Effects of rearing conditions on growth, development and moulting in European lobster (*Homarus gammarus*)

Submitted by **Karen Lewanne Middlemiss** to the University of Exeter as a thesis for the degree of **Master of Science by Research in Biosciences**, **August 2014.**

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

(Signature) ……

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I declare that all work undertaken in the formulation of the above chapters was carried out solely by me and that the co-authors contributions were supervisory in nature providing guidance and assistance whilst overseeing the completion my degree.

Signature.....

Chapter One

General Introduction

Background

Declines in wild populations of European lobster (*Homarus gammarus*) are predominately the result of increased commercial demand on lobster fisheries. This theme is repeated globally in many commercially fished aquatic species. In the later decades of the 1900's the European lobster fishery went into a steep decline (Agnault *et al*., 2007; Browne *et al*., 2001, Moland *et al*., 2001). People drawing a living in small coastal fishing communities are economically dependent on the lobster fishery in part for their income and way of life (Reed *et al*., 2013). Therefore, there is a need to balance socioeconomic factors alongside conservation measures in order to preserve both the species and the fishery for those who depend on it for their livelihood. Over the years several initiatives have been introduced to lobster fisheries as control measures, e.g. gear restrictions, licensing and a ban on removing berried hens (Nicosia & Lavalli, 1999). Another successful alternative conservation measure has been wild stock enhancement programmes managed by the larviculture industry (Nicosia & Lavalli, 1999). This began in earnest in the early 1990's with hatcheries in Norway (Agnault *et al*., 2007, Burton, 2001) and is a technique commonly employed by many regions, including the National Lobster Hatchery in Cornwall, England.

Larviculture is a key tool in the management of natural populations of European lobster and other species such as shrimp and molluscs (Nicosia & Lavalli, 1999). This involves through-growth of vulnerable larval stages to the more robust early juvenile stages in a captive rearing environment (hatcheries) for later release into the wild. Berried hens (egg-bearing female) produce several thousand eggs in each brood, however in nature very few (it is thought <0.005 %) (National Lobster Hatchery, 2014) will survive past the larval stages, due predominantly to predation as a result of their vulnerability during early lifecycle stages. In the first few weeks they grow through three pelagic larval stages (Daniels *et al*., 2010). During this time whilst 'floating' in the top few metres of the ocean currents they are obviously exposed and therefore highly vulnerable to predation. After they have gone through metamorphosis to the first benthic juvenile stage, they develop the ability to swim down and seek shelter in the substrate where they stand a greater chance of survival. This is where larviculture comes into the picture with the release of hatchery grown, juvenile

animals in large numbers, to ‘enhance’ the natural populations of European lobster. It is thought that survival rates of hatchery reared European lobster in Norway are between 30-80 % (Agnalt *et al*., 1999). Therefore, hatcheries potentially play a valuable role in species conservation and regional socio-economic stability and must provide a long-term commitment to stock enhancement programmes in order for them to be successful in the long run. This long term commitment requires constant refinement of animal husbandry and production methods in order to increase outputs and minimise inevitable losses. Larviculture facilities face a number of factors that impact on growth and survival of animals grown in hatcheries.

Limiting factors within crustacean aquaculture facilities commonly include water quality and pathogen control (Daniels *et al.*, 2010, Ritar *et al*., 2006). One of the biggest issues with respect to water quality in the rearing of crustaceans is the availability of calcium and carbonate related to their moult cycle. In order to facilitate growth, crustaceans must shed their exoskeletons. In early developmental stages this happens frequently. Each time marine crustaceans do this, they need to obtain nearly all the calcium and carbonate required to harden their new shells from the surrounding seawater medium (Wheatly, 1999). Commonly, the seawater used in aquaculture facilities is run through a recirculation system which involves treating the water before it comes into contact with the animals. During this treatment period, some of the calcium and carbonate is lost as a result of biofiltration in which nitrifying bacteria, present as part of the water treatment regime, oxidise ammonia into nitrate (Eshchar *et al*., 2006). Calcium and carbonate are also being regularly being removed from the seawater by calcifying animals themselves.

As previously mentioned a second limiting factor to growth and survival are pathogens and are typically introduced to the animal via seawater and food sources. These microbes include bacteria and fungi which live on external surfaces (such as the exoskeleton in crustaceans, which includes the gills), and in the gastrointestinal tract (Bauer, 1981; Daniels *et al*., 2010). They compete for resources and if left to proliferate can lead to reduced physiological efficiencies and animal health. For example, respiratory surfaces covered in microbes will create a larger diffusion distance for oxygen and carbon dioxide to be exchanged across the gills between the water and haemolymph

(Schuwerack *et al*., 2001). This in turn can negatively impact the animal by increasing energy for respiratory function that could be used for other processes such as moulting.

It is common practice for aquaculture facilities to use some form of water treatment to control pathogens (Summerfelt, 2003). This has commonly included the use of ozone and ultraviolet radiation where water is treated to reduce pathogens in the seawater prior to contact with animals, therefore reducing the microbial load (Summerfelt, 2003). There has also been a lot of research on the effectiveness of pre and probiotic feed supplements and water additives for controlling microbial communities (Daniels *et al*., 2010). Varying successes have been achieved depending on species involved, types of rearing systems, e.g. ponds or closed recirculation systems, and general animal husbandry techniques (Maeda *et al*., 1997).

In addition to these aquaculture forms of pathogen control, crustaceans have evolved cleaning mechanisms to help minimise the effects of naturally accumulating epibionts. These mechanisms are grouped as either passive or active and are used to scrape or pick foreign bodies from external surfaces (Bauer, 1981). As mentioned, the presence of epibionts on gill surfaces can impact on overall fitness of the animal and in an effort to control epibionts, European lobster employ passive gill cleaning setae. Moulting also serves a secondary role in microbial control by effectively removing epibionts during ecdysis as part of the growth cycle (Bauer, 1998).

Study Aim

Pathogen control and the effects of water quality form the basis of this body of work and the following section details how each chapter helps to address these limiting factors. These were identified as being critical areas of concern to address in order to improve growth and survival rates in hatchery reared European lobster and the synergies between all three experiments are detailed in the conclusion.

1. Chapter 2 - UV irradiation, ozonation and probiotic water treatments

One of the most commonly understood threats to the success of aquaculture facilities is disease outbreak. Intensive rearing of European lobster in high

density numbers with animals in close contact to each other obviously means that an outbreak will spread quickly between animals. Disease can result in catastrophic losses in aquaculture systems and or decreased growth and survival, therefore pathogen control is vital to the success of any aquaculture facility. Pathogens are typically introduced through the seawater which the animals are cultured in, and food sources (Ritar *et al*., 2006). Much research has been carried out on the most effective water treatment options for various growing conditions (e.g. ponds, recirculating systems) (Ritar *et al*., 2006; Scolding *et al*., 2012) and research into new and improved methods for pathogen control continues. The current study investigated the effects of three forms of water treatment on growth and survival in European lobster; UV irradiation, ozonation and probiotic water additive *Bacillus* spp. Two trials were run; the first used ozone and probiotic treatments and the second added UV to treatment groups. Stage I-IV European lobster were grown in semi-closed recirculation systems and UV light was transmitted into the seawater at a constant rate for the entire treatment period of 18 days. In the ozone treatments, ozone was also administered to the treatment water at a constant dose for 18 days. Probiotic treatment groups received a daily dosage of *Bacillus* spp. spores, incubated and applied directly to the seawater. Growth and survival measurements were taken at intervals during the 18 day treatment period. A two week post-treatment period also assessed growth and survival as well as a salinity challenge test one week post-treatment as a general assessment of fitness. The study has highlighted that on its own the use of a water additive, and in this case *Bacillus* spp., confers no health benefits in comparison to the use of UV or ozone water treatments in a semi-closed recirculation system. No between trial statistical analyses could be made to directly assess the effectiveness of ozone and UV, due to eggs being hatched from different batches between trials and known parental effect on growth. Any differences found could therefore not solely be attributable to treatment effects. However, the ozone treatment group appeared to show far greater survival rates (66 % at 18 days post hatch), higher biomass (60 % at 18 days post hatch) and increased liveweight gain (>5 mg between development stages IV-V), than probiotic. This study addressed the limiting effect of pathogens on growth and survival in early developmental stages of European lobster and

highlighted the effectiveness of UV and or ozone water treatments over a probiotic water additive.

2. Chapter 3 - Gill cleaning mechanisms for pathogen control

Intensive aquaculture results in a high risk of pathogen proliferation and many control methods are employed in an attempt to reduce numbers (discussed in chapter two). As previously discussed, the presence of pathogens not only increases the risk of disease to animals, but can also have a negative impact on respiratory function which can therefore have great consequence to growth and survival rates of hatchery reared animals (Bauer, 1981). Crustaceans have evolved gill cleaning mechanisms in an attempt to rid gill structures of both microbes and fouling from particulate matter (Bauer, 1981) as described below. Respiratory surfaces covered by a proliferation of microbial growth can result in reduced physiological functions related to gas exchange across increased diffusion distances at the gills (Schuwerack *et al*., 2001). The increase in energetic costs required to support respiratory function during this time, takes valuable energy resources away from other functions such as moulting. In an effort to control these pathogens, crustaceans utilise gill cleaning mechanisms (Bauer, 1981). These are common in many aquatic crustacean species but were undescribed in early life stages of European lobster. Using scanning electron microscopy to investigate the branchial chamber in early development stages, results showed that larval and juvenile stages of European lobster possess passive gill cleaning mechanisms. They take the form of setae with rasp like tips that jostle amongst the gill filaments upon locomotion of the pereopods, or by water flow through the branchial chamber, and scrape off any foreign bodies. However, interestingly, they do not develop until larval stage III. The effectiveness of this form of microbial control mechanism is dubious once microbes are present in large numbers on gill structures. The current study investigated the development of pathogens on gill filaments during a moult cycle and found that by mid-cycle filaments were densely covered in pathogens. This would appear to show that setae are too few in numbers to be effective enough to entirely prevent rapid colonisation of gill surfaces. However, they may provide valuable assistance in slowing microbial colonisation down enough to reduce moult frequency. Results also showed that immediately after a moult, the gill filaments were clear of pathogens and this seems to be (as a secondary

product of the growth process) a much more effective control method than the use of passive gill cleaning setae. The exoskeleton includes the outer covering of the gills in crustaceans and therefore when the animal moults, anything attached to the gill surface is also removed during ecdysis.

3. Chapter 4 - Calcium and acid-base fluxes during a moult cycle

Rapid calcification (hardening) of exoskeletons is obviously critical to the survival of newly moulted crustaceans (whose soft bodies offer no protection from predation) and is predominantly dependent on availability of calcium and bicarbonate ions in the surrounding seawater (Wheatly, 1999). These ions are depleted from seawater in two ways: via calcification by the animals themselves and through decreased alkalinity caused by water treatment processes for water quality control (nitrification through biofiltration) (Eshchar *et al*., 2006). Without the rapid hardening of the new exoskeleton, crustaceans are therefore extremely vulnerable to predation due to their soft bodies (as previously mentioned), and are unable to feed until their mouthparts are calcified. In an aquaculture context, intensive larviculture of European lobster results in high density numbers of animals in close contact with each other during larval stages of growth. This ultimately means the opportunity for predation is high and therefore calcification must be rapid to ensure survival.

The flux of calcium and acid-base relevant ions into and out of lobsters correlates well with their growth cycle. The old exoskeleton requires demineralising in preparation for the moult which generates excess free calcium and carbonate ions that need to be stored or excreted (Greenaway, 1985). Then immediately postmoult, calcium and carbonate ions are rapidly taken up from the seawater (or internal stores) to enable mineralisation of the newly produced exoskeleton (Greenaway, 1985). In between these two extremes, during the intermoult periods, the calcium and carbonate balance remains fairly stable (Wheatly, 1999). In early development stages this is a frequent occurrence which then occurs less often once they reach adulthood. Therefore, in order for maximum calcification rates to be achieved immediately postmoult, calcium and carbonate (or bicarbonate) ions must be readily available. Calcium availability is not normally a problem for marine crustacean species as seawater is known to be rich in calcium (~10 mM) (Greenaway, 1985), however

carbonate and bicarbonate availability (collectively known as carbonate alkalinity) can be depleted for several reasons. In intensive aquaculture systems, calcifying crustaceans are constantly removing calcium and nitrification as part of the biofiltration system and this also reduces alkalinity (though not necessarily calcium) (Eshchar *et al*., 2006). All these factors can impact on rates at which remineralisation of the new exoskeleton are achieved. Levels of calcium, $NH_4^{+,}$ HCO_3^- and H^+ fluxes were measured in stage IV megalopa European lobster at intermoult and then juvenile stage V animals during the subsequent 24 hour period immediately postmoult in high and low alkalinity seawater treatments. The current study showed that low alkalinity seawater is a limiting factor in remineralisation of the exoskeleton resulting in a longer time required for hardening of the new shell. Consequences of this in an aquaculture context are that if animals struggle to get enough calcium and/or carbonate to achieve maximum calcification rates, they will require larger energy reserves resulting in less energy available for other physiological processes. Ultimately this could result in reduced survival.

Conclusions

Larviculture is a valuable tool used widely around the world for conservation of marine species through stock enhancement of wild populations. The vulnerable nature of pelagic larval stages of European lobster in the wild can greatly reduce survival rates through to juvenile benthic life stages. When grown in aquaculture facilities, larval survival is therefore greatly enhanced through decreased predation risks in comparison to the natural environment. By releasing more robust benthic juvenile stages they are then able to burrow into the seabed allowing them to avoid predation and therefore enhance their chances of survival. Aquaculture facilities therefore play an important role in enhancing wild stocks of juvenile European lobster. However, these facilities do not come without their own risks to the successful growth of larvae. Disease outbreaks caused by pathogens can result in reduced growth and survival of European lobster and it is critical that pathogens are minimised in captive rearing facilities. Crustaceans employ grooming techniques in an attempt to control external pathogens, however it is essential that animal husbandry techniques also include pathogen control. Moulting as part of the growth cycle comes at a cost to health and survival of animals if poor quality water exists in

aquaculture facilities. Postmoult animals require large amounts of calcium and bicarbonate ions to harden newly formed shells and this predominately comes from the surrounding seawater. Reductions to the availability of these ions in aquaculture facilities can negatively impact on the health of the animals. These factors are essential considerations to the success of captive rearing facilities for European lobster. The need to find new and improved methods for animal husbandry in aquaculture to increase outputs is essential to the conservation efforts of this species through the support of wild stock enhancement programmes.

A chapter summary of experimental conclusions follows:

Chapter Two

This chapter addresses the use of ozonation, UV irradiation and probiotic water treatments for the health and survival of animals. Results showed that ozone water treatment provided benefits over the use of either UV and/or probiotic treatments with increased live weight, survival rates and greater biomass. There were also reduced numbers of pathogens in the seawater treatment groups of ozone and UV than in the probiotic group. Whilst differences in growth and survival were present between treatment groups, an osmoregulatory challenge test resulted in no significant differences in physiological fitness between any treatment groups. Conclusions drawn from these results are that the use of a probiotic water additive does not confer increased health benefits over traditional methods of ozonation and UV irradiation water treatments. The singular use of ozone provided comparatively greater effects on growth and survival than other treatment groups. This has important implications for semi-closed recirculation systems used in aquaculture facilities that might consider replacing traditional methods with the less well researched and understood effects of probiotic water additives.

Chapter Three

This chapter addressed the functional morphology of gill cleaning mechanisms in early life stages of European lobster and quantified the progression of the microbial biofilm throughout a complete moult cycle. The aim of this chapter was to identify how pathogens in the seawater impact on gill structures; identify

mechanisms for pathogen control present in early life stages of European lobster and discuss possible physiological effects on animal health as a result of gill pathogens. Again, this relates back to the need for effective water treatment for the control of seawater pathogens in aquaculture facilities in order to promote increased growth and survival rates. Results showed that development of gill cleaning mechanisms occurs from larval stage III and that during a moult cycle pathogen proliferation steadily increases between 10 and 15 days postmoult with complete removal of all pathogens after the following moult. It was concluded that the presence of gill pathogens (originating from the seawater) would be detrimental to the respiratory health of the animal, particularly in the later stages of a moult cycle when proliferation occurs on gill surfaces just prior to ecdysis. The consequences of respiratory structures being inundated with pathogens are likely to include reduced ability to perform the functions of gas exchange, osmoregulation, ion and acid-base regulation and nitrogenous waste excretion. Impaired function in these physiological processes can contribute to the death of the animal especially during times of increased metabolic activity during ecdysis. In conjunction with chapter two detailing the effectiveness of various water treatments, this chapter provides evidence to support the importance of pathogen control in aquaculture facilities for animal health. It is crucial not only to provide effective water treatment to prevent potential disease outbreaks caused by waterborne pathogens (chapter two), but also to ensure viability of physiological processes to maintain healthy animals.

Chapter Four

The focus of this chapter was on the effects of reduced seawater alkalinity on availability of bicarbonate required for remineralisation of European lobster exoskeleton immediately postmoult. Water quality is an essential consideration for aquaculture facilities as discussed in chapters two and three in relation to pathogen control, but it is also essential for the rates of calcification required by postmoult crustaceans. Crustaceans moult in order to grow and upon doing so the newly formed exoskeleton in European lobster is predominantly calcified by the uptake of calcium and bicarbonate ions from the surrounding seawater. This needs to happen rapidly in order to harden the exoskeleton for the purposes of defence from predators and to allow mouthparts to harden so they can eat. The aim of this study was to identify calcification rates of the newly formed

exoskeleton in differing alkalinity treatments, and to identify the amount of stored minerals postmoult. Results showed that in low alkalinity seawater treatments calcification rates are greatly reduced and only a small portion of total exoskeleton minerals are stored in the newly moulted animal. The conclusion drawn from these results is that European lobsters rely on the surrounding seawater to provide sufficient quantities of calcium and bicarbonate ions for recalcification of the exoskeleton following ecdysis. In aquaculture facilities it is known that the nitrification process performed by biofiltration as part of water treatment reduces seawater alkalinity (and therefore bicarbonate ion availability). In intensive rearing facilities the animals themselves are also removing both calcium and bicarbonate from the seawater for calcification purposes. Therefore, European lobster aquaculture facilities are likely to have reduced alkalinity due to water treatment processes and calcifying animals and this will impact on the rate at which calcification of the new exoskeleton occurs. Findings suggest that in order to facilitate rapid calcification of the newly formed exoskeleton, and therefore potentially increase survival rates, seawater alkalinity in aquaculture facilities needs to be maintained at suitable levels.

Collectively the results from all three experiments highlight the need and effectiveness of various water treatment measures for pathogen control and water quality in order to increase health and survival. It also highlights the need to consider impacts of aquaculture water treatment measures (particularly biofiltration on alkalinity and subsequently calcification) on other aspects of captive reared European lobster health. It is important to consider knock-on effects from animal husbandry techniques and the impact those measures can have on other areas of animal health.

This combined knowledge gained from all three experimental chapters in this study has advanced our understanding of the importance of pathogen control and maintaining seawater chemistry to achieve the best possible results for culture of larval/juvenile European lobster in aquaculture facilities.

References

Agnalt, A. L., Van der Meeren, G. I., Jørstad, K. E., Næss, H., Farestveit, E., Nøstvold, E., Svåsand, T., Korsoen, E., Ydstebo, L., (1999). *Stock enhancement of European lobster (Homarus gammarus): a large-scale experiment off South-western Norway (Kvitsøy)*. In Howell, B., Moksness, E., Svåsand, T., (Eds.), Stock Enhancement and Sea Ranching, pp 401-419. Fishing News Books Ltd, Farnham.

Agnalt, A-L., Kristiansen, T.S., Jorstad, K.E., (2007). Growth, reproductive cycle, and movement of berried European lobsters (*Homarus gammarus*) in a local stock off southwestern Norway. *ICES Journal of Marine Science*, **64**, 288-257.

Bauer, R.T., (1981). Grooming behavior and morphology in the decapod Crustacea. *Journal of Crustacean Biology*, **1**,153-173.

Bauer, R.T., (1998). Gill-cleaning mechanisms of the crayfish *Procambarus clarkii* (Astacidea: Cambaridae): experimental testing of setobranch function. *Invertebrate Biology*, **117**, 129-143.

Browne, R.M., Mercer, J.P., Duncan, M.J., (2001). An historical overview of the Republic of Ireland's lobster (*Homarus gammarus* Linnaeus) fishery, with reference to European and North American (*Homarus americanus* Milne Edwards) lobster landings. *Hydrobiologia*, **465**, 49–62.

Burton, C.A., (2001). The role of lobster (*Homarus* spp.) hatcheries in ranching, restoration and remediation programmes. *Hydrobiologica*, **465**, 45–48.

Daniels, C.L., Merrifield, C.I., Boothroyd, D.P., Davies, S.J., Factor, J.R., Arnold, K.E., (2010). Effect of dietary Bacillus spp. and mannan oligosaccharides (MOS) on European lobster (*Homarus gammarus* L.) larvae growth performance, gut morphology and gut microbiota. *Aquaculture*, **304**, 49–57.

Eshchar, M, Lahav, O., Mozes, N., Peduel, A., Ron, B., (2006). Intensive fish culture at high ammonium and low pH. *Aquaculture*, **255**, 301–313.

Greenaway, P., (1985). Calcium balance and moulting in Crustacea. Biological *Reviews Cambridge Philosophical Society*, **60**, 425-454.

Maeda, M., Nogami, K., Kanematsu, M., Hirayama, K., (1997). The concept of biological control methods in aquaculture. *Hydrobiologia*, **358**, 285–290.

Moland, E., Olsen, E.M., Knutsen, H., Knutsen, J.A., Enersen, S.E., Andre, C., Stenseth, N.C., (2011). Activity patterns of wild European lobster *Homarus gammarus* in coastal marine reserves: implications for future reserve design. *Marine Ecology Progress Series*, **429**, 197–207.

National Lobster Hatchery (2014). Retrieved September 19, 2014, from http://www.nationallobsterhatchery.co.uk/whats-it-all-about/education/lobster-biology/.

Nicosia, F., Lavalli, K., (1999). Homarid lobster hatcheries: their history and role in research, management, and aquaculture. *Marine fisheries review*, **61**, 1-57.

Ritar, A.J., Smith, G.G., Thomas, C.W., (2006). Ozonation of seawater improves the survival of larval southern rock lobster, *Jasus edwardsii*, in culture from egg to juvenile. *Aquaculture*, **261**, 1014-1025.

Reed, M., Courtney, P., Urquhart, J. and Ross, N., (2013). Beyond fish as commodities: Understanding the socio-cultural role of inshore fisheries in England. *Marine Policy*, **37**, 62-68.

Schuwerack, P.M., Lewis, J.W., Jones, P.W., (2001). Pathological and physiological changes in the South African freshwater crab *Potamonautes warreni* Calman induced by microbial gill infestations. *Journal of Invertebrate Pathology*, **77**, 269-279.

Scolding, J.W.S., Powell, A., Boothroyd, D.P., Shields, R.J., (2012). The effect of ozonation on the survival, growth and microbiology of the European lobster (*Homarus gammarus*). *Aquaculture*, **364-365**, 217-223.

Summerfelt, S.T., (2003). Ozonation and UV irradiation - an introduction and examples of current applications. *Aquacultural Engineering*, **28**, 21-36.

Wheatly, M.G., (1999). Calcium homeostasis in Crustacea: The evolving role of branchial, renal, digestive and hypodermal epithelia. *Journal of Experimental Zoology*, **283**, 620-640.

Chapter 2

Combined effects of UV irradiation, ozonation, and the probiotic *Bacillus* spp. on growth, survival, and general fitness in European lobster (*Homarus gammarus*)

Abstract

Bacterial pathogens are a leading cause of disease in hatchery aquaculture systems and preventative methods such as probiotics as feed supplements and water additives is well documented. However, comparisons between the effectiveness of using probiotic water additives over traditional biocontrol methods are less understood. This study assessed the effects of ultraviolet irradiation (UV), ozonation and *Bacillus* spp. as a water additive (probiotic), in the culture of European lobster in a semi-closed recirculation system. Stage I larvae were assigned to one of six treatment groups consisting of 1) ozone, 2) probiotic, 3) probiotic + ozone, 4) probiotic + ozone + UV, 5) ozone + UV, or 6) probiotic + UV, for 18 days. Growth and survival at stages I-V were measured on 1, 6, 11, 18, 24, 31 days post hatch and 1, 18, 24 and 31 days post hatch respectively. Bacterial counts of pathogenic *Vibrio* spp. in culture water were measured at 1, 4, 9, 14, 18 days post hatch. Lobsters were also exposed to a physiological fitness test (low salinity challenge) at megalopa stage IV, 7 days post treatment. Ozone was comparatively more beneficial than probiotic with increased live weight gain in the ozone treatment over probiotic between zoea stage IV-V (>5 mg). Survival rates were higher in the ozone treatment than probiotic on days 18, 24, and 31 (~66, ~117 and ~120%, respectively). There was a greater biomass in the ozone treatment than probiotic on days 18 and 31 respectively (~60 and ~116%, respectively). Total *Vibrio* spp. present in the ozone treatment was 99% less than in the probiotic treated culture water (day 18). Results between UV treatment groups showed significantly lower numbers of *Vibrio* spp. present in probiotic + ozone + UV culture water 4 days post hatch than ozone + UV (~10 fold higher) or UV + probiotic (~15 fold higher) and by day 18 probiotic + ozone + UV was significantly higher than ozone +UV (~8 fold higher). Osmoregulatory challenge test resulted in no significant differences in physiological fitness between any treatment groups. The present study clearly shows the effectiveness of O_3 in aquaculture facilities for control of pathogens in the rearing of European lobster over either a probiotic water additive or UV irradiation.

1. Introduction

Due to the explosive growth of human population and consequent increase in the demand for food, aquaculture and fisheries are one of the fastest growing industries (FAO, 2009). Crustaceans are a large part of the seafood market and demand has increased steadily. King crabs (Urbina et al., 2013) and lobsters are probably the most economically valued crustacean species (FAO, 2014). The European lobster, *Homarus gammarus* (L.), belongs to the order Decapoda, and their natural habitat encompasses a large latitudinal and temperature range. Their habitat extends predominantly along the Eastern Atlantic Ocean, from the cool waters of Norway (~ 2-18 °C) (Moland et al., 2011) to the more temperate waters of the Morocco and Mediterranean coastline (~ 14-25 °C) (Diaz et al., 2001). The European lobster is usually found in depths up to 50 m, but it has been reported to inhabit down to 150 m (Cobb and Castro, 2006). The European lobster is commercially fished throughout its distribution and annual global catches have shown a steady increase since 1950, reaching a maximum of 425 tonnes in 2010 (FAO, 2014). However, a rapid decline to only 223 tonnes was reported in 2011 (FAO, 2014).

Increased catch rates for the closely related *Homarus americanus* (American lobster) have also been seen with catches rising from 83,000 tonnes in 2005 to 139,000 tonnes in 2011 (FAO, 2014). As with many marine species, intensive harvesting of the European lobster has contributed to a general population decline. This has resulted in conservation measures such as enhancement of wild stock populations through hatchery based culturing of larvae for release into coastal marine waters (Daniels et al., 2010, 2013; Contarini et al., 2008).

Bacterial pathogens, frequently found in seawater and food sources, are a leading cause of disease-related mortality common to aquaculture facilities (Goulden et al., 2012; Jithendran et al., 2010; Silva et al., 2013). It has even been suggested that the intensive nature of larviculture has selected for virulent strains of the gram negative genus *Vibrio* which are pathogenic to decapod larvae (Goulden et al., 2012) causing reduced growth and survival (Bourne et al., 2007). Major routes of infection in aquatic species include the gills and gastrointestinal tract (Goulden et al., 2012). Animals are particularly vulnerable after a moult (especially larvae because of the high frequency of moults) when

the new exoskeleton is thinner (Cawthorn, 1997). However, infection from injury is also likely to occur due to their highly cannibalistic behaviour (Scolding et al., 2012). Traditional antimicrobial control methods in aquaculture including the use of antibiotics and chemical treatment of water using UV and ozone (O_3) (Brown & Russo, 1979; Scolding et al., 2012) are being challenged by recent research on the alternative use of probiotics to improve water quality and for pathogen control (Moriarty, 1998). Probiotics are described as microbial supplements that confer health benefits through modulation of bacterial communities (Gatesoupe, 1999).

It has been found that higher doses of UV effectively removed about 98% of heterotrophic bacteria in salmon farming on a recirculating system (Sharrer et al., 2007a). Combined use of UV and O_3 resulted in almost total elimination of bacteria in an Arctic Char freshwater recirculation system (Sharrer et al., 2007b). Ozone also effectively eliminates bacterial and viral pathogens on both the host and in the water (Emerson et al., 1982; Scolding et al., 2012; Sellars et al., 2005). While ozone has been considered to be more effective than UV in some cases (Liltved et al., 1995), it has also been reported that its application could negatively affect host species by causing tissue damage (Ritola et al., 2002). The normal use of O_3 is in application to the water treatment system, with residuals then removed before contact with animals. The simultaneous use of O_3 and UV has also been used and found necessary to control disease outbreak in the American lobster (Fisher et al., 1976). Both, O_3 and UV are clearly efficient at controlling bacterial pathogens, but their real advantages for farming species are not yet clear. The use of both O_3 and UV as an antimicrobial treatment has been effective at controlling bacterial pathogens without harming the host in studies on European lobster (Daniels et al., 2010; Scolding et al., 2012). However, synergies between direct application of probiotics to culture water in combination with both O_3 and UV have not been evaluated in semi-closed recirculating systems.

In recent years growth and survival of many aquatic species, including crustaceans, have significantly improved through the alternative use of probiotics, including the genus *Bacillus* as both a feed supplement and water additive (Balcazar et al., 2006; Cha et al., 2013; de Souza et al., 2012; Daniels et al., 2010, 2013; Decamp et al., 2008; Gatesoupe, 1999; Hai et al., 2009;

Kesarcodi-Watson et al., 2008; Rengipat et al., 2000; Verschuere et al., 2000; Zhang et al., 2011). Probiotics are well accepted as an alternative to traditional bacterial control methods, and its benefits are proposed to be conferred by mechanisms such as competitive exclusion of pathogenic bacteria (e.g. *Vibrio* spp.) from adhesion sites in the gills and gastrointestinal tract, production of inhibitory compounds, improvement of digestive enzymatic activity, nutrient provision, immunostimulants and improved water quality (Balcazar et al*.,* 2006; Cha et al., 2013; Gullian et al., 2004; Irianto & Austin*,* 2002; Merrifield et al*.*, 2010). It is accepted, however, that these mechanisms are not yet well understood. Selection of specific strains within species of probiotics and understanding mechanisms are critical to their successful use so that the host is not negatively affected (Ibnou-Zekri, et al., 2003; Luis-Villasenor et al., 2011; Madsen, 2006), and considerable research has been conducted in this area (de Souza et al., 2012; Gomez-Gil et al., 2000; Merrifield et al., 2010; Newaj-Fyzul et al., 2013). In aquatic species, *Vibrio* spp. present the biggest pathogenic threat, as they grow quickly and adapt to changing environmental conditions (de Souza et al., 2012; Battison et al., 2008; Maeda et al., 1997). *Bacillus* spp. are commercially available gram-positive spore forming bacteria used as probiotics. They have been shown to benefit European lobster when administered as a feed supplement either separately or more effectively in combination with prebiotics (Daniels et al., 2010). Positive results have also been found when used as a water additive in shrimp species (Cha et al., 2013), but to the authors knowledge this has not been tested in the early larviculture stages of European lobster.

Much research has been carried out on the development of effective dietary probiotics to improve growth and survival of European lobster (Daniels, 2011), however, should this study prove successful, there are cost benefits to be made from administering probiotics as a water additive to both improve water quality and provide biocontrol over the traditional and more expensive methods of UV and O_3. Therefore, comparisons between these methods and their use in combination could also identify areas for cost reductions in pathogen control measures for semi-closed recirculation aquaculture systems. This study aims to compare the single and synergistic effects of *Bacillus* spp. administered as a water additive with UV and O_3 to control pathogens in the culture of European

lobster. A change in the microbial community balance in the culture water with the singular use of *Bacillus* spp. could significantly benefit whole animal microbial community resulting in improved growth and survival.

It should be noted that due to animal welfare concerns the NLH could not allow the use of a 'control' in the form of completely untreated seawater and therefore, due to facility restrictions, this was not included as part of the experimental design. All seawater coming into the NLH from the nearby estuary is treated via mechanical filtration and UV irradiation and held in a reservoir prior to use in experiments. Therefore had restrictions not been in place for animal welfare purposes, 'pure seawater' would not have been available for use as a control anyway. Lobsters are never cultured without water treatment in the form of ozone and/or UV irradiation at the NLH. Therefore the ozone (trial 1) and ozone + UV (trial 2) treatment groups were effectively the controls within each trial for comparison with the probiotic treatment groups. Previous studies conducted at the NLH on benefits of ozone water treatments by Scolding et al., (2012) were conducted similarly with the 'control' being UV treated seawater with no ozone. It was hypothesised that *Bacillus* spp. (referred to herein as 'probiotic') would confer supplementary health benefits to the host in addition to traditional biocontrol and water quality methods.

2. Materials and Methods

2.1. Experimental Animals

Experiments were undertaken at the National Lobster Hatchery (NLH) larval rearing research facility in Padstow, North Cornwall, U.K., during October and December 2013, just after the natural breeding season (April-September). Water for the aquaria was pumped from the surrounding Camel Estuary (Padstow, UK -50°32'19.67"N, 4°56'5.85"W) at high tide into a reservoir and treated using glass artificial filter media providing mechanical filtration to 50 µM in addition to UV irradiation before use in aquaria. Ovigerous adult female European lobsters were collected from various locations along the Cornish coast and held in 6 °C cold water storage tanks at the NLH until required. At which point, females were gradually acclimated to 19 °C (approximate 6 °C

increase after every 3-4 days until optimal temperature reached) and placed in a recirculating and aerated broodstock tank at a salinity of 35 PSU and sustained with a diet of blue mussels (*Mytilus edulis*). Larvae hatched overnight from several females creating a pool of larvae which were then treated in a Chloromine –T (Pharmaq, UK) bath for one hour before being transferred to experimental aquaria. Larval stages were classified according to the conventional and pre-established developmental stages as detailed in Charmantier et al., (1991). Stages I-V were used in this study, whereby stages I-III are zoea, stage IV is a megalopa and stage V is a post larvae (PL) or juvenile.

2.2. Rearing systems

2.2.1 Experimental design

Experimental aquaria consisted of three separate semi-closed recirculating systems each supporting 4 replicate 80 L up-welling Kreisel cones (i.e. 12 cones in total) maintained at a water flow rate of ~1800 L h^{-1} per system and each system was randomly assigned to treatment groups. Seawater was provided from the hatchery reservoir. Aeration of cones was maintained to a level which provided dissolved oxygen in the range of 8.1-8.9 mg L^{-1}, and active water mixing as a strategy to limit conspecific contact. Each system contained approximately 600 L of culture water and the setup consisted of; filter sock, protein skimmer, bio-filter, sand filter and ozonation via the protein skimmer and or irradiated by 2 x 55-watt UV steriliser where treatment required. Water tests were carried out every second day to measure total ammonia, NO_2^-, NO_3^-, salinity, pH, temperature and dissolved oxygen (DO) in all systems. A saltwater master test kit (API® Mars Inc.), H_2 ocean salinity refractometer, HQ11d pH and temperature meter (Hatch, Salford, UK) and DO probe (OxyGuard Handy-Alpha, Sterner AquaTech, UK) were used respectively to take measurements. Water chemistry variables were maintained within the following ranges and were consistent between treatments: salinity, 34-35 PSU; pH, 7.9-8.2; temperature, 17-19 °C; total ammonia, 1-5 mg L^{-1}; NO_2^- and NO_3^-, 0-10 mg L^{-1}; DO, 8.1-8.9 mg L^{-1}.

Trial 1: The effects of ozone, probiotic and ozone + probiotic

Each experimental treatment (O_3, probiotic and O_3 + probiotic) was replicated four times (cone C1, 2, 3, and 4). Each treatment was stocked with a total of 2070 lobster larvae (stage I) and cones (4) seeded separately over 4 consecutive days (n=470 cone 1, 500 cone 2, 550 cone 3 and 550 cone 4 in each treatment) maintaining a final density of 6-7 larvae L^{-1} depending on daily larval supply.

Trial 2: The effects of ozone + UV, probiotic + UV, and probiotic + UV + ozone

Experimental conditions matched Trial 1 but with the addition of UV to all systems. After completion of Trial 1, all systems were drained, disinfected, refilled with reservoir water and biofilters reseeded.

2.2.2 System management

Daily larval diet added to each cone consisted of Gemma Micro 500 (Skretting, UK) and frozen red plankton (*Calanus* sp., Tropical Marine Centre, UK). Water management protocol consisted of: 15% water changes daily; fine-mesh filters replaced every second day (to remove organic matter from each cone); filter sock changes and cleaning of protein skimmer cups daily. Approximately 100 non-experimental larvae were added to each system nine days before the start of the trials in order to seed and stabilise the biofiltration system.

2.3. Water treatment factors

2.3.1. Ozone

Systems requiring ozonation were dosed to a set point of 320 mV from an ozone generator (Certizon, Sander, UK) and administered via protein skimmers (Schuran Aquaflotor, Tropical Marine Centre, UK), which remove residuals from the oxidation process to avoid direct contact with the animals, minimising detrimental effects. Oxidation-reduction potential (ORP) was monitored to ensure accurate dosage using frequently calibrated probes

positioned at protein skimmer outflows and connected to ORP controllers (Aqua Bedic, Bissendorf, Germany).

2.3.2. Probiotic

Commercial probiotic *Bacillus* spp. (Sanolife MIL-F, INVE, Belgium) composing strains of *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus* spores (min ≥1 x 10^{-10} CFU g) were germinated and administered in accordance with manufacturer's instructions (initial and daily maintenance dose per treatment 4.5 g and 2.25 g daily respectively).

2.3.3. UV

UV sterilisation involved culture water being recirculated at a rate of ~1800 L h^{-1} per treatment system through two 55-watt UV sterilisers (Tropical Marine Centre (TMC), Gertfordshire, UK) and irradiated prior to entering the rearing tanks and therefore, never directly coming into contact with animals.

2.4. Bacteriology

Culture water was sampled from each treatment system for evaluating the presence and abundance of *Vibrio*-like species on days 1, 4, 9, 14, 18. Colony forming units (CFU's mL^{-1}) were quantified using the spread plate method. Samples underwent five serial dilutions of 10:1 ratio with phosphate buffer solution (PBS) and then 100 µL spread onto thiosulfate citrate bile salt sucrose (TCBS) (Oxoid, UK) agar and incubated at 20 °C for 48 hours. Total numbers of CFU's were counted for each plate (three replicates per sample and three replicates of each dilution (10^{-1} - 10^{-5}). Only dilutions which achieved counts between 20 and 300 CFU's were accepted for analysis.

2.5. Growth and survival

Samples of larval stages I-III and megalopa/juvenile stage IV-V (n=24 per treatment; 6 per replicate cone) were selected on respective days post hatch (dph): 1, 6, 11 (stages I, II and III, respectively) and 18 and 31 (megalopa stage IV and juvenile stage V, respectively) which reflected the average expected intermoult periods. Samples were blotted dry using paper tissue to

ensure consistency between samples before they were weighed using a top loading balance (Acculab VIC-303, VICON, Germany) with precision of 0.001 g. Carapace length (mm) was measured using a Digital Microscope with Expert Prima 1.1 imaging software. Survival data was calculated by counting the total number of individuals at 1, 18, 24, 31 dph, and biomass was then calculated as the factor between the total number of animals and their average wet weight. The different indicators of growth, condition and survival were calculated as described in Table 1.

Table 1. Description of measurements used to describe growth and survival in European lobster.

Measurement	Description
Survival	Final Number (FN)/Initial Number(IN) x 100
Carapace length (mm)	CL measured from base of eye socket to posterior dorsal margin of carapace
Weight (mg)	mg
Live weight gain (mg)	LWG where $W_t - W_0$ where W_t is final weight and W_0 is initial weight
Weight to carapace length ratio	W/CL where W = weight (mg), CL= carapace length (mm)
Biomass (g)	Survival numbers x mean weight

2.6. Osmoregulatory Challenge Test

A common method used to assess the 'fitness' of hatchery reared animals is an osmoregulatory test (Dhert et al., 1992). Stage IV megalopa (7 days after the treatment finished) were exposed to an osmoregulatory challenge test to identify possible differences in fitness between treatment groups. Lethal time (minutes) to 50% mortality (LT50) was determined. Replicate samples were selected from each treatment group (n=24). Animals (n=5 per replicate) were placed in 1 L glass beakers with aerated water at a salinity of 10 PSU which resulted in 100% mortality after ~100 minutes. Mortality was assessed every 5 min, and animals were considered dead when nil pleopod movement was observed and nil response was elicited when provoked by physical stimuli using a metal probe. The percentage of survival during the challenge test for each of the four replicate cones was adjusted to a sigmoidal dose-response curve, whereby the time at which 50% of the larvae where alive (LT50) was calculated. The upper and lower values were fixed to 100% and 0% in the model, as each single data set actually met this assumption. Maximum survival

time and time to 50% mortality was also extracted from the crude survival curves.

2.7. Statistical Analysis

Analysis was carried out using SigmaPlot V.12.1 (Systat Software Inc., USA) and data are represented as the mean ± SD. Data from both trials were analysed using a two-ways ANOVA with time and treatment/stage as factors for survival, biomass, live weight gain, bacterial counts, carapace length, weight/carapace length ratio and weight. Data from the osmoregulatory salinity challenge for maximum survival time and LT50 were analysed using a one-way ANOVA and for survival using a two-ways ANOVA (factors time and treatment). Due to well known variability in parentage of larvae between each trial (different broodstock and a 5 weeks difference in trial starting dates), results from trial 1 and 2 were analysed separately. Therefore, no statistical analysis between the results of the two independent trials was attempted. Data failing assumptions of normal distribution and equal variances underwent square, square root, log and arsine transformations. Data that did not meet parametric assumptions after transformation were then ranked before a two-ways ANOVA was performed. Any significant differences were then subjected to post hoc analysis (Tukey). Survival during the osmotic challenge test was adjusted to a sigmoidal dose-response curve using GraphPad Prism. Differences were considered significant with P value < 0.05.

3. Results

3.1. Growth and Survival

Analysis showed a significant difference in survival between Trial 1 treatments ($P=0.045$) probiotic + O_3, however post hoc analysis failed to identify which days of the O_3 treatment had significantly higher survival (Fig. 1A). There was no significant interaction between factors treatment and days ($P=0.830$). Analysis from Trial 2 treatment groups showed no significant difference in survival (Fig. 1B), ($P=0.660$).

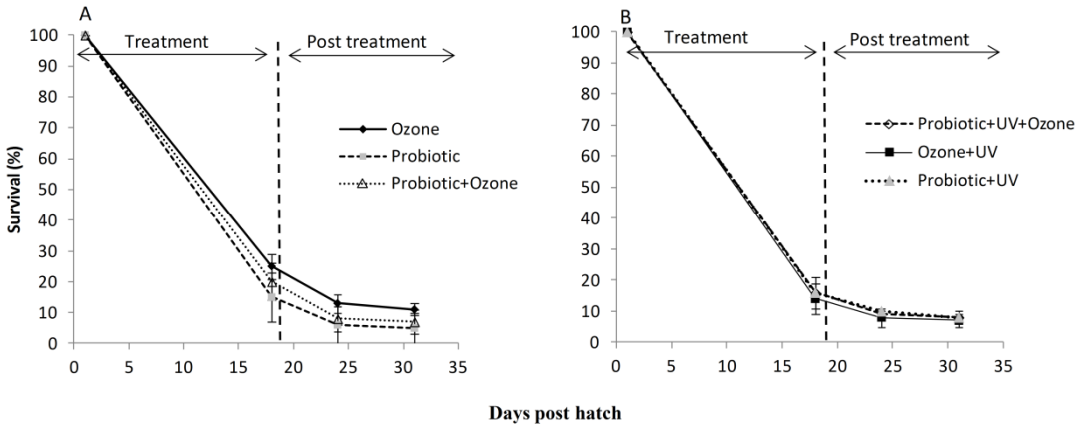

Fig. 1. Survival rates of European lobster 18 dph in (A) Trial 1 treatments: O_3, probiotic and probiotic + O_3 (1A), and (B) Trial 2 treatments: probiotic + UV + O_3, O_3+ UV and probiotic + UV (1B), followed by non-treatment period 18-31 dph. Figures represent the percentage of live animals at each time point. Data represent the mean ± SD.

No differences between any of the treatments in Trial 1 were found for carapace length, weight or weight/carapace length ratio (P=0.112, P=0.862, P=0.537 respectively) at any of the stages evaluated, nor interaction between stage and treatment was present (P=0.446, P=0.605, P=0.705 respectively), (Fig. 2A, C, E). Analysis from Trial 2 treatment groups showed no significant difference in carapace length (P=0.960, Fig. 2B), weight (P=0.885, Fig. 2D), or weight/carapace length ratio (P=0.760, Fig. 3F).

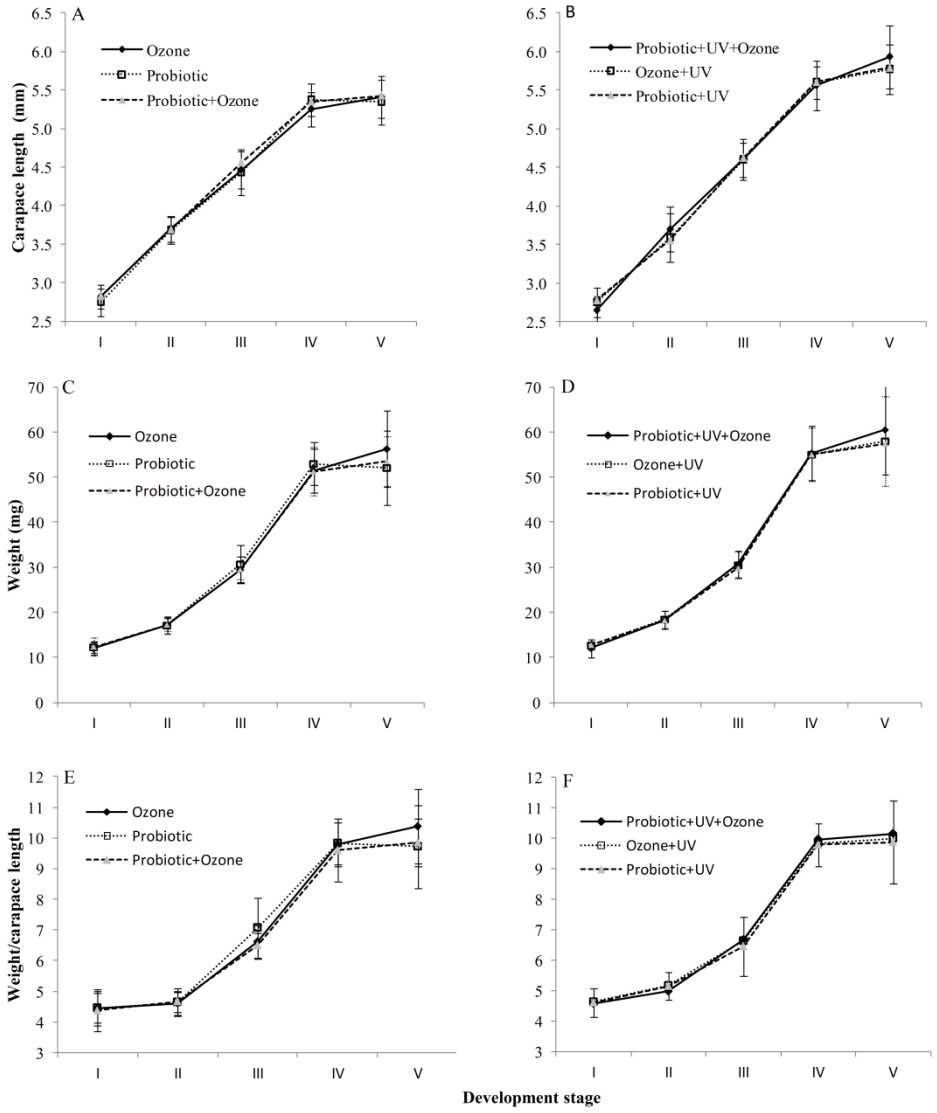

Fig. 2. Growth in European lobster carapace length, weight and weight/carapace length ratio (2A, C and E respectively) at each development stage (I-V) in Trial 1 treatments: O_3, probiotic and probiotic + O_3. Trial 2 treatments: probiotic + UV + O_3, O_3 + UV and probiotic + UV (2B, D and F respectively). All followed by non-treatment period stage IV-V. Data represent the mean ± SD.

No significant differences in the biomass between treatments in Trial 1 was found (P=0.051, Fig. 3A), however analysis showed that live weight gain was significantly higher (Fig. 3C) in the O_3 treatment than the probiotic (P<0.001) and O_3 + probiotic treatments (P=0.031) 18 dph. Analysis from Trial 2 treatment groups showed no significant difference in biomass (P=0.780, Fig. 3B), or live weight gain, (P=0.250, Fig. 3D).

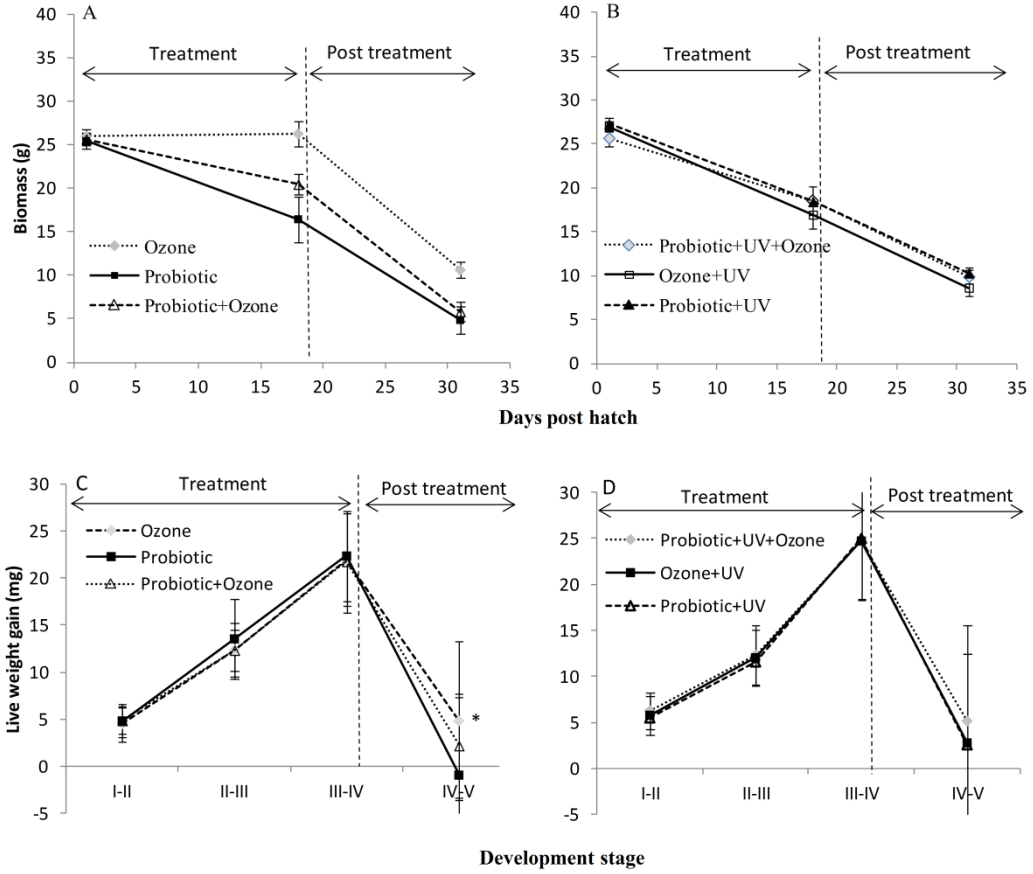

Fig. 3. European lobster biomass at 1 and 18 dph and live weight gain in stages I-IV under different water treatments followed by 13 day non-treatment growth period. Trial 1 treatment groups: probiotic, O_3 and probiotic + O_3 (3A and C respectively) and Trial 2 treatment groups: probiotic + UV + O_3, O_3 + UV and probiotic + UV (3B and D respectively). Data are presented as mean ± SD, and statistical differences in live weight gain ($P<0.05$) signified by an asterisk (*).

3.2. Osmoregulatory challenge test

No significant difference in survival between different treatments, or interactive effect between treatment and time were found in Trial 1 ($P=1.00$) or Trial 2 ($P=0.998$) respectively (Fig. 4A, B). LT50 and maximum survival time also showed no significant difference between treatment groups in Trial 1 ($P=0.911$, $P=0.991$; Fig. 4C, E) and Trial 2 ($P=0.872$, $P=0.730$; Fig. 4D, F) respectively.

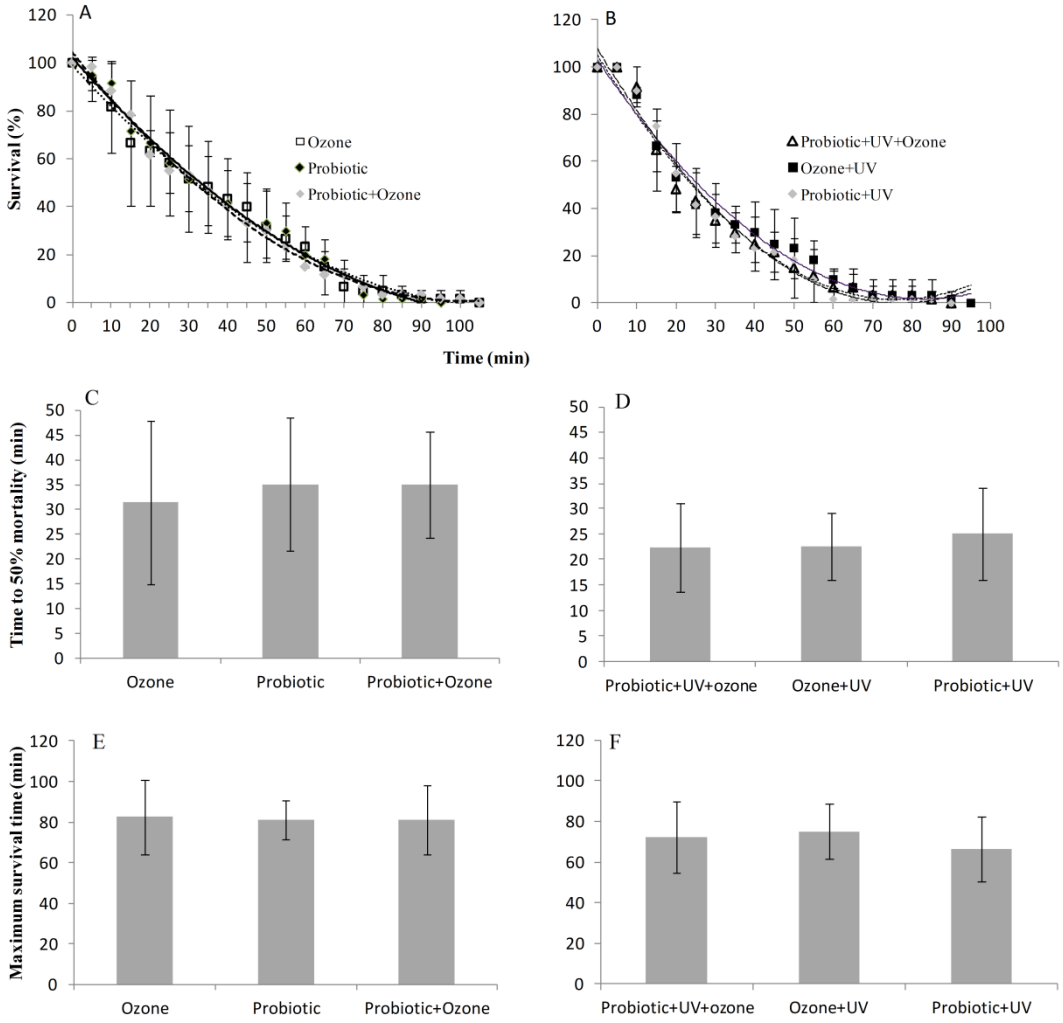

Fig. 4. European lobster osmoregulatory challenge test (4A) survival at five minute intervals in O_3 treatments in Trial 1, and (4B) UV treatments in Trial 2 during exposure to reduced salinity (10 PSU) after previously being maintained at a salinity of 35 PSU. Time to 50% mortality (4C and D respectively) and maximum survival time (4E and F respectively) are also shown. Tests were conducted seven days post-treatment using stage IV megalopa animals. Data represent the mean ± SD.

3.3. Bacteriology

Analysis of the interaction between factors treatment and day showed significantly higher CFU's of *Vibrio* like spp. in the probiotic treatment group without O_3 than those with O_3 in Trial 1 on day 18 ($P<0.001$; Fig. 5A). Significantly lower numbers of *Vibrio* like spp. were present in Trial 2 treatment group probiotic + UV + O_3 on day 4 than all other groups and at 18 dph higher numbers in the same treatment group than O_3 + UV (Fig. 5B).

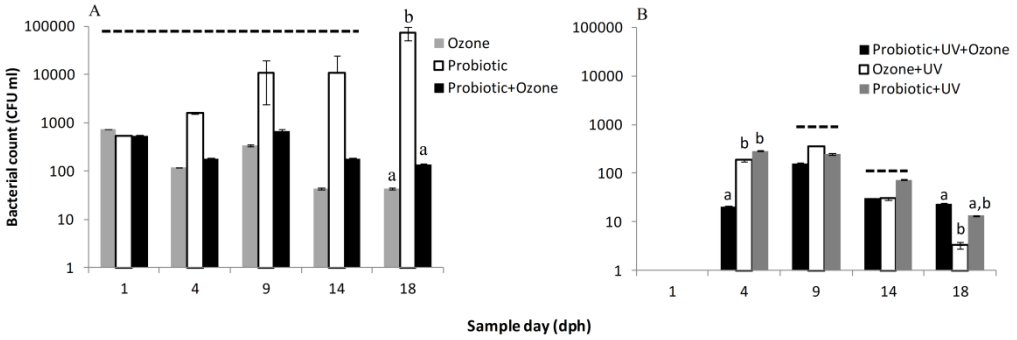

Fig. 5. Bacterial counts of *Vibrio* spp. present in culture water at 1, 4, 9, 14 and 18 dph across (5A) Trial 1 treatment groups: O_3, probiotic and probiotic + O_3, and (5B) Trial 2 treatment groups: probiotic + UV + O_3, O_3+ UV and probiotic + UV. Data represent the mean ± SD and are displayed on the 'y' axis using Log10 scale. Dotted lines on top of the treatments represents no significant difference between treatments on those days post hatch. Different letters represent significant differences between treatments at 18 dph (Trial 1), and 4 and 18 dph (Trial 2).

4. Discussion

The present study revealed that probiotic *Bacillus* spp. when used as a water additive in a semi-closed recirculating system confers no obvious health benefits in growth, survival or fitness in comparison to the traditional antimicrobial methods of O_3 or UV. Furthermore, the exclusive use of *Bacillus* spp. caused detrimental effects on growth and survival when compared to the O_3 treatments (Trial 1). Previous research into improved growth and survival in hatchery reared European lobster has predominantly focused on the use of probiotics as a dietary supplement, and positive growth effects have been demonstrated in larvae with the use of *Bacillus* spp. (Daniels et al., 2010, 2013). Proposed mechanisms for improved growth include changes to gut morphology (increased size and density of microvilli) resulting in greater surface area for nutrient uptake (Daniels et al., 2010), and competitive exclusion of pathogens in the gastrointestinal tract (Daniels et al., 2013). However, comparisons between the effectiveness of using probiotic alternatively as a water additive in European lobster larviculture is less understood.

Proliferation of microbial communities in culture systems is dependent on several factors. These include but are not limited to: water temperature, oxygen content, organic matter and nutrition to provide the right growing conditions, and chance habitat encounters by bacteria – right place, right time (Moriarty and

Body, 1995). Verschuere et al. (2000) describe the opportunity to manipulate the development of microbial communities through the addition of probiotics. They also discuss the need for regular application of probiotics rather than a single dose, to maintain 'artificial dominance' in culture water where microbial communities are well established. A large body of existing research demonstrates the effectiveness of some probiotics as water additives in aquaculture, but this is predominantly in species such as shrimp cultured in pond environments (Moriarty, 1998; Silva et al., 2013; Vine et al., 2006; Zhou et al., 2009). Here, water management does not involve the more technical anti-microbial methods of UV irradiation and O_3 for control of bacterial communities in semi-closed recirculation systems, as was used in the present study. O_3 and UV in aquatic systems are primarily used to maintain pathogens and microorganisms at low levels. In addition to this, UV also plays a double role by eliminating O_3 residuals in the culture water produced from the chemical process of oxidation in the conversion of O_3 to O_2 (Summerfelt, 2003). The latter is dependent on wavelength and energy of the UV light used (Hunter et al., 1998), with 250-260 nm destroying O_3 residuals while 100-400 nm is required to limit microorganism survival (Summerfelt, 2003). Within the current study it is not known how much residual ozone or by-products were present in the non UV treatment systems. However, results from Scolding et al. (2012) on research into the use of O_3 used directly in the culture water with European lobster larvae make it unlikely that any potential residuals in the current study negatively impacted results.

Garrido-Pereira et al. (2013) described UV as an effective water disinfectant. This would explain why the combination of UV and probiotic bacteria showed no benefits as the probiotic bacteria were likely inactivated in the current study within 6 hours of application due to high water turnover through UV's. Therefore, the probiotic would need to be applied every 6 hours in order to maintain appropriate levels within the culture water, which is neither practical nor feasible. It is likely that findings from the current study for the trial that included UV (Trial 2) supports a similar outcome and therefore the use of probiotic with UV has no benefit given it is inactivated soon after application to the culture system. Selected species of probiotics have provided effective biocontrol reducing *Vibrio* pathogens in pond water for shrimp culture by means

of a change in bacterial species composition (Moriarty, 1998). However, Zhou et al. (2009) found no improvement in water quality from the use of a probiotic water additive in the rearing of shrimp larvae (*Penaeus vannamei*). It was found that the addition of probiotic was of no benefit without maintaining traditional water management techniques. Results from Shariff et al. (2001) also found no benefit in growth and survival of pond reared tiger shrimp (*Penaeus monodon*) after the addition of a microbial product. It was suggested that water management protocols such as filtration are essential and that the use of probiotic water additives on their own are not enough to confer improved health benefits. These findings support current results showing that probiotic *Bacillus* spp. was ineffective under the system conditions in the present study at providing improved growth and survival when compared to UV and $O_{3.}$

European lobster larvae treated singularly with probiotic showed a 15% survival rate on completion of the treatment period (18 dph); significantly lower than 25% survival in animals treated with O_3 in Trial 1. This is a similar trend to Scolding et al. (2012) finding improved survival rates for European lobster larvae grown in ozonated treatment over control (UV treated seawater) during an 18 day period. Whilst there is no statistical comparison between trials, results from Trial 2 ranged from 14-16% survival across all three treatments with the addition of UV to each. This could suggest that UV had a negative effect on survival rates, reduced probiotic efficacy or that differences arise from separate broodstock used in each trial, as described below. Hedgecock and Nelson (1978) suggested up to 30% of variance in growth rate in laboratory reared juvenile American lobster resulted from genotype variance related to mixed parentage. However, environmental factors such as stocking density and temperature are believed to have a greater impact on growth and survival rates than genetic factors. Regardless of this, effects of mixed parentage must remain a consideration when discussing results between treatment groups in the current study. In regards to maintaining probiotic efficacy, both UV and O_3 inactivate bacterial pathogens and so it is expected that any possible benefits could have been negated from the use of probiotic combined with either of these biocontrol methods over both trials. Results from Trial 1 clearly show the use of probiotic singularly without O_3, and therefore retaining efficacy, had no effect on growth or survival. This suggests that the combined use of the two

treatments simultaneously is of no value. The present study suggests that ozone used singularly is more effective at increasing survival rates through controlling the bacterial community in the culture water than probiotic on its own.

Measurements of weight, carapace length and weight/carapace length ratio from all treatments in both trials revealed no significant differences. Weight and carapace length in the current study (Trial 1) are similar to results for European lobster larvae after 18 days grown in O_3 treated water (Scolding et al., 2012). Trial 1 results also showed no significant difference in total biomass between treatments, but did show a trend towards higher biomass in the O_3 treatment (69% higher) than probiotic treatment. Trial 2 also showed no significant difference between treatment groups in either biomass or live weight gain. Measurements of biomass in the current study are based on survival rates as discussed in the methods section. Therefore, increased total biomass is directly attributable to the abundance of animals at each given sampling point. Given that results in Trial 1 show significant increase in survival you would expect to see significant differences in biomass also. However, the authors believe that variation in survival between replicate cones resulted in no significant difference after analysis. A significant interactive effect of treatment and time factors resulted in higher live weight gain in O_3 treatment (Trial 1) than either probiotic or probiotic + O_3 in post larval stage V juveniles two weeks post treatment. Although analysis failed to show a significant difference in biomass between treatments (Trial 1), after 18 days in the O_3 treatment results clearly show a trend towards a total biomass ~63% higher than that achieved in the probiotic treatment.

Whilst results from the current study highlight the potential benefits of O_3 and UV for microbial control in semi-closed recirculation aquaculture systems, it is worth considering possible negative impacts from water sterilisation. O_3 is a very effective water steriliser and its success is due to its highly reactive nature in water. It is used both for controlling bacterial pathogens and improving water quality via the breakdown of organic matter, production of O_2 and conversion of toxic nitrites to safer nitrates (Scolding et al., 2012; Summerfelt, 2003; Tango & Gagnon, 2003). However, the by-product oxidants hypobromous acid (HOBr), hypobromite ion (OBr^-) and bromate (BrO_3^-) are toxic to many species

(Crecelius, 1979; Tango and Gagnon, 2003). When administered directly into the culture water O_3 has known detrimental effects to animals causing tissue damage from direct contact (Ritola et al., 2002). However, Scolding et al. (2012) identified acceptable levels of O_3 (400 mV) for direct exposure with European lobster that resulted in improved survival and supports similar findings in southern rock lobster *(Jasus edwardsii)* by Ritar et al. (2006). Conversely, results from Scolding et al. (2012) showed that whilst survival increased, weight and carapace length were significantly reduced approaching metamorphosis to stage IV after 18 days in ozone treatment groups. In relation to effects of sterilisation on nutrition, research by Souza et al. (2010) show direct nutritional uptake from culture water in larvae of the Japanese spiny lobster (*Panulirus japonicus*). Therefore, it is conceivable that results from the current study showing no improved growth parameters in the O_3 treatment over probiotic may be a result of the oxidative effect on water nutrients or production of toxic compounds. It was further proposed that toxic by-products from the oxidation process might decrease calcium availability in the water for carapace development in research on *Litopenaeus vannamei* by Schroeder et al. (2010). However, to the author's knowledge there is no current evidence to support the theory of negative effects of ozone on calcium availability in seawater.

As discussed in Samocha et al. (1998), assessing fitness of post larvae through salinity challenges can benefit *Penaeus vannamei* larviculture and assessing viability can indicate which treatment may be most beneficial for on-growth post-treatment and pre-release into the wild. This is true also in larviculture of the European lobster. The use of a hyposalinity experiment to assess fitness, quality or ability to tolerate change is relevant in homarid lobsters as discussed in Charmantier et al. (2001). European lobster habitat ranges from shallower in shore niches to deeper off shore locations (Cobb and Castro, 2006). Therefore, a salinity and temperature gradient exists for animals that move between shallow and deep waters, as well as pelagic living larvae that develop into benthic juvenile/adults. This requires, at least, some degree of tolerance to changes in salinity and so osmoregulatory challenges are therefore useful for assessing the health of animals. Exposure to an osmoregulatory challenge test to assess post larval quality one week post treatment showed no significant difference between treatment groups within either trial. However, there is a

trend showing decreased time to 50% mortality (LT50) in Trial 2 treatments with UV (22-25 min) when compared to Trial 1 (31-35 min). This trend was also seen in the maximum survival time between treatments in both trials (Trial 1, 81-83 min, Trial 2, 66-75 min). Whilst no statistical comparison was made between trials, it is clear that either: UV affected salinity tolerance, or differences resulted from mixed parentage in each trial as discussed previously. It is recommended that future comparative studies of fitness between UV and O_3 treatments be carried out using larvae from the same broodstock, at a higher salinity and for a longer period to investigate post treatment viability during osmoregulatory challenge tests.

Bacteriology results from water samples from each treatment system were sampled to identify levels of *Vibrio* like spp. in the culture water. As expected the presence of *Vibrio* like spp. was significantly lower (0.05% of total probiotic treatment CFU's) at 18 dph in the Trial 1 ozone treatment group. Similar findings for effective control of *Vibrio* spp. with ozonation of culture water have been found in research on lobster species (Ritar et al., 2006; Scolding et al., 2012). Both trials show a decrease in *Vibrio* spp. at nine days post hatch. In Trial 2, day four showed significantly less *Vibrio* spp. in treatment group probiotic + O_3 + UV than in probiotic + UV. It is believed that the use of UV would have inactivated and, therefore, negated any effect the probiotic may have had and that the differences seen are likely due to the effect of O_3 and UV. Although effective at greatly reducing pathogen abundance, without very high doses UV or O_3 treatment of culture water cannot completely eliminate them. In fact, as discussed in Maeda et al. (1997), changing the microbial community through the use of UV and O^3 can provide the opportunity for some species to thrive. It was shown in research on rearing of crab (*Portunus trituberculatus)* that growth of *Vibrio* spp. was repressed by the addition of another bacterial strain (Nogami et al., 1997). Therefore, competition between bacterial species affects their abundance.

While no between trials statistical comparisons were made for *Vibrio* spp. levels, it appears there was little difference between the mean bacteria levels in either the O_3 (Trial 1) or UV + O_3 (Trial 2). However, when looked at in conjunction with higher survival rates and live weight gain in the O_3 treatment in Trial 1 than in any other treatment in Trial 2, it appears that O_3 is more

beneficial than UV. This would appear to signify that the use of O_3 singularly in the current study as a microbial control is more effective at controlling *Vibrio* spp. and providing health benefits than any of the treatments involving the use of probiotic in the culture water and/or UV, consistent with Liltved et al. (1995). As previously mentioned, the use of UV in conjunction with O_3 removes any possible residuals from the oxidation process. This would support the simultaneous use of both O_3 and UV together for water sterilisation. However, both are used at considerable cost and cost benefit analysis between the two may reveal that the use of O_3 singularly is more effective in terms of cost and benefits to the host animals. This current study may therefore provide beneficial information to aquaculture facilities investigating current animal husbandry practices and their cost effectiveness.

5. Conclusion

The present results suggest that probiotic *Bacillus* spp. used as a water additive conferred no comparative culture benefit to the use of either O_3 or UV, nor benefits as a biocontrol in a semi-closed recirculation aquaculture system. Although O_3 and UV are both highly effective at controlling levels of *Vibrio* spp. in culture water, only O_3 treatment provided significantly higher survival rates and live weight gain in larviculture of European lobster (*H. gammarus*) than waterborne *Bacillus* spp. used as a probiotic water additive in Trial 1. Since O_3 and UV were both effective at reducing pathogens in the culture water, potential differences in growth and survival rates between these treatments might have occurred as a result of parental effect, highlighting the importance of using a pool of larvae from the same batch of parents. It is theorised that residual by-products from the ozonation process may potentially decrease water nutrients and result in decreased growth potential of the cultured organism. However, in relative terms O_3 still provided better results in this study compared to other treatments. Key to improving aquaculture, any possible negative impacts need to be balanced against the gains in survival and growth rates. The use of UV in conjunction with a probiotic is ineffectual in a recirculating system with high turnover rates between animal holding tanks and the biofiltration units. This is because waterborne bacteria are inactivated once irradiated and it would

appear that UV shows a trend towards lower growth and survival statistics than O_3 as a biocontrol method. Positive results have been shown in the use of probiotics as dietary supplements. However, results from the present study clearly show the effectiveness of O_3 in aquaculture facilities for control of pathogens in the rearing of European lobster over either a probiotic water additive or UV.

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7. References

Balcazar, J.L., de Blas, J., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Muzquiz, J.L., (2006). The role of probiotics in aquaculture. *Veterinary Microbiology*, **114**, 173-186.

Battison, A.L., Despres, B.M., Greenwood, S.J., (2008). Ulcerative enteritis in *Homarus americanus*: case report and molecular characterization of intestinal aerobic bacteria of apparently healthy lobsters in live storage. *Journal of Invertebrate Pathology*, **99**, 129-135.

Bourne, D., Hoj, L., Webster, N., Payne, M., Skinderoe, M., Givskov, M., Hall, M., (2007). Microbiological aspects of phyllosoma rearing of the ornate rock lobster *Panulirus ornatus*. *Aquaculture*, **268,** 274–287.

Brown, C., Russo, D.J., (1979). Ultraviolet light disinfection of shellfish hatchery seawater. I. Elimination of five pathogenic bacteria. *Aquaculture*, **17**, 17-23.

Cawthorn, R.J., (1997). Overview of "bumper car" disease-impact on the North American lobster fishery. *International Journal of Parasitology*, **27**, 167-172.

Cha, J.-H., Rahimnejad, S., Yang, S.-Y., Kim, K.-W., Lee, K.-J., (2013). Evaluations of *Bacillus* spp. as dietary additives on growth performance, innate immunity and disease resistance of olive flounder (*Paralichthys olivaceus*) against *Streptococcus iniae* and as water additives. *Aquaculture*, **402-403***,* 50-57.

Charmantier, G., Haond, C., Lignot, J.H., Charmantier-Daures, M., (2001). Ecophysiological adaptation to salinity throughout a life cycle: A review in homarid lobsters. *The Journal of Experimental Biology*, **204**, 967–977.

Cobb, J.S. and Castro, K.M., (2006). Homarus species. In: Phillips, B. (ed.), *Lobsters. Biology, Management, Aquaculture and Fisheries*, pp. 506. Blackwell Publishing Limited.

Contarini, G., Perrella, N., Hickey, J., Ballestrazzi, R., (2008). Hatchery production of European lobster (*Homarus gammarus*, L.): broodstock management and effects of different holding systems on larval survival. *Italian Journal of Animal Science*, **7**, 351-362.

Crecelius, E.A., (1979). Measurements of oxidants in ozonized seawater and some biological reactions. *Journal of the Fisheries Board of Canada*, **36**, 1006-1008.

de Souza, D.M., Suita, S.M., Leite, F.P.L., Romano, L.A., Wasielesky. W., Ballester, E.L.C., (2012). The use of probiotics during the nursery rearing of the pink shrimp *Farfantepenaeus brasiliensis* (Latreille, 1817) in a zero exchange system. *Aquaculture Research*, **43**, 1828–1837.

Daniels, C.L., Merrifield, C.I., Boothroyd, D.P., Davies, S.J., Factor, J.R., Arnold, K.E., (2010). Effect of dietary *Bacillus* spp. and mannan oligosaccharides (MOS) on European lobster (*Homarus gammarus* L.*)* larvae growth performance, gut morphology and gut microbiota. *Aquaculture*, **304**, 49–57.

Daniels, C.L., (2011). *Evaluating the effects of Bacillus spp. and MOS on the culture success, gut microbiota and immune status of juvenile European Lobster Homarus gammarus.* Unpublished doctoral dissertation (Chapter 6), University of Plymouth, Devon, U.K.

Daniels, C.L., Merrifield, D.L., Ringo, E., Davies, S.J., (2013). Probiotic, prebiotic and symbiotic applications for the improvement of larval European lobster (*Homarus gammarus*) culture. *Aquaculture*, **416-417**, 396-406.

Decamp, O., Moriarty, D.J.W., Lavens, P., (2008). Probiotics for shrimp larviculture: review of field data from Asia and Latin America. *Aquaculture Research*, **39**, 334-338.

Diaz, D., Mari, M., Abello, P., Demestre, M., (2001). Settlement and juvenile habitat of the European spiny lobster *Palinurus elephas* (Crustacea: Decopoda: Palinuridae) in the western Mediterranean Sea. *Scientia Marina*, **64**, 347-356.

Dhert, P., Lavens, P., Sorgeloos, P., (1992). Stress evaluation: a tool for quality control of hatchery-produced shrimp and fish fry. *Aquaculture Europe*, **17**, 6–10.

Emerson, M.A., Sproul, D.J., Buck, C.E., (1982). Ozone inactivation of cell-associated viruses. *Journal of Applied and Environmental Microbiology*, **43**, 603-608.

Fisher, W.S., Nilson, E.H., Follett, L.F., Shleser, R.A., (1976). Hatching and rearing lobster larvae (*Homarus americanus*) in a disease situation. *Aquaculture*, **7**, 75-80.

Food and Agriculture Organization of the United Nations (FAO), (2009). State of World Fisheries and Aquaculture. Fisheries and Aquaculture Department: Rome.

Food and Agriculture Organization of the United Nations (FAO), (2014). Fisheries statistical collections CECAF (Eastern Central Atlantic) Capture Production. Available: http://www.fao.org/fishery/statistics/cecaf-capture-production/query/en

Garrido-Pereira, M.A., Braga, A.L., Ferretto da Rocha, A., Sampaio, L.A., Abreu, P.C., (2013). Effect of ultraviolet (UV) radiation on the abundance and respiration rates of probiotic bacteria. *Aquaculture Research*, **44**, 261–267.

Gatesoupe, F.J., (1999). The use of probiotics in aquaculture. *Aquaculture*, **180**, 147–165.

Gomez-Gil, B., Roque, A., Turnbull, J.F., (2000). The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. *Aquaculture*, **191**, 259–270.

Goulden, E.F., Hall, M.R., Bourne, D.G., Pereg, L.L., Hoj, L., (2012). Pathogenicity and Infection Cycle of *Vibrio owensii* in Larviculture of the Ornate Spiny Lobster (*Panulirus ornatus*). *Journal of Applied and Environmental Microbiology*, **78**, 2841-2849.

Gullian, M., Thompson, F., Rodriguez, J., (2004). Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. *Aquaculture*, **233**, 1–14.

Hai, N.V., Buller, N., Fotedar, R., (2009). Effects of probiotics (*Pseudomonas synxantha* and *Pseudomonas aeruginosa*) on the growth, survival and immune parameters of juvenile western king prawns (*Penaeus latisulcatus*, Kishinouye, 1896). *Aquaculture Research*, **40**, 590-602.

Hedgecock, D., Nelson, K., (1978). Components of growth rate variation among laboratory cultured lobsters (*Homarus*). Proceedings of the annual meeting - *World Mariculture Society*, **9**, 125–137.

Hunter, G.L., O'Brien, W.J., Hulsey, R.A., Carns, K.E., Ehrhard, R., (1998). Emerging disinfection technologies: medium-pressure ultraviolet lamps and other systems are considered for wastewater applications. *Water Environment and Technology*, **10**, 40-44.

Ibnou-Zekri, N., Blum, S., Schiffrin, E.J., von der Weid, T., (2003). Divergent patterns of colonization and immune response elicited from two intestinal *Lactobacillus* strains that display similar properties in vitro. *Infection and Immunity*, **71**, 428-436.

Irianto, A., Austin, B., (2002). Probiotics in aquaculture. *Journal of Fish Diseases*, **25**, 633–642.

Jithendran, K.P., Poornima, M., Balasubramanian, C.P., Kulasedarapandian, S., (2010). Diseases of mud crabs (*Scylla* spp.): an overview. *Indian Journal of Fisheries*, **57**, 55-63.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.J., Gibson, L., (2008). Probiotics in aquaculture: The need, principles, and mechanisms of action and screening processes. *Aquaculture*, **274**, 1-14.

Liltved, H., Hekteon, H., Efraimsen, H., (1995). Inactivation of bacterial and viral fish pathogens by ozonation or UV irradiation in water of different salinity. *Aquacultural Engineering*, **14**, 107-122.

Luis-Villasenor, I.E., Macias-Rodriguez, M.E., Gomez-Gil, B., Ascensio-Valle, F., Campa-Cordova, A.I., (2011). Beneficial effects of four *Bacillus* on the larval cultivation of *Litopenaeus vannamei*. *Aquaculture*, **321**, 136-144.

Madsen, K., (2006). Probiotics and the immune response. *Journal of Clinical Gastroenterology*, **40**, 232-234.

Maeda, M., Nogami, K., Kanematsu, M., Hirayama, K., (1997). The concept of biological control methods in aquaculture. *Hydrobiologia*, **358**, 285–290.

Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R.T.M., Bogwald, J., Castex, M., Ringo, E., (2010). The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture*, **302,** 1-18.

Moland, E., Moland, O. E., Knutsen, H., Knutsen, J.A., Enersen, S.E., André, C., Stenseth, N.C., (2011). Activity patterns of wild European lobster *Homarus gammarus* in coastal marine reserves: implications for future reserve design. *Marine Ecology Progress Series*, **429**, 197-207.

Moriarty, D. J. W., and Body, A. G. C., (1995). *Modifying microbial ecology in ponds: the key to sustainable aquaculture, p.1–10*. In Proceedings of Fish Asia ’95 Conference: 2nd Asian Aquaculture and Fisheries Exhibition and Conference. RAI Exhibitions, Singapore.

Moriarty, D.J.W., (1998). Control of luminous Vibrio species in *penaeid* aquaculture ponds. *Aquaculture*, **164**, 351–358.

Newaj-Fyzul, A., Al-Harbi, A.H., Austin, B., (2013). Review: Developments in the use of probiotics for disease control in aquaculture. *Aquaculture*, **416**, in press.

Nogami, K., Hamasaki, Maeda, M., Hirayama, K., (1997). Biocontrol method in aquaculture for rearing the swimming crab larvae *Portunus trituberculatus*. *Hydrobiologia*, **358**, 291–295.

Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., Menasavetab, P., (2000). Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium *Bacillus* S11. *Aquaculture*, **191**, 271–288.

Ritar, A.J., Smith, G.G., Thomas, C.W., (2006). Ozonation of seawater improves the survival of larval southern rock lobster, *Jasus edwardsii*, in culture from egg to juvenile. *Aquaculture*, **261**, 1014-1025.

Ritola, O., Peters, L.D., Livingstone, D.R., Lindstrom-Seppa, P., (2002). Effects of in vitro exposure to ozone and/or hyperoxia on superoxide dismutase, catalase, glutathione and lipid peroxidation in red blood cells and plasma of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Aquaculture Research*, **33**, 165-175.

Samocha, T.M., Guajardo, H., Lawrence, A.L., Castille, F.L., Speed, M., McKee, D.A., Page, K.I., (1998). A simple stress test for *Penaeus vannamei* postlarvae. *Aquaculture*, **165**, 233–242.

Schroeder, J.P., Gartner, Al, Waller, U., Henel, R., (2010). The toxicity of ozone-produced oxidants to the Pacific white shrimp *Litopenaeus vannamei*. *Aquaculture*, **305**, 6-11.

Scolding, J.W.S., Powell, A., Boothroyd, D.P., Shields, R.J., (2012). The effect of ozonation on the survival, growth and microbiology of the European lobster (*Homarus gammarus*). *Aquaculture*, **364-365**, 217-223.

Sellars, M.J., Coman, G.J., Morehead, D.T., (2005). Tolerance of *Penaeus* (*Marsupeneaus*) *japonicus* embryos to ozone disinfection. *Aquaculture*, **245**, 111-119.

Shariff, M., Yusoff, F.M., Devaraja, T.N., Srinivasa-Rao, P.S., (2001). The effectiveness of a commercial microbial product in poorly prepared tiger shrimp, *Penaeus monodon* (Fabricius), ponds. *Aquaculture Research*, **32**, 181-187.

Sharrer, M.J., Summerfelt, S.T., (2007a). Ozonation followed by ultraviolet irradiation provides effective bacteria inactivation in a freshwater recirculating system. *Aquaculture Engineering,* **37,** 180-191.

Sharrer, M.J., Summerfelt, S.T., (2007b). Inactivation of bacteria using ultraviolet irradiation in a recirculating salmonid culture system. *Aquacultural Engineering*, **33**, 135-149.

Silva, E.F., Soares, M.A., Calazans, N.F., Vogeley, J.L., do Valle, B.C., Soares, R., Peixoto, S., (2013). Effect of probiotic in shrimp larviculture. *Aquaculture Research*, **44**, 13-21.

Souza, J.C.R., Strussmann, C.A., Takashima, F., Satoh, H., Sekine, S., Shima, Y., Matsuda, H., (2010). Oral and integumental uptake of free exogenous glycine by the Japanese spiny lobster *Panulirus japonicus* phyllosoma larvae. *Journal of Experimental Biology*, **213**, 1859-1867.

Summerfelt, S.T., (2003). Ozonation and UV irradiation - an introduction and examples of current applications. *Aquacultural Engineering*, **28**, 21-36.

Tango, M.S., Gagnon, G.A., (2003). Impact of ozonation on water quality in marine recirculation systems. *Aquaculture Engineering*, **29**, 125-137.

Urbina, M.A., Paschke, K., Gebauer, P., Cumillaf, J-P., Rosas, P., (2013). Physiological responses of the southern king crab, *Lithodes santolla* (Decapoda: Lithodidae), to aerial exposure. *Comparative Biochemistry and Physiology*, Part A, **166**, 538–545.

Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews*, **64**, 655-671.

Vine, N.G., Leukes, W.D., Kaiser, H., (2006). Probiotics in marine larviculture. FEMS *Microbiol Rev*, **30**, 404–427.

Zhou, X.X., Wang, Y.B., Li, W.F., (2009). Effect of probiotic on larvae shrimp (*Penaeus vannamei*) based on water quality, survival rate and digestive enzyme activities. *Aquaculture*, **287**, 349–353.

Zhang, Q., Tan, B.P., Mai, K.S., Zhang, W.B., Ma, H.M., Ai, Q.H., Wang, X.J., Liufu, Z.G., (2011). Dietary administration of *Bacillus* (*B. licheniformis and B. subtilis*) and isomaltooligosaccharide influences the intestinal microflora, immunological parameters and resistance against *Vibrio alginolyticus* in shrimp, *Penaeus japonicus* (Decapoda: Penaeidae). *Aquaculture Research*, **42**, 943-952.

Chapter 3

Development of branchial grooming setae in larval and juvenile *Homarus gammarus* and their functional relationship to microbial proliferation during a moult cycle

Abstract

The functional morphology of gill cleaning mechanisms is not well described in European lobster (*Homarus gammarus*). Brush like cleaning appendages called setae have evolved in many decapod crustacean species to control the accumulation of epibionts on gill structures that are vital for respiration, osmoregulation, acid-base balance and excretion of metabolic end products. Scanning electron microscopy was used to investigate structural development of the setae during larval and early juvenile stages of *H. gammarus* (larval stages I-III, megalopa stage IV and first juvenile stage V). Branchial cleaning mechanisms were further assessed by quantifying and classifying the progression of the microbial biofilm throughout a complete moult cycle from the megalopa (stage IV) to the first juvenile (stage V). Results showed denticulate (serrated) scales arranged along the distal end of a smooth setal shaft originating proximal to epipodite attachment. Setae occur only from larval stage III. Immediately after shedding (ecdysis) of the old exoskeleton, no epibionts were visible on the new cuticle. Microbial development was first evident on the gill filaments from day 10 postmoult, showing a significant 16-fold increase from day 10 to 15. This research provides new insights into the functional morphology of gill grooming in early developmental stages of *H. gammarus* and the role of moulting in microbial control.

1. Introduction

Gills in Crustacea contribute to several vital physiological processes such as respiration, osmoregulation, ion and acid-base regulation, and nitrogenous waste excretion (Dickson *et al*., 1991). Decapod crustacean gills are complex structures which fall into three distinct morphological categories; phyllobranchiate, dendrobranchiate and trichobranchiate (Boxshall and Jaume, 2009). In crabs for instance, the gills are phyllobranchiate consisting of lamellar structures (Taylor and Greenaway, 1979). Penaeoid and sergestoid shrimps possess dendrobranciate gill structures (Boxshall and Jaume, 2009). Crayfish and lobster gills take on a trichobranchiate form with a central axis from which three columns of several filaments arise, rather than lamellae, creating a large

surface area primarily to enable improved respiratory function (Dickson *et al*., 1991; Spicer and Eriksson, 2003). As a result of bilateral symmetry in crustaceans left and right branchial chambers are located on either side of the cephalothorax and protected by the branchiostegite (an extension of the carapace). In *H. gammarus*, each gill chamber contains 20 trichobranchiate gills and 7 epipodites responsible for respiration and osmoregulation, respectively (Haond *et al*., 1998; Lignot *et al*., 1999; Lignot and Charmantier, 2001). Maintaining viability of these structures is vital in optimising physiological functions, which are known to be reduced by surface microbial infestations (Schuwerack *et al*., 2001). Growth rates were significantly reduced and resting oxygen consumption rates increased in the freshwater crab *Potamonautes warreni* after microbial infestations of the gills (Schuwerack *et al*., 2001). Microbial control is critical not only to the animals health, but also to enhance the chance of surviving environmental challenges such as changes in salinity and oxygen levels.

Decapod crustaceans utilise mechanical cleaning mechanisms in an effort to control microbial pathogens present on specific external surfaces of the animal. These can be passive or active mechanisms (Bauer, 2013) and includes cleaning of olfactory, respiratory and sensory structures (Bauer, 2013). Decapod crustaceans have evolved both passive (i.e. which rely on locomotion or water flow for movement), and active (i.e. cheliped brushing) grooming appendages to remove microbes using complex setae which possess rasp like ultrastructures. This is in addition to the use of reverse water flow through the gills as a mechanism to remove particulate matter (Factor, 1978). These mechanisms have been well described in larval and adult decapod crustacean species (Bauer, 1979, 1981, 1999, 2013; Pohle and Telford, 1981; Lavalli and Factor, 1995). Most crustacean species identified, however, do not possess both (Bauer, 1989), and *H. gammarus* has been described as only possessing a passive gill cleaning mechanism. In the gills, passive setae are often attached to epipodites associated with the pereopods (legs). Movement of pereopods during normal locomotion causes movement of the epipodites, which in turn moves the attached setae amongst the gill filaments. Setae are non-muscular structures and therefore rely on appendage locomotion or water flow through the gill chamber to power their movement. The effectiveness of this passive

form of microbial control is discussed by Bauer (1998) in research on crayfish *Procambarus clarkii*. Bauer found that during times of heavy infestation, gill cleaning setae are relatively ineffectual at removing epibionts, but were effective at removing fouling by particulate matter (e.g. sediment). Active brushing of gill surfaces by the use of chelipeds in the branchial chamber was shown to be highly effective at removal of both epibionts and particulate matter in caridean shrimp (Bauer, 1979). However, as previously discussed, *H. gammarus* only possess a passive gill cleaning mechanism, which is a less effective method of microbial control. In contrast, moulting does provide an effective mechanism for removal of epibionts from gill structures (Corbari *et al*., 2008).

The crustacean growth cycle primarily involves shedding of the exoskeleton (Aiken and Waddy, 1987). A secondary result of moulting is the removal of microbes from the gill cuticle, which forms a part of the exoskeleton (Bauer, 1979). Moulting as a mechanism for pathogen control in decapod crustaceans is likely to be more effective in early developmental stages given the high frequency of moults associated with the rapid growth rates typical of early life stages in animals. However, as mentioned, moulting is a secondary effect of the growth cycle and in adult crustaceans this occurs relatively infrequently in comparison to the rapid development of microbial communities. *H. gammarus* are thought to moult up to 25 times before reaching adulthood after five years, and this rate reduces during adulthood occurring as few as once every two years. Therefore, the reduced frequency renders moulting less effective than in early developmental stages making grooming appendages more critical in later life.

While microbial infestation of respiratory structures has the potential to negatively impact growth and survival of *H. gammarus*, quantitative effects of microbial coverage of gill surfaces during a juvenile *H. gammarus* moult cycle has not previously been documented. Therefore, the aim of the present study was to 1) describe the morphology of gill cleaning setae, 2) characterise the time scale for deposition of microbes during a complete moult cycle, and 3) to show the effectiveness of moulting in microbial control compared to passive gill cleaning setae during early development. This study provides a novel insight into moulting as a gill cleaning mechanism in early developmental stages

coupled with a descriptive examination of the structure and development of epipodial setae in relation to gill epibionts. It also provides valuable insight into a possible shift in our current understanding of the role of moulting in adult *Homarus*. We propose a change in thinking from moulting supporting an indiscriminate growth strategy, to that of improved general health and fitness through total removal of biofilms and pathogens on gill structures. This might be especially important in adults where growth is much reduced and moulting much less frequent.

2. Materials and Methods

2.1. Animals and experimental design

Animals were hatchery reared at ~20 °C in a salinity of ~35 PSU (National Lobster Hatchery, Padstow, North Cornwall, UK). Branchial development and cleaning mechanisms during larval stages were evaluated by sampling and fixing four larvae/juveniles that had recently moulted (within 24 h). This was performed at each stage from zoea (I-III), to megalopa (IV) and juvenile (V). Microbial film development on the gills between moults was evaluated in the megalopa stage by sampling four individuals at 1, 5, 10 and 15 days post moult and after 24 h of moulting to juvenile, stage V. Animals were preserved in 2.5 % glutaraldehyde and kept at 4 °C until required for histology. Histology work was carried out at the Biosciences Department of the University of Exeter, Devon, UK.

2.2. Scanning Electron Microscopy

After removal of the carapace to expose gill structures, samples were placed in osmium for 24 h and then dehydrated using a graded ethanol series (30%, 50%, 75%, 90%, and 100% for 15 minutes at each concentration). Samples were then critical point-dried (E3000, Polaron Equipment Limited, UK) using liquid carbon dioxide (CO_2), stub mounted and coated in gold palladium (20 mA thickness) using a sputter coater (SC510, V.G. Microtech, UK) for analyses using a scanning electron microscope (SEM) (Jeol SEM 6390 at 5 kV acceleration voltage). Scanning electron microscopy was used to identify the

structure of setae on the epipodites within the branchial chamber to provide descriptive analysis for stages I-V. Images were also taken of stage IV (megalopa) and stage V (juvenile) *H. gammarus* larvae (n = 4) at 2000 x magnification from nine different locations on podobranch (outer layer) gill filaments. These locations were the top, middle and bottom anterior bud on the first, third and fifth filament (anterior to posterior respectively), and were selected based on accessibility. Left or right hand chambers were used depending on the position and orientation of gill filaments; the one presenting a more natural position was chosen. For image analysis, a 432 μm^2 macro grid was created using ImageJ analysis software (ImageJ, v1.47). The grid was overlaid centrally at the base of each filament where it connects to the central axis and the presence and abundance of microbes was counted within the grid. Microbes were only counted if the attachment point could be clearly seen within the grid. Microbes were counted and classified only by shape as either filamentous, cocci or rod, as accurate species identification could not be performed from images alone.

2.3. Statistical analysis

All counts from each animal (9 grids) were added together and total microbial density (number/μm^2) was calculated as the total number of microbes divided by the total area measured (i.e. 432 μm^2 per grid x 9 grids = 3888 μm^2 total area). Analysis was performed using SigmaPlot and data represented as mean ±SEM. All data failed assumptions of equal variances and distribution and were analysed using non parametric analysis. Data for microbial density (number/μm^2) were transformed as $\sin\left(\frac{1}{x+1}\right)$ and analysed using Kruskal-Wallis. Total microbial densities of each bacterial type (number/μm2) was analysed using ranked two-ways ANOVA, and significant differences were subjected to a Tukey post hoc analysis. Significant differences were accepted at a $P \leq 0.05$.

3. Results

3.1. Branchial chamber structures and setal cleaning appendages

Scanning electron microscopy of the right branchial chamber in *H. gammarus* stage IV megalopa revealed epipodites with clearly visible setae positioned amongst the gills (Fig. 1). Gills are trichobranchiate in structure and separated by epipodites (flat blade shaped structures) extending into the branchial chamber from the base of the pereopods (legs). Gill filaments bud off from a central axis and gills are wider at the base and distally taper off into a point.

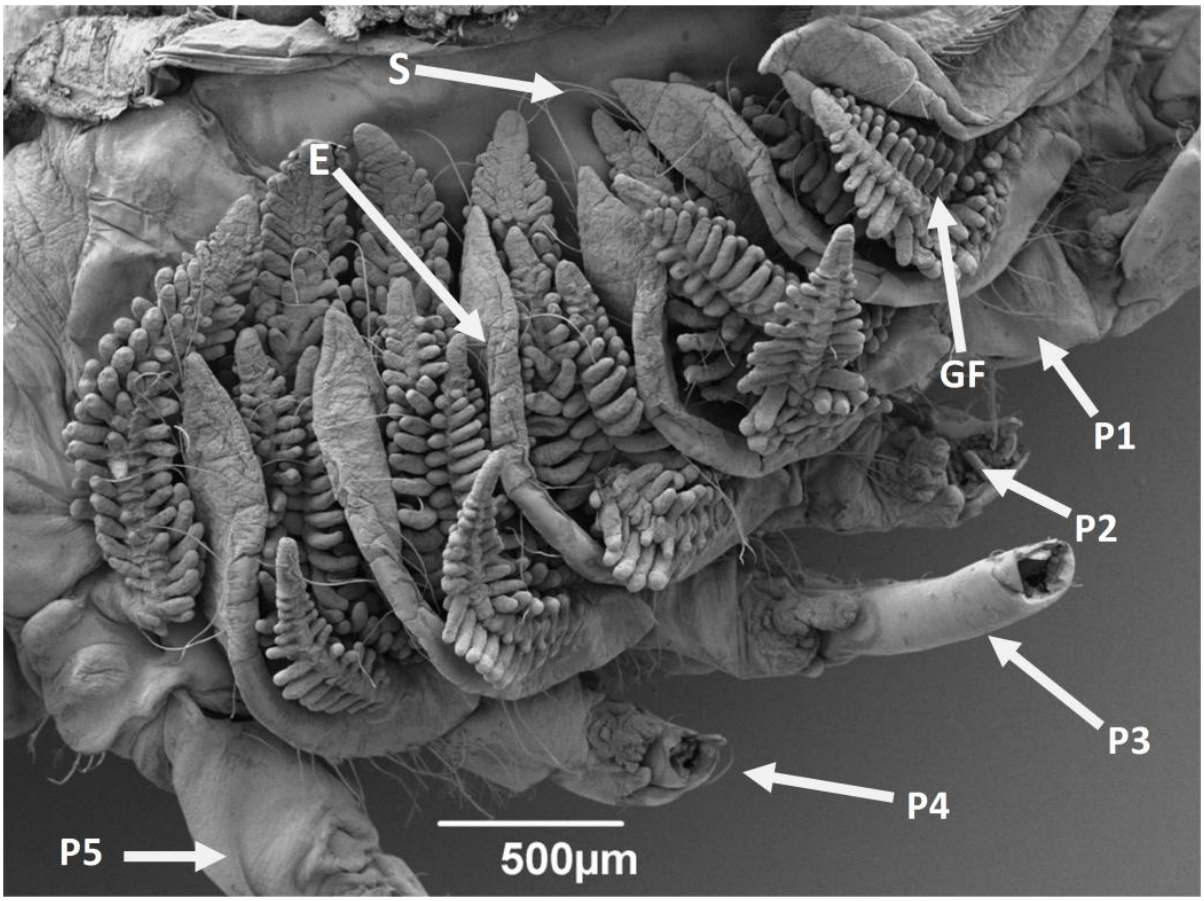

Fig. 1. Right branchial chamber of *H. Gammarus* with branchiostegite (carapace) removed to reveal gills, epipodites and epipodial setae in a stage IV megalopa. Animal is positioned left to right posterior to anterior. P1-P5 pereopods have been removed to provide a clear view of the gill chamber. Abbreviations: GF, gill filaments; E, epipodite; S, epipodial setae.

Setae structures have a smooth shaft proximal to the epipodite attachment point (setal socket, SS; Fig. 2Ai). Distally the setae are three-sided (Fig. 2B), with dentate ultrastructures (Fig. 2C, 2D).

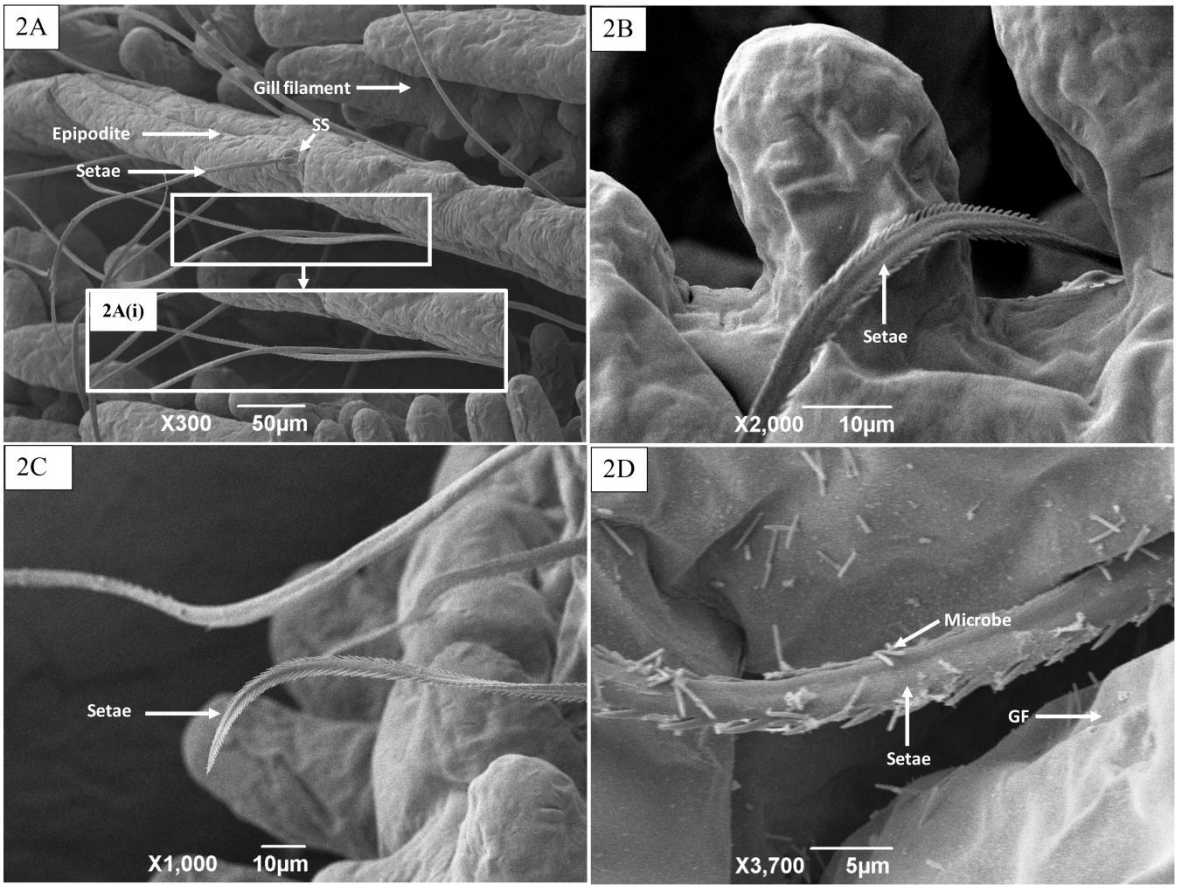

Fig. 2. *Homarus gammarus* epipodial gill cleaning setal structure with microbial growth: 2A, 2A(i), setal socket attachment point; 2B, distal three-sided setal structure; 2C, serrated distal and smooth proximal structure of setae; 2D dentate setal ultrastructure showing epibiont attachment. SS: setal socket, GF: gill filament.

Gill structures were found to be fully formed, although under developed, in *H. gammarus* stage I larvae post hatch (Fig. 3A). The epipodites were also present in stage I larvae, but not yet well developed (Fig. 3A). Gill cleaning setae, however, first appeared in megalopa (stage IV) post larvae onwards (Fig. 1). SEM images detailing the development of setae on the epipodites through stages I-III found no setae present in stage I-II (Fig. 3A, B), and development of setae appears to be delayed until larval stage III (Fig. 3C, C(i)).

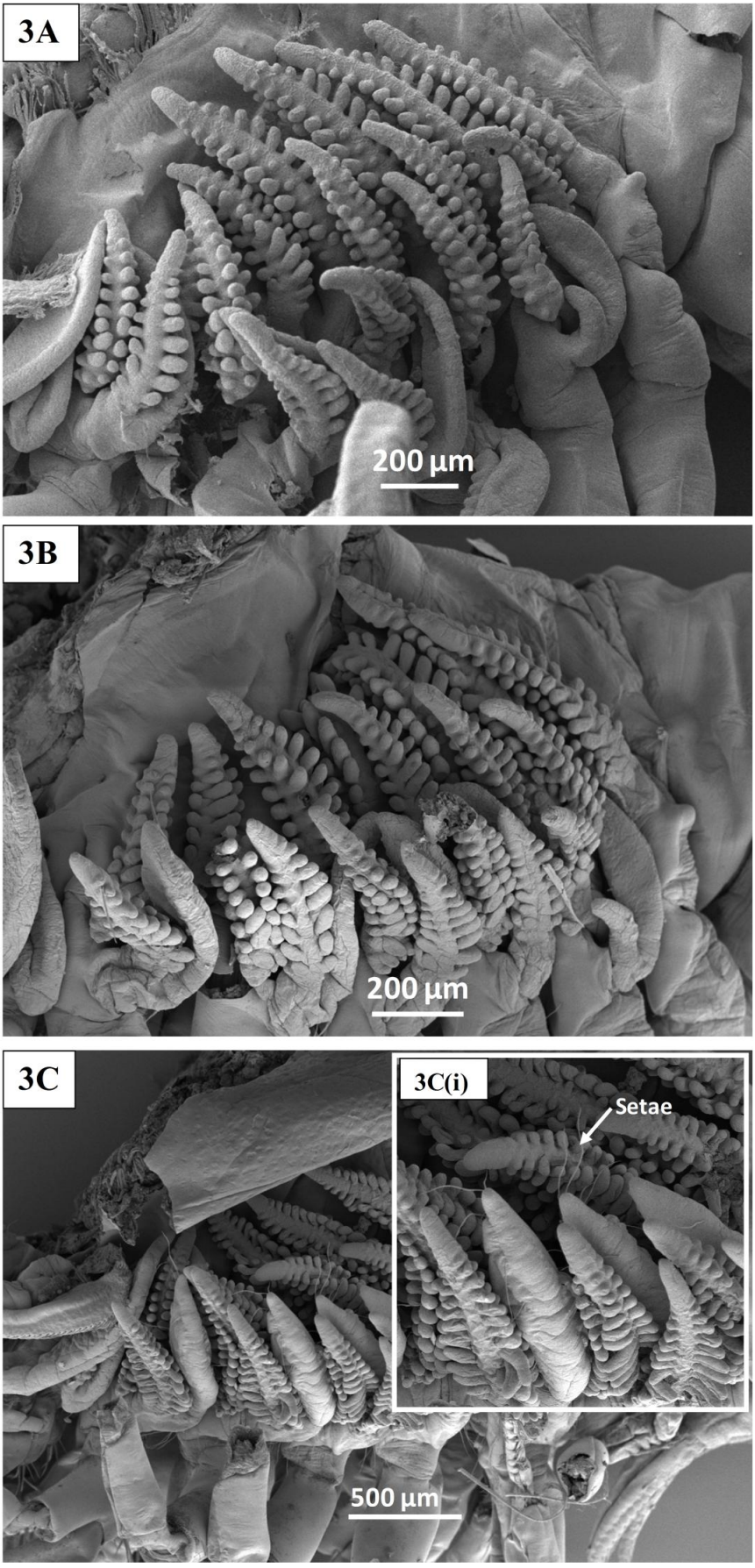

Fig. 3. Setal development through *H. gammarus* larval stages I-III (3A, 3B, 3C) with setae enlarged in panel 3C(i).

3.2. Microbial film proliferation between moults

Microbes were categorised by shape as either cocci, rod or filamentous as detailed in Fig. 4A. SEM analysis clearly showed proliferation of microbes on gill surfaces at 15 days postmoult in *H. gammarus* stage IV megalopa.

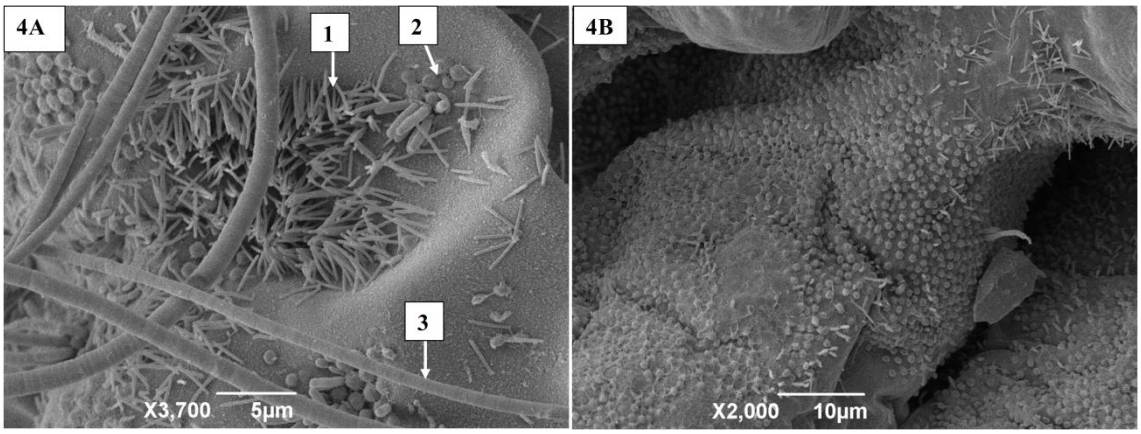

Fig. 4. Classification of microbial growth on *H. gammarus* gills by shape: 4A(1), rod; 4A(2), cocci; 4A(3), filamentous. 4B shows proliferation of microbial pathogens on a stage IV megalopa gill filament 15 days postmoult.

Development of microbial communities on gill structures increased during a moult cycle (Fig. 5A). Analysis of the development of microbial colonisation on gill surfaces between postmoult megalopa (day 1), and postmoult juvenile (day 1), of *H. gammarus* revealed a clear cycle of changes. Microbes were not present on gill filament areas sampled on days 1 and 5, and first quantified after 10 days postmoult (megalopa stage IV), with a furthur 16 fold increase in microbial density observed between days 10 and 15 (Kruskal-Wallis test, $P< 0.001$, Fig. 5A), returning to a completely microbial-free cuticle on day one post moult to juvenile stage V. There were significantly more rod shaped bacteria than filamentous bacteria 10 days postmoult (ANOVA, $P=0.045$), while cocci shaped bacteria were significantly more abundant than filamentous by 15 days postmoult (ANOVA, $P<0.001$, Fig 5B).

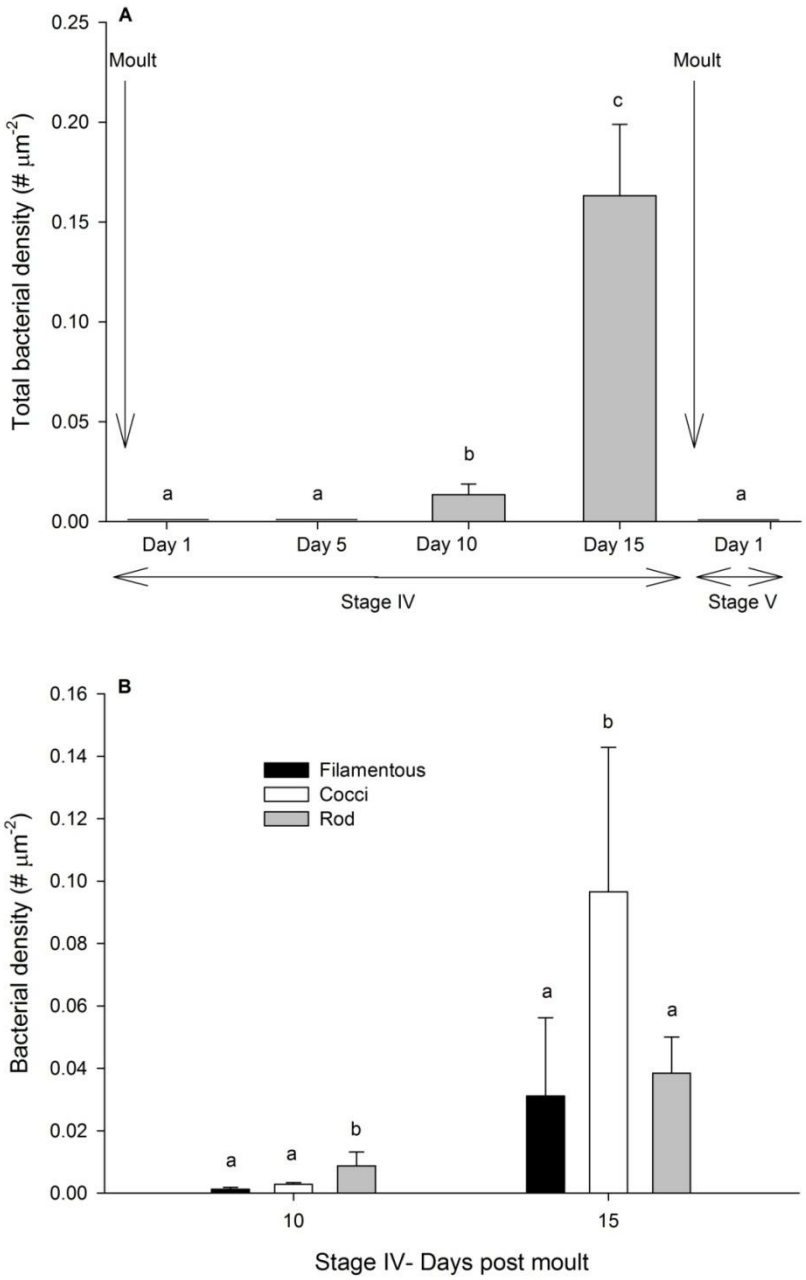

Fig. 5. *Homarus gammarus* microbial count during developmental stage IV-V moult cycle: (A) number of microbes per µm² over a surface area of 3888 µm²; (B) number of individual microbial species on days 10 and 15 postmoult. Data represent means ± SEM. Significant differences represented with letters relate to each day postmoult.

4. Discussion

Growth during the first four developmental stages in *H. gammarus* (larval I-III, megalopa IV) occurs within an approximate three week period in 19 °C seawater and involves three moult cycles (Scolding *et al*., 2010). Between each moult, numbers of epibionts increases on gill surfaces increased exponentially over time. Passive gill grooming setae provide control of debris and epibionts, but are comparatively ineffective when compared to moulting, which was shown to completely remove all foreign bodies in the current study. Setae development only occurs from larval stage III and it can therefore, reasonably be speculated that the lack of setae in the first two growth stages is related to the frequency and effectiveness of moults.

4.1 Gill grooming setae

Crustacea moult predominantly as a function of growth, therefore growth is limited to moulting frequency, i.e. each time an animal goes through a growth period the exoskeleton is shed. Reproduction in crustaceans also requires soft bodied postmoult females (Carlisle, 1957). Rapid growth in hatchery reared (~20 °C optimal growing temperature) larval stages of *H. gammarus* involves moulting approximately every five days from larval stage I through to the megalopa stage IV. Then approximately seven days between megalopa and the first juvenile stage V (Scolding *et al*., 2010). On completion of pelagic larval stages, metamorphosis through to megalopa stage IV results in transition to a benthic habitat. On the ocean floor, newly formed benthic lobsters are exposed to sedimentary conditions and obviously exposed to greater levels of gill fouling than would normally be present in the water column. Exposure to sedimentary environments requires gill grooming appendages in order to maintain normal respiratory function (Bauer, 1979). Arguably this is the most crucial time for the use of established gill grooming setae. Results from the current study show development of gill cleaning setae on the epipodial blade begins at pelagic larval stage III. This is consistent with the above theory that setal appendages are less important during the first two pelagic stages when moult frequency is high and therefore, epibionts and sediment are removed frequently.

Correct identification of setae involves the recognition of typical setal microstructures (i.e. dentate scales). However, Thomas (1970) describes ‘annuluses’ or rings as an important diagnostic feature of setae which helps to differentiate them from other structures such as spines or hairs. As previously discussed, setae form part of the exoskeleton in crustaceans and therefore, during a moult these setae are shed with the old exoskeleton. The presence of a ‘ring’ around the setal shaft marks the point of new setal growth (setagenesis), and this has been discussed in many studied crustacean species (Thomas, 1970; Reaka, 1975; Pohle and Telford, 1981; Mesce, 1993). Images from the current study (larval stage III onwards) did not focus on growth rates, and therefore setagenesis was not observed. However, morphology of the setae ultrastructure in stages III onwards, i.e. dentate scales, clearly identifies structures as epipodial setae.

4.2 Branchial cleaning mechanisms

Gill filaments and epipodites are responsible for respiration (gill filaments) and osmoregulation (epipodites) in *H. gammarus* (Lignot and Charmantier, 2001). Irrigation of gill structures is via the scaphognathite (Factor, 1978). Water is rapidly drawn into and through the branchial chamber from apertures near the base of the pereopods in a posterior to anterior direction and expelled via “anterior excurrent canals” (Wilkens and McMahon, 1972). The containment of gill lamellae or filaments within a confined branchial space increases the potential for trapping waterborne sediment or microorganisms (Bauer, 1979). This is thought to be the driver to the development of grooming mechanisms in decapods to control the negative impact of environmental (i.e. sediment) and biological (i.e epibionts) factors, on physiological functions e.g. sensory and respiratory. Decapod crustaceans periodically utilise reverse water flow through the branchial chamber to flush out debris and in addition have evolved gill cleaning setae (Bauer, 1998, 1999, 2013).

Setae are complex structures and morphology varies greatly between crustacean species (Jacques, 1989). Earliest research on classification of setae using light microscopy was done by Laubmann (1913) in carideans and grouped as dentate, plumose, spiny and sack-shaped. Since then, morphological classification has been refined many times (Thomas, 1970;

Factor, 1978; Pohle and Telford, 1981; Jacques, 1989). When describing setae and associated ultrastructures it is important to consider their form, function and arrangement. Features include identification of the shape and size of the shaft and respective ultrastructures e.g. setules, teeth, spines and scales. The development of SEM images led to more definitive classifications of setae (Thomas, 1970), predominantly on adult animals. Research into passive gill cleaning setae of two decapod species; crayfish (*P. clarkii*) and shrimp (*Rimapenaeus similes*) using SEM has been well described in Bauer (1998, 1999). These examples illustrate multidenticulate scale setules typical of gill grooming setae. Batang and Suzuki (2003), also describe gill cleaning mechanisms in the freshwater crab *Geothelphusa dehaani*. Pohle and Telford (1981) studied setae in laboratory reared larval, megalopa and juvenile crab (*Dissodactylus crillitichelis*), and identified 30 setal groups. Factor (1978) described the setae of larval *Homarus americanus* and provided a detailed diagrammatic of various setal types. Passive gill cleaning setae in the current study on larval and megalopa animals conform to typical decapod crustacean setal structures. In *H. gammarus* the setal shaft is naked proximal to the setal socket and distally is three-sided in shape, with dentate ultrastructures that taper into a pointed tip. On reaching the megalopa stage setae appear long enough to reach amongst adjacent gill filaments. The location and structure of epipodial setae in *H. gammarus* is similar to closely related *H.americanus* (Factor, 1978), describing them as distally serrate and originating from the epipodites.

As discussed, gill grooming can be categorised into either active or passive behaviours, and often involves the use of complex setae (Bauer, 2013). Setal structures act as serrated brushes to scrape away surface debris. Active grooming of gill structures in crustaceans involves the use of specially adapted appendages inserted into the branchial chamber to remove fouling (Bauer, 2013). Examples of this are seen in squat lobsters, king crabs and caridean shrimp (Bauer, 1989, 2013; Pohle, 1989), using cheliped appendages. Passive gill grooming, the focus of the current study on *H. gammarus*, relates to epipodial setae associated with the pereopods, the locomotion of which moves the setae amongst the gill structures. Bauer (2013) does suggest however, that not all passive grooming is purely linked to normal locomotory movements and

that the act of “limb rocking” (movement caused by the animal actively rocking backward and forwards on the pereopods), may be a specific attempt to engage the setae. As earlier discussed, Crustacea possessing active gill cleaning appendages do not possess the more primitive form of passive gill cleaning structures and they are considered mutually exclusive of each other (Bauer, 1989). In the current study, the morphology of lobster gill setae was typical of that in other crustacean species (Bauer, 1989). We also revealed that passive gill cleaning is not present in the first two larval stages in *H. gammarus* and that microbial growth happens somewhere between 5 and 10 days postmoult. However, given the frequency of moulting in early developmental stages, and in particular the role of moulting in removal of epibionts from gill structures, it is assumed that setae are not crucial in microbial control in early life stages.

4.3 Microbial proliferation in the gills

Results showed that the proliferation of microbes on the gills during a moult cycle is not immediate, taking longer than five days after a moult. Furthermore, all epibionts are immediately removed after a moult. This demonstrates the effectiveness of ecdysis in removing microorganisms from gill structures. Research on adult shrimp (Bauer, 1998) supports the hypothesis that in the management of microbial pathogens, epipodite gill cleaning setae are ineffectual on their own. The present study confirms this finding with SEM images clearly showing the proliferation of microbial pathogens over gill filament surfaces. It is therefore evident that passive epipodial setae are not able to completely prevent a rapidly increasing microbial community in the later stages of a moult cycle and that moulting is a more effective mechanism for removal of epibionts. Whilst pathogen control via moulting is a successful secondary effect of the growth cycle, it is also energetically expensive and time consuming because of the complex physiological processes involved (i.e. demineralisation/remineralisation of exoskeleton over several days during each moult). Moulting is also when lobsters are most at risk of predation.

Growth patterns vary in crustacean species and are categorised as either indeterminate (continuous growth during life cycle) or determinate (reaching set point for maximal growth) (Hartnoll, 2001). *Homarus* are indeterminate and continue to grow with age, however as discussed in Hartnoll (2001), the rate at

which crustacean species grow markedly decreases with age as does moult frequency. Regardless of indeterminate growth strategies, growth is not infinite (Hartnoll, 2001). It could be reasonably argued that moulting for the purpose of growth is not necessary after reaching sexual maturity at the age of 5 years. An alternative explanation for moulting beyond this point (at much reduced growth rates), other than reproduction, would be the need for a gill “clean-up” due to the complete removal of microbial biofilms, which is only possible after a moult. That is to say, if adults were to cease growing, they would therefore no longer shed the exoskeleton and as a result would be unable to rid themselves of foreign bodies, therefore moulting could continue as a function of gill cleaning. This would support the concept that the primary driver for a shift in moulting from being solely related to a growth in juveniles to a “gill clean-up” in adults.

As previously discussed, studies on the effectiveness of gill cleaning setae in caridean shrimp showed that passive setae do play an important role in controlling particulate matter, and to a much lesser degree control of epibionts (Bauer, 1998). It is suggested that although setae of megalopa *H. gammarus* in the present study appeared ineffective at epibiont removal once microbial proliferation had occurred on the gill surfaces, they may play an important role in slowing down the time it takes for proliferation to occur. We propose that moulting in adults, albeit less frequent, may shift from solely being a function of growth and reproduction to include an active method of microbial control, and that setae play an important role in increasing the time between moults. This would subsequently increase the time available to gain energy reserves for the next moult by slowing down the rate of microbial growth and coverage of gill surfaces. It may be the case that in crustacean species that possess active gill grooming appendages, there is a relationship between discriminative growth strategies and microbial control within the branchial chamber. This may challenge our understanding of the role that moulting plays in general fitness of crustaceans and would be an area for further investigation. The significance of needing to control microbes on the gill surface is related the vital physiological processes involving these branchial structures; including oxygen consumption and ion regulation. Gas exchange is impaired by the presence of epibionts on the gill surface via reduced surface area and increased diffusion distance between the outer surface of the gill and the haemolymph leading to decreased

respiratory function (Schuwerack *et al*., 2001). Therefore, mechanisms for control of gill epibionts are critical to the health and survival of *H. gammarus*.

5. Conclusion

Gill structures in decapod crustaceans are subjected to excessive amounts of particulate matter from marine sediment and microbial pathogens in their natural environment. The structure and number of gill filaments being confined within a narrow branchial chamber beneath a calcified protective cover provides an effective trap for pathogens and particulate matter. Throughout the moult cycle these increase in abundance. In order to maintain effective gill function (particularly respiratory), epipodial grooming setae attempt to provide control of gill microbial pathogens. These setae develop from larval stage III and are not present in stages I-II in *H. gammarus*. This passive gill cleaning mechanism seems relatively ineffective at removing epibionts in megalopa animals, however may play an important role at reducing the time it takes for microbial proliferation of gill surfaces to occur. This in turn could affect the rate at which moulting occurs. A more effective control method results as a secondary function of ecdysis as part of the growth process with successful removal of all foreign debris and epibionts from the exoskeleton, which includes gill structures. In their natural habitat, *H. gammarus* may have little control over volume of waterborne bacteria or debris they are exposed to and protect their branchial structures via passive or active cleaning appendages, and or reverse flow of water through the gill chamber. Passive gill cleaning setae may slow down the proliferation of microbes on gill surfaces. However, moulting may become more crucial to crustacean survival as the only effective method for complete removal of pathogens on the gill surfaces, as opposed to primarily the facilitation of growth and reproduction.

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7. References

Aiken, D.L., Waddy, S.L., (1987). Molting and growth in crayfish: a review. *Canadian Technical Report on Fisheries and Aquatic Science.* **1587**, 1-34.

Batang, Z.B., Suzuki, H., (2003). Gill-cleaning mechanisms of the amphibious freshwater crab *Geothelphusa dehaani* (Decapoda, Brachyura, Potamidae). *Journal of Crustacean Biology*, **23**, 230-240.

Bauer, R.T., (1979), Antifouling adaptations of marine shrimp (Decapoda: Caridea): gill cleaning mechanisms and grooming of brooded embryos. *Zoological Journal of the Linnaean Society*, **65**, 281-303.

Bauer, R.T. (1981). Grooming behavior and morphology in the decapod Crustacea. *Journal of Crustacean Biology*, **1**, 153-173.

Bauer, R.T., (1989). *Decapod crustacean grooming: Functional morphology, adaptive value, and phylogenetic significance*. In: Felgenhauer, B.E., Watling, L., Thistle, A.B. (eds). Functional morphology of feeding and grooming in Crustacea. Balkema, Rotterdam, Netherlands, pp 49-74.

Bauer, R.T., (1998). Gill-cleaning mechanisms of the crayfish *Procambarus clarkii* (Astacidea: Cambaridae): experimental testing of setobranch function. *Journal of Invertebrate Biology*, **117**,129-143.

Bauer, R.T., (1999). Gill-Cleaning Mechanisms of a Dendrobranchiate Shrimp, *Rimapenaeus similis* (Decapoda, Penaeidae): Description and Experimental Testing of Function. *Journal of Morphology*, **24**,125-139.

Bauer, R.T., (2013), *Adaptive Modification of Appendages for Grooming (Cleaning, Antifouling) and Reproduction in the Crustacea.* In: Watling, L., Thiel, M. (eds). Functional Morphology and Diversity: 1 (The Natural History of the Crustacea). Oxford University Press, New York, chapter 13, pp 337-364.

Boxshall, G.A., Jaume, D., (2009). Exopodites, Epipodites and Gills in Crustaceans. *Arthropod Systematics & Phylogeny*, **67**, 229-254.

Carlisle, D.B., (1957). On the hormonal inhibition of moulting in Decapod Crustacea. II. The terminal anecdysis in crabs. *Journal of the Marine Biological Association of the U.K.*, **36**, 291-307.

Corbari, L., Zbinden, M., Cambon-Bonavita, M-A., Gaill, F., Compere, P., (2008). Bacterial symbionts and mineral deposits in the branchial chamber of the hydrothermal vent shrimp *Rimicaris exoculata*: relationship to moult cycle. *Aquatic Biology,* **1**, 225-238.

Charmantier, G., Charmantier-Daures, M., Aiken, D. E., (1991). Metamorphosis in the Lobster *Homarus* (Decapoda): A Review. *Journal of Crustacean Biology*, **11**, 481-495.

Dickson, J.S., Dillaman, R.M., Roer, R.D., Roye, D.B., (1991). Distribution and characterization of ion transporting and respiratory filaments in the gills of *Procambarus clarkii*. *Biological Bulletin*, **180**, 154-166.

Factor, J.R. (1978). Morphology of the mouthparts of larval lobsters, *Homarus americanus* (Decapoda: Nephropidae), with special emphasis on their setae. *Biological Bulletin*, **154**, 383-408.

Factor, J.R., (1995). *Biology of the Lobster: Homarus americanus*. Academic Press, Sandiago, USA.

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*. *Journal of Experimental Biology*, **201**, 1817-1833.

Hartnoll, R.G., (2001). Growth in Crustacea – twenty years on. *Hydrobiologia,* **449**, 111–122.

Jacques, F., (1989). *The setal system of crustaceans: Types of setae, groupings and functional morphology*. In: Felgenhauer, B.E., Watling, L., Thistle, A.B. (eds). Functional morphology of feeding and grooming in Crustacea. Balkema, Rotterdam, Netherlands, pp 1-14.

Laubmann, A.L. (1913). Untersuchungen uber die Hautsinnesorgane bei decapoden. Krebsen aus der Gruppe der Carididen. *Zoologische Jahrbuecher Abteilung fuer Anatomie und Ontogenie der Tiere*, **35**, 105-159.

Lavalli, K.L., Factor, J.R., (1995). *The feeding appendages*. In: Factor, J.R. (ed). Biology of the Lobster: *Homarus Americanus*. Academic Press, Sandiago, USA, pp 349-392.

Lignot, J.H., Charmantier-Daures, M., Charmantier, G., (1999). Immunolocalization of Na+,K+-ATPase in the organs of the branchial cavity of the European lobster *Homarus gammarus* (Crustacea, Decapoda). *Cell and Tissue Research*, **296**, 417-426.

Lignot, J,H,, Charmantier, G., (2001). Immunolocalization of NA+, K+ -ATPase in the Branchial Cavity During the Early Development of the European Lobster *Homarus gammarus* (Crustacea, Decapoda). *J. Histochem. Cytochem.*, **49**, 1013–1023.

Mesce, K.A., (1993). Morphological and Physiological Identification of Chelar Sensory Structures in the Hermit Crab *Pagurus hirsutiusculus* (Decapoda). *Journal of Crustacean Biology*, **13**, 95-110.

Middlemiss, K.L., Daniels, C.L., Urbina, M.A., Wilson, R.W., (2014). Combined effects of UV irradiation, ozonation, and the probiotic *Bacillus* spp. on growth, survival, and general fitness in European lobster (*Homarus gammarus*). *Aquaculture* (In revision).

Pohle, G., Telford, M., (1981). Morphology and classification of decapod crustacean larval setae: a scanning electron microscope study of *Dissoodactylus crinitichelis* Moreira, 1901 (Brachyura: Pinnotheridae). *Bulletin of Marine Science*, **31**, 736-752.

Pohle, G., (1989). *Gill and embryo grooming in lithodid crabs: Comparative morphology based on Lithodes maja*. In: Felgenhauer, B.E., Watling, L., Thistle, A.B. (eds). Functional morphology of feeding and grooming in Crustacea. Balkema, Rotterdam, Netherlands, pp 75–94.

Reaka, M.L., (1975.) Moulting in stomatopod crustaceans. 1. Stages of the moult cycle, setagenesis, and morphology. *Journal of Morphology*. **146**, 55-80.

Schuwerack, P.M., Lewis, J.W., Jones, P.W., (2001). Pathological and Physiological changes in the South African freshwater crab *Potamonautes warreni* Calman induced by microbial gill infestations. *Journal of Invertebrate Pathology*, **77**, 269-279.

Scolding, J.W.S., Powell, A., Boothroyd, D.P., Shields, R.J., (2012). The effect of ozonation on the survival, growth and microbiology of the European lobster (*Homarus gammarus*). *Aquaculture*, **364-365**, 217-223.

Spicer, J.I., Eriksson, S.P., (2003). Does the development of respiratory regulation always accompany the transition from pelagic larvae to benthic fossorial postlarvae in the Norway lobster *Nephrops norvegicus* (L.)? *Journal of Experimental Marine Biology and Ecology*, **295**, 219–243.

Taylor, H.H., Greenaway, P., (1979). The structure of the gills and lungs of the arid-zone crab, *Holthuisana* (Austrothelphusa) *transversa* (Brachyura: Sundathelphusidae) including observation on arterial vessels within the gills. *Journal of Zoology*, **189**, 359-384.

Thomas, W.J., (1970). The setae of *Austropotambius pallipes* (Crustacea: Astacidae). *Journal of Zoology*, **160**, 91-142.

Wilkens, J.L., McMahon, B.R., (1972). Aspects of branchial irrigation in the lobster *Homarus americanus*. I. Functional analysis of scaphognathite beat, water pressures and currents. *Journal of Experimental Biology*, **56**, 469-479.

Chapter 4

Effects of seawater alkalinity on calcium and acid-base fluxes in juvenile European lobster (*Homarus gammarus*) during a moult cycle

Abstract

Availability of calcium and carbonate ions from surrounding seawater influences the rapid calcification required by newly moulted marine crustaceans for survival postmoult. Calcium content of juvenile European lobster *Homarus gammarus*) was determined during intermoult, immediately postmoult and 24 hours postmoult. The fluxes (excretion or uptake) of NH_4^+ and HCO_3^- were measured at intermoult, 3, 6 and 24 hours immediately postmoult in high and low alkalinity seawater (2000 and 1000 µeq/l). Of the combined total of calcium in the newly moulted animal and shed exoskeleton (exuvia), ~80 % of the calcium was found in the exuvia. The total calcium content of animal and exuvia combined was 44 % less than the intermoult total body calcium. At 24 h postmoult total body calcium was 50 % of the intermoult stage. Net flux of NH_4^+ was the same in both alkalinity treatments groups. Postmoult uptake of HCO_3^- was substantially reduced in the low alkalinity seawater treatment at all three time points (32, 29 and 42 % at 3, 6 and 24 h postmoult, respectively). HCO_3^- uptake in high and low alkalinity seawater peaked at 6 hours with a decrease between 6-24 h postmoult (~20 and 16 % respectively). Exoskeleton calcification was still taking place 24 h postmoult. Net acid excretion (equivalent to net base uptake) was significantly lower in low alkalinity seawater treatments at 3, 6 and 24 h postmoult (~26, 23 and 26 %, respectively). High and low alkalinity seawater treatment net acid fluxes increased over the first 6 h postmoult with a decrease between 6 and 24 h postmoult (~23 and 26 % respectively). Within intensive aquaculture systems, reduced alkalinity is a common occurrence and results showed that low alkalinity substantially slows the calcification rates in juvenile *H. gammarus* after a moult.

1. Introduction

Crustacean growth is dependent on ecdysis (shedding) of the old exoskeleton (exuvia). This involves many complex physiological processes that include; demineralisation of the old exoskeleton, reabsorption and storage of cuticular minerals, limb regeneration, absorption of ion and water components, secretion of the new cuticle, and subsequent recalcification of the new exoskeleton postmoult (Roer & Dillaman, 1984; Aiken & Waddy, 1987). The crustacean exoskeleton mineral matrix consists predominately of calcium

carbonate ($CaCO_3$; Ahearn *et al*., 2004); making up 80% of exoskeleton dry mass (Wheatly, 1997). The regulation of calcium and carbonate are therefore closely linked to the moulting cycle in Crustacea. Availability of Ca^{2+} and HCO_3^- ions in seawater directly affects the rate of recalcification in newly formed crustacean exoskeletons and subsequent growth and survival rates in adults (Greenaway, 1983; Cameron, 1985). A decrease in availability of either ion can cause a negative impact by reducing uptake rates and extending the remineralisation time, making the soft bodied animal more vulnerable to predation. This ion transport process is driven by osmoregulatory structures in the branchial cavity of European lobster, i.e. epipodite and branchiostegite epithelia and gills (Haond, *et al*., 1998).

Crustaceans undergo regular demineralisation/remineralisation processes due to moult frequency associated with the growth cycle. The net reaction that describes all the acid-base changes occurring during calcification is as follows:

$$Ca^{2+} + 2HCO_3^- \Leftrightarrow CaCO_3 + CO_2 + H_2O$$

(from Hoffman *et al*., 2010). So animals that calcify need a reliable source of Ca^{2+} and HCO_3^- ions. Prior to moulting a portion of calcium from the exoskeleton is reabsorbed and stored elsewhere in the body, e.g. gastroliths and hepatopancreas (Greenaway, 1985). A small amount is also stored as raised levels of dissolved Ca^{2+} ions in the haemolymph, but this is negligible in terms of the animal's requirements during calcification (Greenaway, 1985). The amount of reabsorbed and internally stored calcium varies greatly between freshwater and marine species (Graf, 1978; Greenaway, 1985; Wheatly, 1995) (e.g. ~60 % *Orchestia cavimana* (semiterrestrial); 75% *Gecarcinus lateralis* (land crab); 20%, *Panuliris argus* (marine); 15-20%, *Carcinus* (marine). It is suggested by Greenaway (1985) that it is the stored component of calcium that is rapidly mobilised immediately postmoult to calcify feeding appendages (maxillipeds). The rapid hardening of mouthparts is of particular importance to allow animals to feed on their exuvia as a potential additional source of calcium, particularly in fresh water species where calcium availability in the water is usually very low. It is also believed that the stored component helps regulate calcium levels in the haemolymph during the rapid uptake of water during the moult process previously mentioned (Chaisemartin, 1964). In marine species a

high proportion of total body calcium is lost to the exuvia compared to that of freshwater species (92%, *Carcinus*; 25%, *Ligia,* respectively) (Graf,1978). Early quantitative studies of calcium storage in crustaceans during a moult revealed insufficient storage capacity for demineralised exoskeleton components for calcification of the new cuticle (Hecht, 1914). However, in seawater environments internal storage is not such a limiting factor because both calcium and bicarbonate ions are available in abundance from the external environment, with their concentrations typically around 10 and 2 mM, respectively. For bicarbonate ions, in addition to the external seawater source, it can also potentially be provided through the hydration of internal waste CO_2 to form HCO_3^- and H^+ ions (Cameron, 1985). Collectively, the external seawater, internal stores, and ingested material (especially shed exuvia) are all critical components required to fuel recalcification of exoskeletons postmoult.

New postmoult exoskeletons are very soft and unable to provide functional support or nutritional uptake (i.e. protection, food consumption). Animals must therefore rapidly mobilise internal or take up external Ca^{2+} and HCO_3^- in order to harden their exoskeleton to survive (Wheatly, 1999; Wheatly *et al*., 2002). The process begins almost immediately after ecdysis (Greenway 1985). In marine animals these ions are predominately obtained from the water, taken up via the gills and transported into the haemolymph for deposition onto the cuticle. Not surprisingly, calcium fluxes vary greatly at different stages of the moult cycle. Intermoult animals maintain homeostasis with little net movement of calcium in or out (Wheatly, 1997). By contrast, demineralisation of the exoskeleton at premoult results in a large efflux of calcium and bicarbonate, while during post moult there is a rapid influx of calcium and bicarbonate from external sources to aid in hardening the new exoskeleton (Greenaway, 1985; Wheatly *et al*., 2002). Research by Li and Cheng (2012) on white leg shrimp *Litopenaeus vannamei* showed ~18 % decrease in cuticle Ca^{2+} between intermoult and postmoult (113 -107 mg g^{-1} respectively).

In addition to low alkalinity, studies have been done on the effects of low pH seawater on carapace calcification in larval and megalopa stages of *H. gammarus* (Arnold *et al*., 2009). Spicer and Eriksson (2003) also researched carapace calcification in larval, megalopa and juvenile animals in the closely related Norway lobster (*Nephrops norvegicus*). Alkalinity, pH and pCO_2 are

inextricably linked, and therefore the resulting consequence of a change in one parameter is simultaneous change in either/both of the others (i.e. changing pH will by default result in subsequent increase/decrease in pCO_2 and/or alkalinity) (Orr *et al*., 2005). Acid-base regulation is known to be influenced by changes in pCO_2 which also affects internal calcification processes (Orr *et al*., 2005). The effects of 'ocean acidification' caused by rising levels of atmospheric CO_2 has been the subject of intense scrutinisation by scientists in recent years. The focus of the current study was to avoid the issue of pCO_2 and focus on the effects of low alkalinity (i.e. low bicarbonate availability for uptake by lobsters to fuel calcification). Therefore, pCO_2 was kept constant whilst varying alkalinity. As a result, by reducing alkalinity to reproduce aquaculture conditions, pH was also unavoidably reduced. However, it is commonly considered by physiologists that changes to seawater pH within the current range used (~8.1 to ~7.8) would be unlikely to cause the effects observed in this study. Changes in calcification under the conditions used here are therefore almost certainly entirely due to reduced bicarbonate availability (by half) in the seawater for uptake via the gills.

The aim of the current study was to investigate; (1) total body calcium in megalopa intermoult animals, immediate postmoult juvenile animals + exuvia, and 24 h postmoult juvenile animals; (2) uptake rates of Ca^{2+} and HCO_3^- at different times in the moulting cycle between megalopa and juvenile stages of *Homarus gammarus* (European lobster). In addition, the fluxes of these ions during the first 24 hours postmoult were measured in normal and low alkalinity seawater (TA ~2000 and 1000 µeq/l respectively). These growth stages are of particular relevance to the European lobster aquaculture industry that grows benthic larvae stages through to early pelagic juvenile stages for release as part of stock enhancement programmes. Furthermore, the alkalinity of seawater can be dramatically reduced in intensive aquaculture conditions due to the combined effects of biofiltration processes (specifically nitrification) (Eshchar *et al*., 2006), and calcification by the cultured organisms themselves.

2. Methods

2.1 Animals

Animals were grown within the National Lobster Hatchery (NLH; Padstow, North Cornwall, UK) until five day old stage IV megalopa at which point 120 individuals were transferred to the University of Exeter (Devon, UK) and held in the Aquatic Resource Centre within the Biosciences department. A resting period of four days was observed before any experimental work was carried out to allow recovery from any disturbance endured during transportation. The moult cycle was approximately 16 days between stages IV and V. Intermoult animals were sampled 8 days post stage IV metamorphosis. Postmoult sampling was conducted during the first 24 hours immediately after the juvenile stage V moult. Animals were fed a diet of NLH formulated pellet feed once daily and housed in individual cells within a closed recirculation seawater system. Water temperature and salinity were maintained at 21.5° C and 35 PSU respectively. Photoperiod was set at 0800 hours (dawn) and 2000 hours (dusk).

2.2 Experimental setup

For measuring the fluxes of calcium and acid-base equivalents between lobsters and the ambient seawater, individual animals were transferred into 40 mL aerated chambers that were partially submerged in a water bath maintained at 21.5 °C. Flux measurements were carried out in seawater with one of two different total alkalinities; ~2000 µmol/L (control), pH ~8.12 or ~1000 µmol/L (low alkalinity), pH ~7.86. Each treatment was formulated from an artificial sea salt mix (Tropic Marin; TMC, UK), in deionised water. Sufficient hydrochloric acid (1 M) was added to each seawater treatment to achieve the desired total alkalinity and pH values following vigorous overnight aeration (i.e. equilibration with atmospheric CO_2 ~ 400 ppmv). Intermoult animals were measured for 24 hours (n=15) in control seawater only (TA = 2000 µmol/L). To assess the ion flux rates, seawater samples were taken to represent the initial and final conditions within each chamber. Initial samples were taken from the same stock water used to fill the chambers at the same time that each animal was placed in its flux chamber. Final seawater samples were taken at the end of each flux period (actual duration was recorded to the nearest minute). Seawater samples were preserved and stored at 4 °C for titratable alkalinity (see below),

frozen at -20 °C for total ammonia, and immediately diluted (see below) and stored at -20 °C for calcium analysis. Postmoult experiments were carried out on newly moulted animals (n=10) within 30 minutes of moulting. Animals moulted naturally and at different times over a period of 2 days, and they were distributed alternately to chambers containing either control seawater or low alkalinity seawater. This ensured an equal number of animals exposed to each treatment, and limited any bias associated with time of moult. At the start of each flux measurement animals were rinsed (in the relevant seawater) and transferred to their chamber containing clean seawater of the relevant alkalinity. Fluxes were measured from 0 to 3, 3 to 6, and 6 to 24 hours post moult.

Using a separate group of lobsters that were undergoing the same moult, samples were taken for measuring the calcium content of body parts. These included whole bodies sampled during the intermoult (n=15); postmoult whole body (n=8) and exuvia (n=8); and 24 hour postmoult whole body (n=10). Animals were placed in a -80° C freezer to euthanise. They were blotted dry, weighed then dried in a 40° C oven for ~48 hours in pre-dried and weighed centrifuge tubes. Following drying samples were placed in 1.5 ml of 5 % (w/v) sodium hypochlorite (NaOCl) until all organic components were removed leaving only the remaining white inorganic carbonate exoskeleton. Exoskeletons were then rinsed in deionised water three times to remove traces of the hypochlorite, centrifuging and decanting the supernatant between rinses.

The cleaned exoskeletons were then dried (as above), weighed and digested in a volume of 1 M HCl (40 µL acid per mg of sample) as discussed in Walther *et al*., (2011).

2.3 Ion analysis

Seawater samples for measuring titratable alkalinity were preserved (4 µl of 4 % (w/v) mercuric chloride per 10 mL of seawater) and stored at 4 °C until analysis by double titration using Metrohm double titrator (815 Robotic USB Sample Processor XL, Switzerland). Methods for ion analysis were carried out as described in Whittamore *et al*., (2010). Alkalinity was measured by titration of a 20 mL sample to pH 3.89 with 0.02 N HCl whilst gassing with CO_2-free nitrogen, followed by return to the starting pH with 0.02 N NaOH. Ammonia concentration was measured using a modified version of the colourimetric

method of Verdouw *et al*., (1978) using a microplate reader (Infinite® M200 PRO, Tecan, UK). Seawater samples (200 µL) were pipetted into microplates and 25 µL of each of three reagent solutions were added and mixed in between additions in the following order: (1) sodium salicylate, (3.63 M); (2) catalyst and complexing reagent; (sodium nitroprusside (0.67 mM) and sodium citrate (1.19 M)); (3) alkaline hypochlorite; (sodium hydroxide (1 M) and sodium hypochlorite (5% (w/v)).

2.4 Calculations:

Net calcium and acid-base fluxes (positive values representing uptake and negative values representing excretion) were calculated (µeq kg^{-1} h^{-1}) using the following equation:

$$J_X = [([X]_i - [X]_f * V]/(M*t)$$

as described in Wilson and Grosell (2003) where V is the volume of water (l); M is the mass of the lobster (g); t is the duration of the flux period (h); and $[X]_i$ and $[X]_f$ are the ion concentrations in the seawater (µmol L^{-1}) at the beginning and end of the flux period, respectively. Titratable acid fluxes (J_{TA}) were calculated from the above equation using titratable alkalinity measurements (essentially $[HCO_3^- + 2\ CO_3^{2-}]$), but reversing the initial and final values to achieve acid instead of base fluxes. The net flux of acidic equivalents (J_{H+}^{net}) was then calculated as the sum, signs considered, of flux of J_{TA} and the ammonia flux (J_{Amm}) as described by McDonald and Wood (1981). Calcium concentrations in the sea water and total body calcium were measured by ion chromatography (Dionex ICS-1000) and flame photometry (Corning 410), respectively. Seawater samples for calcium analysis were diluted 401-fold (25 µL sample added to 10 ml ultrapure deionised water). Acid-digested body samples for calcium analysis were diluted 201-fold by adding 15 µL sample to 3 mL ultrapure deionised water.

2.5 Statistical analysis

Analysis was carried out using SigmaPlot V.11.0 (Systat Software Inc., USA) and data are presented as the mean ± SEM. Total body Ca^{2+} data was log^{10} transformed and analysed using a one-way ANOVA. Data for H^+, HCO_3^+ and NH_4^+ were analysed using a two-ways ANOVA (factors time and

treatment). Data that did not meet parametric assumptions of equal variance and normal distribution were transformed using sqrt (NH_4^+). All data were subjected to post hoc analysis (Tukey). Differences were considered significant with P value ≤0.05.

3. Results

3.1 Total body calcium

The total body Ca^{2+} in European lobster and exuvia was measured at various intervals during a moult cycle; 1) intermoult (whole animal), 2) immediate postmoult exuvia, 3) immediate postmoult animal (exoskeleton shed), and 4) 24 h postmoult (whole animal). Data shown in Fig. 1A, B. Immediately after moulting the animals retained significantly less total body Ca^{2+} (~90% decrease), than was found during intermoult period ($P<0.05$). Almost three times as much Ca^{2+} was present in the discarded exuvia compared to the freshly moulted body. The combined Ca^{2+} content of the postmoult body plus exuvia was only about 70 % of the Ca^{2+} content of the whole animal during intermoult ($P<0.001$) (Fig. 1B). The mean percentage of both the newly moulted animal and corresponding exuvia (representing 100 % total body Ca^{2+}) was 81 % and 19 % respectively (SEM 8 %), and are significantly different ($P<0.001$). After a 24 h period the newly moulted animal had a four-fold increase in its Ca^{2+} content (compared to the immediate postmoult body), but this was still less than 50 % of the whole animal Ca^{2+} content during the previous intermoult period ($P<0.001$).

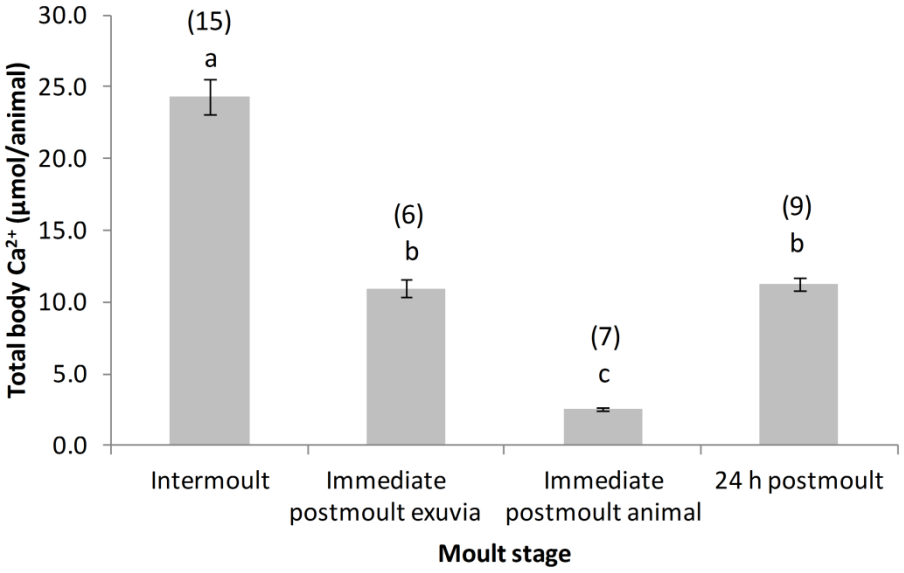

Fig. 3. Total body calcium in European lobster at intermoult (megalopa), exuvia immediately postmoult and whole animal immediately postmoult and 24 h postmoult (juvenile). Numbers in parenthesis () are sample sizes. Data presented as the mean ± SEM. Significant differences represented by letters and significance accepted at P≤0.05.

3.2 Acid-base fluxes

Net H^+ flux is calculated from the difference between measured NH_4^+ and HCO_3^- fluxes. During intermoult periods (Fig. 2A, B, C), lobsters exhibited low excretion rates of both NH_4^+ and of HCO_3^-. Bicarbonate (base) excretion was ~70 % higher than the NH_4^+ (acid) excretion rate, and the difference between these two variables therefore culminates in a positive net H^+ flux (representing net base excretion or acid uptake). In the first three hours immediately postmoult, NH_4^+ excretion was five-fold higher than the intermoult rate (Fig. 2A). After peaking during the first 3 hours postmoult, ammonia excretion rate then steadily declined at each subsequent flux period postmoult in relation to the 3 h postmoult value, by ~60 % (3 to 6 h ($P<0.001$)) and a further ~34 % (6 to 24 h postmoult ($P=0.014$)), approaching the intermoult excretion rates. Low alkalinity had no effect on net NH_4^+ excretion rates between treatments ($P=0.087$).

Figure 2B shows that during postmoult periods HCO_3^- fluxes were reversed in comparison to intermoult (i.e. HCO_3^- uptake instead of excretion), and were of much greater magnitude (~6 fold peak increase in high alkalinity seawater treatment 3-6 h postmoult). This peak was also observed for the same time

period (3-6 h postmoult) in the low alkalinity seawater treatment. Under low alkalinity conditions, net bicarbonate uptake rates were substantially smaller than high alkalinity conditions (32, 29 and 42 % respectively for the 0-3, 3-6 and 6-24 h periods). There was no interactive effect between time and treatment ($P=0.080$) in high and low alkalinity HCO_3^- treatment groups. At 0-3 h postmoult there was significantly lower influx of HCO_3^- in the high alkalinity treatment ($P=0.027$) than at 3-6 h postmoult (Fig. 2B). At 0-3 and 3-6 h postmoult there was a significantly higher influx of HCO_3^- in the high alkalinity treatment group than low alkalinity ($P=0.029$ and $P<0.001$ respectively). In both high and low treatment groups, HCO_3^- showed an increase between 3 and 6 h postmoult and then a decrease at 6-24 h postmoult, however rates were still higher than at the 0-3 h time point.

Rates of HCO_3^- uptake were substantially larger than the amount of NH_4^+ excretion resulting in HCO_3^- having a far greater impact on net H^+ fluxes (Fig. 2C). Subsequently, during postmoult periods, all animals displayed large net acid excretion rates (i.e. negative net H^+ fluxes, equivalent to large net base uptake). Analysis showed significant differences between high and low alkalinity treatment groups at 0-3, 3-6 and 6-24 h postmoult ($P=0.016$, $P=0.023$, $P=0.043$), respectively (Fig. 2C). There were no interactive effect of factors time and treatment ($P=0.954$). Between 6 and 24 h postmoult, analysis showed a significant difference in net H^+ uptake ($P=0.016$) between these time points, however post hoc analysis failed to identify which treatment group was significantly different (Fig. 2C). Net H^+ fluxes were highest from 3-6 h postmoult and then from 6-24 h they decreased to below the 0-3 h rate in both alkalinity treatment conditions.

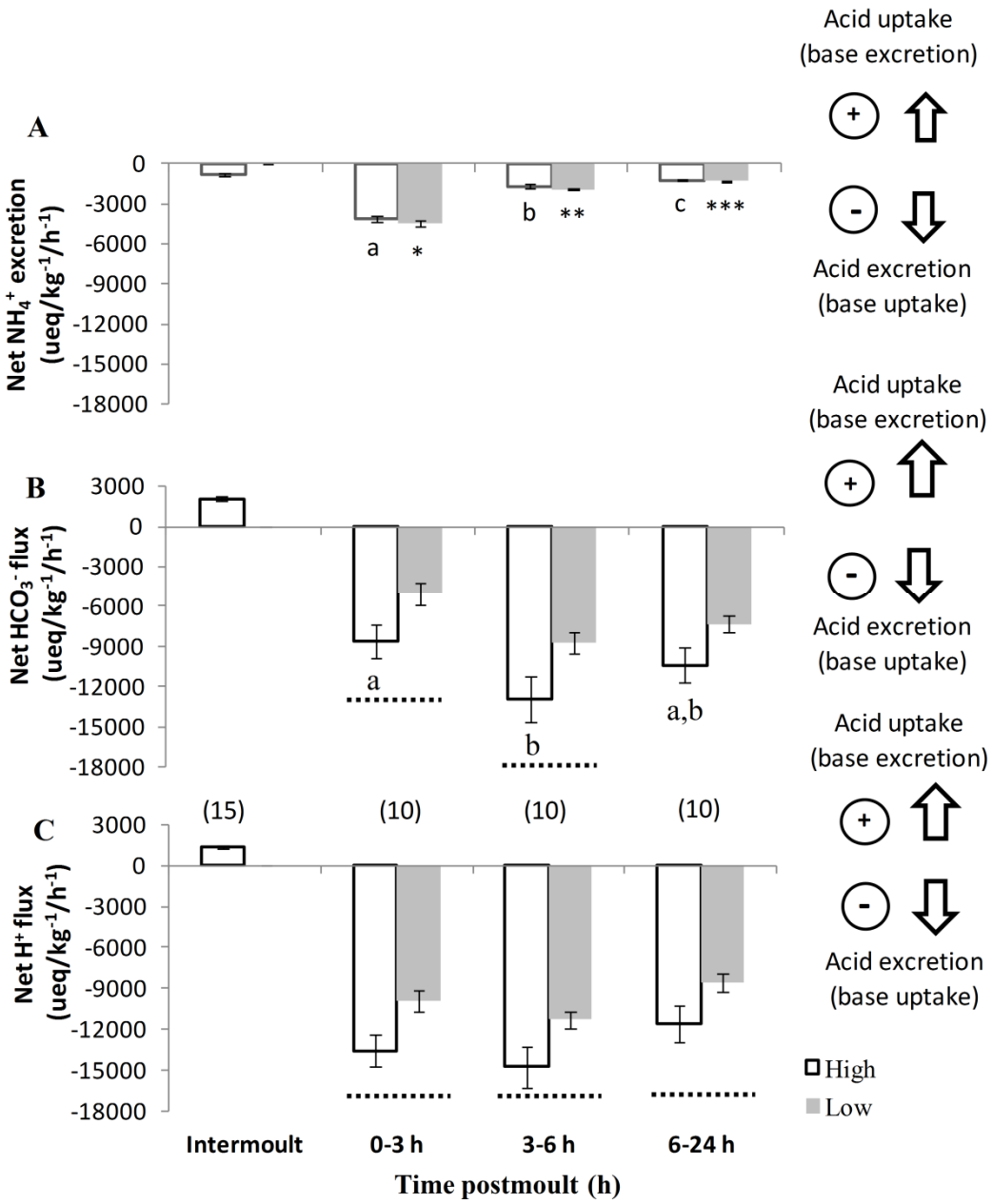

Fig. 4. Net NH_4^+ flux (A), net HCO_3^- flux (B) and H^+ flux (C) in intermoult (megalopa), and 0-3, 3-6, and 6-24 h postmoult (juvenile) European lobsters in high and low alkalinity seawater treatments. Numbers in parenthesis represent sample size (). Data represent the mean ± SEM. Significant differences between time points in high alkalinity treatment groups indicated by asterisks (*) and between treatment groups at each time point as indicated with dotted line.

4. Discussion

Crustacean species moult as part of the growth process and in doing so newly formed exoskeletons require calcification. This occurs through the uptake of Ca^{2+} from the surrounding water and to a lesser degree (in marine crustaceans), mobilisation of Ca^{2+} stores (e.g. gastroliths), stored as a result of the premoult demineralisation process. The major component of the exoskeleton matrix is calcium carbonate ($CaCO_3$), therefore Ca^{2+} and CO_3^{2-} availability are critical to shell hardening. These can become a limiting factor in the natural environment and in aquaculture facilities, depending on environmental conditions (e.g. changes to salinity and alkalinity). In freshwater environments the level of the cation Ca^{2+} varies enormously from < 50 µM to > 5 mM (depending on factors such as underlying geology and rainfall), whereas in marine environments Ca^{2+} levels are far higher and more consistent, typically being ~10 mM (Greenaway, 1985). Therefore, in marine environments calcium availability is not normally a limiting factor. However, in recirculating marine aquaculture systems, where animals are grown in large numbers and moult frequently (e.g. *Homarus gammarus*), calcium availability in the recirculating seawater can be reduced through Ca^{2+} uptake by moulting calcifiers.

The availability of the anion required for exoskeleton calcification is more complicated. This is because metabolism can generate these anions endogenously from the hydration of metabolic waste CO_2 (forming HCO_3^- and H^+ ions) and the subsequent dissociation of HCO_3^- to CO_3^{2-} and an additional H^+ ion. Providing excessive accumulation of the resulting protons can be avoided (by acid excretion) animals can therefore generate their own carbonate ions without the need for an exogenous source. This endogenous source can therefore act as a supplementary supply, in addition to seawater HCO_3^- ions that can be taken up via the gills. However, the rates of calcification and therefore need for carbonate ions, probably exceed the potential supply via metabolic CO_2 production during the immediate post moult period (Cameron and Wood, 1985). Therefore, HCO_3^- uptake from external seawater is probably quantitatively the most important source for calcification. However, the availability of HCO_3^- ions in seawater is typically ~5 times lower than for Ca^{2+} ions and much less stable than Ca^{2+} in natural marine environments. The availability of HCO_3^- ions is often even lower in recirculating seawater systems.

This is partly due to the fact that microbially-mediated nitrification (conversion of ammonia to nitrate) within the biofiltration systems also consumes HCO_3^- ions (Eshchar *et al*., 2006). As a result the alkalinity of seawater in recirculating systems declines over time if seawater replacement rates are not high enough. In addition, HCO_3^- is obviously removed by moulting marine calcifiers themselves, which can also negatively contribute to availability over time.

4.1 Total body calcium

The current study showed that immediate postmoult juvenile *H. gammarus* lost around 90 % of total Ca^{2+} content present at intermoult to either the exuvia or the surrounding water, leaving only 10 % in the newly moulted animal. This is similar to findings by Graf (1978) in *Carcinus maenus*, supporting the knowledge that marine crustaceans readily acquire the required amounts of Ca^{2+} from surrounding water and not from Ca^{2+} stores. Also, the combined sum of Ca^{2+} in the newly moulted animal and the shed exuvia was 44 % less than in the intermoult animal indicating that around 66% of Ca^{2+} during the demineralisation process is lost to the surrounding medium. At 24 h postmoult, total body Ca^{2+} levels were still <50 % of intermoult animals showing that calcification of the new exoskeleton continued beyond 24 h postmoult. This corresponds with the continued rates of HCO_3^- uptake 24 h postmoult as discussed below in section 5.2. Increased time for calcification results in higher energetic costs reducing energy required for other physiological processes such as growth (Keppel *et al*., 2012). Slower calcification also means a longer period of vulnerability to predators while the exoskeleton is not sufficiently hardened to offer good protection.

Tolerance to low pH or alkalinity, in respect to calcification, varies greatly among benthic crustacean species (Ries *et al*., 2009). It is suggested that this may be related to pH regulation at calcification sites (i.e. in the cuticle) (Ries *et al*., 2009). Research by Arnold *et al*. (2009) showed that mineralisation of the carapace in *H. gammarus* was decreased in low pH environments induced by elevated CO_2, and they concluded that this was due to internal (haemoloymph) acidosis caused by the elevated external CO_2 rather than reduced external seawater pH itself. However, rates of calcification in juvenile American lobster

(*Homarus americanus*), closely related to *H. gammarus*, were not reduced in treatments using CO_2- induced pH levels similar to that used by Arnold *et al*., (2009) in *H. gammarus* (Ries *et al*., 2009). Results from the current study for juvenile *H. gammarus* show a reduced rate of calcification in low seawater alkalinity (and low pH) treatments in which CO_2 was kept constant. Reduced seawater alkalinity in a recirculating aquaculture environment could therefore result in delayed hardening of the exoskeleton. This could potentially result in higher postmoult mortalities as a result of enhanced cannibalism whilst the soft bodied animals are unable to defend themselves. However, alkalinity of seawater in aquaculture systems can be controlled through management of water quality (i.e. enrichment of rearing water with calcium, or more likely with bicarbonate as the greater limiting factor). Obviously, upon release of cultured lobster into their natural environment they would then be faced with any of the prevailing natural environmental pressures. Over the coming centuries this would include ocean acidification which will not change Ca^{2+} availability, but will reduce the concentration of CO_3^{2-} ions, whilst HCO_3^- would be largely unchanged (in fact slightly increased). It is not yet known whether such changes would influence calcification in lobster larvae. Ocean acidification conditions have been shown to reduce calcification in some marine calcifiers but increase it in others (Beardall and Raven, 2013; Wood *et al*., 2008), so it is difficult to predict how lobsters will respond based on our current understanding.

4.2 Acid-base fluxes

Calcification in *Homarus* species is known to take place rapidly during the first one or two days postmoult (Horne and Tarsitano, 2007). This rapid calcification rate is critical in newly moulted animals, whose soft bodies and mouthparts are unable to be used for protection or feeding immediately postmoult. Length of time required to calcify a new exoskeleton is dependent on two factors: availability of calcium and carbonate in the medium and their rate of uptake (Greenaway, 1985, Neufeld and Cameron, 1994). Therefore, in order to achieve maximal uptake rates, high concentrations of these ions must be available in the water, and reduced amounts will result in a longer calcification time. As discussed above, carbonate ions (CO_3^{2-}) for exoskeleton calcification can be generated endogenously. However, a key source during rapid postmoult calcification is via the uptake of external seawater bicarbonate ions (HCO_3^-),

which subsequently dissociate internally to liberate CO_3^{2-} ions at the site of calcification (Giraud, 1981; Henry and Cameron, 1983). Therefore, when seawater alkalinity (essentially equivalent to the sum of HCO_3^- + $2CO_3^{2-}$ ion concentrations) is reduced this reduces HCO_3^- uptake at the gills and subsequently the availability of internal CO_3^{2-} ions for calcification. The current study investigated effects of changing seawater alkalinity on rates of HCO_3^- uptake at intervals (0-3, 3-6 and 6-24 h), during the first day immediately after moulting. In low alkalinity seawater, rates of calcification were dramatically reduced due to reduced HCO_3^+ uptake (32-42 % across the three flux periods postmoult). Net H^+ excretion was also much lower in the low alkalinity seawater during all three flux periods, in line with decreases in uptake of HCO_3^- (NH_4^+ excretion was unaffected by seawater alkalinity so had no influence on the net H^+ excretion rates). Similar results were found in adult blue crab (*Callinectes sapidus*) by Cameron (1985), which showed that reduced HCO_3^- in seawater led to reduced influx of HCO_3^- and net efflux of H^+ ions. This would clearly translate into low alkalinity environments causing a dramatic increase in the time it takes to complete calcification. This is very likely to be detrimental to the successful culture of early development stages in lobsters and other crustacean. This would be for two reasons. Firstly, slow hardening of the mouthparts may delay the initiation of feeding which would result in metabolically active larval/juvenile animals quickly depleting energy reserves. Therefore, a fast return to active feeding will be essential for their subsequent survival postmoult. Secondly, lobsters are highly cannibalistic, and larvae that are slower to harden their exoskeletons will be even more prone to predation by their intermoult peers in intensive aquaculture situations. The data presented here therefore supports the monitoring and management of seawater chemistry, particularly alkalinity but also calcium concentrations, with a view to maximising the speed of postmoult calcification and subsequent larval survival.

5. Conclusion

Crustaceans require vast amounts of calcium and carbonate to harden their new exoskeletons after ecdysis and in *H. Gammarus* these are largely acquired from surrounding seawater. Prior to and during a moult, most of the bodily calcium present during intermoult periods is lost either to the shed exuvia or via excretion to the surrounding medium. Rates of recalcification depend on the availability of both calcium and carbonate, the latter of which is significantly reduced in low alkalinity seawater. Corresponding rates of bicarbonate uptake (equivalent to H^+ excretion) are also reduced in low alkalinity seawater. These factors result in slower calcification rates and hence prolonged time to complete mineralisation (and therefore hardening) of the exoskeleton. Such a delay will result in increased use of vital and limited energy resources in these larval animals, which may impact on other physiological processes involved after a moult and could ultimately reduce survival especially if restoration of normal feeding behaviour is also delayed. Seawater is normally high in calcium ions, however during the aquaculture of *H. gammarus* in larviculture facilities, calcium and carbonate availability can be depleted from the seawater by nitrification within the biofiltration system, as well as through uptake by the animals. This study shows the significance of maintaining alkalinity in larviculture systems to ensure that maximum calcification rates are achieved quickly to preserve the health of the animal and ultimately increasing survival rates.

6. References

Ahearn, G.A., Mandal, P.K., Mandal, A., (2004). Calcium regulation in crustaceans during the molt cycle: a review and update. *Comparative Biochemistry and Physiology Part A*, **137***,* 247–257.

Aiken, D. L. & Waddy, S. L., (1987). Molting and growth in crayfish: a review. *Canadian Technical Report of Fisheries and Aquatic Sciences*, **1587**, 1-34.

Arnold, K.E., Findlay, H.S., Spicer, J.I., Daniels, C.L., Boothroyd, D., (2009). Effect of CO_2-related acidification on aspects of the larval development of the European lobster, *Homarus gammarus* (L.). *Biogeosciences*, **6**, 1747–1754.

Beardall, J., Raven, J.A., (2013). Calcification and ocean acidification: new insights from the coccolithophore *Emiliania huxleyi*. *New Phytologist*, **199**, 1-3.

Cameron, J.N. and Wood, C.M., (1985). Apparent H^+ excretion and CO_2 dynamics accompanying carapace mineralization in the blue crab (*Callinectes sapidus*) following moulting. *Journal of Experimental Biology*, **114**, 181-196.

Chaisemartin, C., (1964). Importance des gastrolithes dans 1'economie du calcium chez Astacus pallipes Lereboullet. *Vie Milieu*, **15**, 457-474.

Eshchar, M, Lahav, O., Mozes, N., Peduel, A. and Ron, B., (2006). Intensive fish culture at high ammonium and low pH. *Aquaculture*, **255**, 301–313.

Findlay, H.S., Kendall, M.A., Spicer, J.I. and Widdicombe, S., (2009). Future high CO_2 in the intertidal may comprise adult barnacle *Semibalanus balanoides* survival and embryonic development. *Marine Ecology Progress Series*, **389**, 193–202.

Giraud, M-M., (1981). Carbonic anhydrase activity in the integument of the crab *Carcinus maenas* during the intermolt cycle. *Comparative Biochemistry and Physiology*, **69**, 381–387.

Graf, F., (1978). Les sources de calcium pour les crustaces venant de muer. *Archives of Zoological Experimental Genetics*, **119**, 143-161.

Greenaway, P., (1983). Uptake of calcium at the postmolt stage by the marine crabs *Callinectes sapidus* and *Carcinus maenas*. *Comparative Biochemistry and Physiology*, **75A**, 181-184.

Greenaway, P., (1985). Calcium balance and moulting in Crustacea. *Biological Reviews Cambridge Philosophical Society*, **60**, 425-454.

Hecht, S., (1914). Note on the absorption of calcium during the molting of the blue crab, *Callinectes sapidus*. *Science*, **39**, 108.

Henry, R.P. and Cameron, J.N., (1983). The role of carbonic anhydrase in respiration, ion regulation and acid-base balance in the aquatic crab *Callinectes sapidus* and the terrestrial crab *Gecarcinus lateralis*. *Journal of Experimental Biology*, **103**, 205–223.

Horne, F.R. and Tarsitano, S.F., (2007). *The Mineralization and Biomechanics of the Exoskeleton*. In Lavalli, K.L. and Spanier, E. (Eds), The Biology and Fisheries of the Slipper Lobster, pp 183-198. CRC Press: USA.

Keppel, E.A., Scrosati, R.A. and Courtenay, S.C., (2012). Ocean acidification decreases growth and development in american lobster (*Homarus americanus*) larvae. *Journal of Northwest Atlantic Fisheries Science*, **44**, 61–66.

Li, C-H., Cheng, S-Y., (2012). Variation of calcium levels in the tissues and haemolymph of *Litopenaeus vannamei* at various molting stages and salinities. *Journal of Crustacean Biology*, **32**, 101-108.

McDonald, D.G., Wood, C.M., (1981). Branchial and renal acid and ion fluxes in the rainbow trout*, Salmo gairdneri*, at low environmental pH. *Journal of Experimental Biology*, **93**, 101-118.

Neufeld, D.S., Cameron, J.N., (1994). Effect of the external concentration of calcium on the postmoult uptake of calcium in blue crabs (*Callinectes sapidus*). *Journal of Experimental Biology*, **188**, 1-9.

Orr, J.C., Fabry, V.J., Aumont, O., Bopp, L., Doney, S.C., Feely, R.A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R.M., Lindsay, K., Maier-Reimer, E., Matear, R., Monfray, P., Mouchet, A., Najjar, R.G., Plattner, G-K., Rodgers, K.B., Sabine, C.L., Sarmiento, J.L., Schlitzer, R., Slater, R.D., Totterdell, I.J., Weirig, M-F., Yamanaka, Y., Yool, A., (2005). Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature*, **437**, 681-686.

Ries, J.B., Cohen, A.L. and McCorkle, D.C., (2009). Marine calcifiers exhibit mixed responses to CO_{2-} induced ocean acidification. *Geology*, **37**, 1131-1134.

Roer, R., Dillaman, R., (1984). The structure and calcification of the crustacean cuticle. *American Zoologist*, **24**, 893-909.

Spicer, J.I. and Eriksson, S.P., (2003). Does the development of respiratory regulation always accompany the transition from pelagic larvae to benthic fossorial postlarvae in the Norway lobster *Nephrops norvegicus* (L.)? *Journal of Experimental Marine Biology and Ecology*, **295**, 219–243.

Verdouw, H., Van Echetld, C.J.A., Dekkers, E.M.J., (1978). Ammonia determination based on indophenols formation with sodium salicylate. *Water Research*, **12**, 399-402.

Walther, K., Sartoris, F.J., Portner, H.O., (2011). Impacts of temperature and acidification on larval calcium incorporation of the spider crab *Hyas araneus* from different latitudes (54° vs. 79°N). *Marine Biology*, **158**, 2043–2053.

Wheatly, M.G., Ayers, J., (1995). Scaling of calcium, inorganic contents, and organic contents to body mass during the moulting cycle of the fresh-water crayfish *Procambarus clarkii* (Girard). *Journal of Crustacean Biology*, **15**, 409-417.

Wheatly, M.G., (1997). Crustacean models for studying calcium transport: the journey from whole organisms to molecular mechanisms. *Journal of the Marine Biology Association of the U.K.*, **77**, 107-125.

Wheatly, M.G., (1999). Calcium homeostasis in Crustacea: The evolving role of branchial, renal, digestive and hypodermal epithelia. *Journal of Experimental Zoology*, **283**, 620-640.

Wheatly, M.G. Zanotto, F.P., Hubbard, M.G., (2002). Calcium homeostasis in crustaceans: subcellular Ca dynamics. *Comparative Biochemistry and Physiology* Part B, **132**, 163–178.

Whittamore, J. M., Cooper, C. A., Wilson, R. W., (2010). HCO_{3-} secretion and $CaCO_3$ precipitation play major roles in intestinal water absorption in marine teleost fish in vivo. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, **298**, 877-886.

Wilson, R.W., Grosell, M. (2003). Intestinal bicarbonate secretion in marine teleost fish - source of bicarbonate, pH sensitivity, and consequences for whole animal acid-base and calcium homeostasis. *Biochimica Et Biophysica Acta (bba) - Biomembranes*, **1618**, 163-174.

Wood, H.L., Spicer, J.I., Widdicombe, S., (2008). Ocean acidification may increase calcification rates, but at a cost. *Proceedings of the Royal Society B: Biological Sciences*, **275**, 1767-1773.