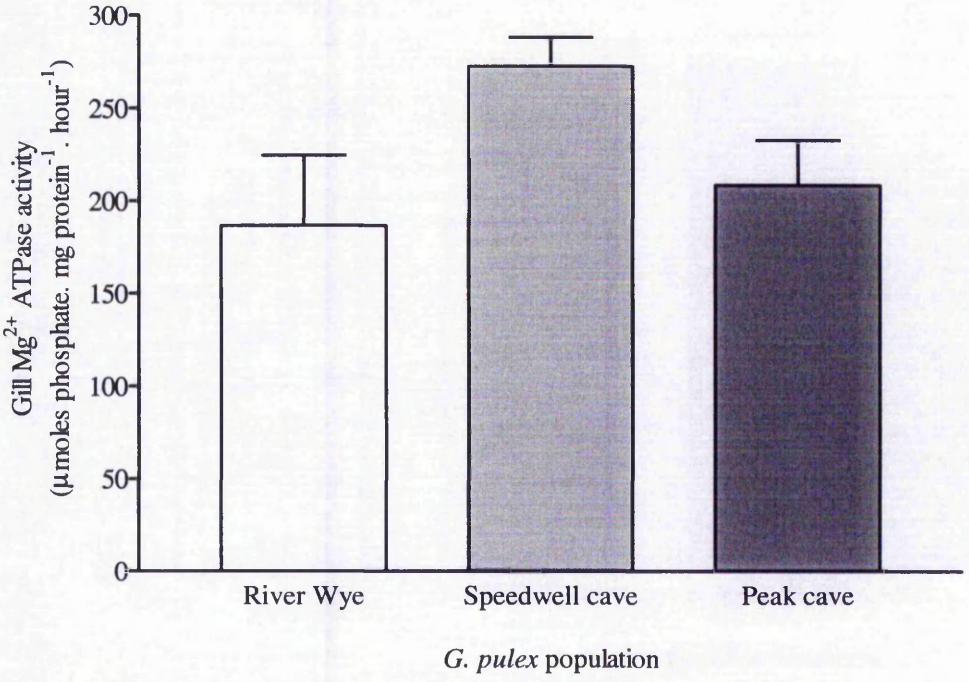


Figure 8. 2. 10. Gill Mg^{2+} ATPase activity in *G. pulex* acclimated to fresh water; comparisons with two isolated cave populations (mean ± SE, n=3). No significant difference between population means. Hepes buffer used in assay solution.

8. 3. Discussion

8. 3. 1. Oxygen consumption

Oxygen consumption rates are considered to be a good indicator of aerobic metabolic activity (Malard & Hervant, 1999). A lowered metabolic rate has been suggested as an adaptive feature of cave-dwelling organisms (Culver *et al.*, 1995). For example, the oxygen consumption rates of the hypogean crustaceans *S. virei*, *N. virei* and *N. rhenorhodanensis* in normoxia were found to be 1.6 to 4.5 times lower than the epigean *G. fossarum* and *A. aquaticus* (Hervant *et al.*, 1995, 1996, 1997a, 1998). However, in this study, the oxygen consumption rates of the two hypogean populations of *G. pulex* were not lower than that found in the epigean population. In fact, the oxygen consumption of the Peak cave population was markedly greater than that found in the other two *G. pulex* populations. This increased oxygen consumption of the Peak cave population corresponds with that reported for hypogean and epigean populations of *G. minus* (Culver & Poulson, 1971). Two hypogean populations of *G. minus* were found to have oxygen consumption rates 3 to 4 times higher than those measured in the epigean population.

The biological significance of the higher oxygen consumption rates found in the Peak cave population is unclear. However, previous authors have suggested that high food abundance in the animal's natural habitat has resulted in the higher metabolic rates of the hypogean animals (Culver & Poulson, 1971). The Peak cave is fed by percolated rainwater, which filters through limestone bedrock and overlying soil and contains little food for the gammarids (Wood *et* al., 2002). The presence of ammonium in the haemolymph of only hypogean *G. pulex,* suggests that these animals have been starved for an extended period, supporting the view that food availability is low. Therefore, high food availability in the Peak cave waters is unlikely to explain the higher oxygen consumption rates found.

8. 3. 2. Haemolymph ion concentration

Marked differences in haemolymph ion concentrations were found between the epigean and the two hypogean populations of *G. pulex*. The haemolymph sodium concentration for both hypogean populations of *G. pulex* was significantly lower than that found in the epigean population. Similar differences in haemolymph potassium concentration were also seen.

The low haemolymph sodium concentration seen in both hypogean populations of *G. pulex* may be an adaptation of the amphipod to its cave environment. Cave waters are often characterised as oxygen and food limiting environments, and animals are often exposed to periods of severe hypoxia (Malard & Hervant, 1999) and food deprivation (Culver *et al.*, 1995). Therefore, any attempt to reduce the energy expenditure of a hypogean organism would be most beneficial. Sodium is an important ion making up the majority of the haemolymph ionic concentration in many crustaceans including *G. pulex*. The maintenance of a constant haemolymph sodium concentration is an active energy demanding process. It has been previously found that *G. pulex* use 11% of their daily energy budget on osmoregulation (Sutcliffe, 1984). Reducing the ionic gradient between the external medium and the internal body fluids could potentially reduce this energy demand, which would have clear benefits for animals inhabiting oligotrophic waters. Therefore, the reduction in haemolymph sodium concentration in *G. pulex* could be an indication of an adaptive strategy by *G. pulex* to life in oxygen and food deficient waters.

There were no significant differences between haemolymph magnesium concentration of the two hypogean populations and the epigean population of *G. pulex*. Mg^{2+} ATPase activity was also found not to differ between hypogean and epigean populations.

Significantly lower haemolymph calcium concentrations were found in the Speedwell cave population when compared to the epigean population. In crustaceans, including *G. pulex*, haemolymph calcium concentrations are strongly influenced by the developmental stage of the animal (Wright, 1980). Although recently moulted animals were not used in these experiments, animals two to three days prior to moulting can lose more than 40% of their body calcium. Therefore, animals in pre-moult may be more likely to have significantly lower haemolymph calcium concentration than those in the intermoult stage. Consequently, the differences in haemolymph calcium concentrations observed between the hypogean and epigean populations, may simply reflect differences in the developmental stages of the two groups.

The significant changes in calcium levels during the moult cycle highlight the importance of environmental calcium concentrations in *G. pulex.* Since around 50% of body calcium is lost upon moulting, the rapid uptake of calcium from the immediate environment by newly moulted individuals is essential for survival (Culver *et al.*, 1995). The absence of *G. minus* from acidic waters has been suggested to be the result of physiological stress, caused through the reduced rates of calcium uptake after moults, and the general poor food quality (Glazier *et al.*, 1992). The Speedwell Cave waters were markedly more acidic (pH 7.21) than that of the River Wye (pH 8.03, Table 8. 3. 2. 1.). Therefore, the difference in calcium concentrations found in this study between the hypogean and epigean *G. pulex* populations

Table 8. 3. 2. 1. Physico-chemical data of the Peak-Speedwell Cavern and River Wye waters where populations of *G. pulex* were collected.

	Temperature (^{0}C)	Conductivity (μS)	Dissolved Oxygen (mg. l^{-1})	pH
Speedwell Cave	8.4	376	6.85	7.21
Peak Cave	8.18	297	7.32	7.25
River Wye	12.05	357	7.75	8.03

may reflect the differences in calcium uptake between the two groups, as a result of the relative acidity of the Speedwell Cave waters.

Both hypogean and epigean populations of *G. pulex* were not fed during their 5 day acclimation period, prior to experiments. However, despite the 5 day starvation period in all *G. pulex* populations, ammonium ions were only detected in the haemolymph of the hypogean populations. The presence of ammonium ions in the haemolymph of the hypogean populations may represent the low food availability in the Peak Cave waters, leading to the use of amino acids as an energy source. The Peak-Speedwell cave waters are characteristic of many cave environments with low food availability (Wood *et al.*, 2002). Consequently, a longer period of starvation experienced by the hypogean populations may have resulted in these animals utilising some of their proteins by metabolising their amino acids for energy production, leading to the presence of ammonium ions in the haemolymph as a waste product. The period of starvation experienced by the hypogean *G. pulex* populations resulting in the haemolymph ammonium concentrations was not known. However, in the hypogean crustaceans *N. virei* and *N. rhenorhodanensis* significant reductions in protein concentrations were found to occur after 30 days of starvation (Hervant *et al.*, 1999).

Increased duration of starvation in the two hypogean *G. pulex* populations may be partly responsible for the lower haemolymph ion concentrations found in these populations. Starvation has been implicated in the reduction of haemolymph ion concentrations in a number of crustaceans, including; *Daphnia*, *Branchipus* (Krogh, 1939); *Triops* (Parry, 1961) and *Corophium* (McLusky, 1970). The lower haemolymph ion concentrations in these animals were partly caused by the reduction in ions obtained from food (Sutcliffe, 1971a). In addition, significantly faster rates of sodium uptake were found in fed rather than starved *Aëdes* larvae (Stobbart, 1960). These faster sodium uptake rates were

attributed to the increased synthesis of transport enzymes in fed *Aëdes* larvae (Stobbart, 1967).

8. 3. 3. Water permeability and sodium flux

The differences in the haemolymph sodium ion concentration were investigated further in all three populations of *G. pulex*. The reduction of the haemolymph sodium concentration in the two hypogean populations may be simply due to an increase in the water uptake in these two groups, since increased water content would subsequently reduce ion concentration. However, no significant difference in $t_{1/2}$ was found between the epigean and hypogean *G. pulex* populations. In addition, the osmotic water flow (O_s) was calculated as shown in equation 8. 3. 3. 1., taken from Lockwood *et al.*, (1973). Analysis of these calculations revealed no significant difference between the osmotic water flows of the three *G. pulex* populations. Therefore, it is likely that the reduction in haemolymph sodium concentration found in the two hypogean populations, may be caused by either a reduction in sodium uptake or an increase in sodium loss. The sodium influxes of the two hypogean populations of *G. pulex* were not significantly different from that of the epigean population, despite the differences in haemolymph sodium concentration.

$$\text{Water Flux } (f) = 100\ (\ln 2 / t_{1/2})$$

$$\left(\frac{M_m - M_\alpha}{M_m} \right) f = O_s \qquad \text{equation 8. 3. 3. 1.}$$

Where, M_m = mole fraction of water in medium
M_α = mole fraction of water in haemolymph
O_s = osmotic water flow

Note: mole fraction was taken as 55.556/ (55.556 + x), where x is the osmolal concentration

Cave environments are often characterised as oligotrophic with low oxygen and food content (Malard & Hervant, 1999). Hypogean organisms have been found to survive long

periods of severe hypoxia and food deprivation by altering their physiological energy budgets. Levels of enzymatic activity in hypogean species (*S. virei*, *N. virei* and *N. rhenorhodanensis*) were 1.2 to 8.6 times lower than in epigean species for the main key regulatory enzymes involved in the Krebs cycle and glycolysis (Hervant, 1996). Gill Na^+, K^+-ATPase is an active transporter, which requires energy to transport sodium across its concentration gradient. In order to reduce energy expenditure in energy limiting environments, hypogean organisms may be capable of reducing gill Na^+, K^+-ATPase activity. Such a reduction in gill Na^+, K^+-ATPase may be responsible for the lower haemolymph sodium concentrations in the hypogean *G. pulex* populations. However, despite this theory, no significant difference in gill Na^+, K^+-ATPase activity was found between any of the three populations, suggesting that the reduction in haemolymph sodium was not caused by a reduction in active sodium uptake in *G. pulex*.

In addition to Na^+, K^+-ATPase, an apical V-type H^+-ATPase has been found in the gills of several freshwater crustacea, including the crayfish *Cherax destructor* (Zare & Greenaway, 1998), and the Chinese mitten crab *Eriocheir sinensis* (Onken, 1999; Riestenpatt *et al.*, 1996; Onken & Putzenlechner, 1996; Riestenpatt *et al.*, 1994). By pumping hydrogen ions outwards from the cytoplasm into the external medium, this transport enzyme has been found to play an important role in driving the uptake of sodium in dilute media. Although, an apical V-type H^+-ATPase has not yet been identified in the gills of *G. pulex*, it has been suggested that the enzyme is ubiquitous to freshwater crustaceans (Onken & Riestenpatt, 1998). The effects of food limiting cave environments on the activity of this enzyme may provide partial explanation for the lower haemolymph sodium concentrations exhibited by the hypogean *G. pulex* populations.

The lower haemolymph sodium concentration in hypogean *G. pulex* may be caused by an increase in the rate of sodium efflux from the body when compared to epigean populations.

Epigean populations of *G. pulex* are able to actively reclaim sodium ions from their own urine (Lockwood, 1961). Reclaiming these ions from the urine enables epigean *G. pulex* to produce urine hypotonic to its body fluids, thereby limiting the loss of sodium from the body. Hypogean populations, which have lost the ability and/ or the energy to actively reclaim ions from their urine, may result in lower haemolymph sodium concentrations. The antennal gland of crustaceans is believed to play an important role in the reabsorption of sodium and chloride from the urine through the action of Na^+, K^+-ATPase (Sarver *et al.*, 1994; Horiuchi, 1980). In the freshwater crayfish *Procambarus clarkii* antennal gland Na^+, K^+-ATPase was responsible for renal salt reabsorption and, thus, the production of dilute urine (Sarver *et al.*, 1994). Similar mechanisms of renal salt reabsorption by antennal gland Na^+, K^+-ATPase may be involved in the production of dilute urine in *G. pulex*. Since this is an energy demanding process, differences in the energy budgets between epigean and hypogean *G. pulex* populations, caused by the food deficient cave environment, may result in differences in renal salt reabsorption, which could potentially influence haemolymph ion concentrations.

CHAPTER NINE

General Discussion

The preceding chapters have dealt with many aspects of osmoregulation in gammarid amphipods, focusing particularly on gammarids from fresh and brackish water habitats. This chapter aims to bring to together the findings of the present study, and to compare these results with those previously known on osmoregulation in gammarids and other crustaceans. The ionic concentration of the animals' natural environment is believed to be of crucial importance in effecting the osmoregulatory mechanisms of crustaceans (Péqueux, 1995). This was certainly found to be true for the gammarids in this study. The effect of habitat ionic concentration on osmoregulation has been suggested from the present study to influence competitive interactions between gammarids as well as potentially causing evolutionary divergence in subspecies of *G. duebeni*. In addition, the effects of a variety of stressors on the osmoregulation of the highly freshwater adapted *G. pulex* will be considered.

9. 1. Adaptation to fresh water

The ability of hyper-regulating crustaceans to maintain stable ion concentrations when acclimated to dilute media involves the co-ordinated activities of a number of different mechanisms. These mechanisms include; 1) reduction in body surface permeability to water and ions, 2) increased levels of active ion uptake (principally Na^+ and Cl^-), 3) reduction in the levels of water and ion loss from the urine, and 4) cell tolerance to low internal body fluids (Dawson, 1982). The ability to alter the first three mechanisms and to tolerate the fourth differs between species, particularly between crustaceans from fresh and brackish water habitats. Freshwater crustaceans are able to tolerate lower internal ion concentrations and often maintain a lower haemolymph ion concentration than those of either brackish or marine origin when acclimated to dilute media. For example, the

freshwater amphipod *G. pulex* maintains its haemolymph ion concentration at a much lower concentration than that exhibited by the brackish water *G. duebeni duebeni* (Fig. 4. 1. 2. 1.). The lower haemolymph ion concentration in dilute media reduces the haemolymph – external medium ionic gradient. This potentially reduces water and ion loss down its concentration gradient, decreasing the active replacement of these lost ions, and provides *G. pulex* with an energetic advantage over brackish water species, such as *G. d. celticus* in dilute media.

In contrast to the freshwater adaptative mechanisms, higher haemolymph ion concentrations exhibited by brackish water gammarids (*G. d. duebeni*, *G. tigrinus* and *G. zaddachi*) are a compromise between reducing energy expenditure in dilute media, and the ability to survive rapid increases in external salinity. By maintaining higher haemolymph ion concentration whilst in dilute media, brackish water gammarids can tolerate sudden increases in external salinities, up to 50% sea water, before haemolymph is forced hypotonic to the external medium (Bolt *et al.*, 1980).

The model of active sodium uptake in the gills of freshwater crustaceans involves an apical H^+-ATPase, enzyme, which actively pumps hydrogen ions out from the cytoplasm into the external medium. This creates an electrochemical gradient to drive sodium movement across the apical membrane *via* Na^+ channels. In addition, two populations of carbonic anhydrase (CA) are involved. The first population on the basolateral membrane dehydrates HCO_3^- to CO_2, the gas diffuses into the cytoplasm across the basolateral membrane. In the cytoplasm the second CA population rehydrates CO_2 to form H^+ and HCO_3^-. Hence, CA provides the H^+ ion for the apical H^+-ATPase aiding in sodium uptake and HCO_3^- for the apical Cl^-/ HCO_3^- antiporter used for chloride uptake. Gill Na^+, K^+-ATPase on the basolateral membrane pumps sodium ions out of the cytoplasm into the haemolymph, maintaining low cellular sodium concentrations, thus contributing to the maintenance of the

electrochemical gradient for the inward movement of sodium (Fig. 1. 3., Onken, 1999; Riestenpatt *et al.*, 1996; Onken & Putzenlechner, 1996; Riestenpatt *et al.*, 1994).

9. 1. 1. Energy budgets

Osmoregulatory energy budgets have been an underlying theme in many of the present studies. This has been particularly true regarding the competitive interactions between invading and native amphipod species (Chapter 4), as well as investigations into the effects of stressors; copper, parasites and caves (Chapters 6, 7 & 8 respectively) on osmoregulation. Due to the active uptake of ions required to maintain haemolymph ion concentration hyper-osmotic to the external environment, osmoregulation is an energy demanding process. *G. pulex* spends about 11% of its daily energy budget on osmoregulation when acclimated to fresh water (Sutcliffe, 1984). It would be expected that gammarids less adapted to fresh water than *G. pulex*, such as brackish water *G. d. duebeni* or *G. tigrinus* would need to use considerably more of their daily energy budget on osmoregulation. This is certainly indicated by the significantly higher rates of sodium uptake exhibited by the more brackish water *G. tigrinus* and *G. d. duebeni*, when compared to the more freshwater adapted *Dikerogammarus villosus* and *G. pulex* (Fig. 4. 1. 2. 6.).

From these results, sodium influx of the brackish water gammarids was about six times that of the freshwater species when acclimated to fresh water. Since sodium influx in fresh water would be almost exclusively through active uptake processes, it might be expected that the energy demand on these brackish water species is substantially higher than the 11% estimate for *G. pulex*.

Due to the large differences in sodium influx between the gammarids from fresh and brackish water habitats when acclimated to fresh water, it would be expected that similar differences in the gill Na^+, K^+-ATPase activity, might also occur. Investigations into gill

Na^+, K^+-ATPase activity, showed salinity responsive behaviour in all gammarids investigated, with maximum activity found in all species acclimated to their most dilute media (Chapter 3. 2.). This coincided with the significantly higher haemolymph-external medium ionic gradient and the significantly higher sodium influx in dilute media. This study does clearly show that gill Na^+, K^+-ATPase is involved in active sodium regulation in gammarids. However, often differences in gill Na^+, K^+-ATPase activity were not found between the species despite large differences in sodium influx. Even when significant differences in gill Na^+, K^+-ATPase activity were found they were not of the same magnitude as that found between sodium influx rates. This may suggest the involvement of a second transport enzyme other than Na^+, K^+-ATPase, such as the apical H^+-ATPase enzyme. The involvement of gill H^+-ATPase was not investigated in this study. It has been found to play a crucial role in sodium uptake in the gills of the freshwater crab *E. sinensis* (Riestenpatt *et al.*, 1996; Onken & Riestenpatt, 1998). The involvement of H^+-ATPase may be suggested to play a similar role in the fresh and brackish water gammarids when acclimated to dilute media, since gill Na^+, K^+-ATPase activity fails to fully account for the measured differences in sodium influx between the species. In future experiments the involvement of H^+-ATPase in ion uptake in gammarid gills should be determined. In addition, CA has been found to be actively involved in the gills of crustaceans, providing the counter ions H^+ and HCO_3^- for the uptake of sodium and chloride respectively (reviewed in Henry, 1998). It is likely that CA provides a similar function in the gills of gammarid species. Its involvement in ion regulation of gammarids should be considered in future work.

It should be noted that measurements of gill Na^+, K^+-ATPase activity in gammarids were partly affected by the buffer used in the assay solution. Imidazole was first used as the buffer in the assay solution (Chapter 3). Although, enzyme activity was detected in these experiments, it was found in the later experiments (Chapter 6), when hepes replaced

imidazole as the buffer, that enzyme activity increased by approximately three fold. Imidazole is a weak metal chelating agent, and has been suggested to inhibit certain metallo-enzymes, such as carbonic anhydrase, possibly by stripping the essential zinc metal from the enzyme important for the correct functioning of the enzyme (Maren, 1967). It was not predicted that imidazole would have any inhibitory effect on gill Na^+, K^+-ATPase, particularly since imidazole has been previously used to measure gill Na^+, K^+-ATPase activity in other crustaceans. These include: the freshwater shrimp *Macrobrachium olfersii* (Lima *et al.*, 1997), the blue crab *Callinectes sapidus* (Towle *et al.*, 1976), the euryhaline crab *Chasmagnathus granulata* (Schleich *et al.*, 2001), and the giant fresh water prawn *Macrobrachium rosenbergii* (Wilder *et al.*, 2000). The enzyme activities determined in chapter 3 might therefore be underestimations. It is strongly recommended in future experiments, that hepes buffer be used (as in section 2. 2. 4.) instead of imidazole to measure gill Na^+, K^+-ATPase activity in gammarids. This partial inhibition of gill Na^+, K^+-ATPase activity by imidazole may also explain why significant differences were not found between gammarids from fresh and brackish water habitats, despite large differences in sodium influx.

9. 1. 1. 1. Competitive interactions

Irish invasion

The differences in the energetic costs of osmoregulation between the freshwater species *G. duebeni celticus* and *G. pulex*, was suggested as an additional factor in the replacement of the former by the latter in the fresh waters of Northern Ireland (Chapter 4. 2.). The freshwater *G. d. celticus* has been isolated from sea water influences for thousands of years and has become increasingly adapted to the freshwater environment (Sutcliffe, 2000). Despite this apparent adaptation to fresh water in *G. d. celticus*, its osmoregulatory ability was found to be significantly less adapted to fresh water than *G. pulex*. This can be seen in comparisons of haemolymph sodium concentration, sodium influx and gill Na^+, K^+-ATPase

activity between *G. d. celticus* and *G. pulex* (Chapter 4. 2. 2.). It was previously suggested that predatory interactions between these two species occur by mutual but differential predation in favour of *G. pulex* in the freshwater habitats of Northern Ireland (Dick, 1996a). The energetic advantage that *G. pulex* appears to have over *G. d. celticus* in fresh waters is likely to influence these predatory interactions due to the increased fitness of the former over the latter. By significantly reducing the energy demands of osmoregulation in fresh water, additional short term energy may be available to *G. pulex*, potentially increasing its activity and / or competitive advantage. This could ultimately lead to the displacement of *G. d. celticus* by *G. pulex*, which has been gradually taking place in many freshwater bodies of Northern Ireland (Hynes, 1955; Pinkster, 1975; Pinkster *et al.,* 1977; Dick *et al.*, 1993; Dick, 1996a; Dick *et al.*, 1999; McNeil *et al.*, 1997). However, in certain water bodies of Northern Ireland and The Isle of Man, *G. d. celticus* has been found to resist displacement by *G. pulex* (Dick *et al.*, 1997). Although, in these water bodies, the ionic compositions were not measured, slight increases in salinity might reduce the energetic advantage of *G. pulex* and effect the competitive interactions between itself and *G. d. celticus*.

Dutch Invasion

In the fresh and oligohaline waters of The Netherlands, the invading amphipod *D. villosus* was found to replace the pre-existing amphipods *G. d. duebeni* and *G. tigrinus*. In addition to the predatory advantage found in favour of *D. villosus* over both *G. d. duebeni* and *G. tigrinus* (Dick & Platvoet, 2000), the present study (Chapter 4. 1.) identified a distinct osmoregulatory advantage. From measurements of haemolymph sodium concentration, sodium influx and Na^+, K^+-ATPase, *D. villosus* was significantly better adapted to fresh and oligohaline waters than either *G. tigrinus* or *G. d. duebeni*. Consequently in the natural fresh/ oligohaline waters of the Netherlands, *D. villosus* would be likely to have a distinct energetic advantage over the native gammarids.

In comparison to *G. pulex* from The Netherlands, *D. villosus* was less adapted to fresh water. Although haemolymph sodium concentrations were comparable between the two amphipods, significantly higher sodium influx rates were recorded in *D. villosus* (Fig. 4. 1. 2. 6.). This would therefore suggest that significantly more energy is required by *D. villosus* to maintain haemolymph sodium concentration than *G. pulex* in fresh water. In addition, it has been previously shown that reduced fitness of *D. villosus* occurs after several weeks of acclimation to fresh water (Brujis *et al.*, 2001). This may be due to the inability of *D. villosus* to actively replace ions lost to the low ion external environment. As yet there have been no reports concerning the displacement of *G. pulex* by *D. villosus* from freshwater bodies with low ion content. In the Moselle River, populations of *D. villosus* and *G. pulex* were found to co-exist (Devlin *et al.*, 2001). The population of *D. villosus* from the Moselle River were significantly smaller than those from Dutch and German waters. Differences in the energetic costs of osmoregulation in fresh water by *D. villosus* may provide partial explanation for the reduced body size. In such a situation the predatory advantage *D. villosus* appears to hold over competing gammarids may be significantly reduced.

Therefore, in low ion freshwater bodies differences in osmoregulatory physiology may be an important factor influencing species interactions. It is suggested under such circumstances *G. pulex* may be able to resist invasion by *D. villosus* due to its osmoregulatory mechanisms being more adapted to fresh water. Whether *G. pulex* can resist displacement by *D. villosus* in waters of low ion content remains to be seen. Continued monitoring of the freshwater bodies of Western Europe will in time answer this question.

A crucial point in *D. villosus* invasion concerns its survival in ballast water of ocean going vessels, potentially resulting in its global distribution. It was previously reported that *D. villosus* could potentially survive incomplete ballast water exchange up to 50% sea water

(Brujis *et al.*, 2001). In the present study, rapid changes in sodium influx occurred 3 to 4 hours after instant transfer from fresh water to 50% sea water. However, this change in sodium influx was not enough to prevent the 100% mortality of *D. villosus* within 24 hours after transfer. The inability to survive such rapid salinity change is a consequence of the species adaptation to dilute media. This adaptation includes the relatively low haemolymph ion concentrations and low sodium influx in dilute media as well as the inability to rapidly alter its $t_{1/2}$ after such rapid salinity transfer. This study therefore fails to support the suggestion that *D. villosus* can survive incomplete ballast water transfer up to 50% sea water. The inability to survive rapid fluctuations in salinity, also suggests that *D. villosus* is unlikely to displace euryhaline gammarids, such as *G. d. duebeni* from estuarine waters that experience changing salinity regimes.

9. 1. 2. Evolutionary divergence

Increased freshwater adaptation in *G. d. celticus* has resulted in its inability to alter $t_{1/2}$ when exposed to increased sea water concentration (Lockwood *et al.*, 1973; Bolt *et al.*, 1980; Lockwood & Bolt, 1989) confirmed in this study (Chapter 5). Further freshwater adaptation exhibited by *G. d. celticus* included a significantly lower sodium influx rate than the subspecies *G. d. duebeni*. This lower sodium influx rate occurred despite no significant reduction in haemolymph sodium concentration in fresh water. The significantly lower sodium influx rate in *G. d. celticus* than *G. d. duebeni* was a reflection of the significantly lower sodium efflux rates exhibited by the former than the latter in fresh water. The ability to lower sodium efflux may be attributed to the increased ability of *G. d. celticus* to reclaim sodium ions from the urine *via* the action of antennal gland Na^+, K^+-ATPase. Due to the difficulties in extracting antennal gland enzyme in gammarids, the activity of antennal gland Na^+, K^+-ATPase was not determined in this study. However, previous reports have implicated antennal gland Na^+, K^+-ATPase in sodium and chloride reabsorption of freshwater crustaceans (Sarver *et al.*, 1994; Horiuchi, 1980). It is known that *G. pulex* and

G. d. duebeni, unlike marine gammarids, can produce hypo-osmotic urine in relation to their body fluids (Lockwood, 1961). Therefore, it is likely that similar mechanisms of salt reabsorption by antennal gland Na^+, K^+-ATPase exist in other fresh and brackish water gammarids, particularly those exhibiting increased freshwater adaptation. This increased ability to reclaim ions lost to urine appears to be a further osmoregulatory mechanism influenced through freshwater adaptation.

Although *G. d. celticus* has demonstrated increased freshwater adaptation, it has still retained the ability to survive high salinity acclimation to at least full strength sea water. Such ability may be related to the fact that *G. d. celticus* still maintains its haemolymph ion concentration significantly higher (cf. to freshwater *G. pulex*) when acclimated to dilute media. However, *G. d. celticus* was found to be less able to tolerate sudden salinity changes (fresh water to 100% sea water or vice versa) when compared to the more brackish water species *G. d. duebeni*. Differences in cell tolerance levels between the subspecies may partly be responsible for the poor survival of *G. d. celticus* after rapid transfer experiments. However, the significantly slower changes to the sodium uptake mechanism combined with the inability of *G. d. celticus* to alter its $t_{1/2}$ are key factors resulting in the inability to actively regulate ion concentrations within cell tolerance limits. Such inability to tolerate rapid changes in external salinity might not be so surprising, particularly since *G. d. celticus* does not experience such changes in salinity in its natural freshwater habitat, unlike that of the brackish water form *G. d. duebeni*.

Freshwater adaptation was also exhibited by the 'Lizard' population of *G. duebeni*, with significantly lower rates of sodium influx than those of the more brackish water form *G. d. duebeni*. However, *G. duebeni* (Lizard) has retained the ability to alter its $t_{1/2}$ with respect to the external salinity despite the apparent adaptation to its natural freshwater habitat. In their natural freshwater environment, *G. duebeni* (Lizard) can be subject to raised salinities

through sea spray. It could be argued that since *G. duebeni* in these waters is occasionally exposed to higher salinities, it has retained some of the osmoregulatory features of its once brackish water form. This study has highlighted the influence of the ionic concentration of the natural habitat in inflicting profound physiological effects on the osmoregulatory mechanisms of inhabiting species. The increased adaptation to fresh water in *G. d. celticus* has led to evolutionary divergence from the brackish water form *G. d. duebeni*. Due to the natural habitat of the 'Lizard' population of *G. duebeni*, its osmoregulatory physiology appears to be a compromise between freshwater adaptation and the ability to survive sudden if only occasional surges in external salinity.

9. 2. Haemolymph magnesium concentration

In addition to the main ions sodium and chloride, measurements of haemolymph magnesium concentration revealed some interesting differences between the species and/or subspecies of gammarids. As mentioned in previous chapters, haemolymph magnesium concentrations have been correlated with activity levels in several crustaceans (Morritt & Spicer, 1993; Frederich *et al.*, 2000). Such reductions in activity levels due to high levels of haemolymph magnesium have been suggested to restrict the geographical distribution of many crustaceans (Frederich *et al.*, 2000). In the present study it has also been suggested that haemolymph magnesium concentrations may restrict the distribution of gammarids at the population level. In the 'Lizard' population of *G. duebeni* (Chapter 5), haemolymph magnesium concentrations (30 to 40mM) were the highest recorded for any of the gammarids in this study. This population of *G. duebeni* were collected in a small isolated fresh water stream where the water ion content becomes occasionally raised due to sea spray. It was suggested that the absence of competitors experienced by the 'Lizard' population, may have reduced the requirement for high levels of activity and that the haemolymph magnesium contributed more to the ionic strength of the haemolymph.

Furthermore, this study has also suggested the influence of haemolymph magnesium concentrations in effecting competitive interactions between gammarid species. Significantly higher haemolymph magnesium concentrations were found in *G. d. celticus* than *G. pulex* in fresh water. The result of the competitive interactions between these two species is likely to be in favour of the more active species i.e. *G. pulex*. In the Irish fresh waters, *G. pulex* was found to have a predatory advantage over *G. d. celticus* resulting in the displacement of the latter by the former (Dick *et al.*, 1990a; Dick *et al.*, 1990b; Dick *et al.*, 1994; Dick *et al.*, 1996a). Such a predatory advantage exhibited by *G. pulex* over *G. d. celticus* may be influenced partly by the differences in the activity levels between the two species.

It should be noted however, that haemolymph magnesium concentrations were significantly higher in freshwater acclimated *D. villosus* compared to the other Dutch gammarids. The low activity levels suggested by the high magnesium levels, appears contrary to its behaviour as an aggressive rapid invader of Western Europe. In future work it is suggested that an attempt to correlate haemolymph magnesium concentration with activity levels in gammarids should be investigated. This may provide further understanding of the competitive interactions between gammarids, such as within the Irish and Dutch invertebrate communities in this study.

9. 3. Stress effects on osmoregulation

One major aspect of this thesis has been to investigate the effects of a variety of stressors on the osmoregulation of *G. pulex*. These stressors include: 1) the toxic effects of copper exposure, 2) the effects of endoparasitic infection, and 3) the influence of oxygen & food deficient cave environments. Although all these stressors were found to have significant effects on osmoregulation in *G. pulex*, they were found to influence the osmoregulatory mechanisms in profoundly different ways. Differences in haemolymph ion concentration,

particularly for sodium, proved to be a very useful method in identifying stressor effects on osmoregulation. This encouraged the osmoregulatory mechanisms to be investigated in an attempt to explain such changes in haemolymph ion concentration.

In both hypogean and copper exposed *G. pulex* significant reductions in haemolymph sodium concentration were found in comparison to control animals. The low haemolymph sodium concentration in hypogean *G. pulex* was believed to be due to a long term adaptation of these cave dwelling animals to their potentially nutrient deficient environment. Although the nutrient availability was not measured in this study, the presence of ammonium in the haemolymph of only hypogean animals was an indication that these animals were starved for a longer time than the epigean population. Despite this fall in haemolymph sodium concentration, measurements of sodium influx and gill Na^+, K^+-ATPase failed to show any significant difference from that of the epigean population. This led to the suggestion that differences in sodium efflux may be responsible. The production hypo-osmotic urine through the active reclamation of sodium and chloride by antennal gland Na^+, K^+-ATPase is believed to reduce ion loss *via* the urine. Although gill Na^+, K^+-ATPase appeared to be unaffected by the apparent energy deficient cave environment, it was suggested that the antennal gland Na^+, K^+-ATPase may be more sensitive to such low energy availability.

In contrast, exposure to copper clearly demonstrated that significant inhibition of gill Na^+, K^+-ATPase was responsible for the reduction in sodium influx and the subsequent lowering of the haemolymph sodium concentration. In addition, although not measured in this study, it was suggested that inhibition of antennal gland Na^+, K^+-ATPase might also contribute to the lowering of haemolymph sodium concentration, by the inability to reabsorb ions from the urine.

The difference between these two stressors on the osmoregulation of *G. pulex*, is that the hypogean animals have adapted gradually over thousands of years to the subterranean environment (Culver *et al.*, 1995). In these hypogean animals, the lowering of the haemolymph sodium concentration is an adaptive strategy designed to reduce the energy demands for active replacement of lost ions. A lower haemolymph sodium concentration would also benefit from a small haemolymph-external medium osmotic gradient, which as long as body permeability is not altered would reduce passive ion loss to the external environment. Consequently, by reducing passive ion loss, the energy requirements for active ion uptake are likely to be reduced. Such an adaptation to low energy environments would benefit hypogean animals, and is likely to increase the overall fitness of the hypogean *G. pulex*. A disadvantage in maintaining particularly low haemolymph ion concentrations in fresh water is the inability to survive relative small changes in external salinity. In the hypogean animals, exposure to salinities around 30% sea water is likely to force the haemolymph hypotonic with respect to the external medium. Hence, the hypogean *G. pulex* is unlikely to survive such salinity acclimation, unlike epigean *G. pulex* populations.

In contrast, the reduction in haemolymph sodium concentration as a result of copper toxicity is not an adaptive strategy like that exhibited by hypogean *G. pulex*, but merely a toxic effect on the active uptake mechanism of sodium. In this case, copper exposure would eventually result in death, due to the inability to maintain haemolymph sodium concentration to within cell tolerance limits. However, short-term adaptation was exhibited by *G. pulex* following pre-exposure to sub-lethal copper concentrations. Increased synthesis of new enzyme triggered by sub-lethal copper pre-exposure was thought to be responsible for reducing *in vitro* copper toxicity to gill Na^+, K^+-ATPase activity.

The effects of the endoparasite *P. minutus* on the osmoregulation of *G. pulex*, differed from that caused by either long term adaptation to cave environments or acute copper exposure. Parasite effects on the neuroendocrine modulation, believed to be responsible for behavioural changes in the host have been well documented (Marriott *et al.*, 1989; Maynard *et al.*, 1996; Bakker *et al.*, 1997). It was suggested in this present study (Chapter 7) that changes in the neuroendocrine system, were responsible for the alteration in haemolymph sodium regulation in *G. pulex* at 15% sea water. The lower sodium efflux in infected *G. pulex* was potentially caused by the increased ability of the antennal glands to actively reclaim sodium ions from the urine. It was suggested that the increased expression of serotonin may have increased Na^+, K^+-ATPase activity in the antennal glands of *G. pulex*, which was responsible for the increased reclamation of sodium from the urine.

The involvement of serotonin in sodium regulation has been previously demonstrated in the shore crab *Carcinus maenas* (Sommer & Mantel, 1988) and the Chinese spider crab *Eriocheir sinensis* (Trausch *et al.* 1989; Bianchini & Gilles, 1990). In these studies serotonin was found to increase Na^+, K^+-ATPase in the gills. Further work is required to determine the involvement of serotonin on sodium regulation in *G. pulex*. Since serotonin stimulates Na^+, K^+-ATPase activity in other crustacea it may be expected that Na^+, K^+-ATPase activity in *G. pulex* is also stimulated to at least some extent.

In conclusion, this study has highlighted the importance of the natural environment in influencing the osmoregulatory mechanisms in gammarids. Freshwater gammarids generally maintain lower haemolymph ion concentrations and therefore lower the haemolymph-external medium ionic gradient and subsequently reduce the osmoregulatory energy demands. This reduction in energy expenditure was taken even further in the hypogean freshwater *G. pulex* believed to be due to the energy deficient cave environment. Although freshwater gammarids reduce energy expenditure in this way, it prevents them

from tolerating rapid changes in salinity. Brackish water gammarids in dilute media maintain significantly higher haemolymph ion concentrations, which increase the energy demand on osmoregulation. Although energy demands are elevated, a high haemolymph ion concentration enables gammarids to survive rapid salinity increases. This is therefore a compromise between the ability to tolerate such low ion content as well as being able to survive sudden increases in external salinity. These differences in osmoregulatory adaptation, to either fresh of brackish water, influence the competitive interactions between species, as shown in the this study with the gammarid communities of Northern Ireland and The Netherlands. In addition, long term adaptation to either fresh or brackish waters can lead to both morphological and physiological distinctions between isolated populations of the same species. This has been demonstrated in this study with isolated populations of *G. duebeni*, resulting in significant differences in their osmoregulatory physiology.

Summary

Characterisation (Chapter Three)

1. Gill Na^+, K^+-ATPase was characterised in the freshwater amphipod *G. pulex*. The optimal ion and co-factor requirements were recorded as;

   Ouabain = 10mM (K_i =3.25) sodium = 100mM

   Potassium = 15mM (K_m = 3.85) Magnesium = 10mM (K_m = 3.2mM)

   ATP = 5mM (K_m = 0.37) pH = 7.2 **(Section 3. 2. 1.).**

2. Characterisation of gill Na^+, K^+-ATPase with respect to sodium in other gammarids (*G. tigrinus*, *G. duebeni celticus* & Irish population of *G. pulex*) revealed identical optimum sodium concentrations to that of *G. pulex* (i.e. 100mM Na). It was therefore, assumed that comparisons could be made between the gill Na^+, K^+-ATPase activity of gammarid species. **(Section 3. 2. 1.).**

3. The involvement of gill Na^+, K^+-ATPase in the osmoregulation of gammarids has been demonstrated, with enzyme activity strongly influenced by the salinity of the external medium. Highest activity levels were found in gammarids when acclimated to their most dilute media. **(Section 3. 2. 2.).**

4. In fresh water, there was a tendency for brackish water gammarids to have higher activity levels than the more freshwater adapted species. **(Section 3. 2. 2.).**

Invasive Species (Chapter Four)

The Netherlands

5. Haemolymph ion concentrations and sodium influx rates in *D. villosus* were similar to that of *G. pulex* and significantly lower than that of the more brackish water gammarids *G. d. duebeni* and *G. tigrinus* from The Netherlands. **(Section 4. 1. 2.).**

6. *D. villosus* was found to significantly decrease its $t_{1/2}$ with increased seawater acclimation to 50% sea water. **(Section 4. 1. 2. 2.).**

7. The osmoregulatory physiology of *D. villosus* is likely to provide it with an energetic advantage over *G. d. duebeni* and *G. tigrinus* in the oligohaline waters of The Netherlands. **(Section 4. 1. 3. 5.).**

8. Rapid transfer of *D. villosus* from fresh water to 50% sea water resulted in 100% mortality within 24 hours after transfer, suggesting that *D. villosus* may not survive incomplete ballast water exchange of 50% sea water and greater.

(Section 4. 1. 3. 5.).

Northern Ireland

9. *Gammarus pulex* maintained a significantly lower haemolymph ion concentration and a significantly lower rate of sodium influx than *G. d. celticus* when acclimated in fresh water. **(Section 4. 2. 2.).**

10. The increased freshwater adaptation of *G. pulex* than *G. d. celticus* would provide the former with an energetic advantage over the latter. This was suggested to influence the competitive interactions between the two freshwater gammarids in favour of *G. pulex* in Northern Ireland. **(Section 4. 2. 3. 5.).**

G. duebeni – evolutionary divergence (Chapter Five)

11. Haemolymph ion concentrations were maintained at significantly higher levels in the brackish water *G. d. duebeni* than either of the two freshwater forms (*G. d. celticus* & *G. duebeni* ('Lizard' population)). **(Section 5. 2. 1.).**

12. The two brackish water populations of *G. d. duebeni*, and the freshwater 'Lizard' population *G. duebeni* were found to decrease their $t_{1/2}$ with increasing seawater acclimation. In contrast, the Irish freshwater *G. duebeni celticus* was unable to alter its $t_{1/2}$ with respect to increasing salinity. **(Section 5. 2. 2.).**

13. In fresh water, the sodium influx rates were significantly higher in the brackish water *G. duebeni duebeni* than the two freshwater forms (*G. d. celticus* & 'Lizard' population *G. duebeni*). **(Section 5. 2. 3.).**

14. All brackish and freshwater forms of *G. duebeni* were found to alter their sodium influx rates after rapid transfer to either hyper- or hypo-osmotic medium. However, faster rates of change in sodium influx were found in the brackish *G. duebeni duebeni* (2-4hrs after transfer) than that of the freshwater *G. d. celticus* (8hrs after transfer). **(Section 5. 2. 4.).**

15. Rapid changes in $t_{1/2}$ occurred within 4 hours following instant transfer to hypo-osmotic media in *G. d. duebeni* from The Netherlands. **(Section 5. 2. 4.).**

16. Overall the differences in the osmoregulatory physiology between freshwater and brackish water forms could be explained in terms of their increased adaptation to the ionic concentration of their natural habitats. **(Section 5. 3. 4.).**

Copper toxicity (Chapter Six)

17. *In vivo* copper exposure to 100 and 1000 $\mu g.\ l^{-1}$ copper caused a significant fall in haemolymph sodium concentration to approximately 70% of control values within 4 hours of copper exposure. This reduction coincided with a similar reduction in sodium influx rate for the same duration and concentration of *in vivo* copper exposure. **(Section 6. 2.).**

18. *In vitro* copper exposure to $100\mu g.\ l^{-1}$ and $1000\mu g.\ l^{-1}$ copper significantly inhibited gill Na^+, K^+-ATPase activity to 60% and 10% of control values respectively. It was suggested that the inhibition of gill Na^+, K^+-ATPase was responsible for the reduction in sodium influx rates resulting in the fall in haemolymph sodium concentration in copper exposed *G. pulex*. **(Section 6. 2.).**

19. Treatment with 1mM DTT significantly reversed the inhibitory effects of $1000\mu g.l^{-1}$ copper on gill Na^+, K^+-ATPase activity to a greater extent than with DTPA. It was concluded that inhibition of gill Na^+, K^+-ATPase enzyme by copper exposure, was primarily as a result of the oxidation of the –SH groups leading to disruption of the 3D structure , affecting functional activity. **(Section 6. 2. 3.).**

20. Pre-exposure to sub-lethal copper concentrations (100μg. l^{-1}) for one and five days significantly reduced *in vitro* copper toxicity to gill Na^+, K^+-ATPase.

(Section 6. 2. 4.).

Parasite infection (Chapter Seven)

21. Haemolymph sodium concentrations were significantly higher in *G. pulex* infected with one or more cystacanths of *P. minutus* when acclimated to 15% sea water compared to uninfected *G. pulex*. **(Section 7. 2. 2.).**

22. A significant reduction in sodium efflux in infected animals at 15% sea water was thought to be responsible for the higher haemolymph sodium concentration at this salinity. **(Section 7. 2. 3.).**

23. The changes in sodium regulation exhibited by *G. pulex* infected with cystacanths of *P. minutus* were thought to reflect alterations in the serotonergic modulation.

(Section 7. 3. 3.).

Cave environment (Chapter Eight)

24. The two hypogean populations (Speedwell cave & Peak cave) were found to have significant reductions in haemolymph sodium, potassium, and calcium concentration when compared to the epigean population (River Wye).

(Section 8. 2. 3.).

25. No significant difference in $t_{1/2}$, sodium influx or gill Na^+, K^+-ATPase activity was found. It was suggested that an increase in the sodium efflux rate of hypogean populations of *G.* pulex might be responsible for their lower haemolymph sodium concentrations. **(Section 8. 2. 4.).**

REFERENCES

Ahl, J. S. B., & Brown, J. J. 1991. The effect of juvenile hormone III, methyl farnesoate, and methoprene on Na/K-ATPase activity in larvae of the brine shrimp *Artemia*. *Comparative Biochemistry and Physiology*. Vol. **100A**, pp. 155-158.

Albers, R. W. 1976. *The (sodium plus potassium)-transport ATPase.* In: The enzymes of Biological membranes. Vol. 3. (Ed: Martonosi, A) J Wiley and Sons, London, 283-301 pp.

Amat, F., Gozalbo, J. C., Navarro, J. C., Hontoria, F., & Varó, I. 1991. Some aspects of *Artemia* biology affected by cestode parasitism. *Hydrobiologia*, Vol. **212**, pp. 39-44.

Bakker, T. C. M., Mazzi, D., & Zala, S. 1997. Parasite induced changes in behaviour and colour make *Gammarus pulex* more prone to fish predation. *Ecology*, Vol. **78**, pp. 1098-1104.

Bambang, Y., Thuet, P., Charmantier-Daures, M., Trilles, J.P., & Charmantier, G., 1995. Effect of copper on survival and osmoregulation of various developmental stages of the shrimp *Penaeus japonicus* Bate (Crustacea, Decapoda). *Aquatic Toxicology*. Vol. **33**, pp. 125-139.

Barnes, R. D. 1987. *Invertebrate Zoology 5th edition.* Saunders College Publishing, USA.

Barra, J. A., Péqueux,. A., & Humbert, W. 1983. A morphological study on gills of a crab acclimated to fresh water. *Tissue & Cell*. Vol. **15**, pp. 583-596.

Barradas, C., & Péqueux, A. 1996. Uptake of mercury by the gills of the fresh water Chinese crab *Eriocheir sinensis* (Milne-Edwards). *Comparative Biochemistry and Physiology*. Vol. **113C**, pp. 157-160.

Barradas, C., DunerlErb, S., Lignon, J., & Péqueux, A. 1999. Superimposed morphofunctional study of ion regulation and respiration in single gill filaments of the crayfish *Astacus leptodactylus*. *Journal of Crustacean Biology*, Vol. **19**, pp. 14-25.

Beadle, L. C., & Cragg, J. B. 1940. Studies on the adaptation to salinity of *Gammarus sp.* 1. Regulation of the blood and tissues and the problem of adaptation to fresh water. *Journal of Experimental Biology*. Vol. **17**, pp. 153-163.

Bentley, C. R., & Hurd, H. 1993. *Pomphorhynchus laevis* (Acanthocephala): elevation of haemolymph protein concentration in the intermediate host, *Gammarus pulex* (Crustacea: Amphipoda). *Parasitology*, Vol. **107**, pp. 193-198.

Bianchini, A., & Gilles, R. 1990. Cyclic-AMP as a modulator of NaCl transport in gills of the euryhaline Chinese crab *Eriocheir sinensis*. *Marine Biology*. Vol. **104**, pp. 191-195.

Bij de Vaate, A., & Klink, A. G. 1995. *Dikerogammarus villosus* Sowinsky (Crustacea: Gammaridae) a new immigrant in the Dutch part of the Lower Rhine. *Lauterbornia*, Vol. **20**, pp.51-54.

Bird, L. M. 1989. *The effects of saline pumping water on freshwater invertebrate communities.* PhD Thesis (unpublished). Nottingham Polytechnic (Nottingham Trent University).

Blockwell, S.J., Taylor, E.J., Jones, I., & Pascoe, D., 1998. The influence of fresh water pollutants and interactions with *Asellus aquaticus* (L.) on the feeding activity of *Gammarus pulex. Archive of. Environmental Contamination and Toxicology*. Vol. **34**, pp. 41-47.

Boitel, F., & Truchot, J.P. 1989. Effects of sublethal copper levels on haemolymph acid-base balance and ion concentrations in the shore crab *Carcinus maenas* kept in undiluted seawater. *Marine Biology*. Vol. **103**, pp. 495-501.

Bollache, L., Gambade, G., & Cézilly, F. 2001. The effects of two acanthocephalan parasites, *Pomphorhynchus laevis* and *Polymorphus minutus*, on pairing success in male *Gammarus pulex* (Crustacea: Amphipoda). *Behavioural Ecology and Sociobiology*, Vol. **49**, pp. 296-303.

Bolt, S. R. L. 1983. Haemolymph concentration and apparent permeability in varying salinity conditions of *Gammarus duebeni, Chaetogammarus marinus* and *Gammarus locusta. Journal of Experimental Biology*. Vol. **107**, pp. 129-140.

Bolt, S. R. L. 1985. Urine clearance rates and apparent permeability of *Gammarus-duebeni* exposed to varying conditions. *Journal of Experimental Biology*, Vol. **114**, pp. 673-678.

Bolt, S. R. L. 1986. Variation of water permeability in selected euryhaline amphipods. *Porcupine Newsletter*, Vol. **3**, pp. 178-180.

Bolt, S. R. L. 1989. *Invertebrate permeability with whole organisms*. In: Water transport in Biological membranes Vol. II, Chapter 12. (Ed. Beuga) CRC Press 233-246 pp.

Bolt, S. R. L., Dawson, M. E., Inman, C. B. E., & Lockwood, A. P. M. 1980. Variation of apparent permeability to water and sodium transport in *Gammarus duebeni* exposed to fluctuating salinities. *Comparative Biochemistry and Physiology*, Vol. **67B**, pp. 465-473.

Bousfield, E. L. 1958. Freshwater crustaceans of glaciated North America. In: Pinkster, S., Smit, H., Brandse-de Jong, N. 1977. The introduction of the alien amphipod *Gammarus tigrinus* Sexton, 1939, in the Netherlands and its competition with indigenous species. *Crustaeana Supplement* Vol. **4**, pp. 91-105.

Bousfield, E. L. 1973. *Shallow-water Gammaridean Amphipoda of New England.* Cornell University Press, Ithaca and London.

Brouwer, M., & Brouwer, T. H. 1998. Biochemical defence mechanisms against copper-induced oxidative damage in the blue crab, *Callinectes sapidus*. *Archives of Biochemistry and Biophysics*. Vol. **351**, pp. 257-264.

Brown, A. F., & Thompson, D. B. A. 1986. Parasite manipulation of host behaviour: acanthocephalans and shrimps in the laboratory. *Journal of Biological Education*, Vol. **20**, pp. 121-127.

Brujis, M. C. M., Kelleher, B., van der Velde, G., & bij de Vaate, A. 2001. Oxygen consumption, temperature and salinity tolerance of the invasive amphipod *Dikerogammarus villosus*: indicators of further dispersal via ballast water transport. *Archive fur Hydrobiologie*, Vol. **152**, pp. 633-646.

Bryan, G. W., & Langston, W. J. 1992. Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: a review. *Environmental Pollution*. Vol. **76**, pp. 89-131.

Bubel, A. 1976. Histological and electron microscopical observations on the effects of different salinities and heavy metal ions, on the gills of *Jaera nordmanni* (Rathke) (Crustacea, Isopoda). *Cell and Tissue Research*. Vol. **167**, pp.65-95.

Bulnheim, H. P. 1985. Genetic differentiation between natural-populations of *Gammarus-tigrinus* (Crustacea, Amphipoda) with reference to its range extension in European continental waters. *Archive Fur Hydrobiologie,* Vol. **102**, pp. 273-290.

Carmack, M., & Kelley, C., 1968. Synthesis of the optically active Cleland reagent [(-)-1,4-Dithio-L_g-threitol]. *Journal of Organic Chemistry*. Vol. **33**, pp. 2171-2174.

Chandler, A. C., & Read, C. P. 1961. Introduction to Parasitology (Tenth edition). Wiley & Sons, New York. pp.822.

Cherian, M. G. & Goyer, R. A. 1978. Metallothioneins and their role in the metabolism and toxicity of metals. *Life Sciences*. Vol. **23**, pp. 1-10.

Compère, P., Wanson, S., Péqueux, A., Gilles, R., & Goffinet, G. 1989. Ultrastructural changes in the gill epithelium of the green crab *Carcinus maenas* in relation to external salinity. *Tissue & Cell*. Vol. **21**, pp. 299-318.

Conlan, K. E. 1994. Amphipod crustaceans and environmental disturbance: a review. *Journal of Natural History*. Vol. **28**, pp. 519-554.

Conte, F. P. 1984. Structure and function of the crustacean larval salt gland. *International Review of Cytology*. Vol. **91**, pp. 45-106.

Cornell, J. C. 1979. Salt and water balance in two marine spider crabs, *Libinia emarginata* and *Pugettia producta* II. Apparent water permeability. *Biological bulletin of Marine Biology Laboratories, Woods Hole.* Vol. **157**, pp. 422-433.

Corotto, F. S. & Holliday, C. W. 1996. Branchial Na, K-ATPase and osmoregulation in the purple shore crab, *Hemigrapsus nudus* (Dana). *Comparative Biochemistry and Physiology.* Vol. **113A**, pp. 361-368.

Costello, M. J. 1993. Biogeography of alien amphipods occurring in Ireland, and interactions with native species. *Crustaceana*, Vol. **65**, pp.287-299.

Crane, M., & Maltby, L. 1991. The lethal and sublethal responses of *Gammarus pulex* to stress: sensitivity and sources of variation in an *in situ* bioassay. *Environmental Toxicology and Chemistry.* Vol. **10**, pp. 1331-1339.

Crompton, D. W. T., & Harrison, J. G. 1964. Observations of *Polymorphus minutus* from a wildfowl reserve in Kent. *Parasitology*, Vol. **55**, pp. 345-355.

Crompton, D. W. T., & Joyner, S. M. 1980. *Parasitic worms*. Wykeham Publications Ltd, London. pp. 207.

Culver, D. C. 1985. Trophic relationships in aquatic cave environments. *Stygologia.* Vol. **1**, pp. 43-53.

Culver, D. C., & Poulson, T. L. 1971. Oxygen consumption and activity in closely related amphipod populations from cave and surface habitats. *American Midland Naturalist.* Vol. **85**, pp. 74-84.

Culver, D. C., Kane, T. C., & Fong, D. W. 1995. Adaptation and natural selection in caves: Evolution of *Gammarus minus*. Harvard University Press, Cambridge, London. 223 pp.

Dainty, J. & House, C. R. 1966. 'Unstirred layers' in frog skin. *Journal of Physiology,* Vol. **182**, pp. 66-78.

Danielopol, D. L., Creuzé des Châtelliers, M., Mösslacher, F., Pospisil, P., & Popa, R. 1994. Adaptation of crustacea to interstitial habitats: A practical agenda for ecological studies. In: Gibert, J., Danielopol, D. L., Stanford, J. A. (eds). Ground Water Ecology. San Diego: Academic Press. 217-243 pp.

Danielopol, D. L., Dreher J., Gunatilaka, A., Kaiser, M., Niederreiter, R., Pospisil, P., Creuzé des Châtelliers, M., & Richter, A. 1992. Ecology of organisms living in a hypoxic groundwater environment at Vienna (Austria); methodological questions and preliminary results. *Proceedings of the First International Conference on Groundwater Ecology* (eds. Stanford, J. A., & Simons, J. J.). pp. 79-90. American Water Resources Association, Bethesda, MD.

Dawson, M. E. 1982. Aspects of osmoregulation in the Amphipod. *Gammarus duebeni:* the effects of changing salinity and some potential pollutants, Ph.D. Thesis, University of Southampton, U. K.

Dennert, H., Dennert, A. L., Kant, P., Pinkster, S., & Stock, J. H. 1969. Upstream and downstream migrations in relation to the reproductive cycle and to environmental factors in the amphipod *Gammarus zaddachi. Bijdragen tot de Dierkund*, Vol. **39**, pp. 11-43.

Devlin, S., Beisel, J. N., Bachmann, V., & Moreteau, J. C. 2001. *Dikerogammarus villosus* (Amphipoda: Gammaridae): another invasive species newly established in the Moselle river and French hydrosystem. *Annual Limnology.* Vol. **37**, pp. 21-27.

Dick, J. T. A. 1989. Assessments and decisions during mate choice in *Gammarus pulex* (Crustacea: Amphipoda). PhD thesis, The Queens University Belfast, UK.

Dick, J. T. A. 1992. The nature and implications of differential predation between *Gammarus pulex* and *G. duebeni celticus. Journal of Zoology, London.* Vol. **227**, pp. 171-183.

Dick, J. T. A. 1995. The cannibalistic behaviour of two *Gammarus* species (Crustacea: Amphipoda). *Journal of Zoology, London.* Vol. **236**, pp. 697-706.

Dick, J. T. A. 1996a: Post-invasion amphipod communities of Lough Neagh, Northern Ireland: Influences of habitat selection and mutual predation. *Journal of Animal Ecology*, Vol. **65**, 756-767.

Dick, J. T. A. 1996b. Animal introductions and their consequences for freshwater communities. *Disturbance and Recovery of Ecological Systems* (eds. A. A. Myers & P. Giller). pp. 47-58. Royal Irish Academy, Dublin.

Dick, J. T. A., & Platvoet, D. 1996: Intraguild predation and species exclusions in amphipods: The interaction of behaviour, physiology and environment. *Freshwater Biology*, Vol. **36**, pp. 375-383.

Dick, J. T. A., & Platvoet, D. 2000. Invading predatory crustacean *Dikerogammarus villosus* eliminates both native and exotic species. *Proceedings of the Royal Society of London*, Vol. **267B**, pp.977-983.

Dick, J. T. A., & Platvoet, D. 2001. Predicting future aquatic invaders; the case of *Dikerogammarus villosus. Aquatic Nuisance Species Digest, Freshwater Foundation*, Vol. **4**, pp.25-27.

Dick, J. T. A., Platvoet, D., & Kelly, D. W. 2002. Predatory impact of the freshwater invader *Dikerogammarus villosus* (Crustacea: Amphipoda). *Canadian Journal of Fisheries and Aquatic Science.* Vol. **59**, pp. 1078-1084.

Dick, J. T. A., Irvine, D. E., & Montgomery, W. I. 1994. Range expansion of the alien freshwater amphipod *Gammarus pulex* (L.) in the River Lagan. *Irish Naturalists Journal*. Vol. **24**, pp. 403-404.

Dick, J. T. A., Nelson, N., & Bishop, J. D. D. 1997. Introduction experiments with *Gammarus* spp. (Crustacea: Amphipoda) in the Isle of Man (British Isles), 1949-1995. *Journal of Zoology, London*. Vol. **242**, pp. 209-216.

Dick, J. T. A., Elwood, R. W., & Irvine, D. E. 1990a. Displacement of the native Irish freshwater amphipod *Gammarus duebeni* by the introduced *Gammarus pulex*. *Irish Naturalists Journal*. Vol. **23**, pp.313-316.

Dick, J. T. A., Irvine, D. E., & Elwood, R. W. 1990b. Differential predation by males on moulted females may explain the competitive displacement of *Gammarus duebeni* by *G. pulex* (Crustacea; Amphipoda). *Behavioural Ecology and Sociobiology*. Vol. **26**, pp. 41-45.

Dick, J. T. A., Montgomery, I., & Elwood, R. W. 1993. Replacement of the indigenous amphipod *Gammarus-duebeni-celticus* by the introduced *Gammarus-pulex* - differential cannibalism and mutual predation. *Journal of Animal Ecology* Vol. **62**, pp. 79-88.

Dick, J. T. A., Montgomery, I., & Elwood, R. W. 1999. Intraguild predation may explain an amphipod replacement: evidence from laboratory populations. *Journal of Zoology, London*. Vol. **249**, pp. 463-468.

Dorgelo, J. 1974. Comparative ecophysiology of gammarids (Crustacea: Amphipoda) from marine, brackish and fresh-water habitats exposed to the influence of salinity-temperature combinations, 1. Effect on survival. *Hydrobiological Bulletin*, Vol. **8**, pp. 90-108.

Dunel-Erb, S., Barradas, C., & Lignon, J. 1997. Morphological evidence for the existence of two distinct types of mitochondria rich cells in the gills of the crayfish *Astacus leptodactylus* Eschscholtz. *Acta Zoologica*, Vol. **78**, pp. 195-203.

Dunn, A. M. 1995. Evidence for the displacement of *Gammarus duebeni* by *Gammarus pulex* (Amphipoda) in a fresh-water site in Brittany, France. *Crustaceana*, Vol. **68**, pp. 912-914.

Eckhardt, E., Pierrot, C., Thuet, P., van Herp, F., Charmantier-Daures, M., Trilles, J. P. & Charmantier, G. 1995. Stimulation of osmoregulating processes in the perfused gill of the crab *Pachygrapsus marmoratus* (Crustacea, Decapoda) by a sinus gland peptide. *General Comparative Endocrinology*. Vol. **99**, pp. 169-177.

Ewing, R. D., Peterson, G. L., & Conte, F. P. 1974. Larval salt gland of *Artemia salina* nauplii – Localisation and characterisation of the sodium + potassium-activated ATPase. *Journal of Comparative Physiology*. Vol. **88**, pp. 217-234.

Forsman, B. 1951. Studies on *Gammarus duebeni* Lillj. with notes on some rock pools organisms in Sweden. *Zoologiska Bidrag fran Uppsala*, Vol. **29**, pp. 215-237.

Frederich, M., Sartoris, F. J., Arntz, W. E., & Pörtner, H. O. 2000. Haemolymph Mg^{2+} regulation in decapod crustaceans: Physiological correlates and ecological consequences in polar areas. *The Journal of Experimental Biology*. Vol. **203**, pp. 1383-1393.

Genovese, G., Luquet, C. M., Paz, D. A., Rosa, G. A., & Pellerano, G. N. 2000. The morphometric changes in the gills of the estuarine crab *Chasmagnathus granulatus* under hyper- and hypo-regulation conditions are not caused by proliferation of specialised cells. *Journal of Anatomy*, Vol. **197**, pp. 239-246.

Gerhardt, A., 1995. Monitoring behavioural responses to metals in *Gammarus pulex* (L) (Crustacea) with impedance conversion. *Environmental Science Pollution Research*. Vol. **2**, pp. 15-23.

Gibert, J., & Mathieu, J. 1980. Relations entre les teneurs en protéines, glucides et lipides au cours du jeûne expérimental chez deux espèces de *Niphargus* peuplant des biotopes différents. *Crustaceana Supplement*. Vol. **6**, pp.137-147.

Glazier, D. S., Horne, M. T., & Lehman. 1992. Abundance, composition and reproductive output of *Gammarus minus* (Crustacea: Amphipoda) in ten cold springs differing in pH and ionic content. *Freshwater Biology*. Vol. **28**, pp. 149-163.

Gledhill, T., Sutcliffe, D. W., & Williams, W. D. 1993. British freshwater Crustacea: Malacostraca: A key with ecological notes. *Scientific publication of Freshwater Biological Association*. No. 52, pp. 1-173.

Glynn, I. M. 1985. *The Na+, K+-Transporting Adenosine Triphosphatase.* In. The enzymes of biological membranes (2nd edition) Vol. 3. (Ed: Martonosi, A). Plenum Press. NY & London pp. 35-114.

Glynn, I. M., & Karlish, S. J. D. 1975. The sodium pump. *Annual Review of Physiology*, Vol. **37**, pp.13.

Hadley, N. F. 1985. The adaptive role of lipids in biological systems, Wiley & Sons, New York.

Hannan, J. & Evans, D. H. 1973. Water permeability in some euryhaline decapods and *Limulus polyphemus. Comparative Biochemistry and Physiology,* Vol. **44A**, pp. 1199.

Hansen, J.I., Mustafa, T., & Depledge, M., 1992. Mechanisms of copper toxicity in the shore crab, *Carcinus maenas*. I. Effects on Na, K- ATPase activity, haemolymph

electrolyte concentrations and tissue water contents. *Marine Biology*, Vol. **114**, pp. 253-257.

Haond, C., Flik, G., & Charmantier, G. 1998. Confocal laser scanning and electron microscopical studies on osmoregulatory epithelia in the branchial cavity of the lobster *Homarus gammarus*. *The Journal of Experimental Biology*. Vol. **201**, pp. 1817-1833.

Hassall, M., & Dangerfield, J.M., 1990. Density dependant processes in the population dynamics of *Armadillium vulgare* (Isopoda: Oniscidae). *Journal of Animal Ecology*, Vol. **59**, pp. 941-958.

Helluy, S., & Holmes, J. C. 1990. Serotonin, octopamine, and the clinging behaviour induced by the parasite *Polymorphus paradoxus* (Acanthocephala) in *Gammarus lacustris* (Crustacea). *Canadian Journal of Zoology*. Vol. **68**, pp. 1214-1220.

Henry, R. P. 1996. Multiple roles of carbonic anhydrase in cellular transport and metabolism. *Annual Review of Physiology*. Vol. **58**, pp. 523-528.

Henry, R. P. 1998. Multiple functions of carbonic anhydrase in the crustacean gill. *The Journal of Experimental Zoology*. Vol. **248**, pp. 19-24.

Hervant, F. 1996. The activities of enzymes associated with the intermediary and energy metabolism in hypogean and epigean crustaceans. *Biochemistry*. Vol. **319**, pp.1071-1077.

Hervant, F., Mathieu, J., & Barré, H. 1999. Comparative study on the metabolic responses of subterranean and surface-dwelling amphipods to long-term starvation and subsequent refeeding. *The Journal of Experimental Biology*. Vol. **202**, pp. 3587-3595.

Hervant, F., Mathieu, J., & Messana, G. 1997a. Locomotory, ventilatory and metabolic responses of the subterranean *Stenasellus virei* (Crustacea: Isopoda) to severe hypoxia and subsequent recovery. *Comptes Rendus de l'Academie des Sciences de Paris, Sciences de la Vie*. Vol. **320**, pp. 139-148.

Hervant, F., Mathieu, J., Barré, H., Simon, K., & Pinon, C. 1997b. Comparative study on the behavioural, ventilatory, and respiratory responses of hypogean and epigean crustaceans to long-term starvation and subsequent feeding. *Comparative Biochemistry and Physiology*. Vol. **118A**, pp.1277-1283.

Hervant, F., Mathieu, J., & Messana, G. 1998. Oxygen consumption and ventilation in declining oxygen tension and post hypoxic recovery in epigean and hypogean aquatic crustaceans. *Journal of Crustacean Biology*. Vol. **18**, pp. 717-727.

Hervant, F., Mathieu, J., Garin, D., & Frémient, A. 1995. Behavioural ventilatory and metabolic responses to severe hypoxia and subsequent recovery of the hypogean

Niphargus rhenorhodanensis and the epigean *Gammarus fossarum* (Crustacea: Amphipoda). *Physiological Zoology*. Vol. **68**, pp. 223-244.

Hervant, F., Mathieu, J., Garin, D., & Frémient, A. 1996. Behavioural ventilatory and metabolic responses of the hypogean amphipod *Niphargus virei* and the epigean isopod *Asellus aquaticus* to severe hypoxia and subsequent recovery *Physiological Zoology*. Vol. **69**, pp. 1227-1300.

Holliday, C. W. 1985. Salinity-induced changes in gill Na, K-ATPase activity in the Mud fiddler crab, *Uca pugnax. Journal of Experimental Zoology.* Vol. **233**, pp. 199-208.

Holliday, C. W. 1988: Branchial Na/K-ATPase and osmoregulation in the Isopod, *Idotea wosnesenkii. Journal of. Experimental. Biology*. Vol. **136**, pp. 259-272.

Holliday, C. W., Roye, D. B., & Roer, R. D. 1990. Salinity-Induced changes in branchial Na/K-ATPase activity and transepithial potential difference in the Brine Shrimp, *Artemia salina. Journal of Experimental Biology*. Vol. **151**, pp. 279-296.

Holsinger, J. R. 1988. Troglobites: the evolution of cave-dwelling organisms. *American Science*. Vol. **76**, pp. 146-153.

Holsinger, J. R. & Culver, D. C. 1970. Morphological variation in *Gammarus minus* Say (Amphipoda, Gammaridae), with emphasis on subterranean forms. *Postilla*, Vol. **146**, pp. 1-24.

Horiuchi, S. 1980. Characterization of antennary gland Na, K-ATPase in the freshwater crayfish, *Procambarus clarkii* Girard. *Comparative Biochemistry and Physiology*. Vol. **65B**, pp. 391-394.

Hough, A. R., & Naylor, E. 1992. Biological and physical aspects of migration in the estuarine amphipod *Gammarus zaddachi. Marine Biology*. Vol. **112**, pp. 437-443.

Hume, R. J. & Berlind, A. 1976. Heart and scaphognathite rate changes in a euryhaline crab, *Carcinus maenas*, exposed to a diluted medium. *Biological Bulletin of Marine Biology Laboratory, Woods Hole*, Vol. **150**, pp.241-254.

Hüppop, K. 1985. The role of metabolism in the evolution of cave animals. *Bulletin of National Speleology Society*. Vol. **47**, pp. 136-146.

Hynes, H. B. N. 1954. The ecology of *Gammarus duebeni* Lilljeborg and its occurrence in fresh water in Western Britain. *Journal of Animal Ecology*. Vol. **23**, pp. 38-84.

Hynes, H. B. N. 1955a. The reproductive cycle of some British freshwater Gammaridae. *Journal of Animal Ecology*. Vol. **24**, pp. 352-387.

Hynes, H. B. N. 1955b. Distribution of some freshwater Amphipoda in Britain. *Verhandlungen der Internationalen Vereinigung für theoretische und angewandte Limnologie*, Vol. **12**, pp. 620-628.

Hynes, H. B. N., Macan, T. T., & Williams, W. D. 1960. Key to the British species of Crustacea Malacostraca occurring in freshwater. *Scientific publication of Freshwater Biological Association.* Vol. **19**, pp. 1-36.

Jazdzewski K., & Konopacka, A. 1988. Notes on the gammaridean amphipoda of the Dniester river basin and eastern Carpathians. *Crustaceana Supplement*, Vol. **13**, pp. 72-89.

Jazdzewski, K. 1980. Range extensions of some gammaridean species in European inland waters caused by human activity. *Crustaceana Supplement*, Vol. **6**, pp. 84-107.

Kamemoto, F. I. 1991. Neuroendocrinology of osmoregulation in crabs. *Zoological Science.* Vol. **8**, pp. 827-833.

Karaman, G. S., & Pinkster, S. 1987: Fresh-water gammarus species from Europe, north-Africa and adjacent regions of Asia (Crustacea, Amphipoda). *Bijdragen tot De Dierkund*, Vol. **57**, pp. 207-260.

Kedwards, T.J., Blockwell, S.J., Taylor, E.J., & Pascoe, D., 1996. Design of an electronically operated flow-through respirometer and its use to investigate the effects of copper on the respiration rate of the amphipod *Gammarus pulex* (L.). *Bulletin of Environmental Contamination and Toxicology*. Vol. **57**, pp. 610-616.

Kennedy, C. R., & Rumpus, A. 1977. Long term changes in the size of the *Pomphorhynchus laevis* (Acanthocephala) population in the River Avon. *Journal of Fish Biology*, Vol. **10**, pp. 35-42.

Kikuchi, S., & Shiraishi, K. 1997. Ultrastructure and ion permeability of the two types of epithelial cell arranged alternately in the gill of the freshwater branchiopod *Caenestheriella gifuensis* (Crustacea). *Zoomorphology*, Vol. **117**, pp. 53-62.

King, V. 1969. A study of the mechanism of water transfer across frog skin by a comparison of the permeability of the skin to deuterated and tritiated water. Cited in: Rasmussen, A. D.; Andersen, O. 1996: Apparent water permeability as a physiological parameter in crustaceans. *Journal of Experimental Biology* Vol. **199**, pp. 2555-2564.

Koop, J. H. E.; & Grieshaber, M. K. 2000: The role of ion regulation in the control of the distribution of *Gammarus tigrinus* (Sexton) in salt-polluted rivers. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology.* Vol. **170**, pp. 75-83.

Krogh, A. 1939. *Osmotic regulation in aquatic animals*. Cambridge University Press.

Lawson, S.L., Jones, M.B., & Moate, R.M., 1995. Effect of copper on the ultrastructure of the gill epithelium of *Carcinus maenas* (Decapoda: Brachyura). *Marine Pollution Bulletin.* Vol. **31**, pp. 63-72.

Li. J., Quabius, E. S., Wendelaar Bonga, S. E., Flik, G., & Lock, R. A. C. 1998. Effects of water-borne copper on branchial chloride cells and Na^+/K^+-ATPase activities in Mozambique tilapia (*Oreochromis mossambicus*). *Aquatic Toxicology*. Vol. **43**, pp. 1-11.

Lignon, J. M., & Péqueux, A. 1990. Permeability properties of the cuticle and gill ion exchanges in decapod crustaceans. In: Truchot, J. P., & Lahlou, B. (eds.). *Animal nutrition and transport processes. 2. Transport, respiration and excretion: comparative and environmental aspects. Comparative Physiology*. Vol. **6**, pp. 14-27. Basel, Karger.

Lima, A. G., McNamara, J. C., & Terra, W. R. 1997. Regulation of hemolymph osmolytes and gill Na/K-ATPase activities during acclimation to saline media in the freshwater shrimp *Macrobrachiumn olfersii* (Wiegmann, 1836; Decapoda Palaemonidae). *Journal of Experimental Marine Ecology*. Vol. **215**, pp. 81-89.

Lin, S. C., Liou, C. H., & Cheng, J. H. 2000. The role of the antennal glands in ion and body volume regulation of cannulated *Penaeus mondon* reared in various salinity conditions. *Comparative Biochemistry and Physiology*. Vol. **127A**, pp. 121-129.

Lockwood, A. P. M. 1961. The urine of *Gammarus duebeni* and *G. pulex. The Journal of experimental biology*. Vol. **38**, pp. 647-658.

Lockwood, A. P. M. 1964. Activation of the sodium uptake system at high blood concentrations in the amphipod, *Gammarus duebeni. Journal of Experimental Biology*. Vol. **42**, pp. 59-69.

Lockwood, A. P. M. 1986. Water regulation in a crustacean inhabiting variable salinity environments. *Porcupine Newsletter*, Vol. **3**, pp. 174-177.

Lockwood, A. P. M. 1992. *Gammarus duebeni*: A flexible phenotype. Quantified Phenotypic Responses in Morphology and Physiology. Proceedings of the twenty seventh European Marine Biology Symposium. pp. 215-227.

Lockwood, A. P. M., & Bolt, S. R. L. 1989. Physiology of crustacea from difficult environments. *Transactions of the Royal Society of Edinburgh: Earth Sciences*, Vol. **80**, pp.285-292.

Lockwood, A. P. M.; Bolt, S. R. L. & Dawson, M. E.1982: *Water exchange across crustacean gills.* In. Gills. Society for experimental biology seminar series 16. (Ed. Houlihan, Rankin & Shuttleworth) Cambridge University Press. pp. 129-147.

Lockwood, A. P. M., Inman, C. B. E., & Courtenay, T. H. 1973. The influence of environmental salinity on the water fluxes of the amphipod crustacean *Gammarus duebeni. Journal of Experimental Biology*. Vol. **58**, pp. 137-148.

Lockwood, A. P. M. & Inman, C. B. E. 1973. The blood volume of some amphipod crustaceans in relation to the salinity of the environment they inhabit. *Comparative Biochemistry and Physiology*, Vol. **44A**, pp. 935–941.

Lovett, D. L., Verzi, M. P., Clifford, P. D., & Borst, D. W. 2001. Hemolymph levels of methyl farnesoate increase in response to osmotic stress in the green crab, *Carcinus maenas*. *Comparative Biochemistry and physiology A - molecular and integrative physiology*. Vol. **128**, pp 299-306.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. 1951: Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*. Vol. **193**, pp. 265-275.

Lucu, C. 1990. Ionic regulatory mechanisms in crustacean gill epithelia. *Comparative. Biochemistry and Physiology*. Vol. **97A**, pp. 297-306.

Lucu, C., & Devescovi, M. 1999. Osmoregulation and branchial Na^+, K^+-ATPase in the lobster *Homarus gammarus* acclimated to dilute seawater. *Journal of Experimental Marine Biology and Ecology*, Vol. **234**, pp. 291-304.

Lucu, C., & Flik, G. 1999. Na^+, K^+-ATPase and Na^+/Ca^{2+} exchange activities in gills of hyperregulating *Carcinus maenas*. *American Journal of Physiology*. Vol. **276**, pp. R490-R499.

Luquet, C. M., Rosa, G. A., Ferrari, C. C., Genovese, G., & Pellerano, G. N. 2000. Gill morphology of the intertidal estuarine crab *Chasmagnathus granulatus* Dana, 1851 (Decapoda, Grapsidae) in relation to habitat and respiratory habits. *Crustaceana*. Vol. **73**, pp. 53-67.

MacNeil, C., Dick, J. T., & Elwood, R. W. 1997: The trophic ecology of freshwater *Gammarus* spp. (Crustacea: Amphipoda): Problems and perspectives concerning the functional feeding group concept. *Biological Reviews of the Cambridge Philosophical Society*. Vol. **72**, pp. 349-364.

Maetz, J. 1968. Salt and water metabolism. In *Perspectives in Endocrinology. Hormones in the lives of lower vertebrates,* (Ed. Barrington & Jorgensen) Academic press, New York, 47-162.

Malard, F., & Hervant, F. 1999. Oxygen supply and the adaptations of animals in groundwater. *Freshwater Biology*, Vol. **41**, pp. 1-30.

Maltby, L. & Crane, M. 1994. Responses of *Gammarus pulex* (Amphipoda, Crustacea) to metalliferous effluents: identification of toxic components and the importance if inter-population variation. *Environmental Pollution*, Vol. **84**, pp. 45-52.

Mantel, L. H., & Farmer, L. L. 1983. Osmotic and ionic regulation. In: Bliss, D. E., Mantel, L. H. editors. The Biology of Crustacea, Vol. 5, London: Academic Press, pp. 54-

126.

Maren, T. H. 1967. Carbonic anhydrase: Chemistry, physiology and inhibition. *Physiological Review*. Vol. **47**, pp. 595-781.

Marriott, D. R., Collins, M. L., Paris, R. M., Gudgin, D. R., Barnard, C. J., McGregor, P. K., Gilbert, F. S., Hartley, J. C., & Behnke, J. M. 1989. Behavioural modifications and increased predation risk of *Gammarus pulex* infected with *Polymorphus minutus*. *Journal of Biological Education*, Vol. **23**, pp. 135-141.

Martinez, C. B. R., Alvares, E. P., Harris, R. R., & Santos, M. C. F. 1999. A morphological study on posterior gills of the mangrove crab *Ucides cordatus*. *Tissue & Cell*. Vol. **31**, pp. 380-389.

Maund, S. J., Taylor E. J., & Pascoe, D., 1992. Population responses of the fresh-water amphipod crustacean *Gammarus pulex* (L) to copper. *Freshwater Biology*, Vol. **28**, pp. 29-36.

Maynard, B. J., DeMartini, L., & Wright, W. G. 1996. *Gammarus lacustris* harbouring *Polymorphus paradoxus* show altered patterns of serotonin-like immunoreactivity. *Journal of Parasitology*. Vol. **82**, pp. 663-666.

McCahon, C. P., Maund, S. J., & Poulton, M. J. 1991. The effects of the acanthocephalan parasite (*Pomphorhynchus laevis*) on the drift of its intermediate host (*Gammarus pulex*). *Freshwater Biology*, Vol. **25**, pp. 507-513.

McGeer, J. C., Szebedinszky, C., McDonald, D. G., & Wood, C. M. 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1: Ionoregulatory disturbance and metabolic costs. *Aquatic Toxicology*. Vol. **50**, pp. 231-243.

McLusky, D. S. 1970. Osmoregulation in *Corophium volutator* – the effect of starvation. *Comparative Biochemistry and Physiology*. Vol. **35**, pp. 303-306.

McLusky, D. S., Bryant, V., & Campbell, R. 1986. The effects of temperature and salinity on the toxicity of heavy metals to marine and estuarine invertebrates. *Oceanography Marine Biology Annual Review*. Vol. **24**, pp. 481-520.

Morris, S. 2001. Neuroendocrine regulation of osmoregulation and the evolution of air breathing in decapod crustaceans. *Journal of Experimental Biology*, Vol. **204**, pp. 979-989.

Morritt, D. & Spicer, J. I. 1993. A brief examination of the function and regulation of extracellular magnesium and its relationship to activity in crustacean arthropods. *Comparative Biochemistry and Physiology*, Vol. **106A**, pp.19-23.

Nesemann, H., Pöckl, M., & Wittmann, K. J. 1995. Distribution of epigean Malacostraca in the middle and upper Danube (Hungary, Austria, Germany). *Miscellanea Zoologica Hungarica*, Vol. **10**, pp. 49-68.

Neufeld, G. J., Holliday, C. W., & Pritchard, J. B. 1980. Salinity adaptation of gill Na, K-ATPase in the blue crab, *Callinectes sapidus*. *Journal of Experimental Zoology*. Vol. **211**, pp. 215-224.

Nonnotte, L., Boitel, F., & Truchot, J.P., 1993. Waterborne copper causes gill damage and haemolymph hypoxia in the shore crab *Carcinus maenas*. *Canadian Journal of Zoology*, Vol. **71**, pp. 1569-1576.

Onken, H. 1999. Active NaCl absorption across the split lamellae of posterior gills of Chinese crabs (*Eriocheir sinensis*) adapted to different salinities. *Comparative Biochemistry and Physiology*. Vol. **123A**, pp. 377-384.

Onken, H. & Riestenpatt, S. 1998. NaCl absorption across the split gill lamellae of hyper regulating crabs: transport mechanisms and their regulation. *Comparative Biochemistry and Physiology*. Vol. **119A**, pp. 883-893.

Onken, H. & Riestenpatt, S. 2002. Ion transport across posterior gills of hyperosmoregulating shore crabs (*Carcinus maenas*): amiloride blocks the cuticular Na^+ conductance and induces current noise. *The Journal of Experimental Biology*. Vol. **205**, pp. 523-531.

Onken, H., & McNamara, J. C. 2002. Hyperosmoregulation in the red freshwater crab *Dilocarcinus pagei* (Bachyura, Trichodactylidae): structural and functional asymmetries of the posterior gills. *The Journal of Experimental Biology*. Vol. **205**, pp. 167-175.

Onken, H., & Putzenlechner, M. 1996. A V-ATPase drives active, electrogenic and Na^+-independent Cl^- absorption across the gills of *Eriocheir sinensis*. *The Journal of Experimental biology*. Vol. **198**, pp. 767-774.

Onken, H., & Riestenpatt, S. 1998. NaCl absorption across the split gill lamallae of hyperregulating crabs: Transport mechanisms and their regulation. *Comparative Biochemistry and Physiology*. Vol. **119A**, pp. 883-893.

Pantin, C. F. A. 1946. *Notes on Microscopical Techniques for Zoologists*. Cambridge University Press, UK.

Parry, G. 1961. Chloride regulation in *Triops*. *Nature*. London. Vol. **192**, pp. 468-469.

Pascoe, D., Kedwards, T. J., Blockwell, S. J. & Taylor, E. J. 1995. *Gammarus pulex* (L.) Feeding Bioassays – Effects of Parasitism. *Bulletin of Environmental Contamination and Toxicology*, Vol. **55**, pp. 629-632.

Pedersen, T.V., & Bjerregaard, P. 2000. Cadmium influx and efflux across perfused gills of the shore crab, *Carcinus maenas*. *Aquatic. Toxicology*. Vol. **48**, pp. 223-231.

Péqueux, A. 1994. Ecophysiology of salt acclimatisation in crustaceans: A mini review. *Belgium Journal of Zoology*, Vol. **124**, pp. 49-60.

Péqueux, A. 1995. Osmotic regulation. *Journal of Crustacean Biology,* Vol. **15**, pp. 1-60.

Pinkster, S. 1975. The introduction of the alien amphipod *Gammarus tigrinus* Sexton 1939 (Crustacea: Amphipoda) in the Netherlands and its competition with indigenous species. *Hydrobiological Bulletin*, Vol. **9**, pp. 131-138.

Pinkster, S., Dennert, A. L., Stock, D., & Stock, J. H. 1970. The problem of European freshwater populations of *Gammarus duebeni* Liljeborg, 1852. *Bijdragen tot de Dierkund.* Vol. **40**, pp. 116-147.

Pinkster, S., Scheepmaker, M., Platvoet, D., & Broodbakker, N. 1992. Drastic changes in the amphipod fauna (Crustacea) of Dutch inland waters during the last 25 years. *Bijdragen tot de Dierkund.* Vol. **61**, pp.193-204.

Pinkster, S., Smit, H., & Brandse-de Jong, N. 1977. The introduction of the alien amphipod *Gammarus tigrinus* Sexton, 1939, in the Netherlands and its competition with indigenous species. *Crustaceana Supplement* Vol. **4**, pp. 91-105.

Plaistow, S. J., Troussard, J. P., & Cézilly, F. 2001. The effects of the acanthocephalan parasite *Pomphorhynchus laevis* on the lipid and glycogen content of its intermediate host *Gammarus pulex*. *International Journal for Parasitology*, Vol. **31**, pp. 346-351.

Postel, U., Petraush, G., Riestenpatt, S., Weihrauch, D., Malykh, J., Becker, W., & Siebers, D., 1998. Inhibition of Na^+/K^+ -ATPase and of active ion-transport functions in the gills of the shore crab *Carcinus maenas* induced by cadmium. *Marine Biology*. Vol. **130**, pp. 407-416.

Potts, W. T. W., & Parry, G. 1964. *Osmotic and ionic regulation in animals*. Pergamon, Oxford, 423 pp.

Poulton, M. J., & Thompson, D. J. 1987. The effects of the acanthocephalan parasite *Pomphorhynchus laevis* on mate choice in *Gammarus pulex*. *Animal Behaviour*, Vol. **35**, pp. 1577-1579.

Rainbow, P. S. 1992. The significance of accumulated heavy metal concentrations in marine organisms. In: Proceedings of Bioaccumulation Workshop. Water Board and Australian Marine Sciences Association Inc., Sydney, 13 pp.

Rankin, J. C. & Davenport, J. 1981. *Animal osmoregulation,* Blackie, Glasgow.

Rasmussen, A. D., & Andersen, O. 1996: Apparent water permeability as a physiological parameter in crustaceans. *Journal of Experimental Biology*. Vol. **199**, pp. 2555-2564.

Rathmayer, M., & Siebers, D. 2001. Ionic balance in the freshwater-adapted Chinese crab, *Eriocheir sinensis*. *Journal of Comparative Physiology*, Vol. **171B**, pp. 271-281.

Reid, D. M. 1939. On the occurrence of *Gammarus duebeni* (Lillj.) (Crustacea, Amphipoda) in Ireland. *Proceedings of the Royal Irish Academy B.* Vol. **45**, pp. 207-214.

Reisser, C. E., & Forward, R. B. 1991. Effect of salinity on osmoregulation and survival of rhizocephalan parasite, *Loxothylacus panopaei*, and its crab host, *Rhithropanopeus harrisii*. *Estuaries*, Vol. **14**, pp. 102-106.

Reynolds, C. D. 2001. *The effects of coal mine closure on macroinvertebrate communities*. PhD Thesis (unpublished). The Nottingham Trent University.

Riestenpatt, S., Onken, H., & Siebers, D. 1996. Active absorption of Na+ and Cl- across the gill epithelium of the shore crab *Carcinus maenas*: voltage-clamp and ion-flux studies. *The Journal of Experimental Biology*. Vol. **199**, pp. 1545-1554.

Riestenpatt, S., Zeiske, W., & Onken, H. 1994. Cyclic AMP stimulation of electrogenic uptake of Na^+ and Cl^- across the gill epithelium of the Chinese crab *Eriocheir sinensis*. *The Journal of Experimental Biology*. Vol. **188**, pp. 159-174.

Roesijadi, G. 1996. Metallothionein and its role in toxic metal regulation. *Comparative Biochemistry and Physiology*. Vol. **113C**, pp. 117-123.

Rumpus, A. E., & Kennedy, C. R. 1974. The effect of the Acanthocephalan *Pomphorhynchus laevis* upon the respiration of its intermediate host, *Gammarus pulex*. *Parasitology*, Vol. **68**, pp. 271-84.

Sarver, R. G., Flynn, M. A., & Holliday, C. W. 1994. Renal Na, K-ATPase and osmoregulation in the crayfish, *Procambarus clarkii*. *Comparative Biochemistry and Physiology*. Vol. **107**, pp. 349-356.

Schleich, C. E., Goldemberg, L. A., & Lopez Mananes, A. A. 2001. Salinity dependent Na^+-K^+ ATPase activity in gills of the euryhaline crab *Chasmagnathus granulata*. *General Physiology & Biophysiology*. Vol. **20**, pp. 255-266.

Sexton, E. W. 1928. On the rearing and breeding of *Gammarus* in laboratory conditions. *Journal of the Marine Biological Association U. K.* Vol. **15**, pp. 33-35.

Shaw, J., & Sutcliffe, D. W. 1961. Studies on sodium balance in *Gammarus duebeni* Lilljeborg and *G. pulex* (L.). *Journal of Experimental Biology*. Vol. **38**, pp.1-15.

Sheader, M. 1983. The reproductive biology and ecology of *Gammarus duebeni* (Crustacea: Amphipoda) in Southern England. *Journal of the Marine Biological Association. UK*. Vol. **63**, pp. 517-540.

Shires, R., Lane, N. J., Inman, C. B. E., & Lockwood, A. P. M. 1994. Structural-changes in the gill cells of *Gammarus-duebeni* (Crustacea, Amphipoda) under osmotic-stress -

with notes on microtubules in association with the septate junctions. *Tissue & Cell* Vol. **26**, pp. 767-778.

Shires, R., Lane, N. J., Inman, C. B. E., & Lockwood, A. P. M. 1995. Microtubule systems associated with the septate junctions of the gill cells of 4 gammarid amphipods. *Tissue & Cell.* Vol. **27**, pp. 3-12.

Siebers, D., Hentschel, J., Bottcher, K., & Lucu, C. 1992. Mitochondrial ATPase in the gills of the shore crab *Carcinus maenas*. *Helgolander Meersunters*. Vol. **46**, pp. 435-445.

Siebers, D., Winkler, A., Leweck, K., & Madian, A. 1983. Regulation of sodium in the shore crab *Carcinus maenas*, adapted to environments of constant and changing salinities. *Helgolander Meersunters*. Vol. **36**, pp. 303-312.

Siebers, D., Winkler, A., Lucu, C., Thedens, G., & Weichart, D. 1985. Na-K-ATPase generates an active transport potential in the gills of the hyperregulating shore crab *Carcinus maenas*. *Marine. Biology*. Vol. **87**, pp. 185-192.

Siebers, D., Winkler, A., Leweck, K., & Madian, A. 1983. Regulation of sodium in the shore crab *Carcinus maenas*, adapted to environments of constant and changing salinities. *Helgolander. Meersunters*. Vol. **36**, pp. 303-312.

Smith, R. I. & Rudy, P. P. 1972. Water exchange in the crab *Hemigrapsus nudus* measured by use of deuterium and tritium oxides as tracers. *Biological Bulletin of the Marine Biology Laboratories, Woods Hole,* Vol. **143**, pp. 234-246.

Smith, R. W., Blaney, S. C., Dowling, K., Sturm, A., Jönsson, M., & Houlihan, D. F. 2001. Protein synthesis costs could account for the tissue-specific effects of sub-lethal copper on protein synthesis in rainbow trout (*Oncorhyncus mykiss*). *Aquatic Toxicology*. Vol. **53**, pp. 265-277.

Soegianto, A., Charmantier-Daures, M., Trilles, J.P., & Charmantier, G., 1999. Impact of copper on the structure of the gills and epipodites of the shrimp *Penaeus japonicus* (Crustacea: Decapoda). *Journal of Crustacean Biology*. Vol. **19**, pp. 209-223.

Sola, F., Isaia, J., & Mansoni, A., 1995. Effects of copper on gill structure and transport function in the rainbow trout, *Oncorhynchus mykiss*. *Journal of Applied Toxicology*. Vol. **15**, pp. 391-398.

Sommer, M. J., & Mantel, L. H. 1988. Effect of dopamine, cyclic-AMP, and pericardial organs on sodium uptake and Na^+, K^+-ATPase activity in gills of the green crab *Carcinus maenas* (L). *Journal of Experimental Zoology*. Vol. **248**, pp. 272-277.

Spanings-Pierrot, C., Soyez, D., van Herp, F., Gompel, M., Skaret, G., Grousset, E., & Charmantier, G. 2000. Involvement of crustacean hyperglycemic hormone in the control of gill ion transport in the crab *Pachygrapsus marmoratus*. *General and Comparative Endocrinology*, Vol. **119**, pp. 340-350.

Sparkies, T. C., Keogh, D. P., & Pary, R. A. 1996. Energetic costs of mate guarding behaviour in male stream-dwelling isopods. *Oecologia*, Vol. **106**, pp. 166-171.

Stobbart, R. H. 1960. Studies on the exchange and regulation of sodium in the larva of *Aëdes aegypti* (L.). II. The net transport and the fluxes associated with it. *The Journal of Experimental Biology*. Vol. **37**, pp. 594-608.

Stobbart, R. H. 1967. The effect of some anions and cations upon the fluxes and net uptake of chloride in the larva of *Aëdes aegypti* (L.), and the nature of the uptake mechanisms for sodium and chloride. *The Journal of Experimental Biology*. Vol. **47**, pp. 35-57.

Stock, J. H. & Pinkster, S. 1970. Irish and French freshwater populations of *Gammarus duebeni* subspecifically different from brackish water populations. *Nature, London.* Vol. **228**, pp. 874-875.

Stock, J. H. 1993. How endangered is *Gammarus duebeni celticus*, a native freshwater amphipod in western Brittany (France)? *Archive fur hydrobiologia*. Vol. **128**, pp. 415-421.

Strange, C. D., & Glass, G. B. 1979. The distribution of freshwater gammarids in Northern Ireland. *Proceedings of the Royal Irish Academy (B)*. Vol. **79**, pp. 145-153.

Sures, B., Siddall, R., & Taraschewski, H. 1999. Parasites as accumulation indicators of heavy metal pollution. *Parasitology Today*. Vol. **15**, pp. 16-21.

Sutcliffe, D. W. & Shaw, J. 1967. The sodium balance mechanism in the freshwater amphipod, *Gammarus Lacustris* sars. *Journal of Experimental Biology* Vol. **46**, pp. 519-528.

Sutcliffe, D. W. 1967a. Sodium regulation in the amphipod *Gammarus duebeni* from brackish-water and fresh-water localities in Britain. *Journal of Experimental Biology*, Vol. **46**, pp. 529-550.

Sutcliffe, D. W. 1967b. Sodium regulation in the freshwater amphipod, *Gammarus pulex*. *Journal of Experimental Biology*, Vol. **46**, pp. 499-518.

Sutcliffe, D. W. 1970. Experimental populations of *Gammarus duebeni* in freshwater with a low sodium content. *Nature*, Vol. **228**, pp. 875-876.

Sutcliffe, D. W. 1971a. Sodium influx and loss in freshwater and brackish water populations of the amphipod *Gammarus duebeni* Lilljeborg. *Journal of Experimental Biology*, Vol. **54**, pp. 255-268.

Sutcliffe, D. W. 1971b. Regulation of water and some ions in gammarids (Amphipoda). I. *Gammarus duebeni* Lilljeborg from brackish water and fresh water. *Journal of Experimental Biology*, Vol. **55**, pp. 325-344.

Sutcliffe, D. W. 1971c. The effects of calcium on sodium influx and loss in *Gammarus* (Amphipoda). *Journal of Experimental Biology*, Vol. **54**, pp. 485-496.

Sutcliffe, D. W. 1971d. Regulation of water and some ions in gammarids (Amphipoda). II. *Gammarus pulex* (L.). *Journal of Experimental Biology*, Vol. **55**, pp. 345-355.

Sutcliffe, D. W. 1972. An examination of subspecific differences in the merus of the fifth walking leg of the amphipod *Gammarus duebeni* Lilljeborg. *Freshwater Biology,* Vol. **2**, pp. 203-216.

Sutcliffe, D. W. 1978. Water chemistry and osmoregulation in some arthropods, especially Malacostraca. *Freshwater Biological Association Annual Report.* Vol. **46**, pp. 57-69.

Sutcliffe, D. W. 1984. Quantitative aspects of oxygen-uptake by *gammarus* (Crustacea, Amphipoda), a critical review. *Freshwater Forum.* Vol. **14**, pp. 443-489.

Sutcliffe, D. W. 1993. Reproduction in *Gammarus* (Crustacea, Amphipoda): Female strategies. *Freshwater Forum*, Vol. **3**, pp. 26-64.

Sutcliffe, D. W. 2000. Subspecies, morphs and clines in the amphipod *Gammarus duebeni* from fresh and saline waters. *Freshwater Forum.* Vol. **13**, pp. 60-75.

Taraschewski, H. 1989. Host-parasite interface of *Paratenuisentis ambiguus* (Ecoacanthocephala) in naturally infected eel and laboratory-infected sticklebacks and juvenile carp and rainbow trout. *Journal of Parasitology*. Vol. **75**, pp. 911-919.

Taraschewski, H. 2000. Host-parasite interactions in acanthocephala. *Advances in Parasitology*. Vol. **46**, pp. 1-179.

Taylor, E. J., Maund, S.J., & Pascoe, D., 1991. Toxicity of four common pollutants to the freshwater macroinvertebrates *Chironomus riparus* meigen (insecta, diptera) and *Gammarus pulex* (L) (Crustacea, amphipoda). *Archive of Environmental Contamination & Toxicology*. Vol. **21**, pp. 371-376.

Taylor, E. J., Rees, E.M., & Pascoe, D., 1994. Mortality and a drift-related response of the freshwater amphipod *Gammarus pulex* (L) exposed to natural sediments, acidification and copper. *Aquatic Toxicology*. Vol. **29**, pp. 83-101.

Taylor, H. H., & Anstiss, J. M. 1999. Copper and haemocyanin dynamics in aquatic invertebrates. *Marine and Freshwater Research.* Vol. **50**, pp. 907-931.

Taylor, L. N., Baker, D. W., Wood, C. M., & McDonald, G. 2002. An *in vitro* approach for modelling branchial copper binding in rainbow trout. *Comparative. Biochemistry and Physiology*. Vol. **133C**, pp. 111-124.

Terrell-Nield, C. 1995. The effects of coal mine drainage water on stream quality during and after cessation of mining activity. In: *Long Term Impact of Pollution in UK*

Freshwaters. The Institute of Fisheries Management Symposium, Nottingham 1994. p. 1-20.

Therien, A. G. & Blostein, R. 2000. Mechanisms of sodium pump regulation. *American Journal of Physiology*. Vol. **279**, pp. C541-C566.

Thienemann, A. 1913. Die Salzwassertierwelt Westfalens. *Verh. Deutsch. Zool. Ges.* In: Sutcliffe, D. W. 1967: Sodium regulation in the fresh-water amphipod, *Gammarus pulex. Journal of Experimental Biology*, Vol. **46**, pp. 499-518.

Towle, D. W., & Holleland, T. 1986. Ammonium ion replaces K^+ in ATP-dependent Na^+ transport by membrane-vesicles from crab gill. *American Zoologist*. Vol. **26** pp. A77.

Towle, D. W., & Taylor, D. D. 1976: Effect of NH_4^+ and K^+ on Na^+ transport ATPase activity of blue crab gill. *American Zoologist*. Vol. **16**, pp. 224.

Towle, D. W., Gilman, M. E., & Hempel, J. D. 1977. Rapid modulation of Na-K-dependent ATPase activity during acclimation of the killifish *Fundulus heteroclitus* to salinity change. *Journal of Experimental Zoology*. Vol. **202**, pp. 179-186.

Towle, D. W., Paulsen, R. S., Weihrauch, D., Kordylewski, M., Salvador, C., Lignot, J. H., & Spanings-Pierrot, C. 2001. Na^+, K^+-ATPase in gills of the blue crab *Callinectes sapidus*: cDNA sequencing and salinity-related expression of α-subunit mRNA and protein. *The Journal of Experimental Biology*. Vol. **204**, pp. 4005-4012.

Towle, D. W., Rushton, M. E., Heidysch, D., Magnani, J. J., Rose, M. J., Amstutz, A., Jordan, M. K., Shearer, D. W., & Wu, W. S. 1997. Sodium/ proton antiporter in the euryhaline crab *Carcinus maenas*: Molecular cloning, expression and tissue distribution. *The Journal of Experimental Biology*. Vol. **200**, pp. 1003-1014.

Trausch, G., Forget, M. C., & Devo, P. 1989. Bioamines-stimulated phosphorylation and Na^+-K^+ ATPase in the gills of the Chinese crab (*Eriocheir sinensis*). *Comparative. Biochemistry and Physiology*. Vol. **94B**, pp. 487-492.

Truesdale, G. A., Downing, A. L., & Lowden, G. F. 1955. The solubility of oxygen in pure water and sea water. *Journal of Applied Chemistry*. Vol. **5**, pp. 53-62.

Urawa, S. 1993. Effects of *Ichthyobodo necator* infections on seawater survival of juvenile chum salmon (*Oncorhynchus keta). Aquaculture*, Vol. **110**, pp. 101-110.

van der Velde, G., Rajagopal, S., Kelleher, B., Musko, I. B. & Bij de Vaate, A. 2000. Ecological impact of crustacean invaders: general considerations and examples from the Rhine river. *Proceedings of the Fourth International Crustacean Congress, Amsterdam, The Netherlands*. July 20-24, Vol. 2 (ed. J. C. von Vaupel Klein & F. R. Schram), pp. 3-34. Rotterdam, the Netherlands: Balkema.

Vawter, A. T., Fong, D. W., & Culver, D. C. 1987. Negative phototaxis in surface and cave populations of the amphipod *Gammarus minus*. *Stygologia*, Vol. **3**, pp. 83-88.

Verweij, P. D., Driessen, W. L., Reedijk, J., Rowatt, B. J., & Sherrington, D. C. 1990. Selective and rapid uptake of copper (II) by a novel chelating resin containing imidazole groups. *Reactive Polymers*. Vol. **13**, pp. 83-92.

Waltham, A. C., Simms, M. J., Farrant, A. R., & Goldie, H. S. 1997. *Karst and Caves of Great Britain*. Joint Nature Conservation Committee, Chapman & Hall, London, pp. 358.

Wang, J. H., Robinson, C. V., & Edelman, I. S. 1953. Self diffusion and structure of liquid water. III. Measurements of self diffusion of liquid water with H^2, H^3 and 0^{18} as tracers. *Journal of the American Chemical Society*, Vol. **75**, pp. 466-470.

Ward, P. I. 1986. A comparative field study of the breeding behaviour of a stream and a pond population of *Gammarus pulex* (Amphipoda). *Oikos*, Vol. **46**, pp. 29-36.

Watson, T.A., & Beamish, F.W.H. 1981. Effects of zinc on branchial adenosine triphosphatase enzymes in vitro from rainbow trout, *Salmo gairdneri*. *Comparative Biochemistry and Physiology*. Vol. **68C**, pp. 167-173.

Weeks, J.M., Jensen, F.B., & Depledge, M.H., 1993. Acid-base status, haemolymph composition and tissue copper accumulation in the shore crab *Carcinus maenas* exposed to combined copper and salinity stress. *Marine Ecology Progress Series*, Vol. **97**, pp. 91-98.

Wilder, M. N., Huong, D. T. H., Atmomarsono, M., Hien, T. T. T., Phu, T. Q., & Yang. W. J. 2000. Characterisation of Na/K-ATPase in *Macrobrachium rosenbergii* and the effects of changing salinity on enzymatic activity. *Comparative Biochemistry and Physiology*. Vol. **125A**, pp. 377-388.

Winkler, A. 1986. Effects of inorganic sea water constituents on branchial Na-K-ATPase activity in the shore crab *Carcinus maenas*. *Marine Biology*. Vol. **92**, pp. 537-544.

Wood, , P. J., Gunn, J., & Perkins, J. 2002. Pollution of subterranean geo-ecosystems – Out of sight out of mind. *Archiv fur hydrobiologie*. Vol. **155**, pp. 223-237.

Wright, D. A. 1980. Cadmium and calcium interactions in the freshwater amphipod *Gammarus pulex*. *Freshwater Biology*. Vol. **10**, pp.123-133.

Zare, S., & Greenaway, P. 1998. The effect of moulting and sodium depletion on sodium transport and the activities of Na^+ K^+-ATPase, and V-ATPase in the freshwater Crayfish *Cherax destructor* (Crustacea: Parastacidae). *Comparative Biochemistry and Physiology*. Vol. **119A**, pp. 739-745.

Zichittella, A.E., Shi, H.G., & Arguello, J.M., 2000. Reactivity of cysteines in the transmembrane region of the Na, K-ATPase α subunit probed with Hg^{2+}. *Journal of Membrane Biology*. Vol. **177**, pp. 187-197.