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Bolstering the wild populations of white-clawed crayfish *Austropotamobius pallipes*, through captive breeding, rearing and release of juveniles into favourable *in-situ* habitats

Jennifer Anne Nightingale

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of PhD in the Faculty of Life Sciences.

School of Biological Sciences

University of Bristol

July 2020

Word count 35,544

Abstract

The white-clawed crayfish *Austropotamobius pallipes,* is a freshwater crustacean native to Europe and is endangered throughout its range due to habitat fragmentation, pollution, competition with the invasive American signal crayfish *Pacifastacus leniusculus,* and the associated disease, crayfish plague (*Aphanomyces astaci*). Production of captive-born *A. pallipes* for wild release into ark sites is a recognised conservation measure to help halt the decline of this species within the UK.

The aim of this thesis was to optimise aquaculture methodologies to maximise *ex-situ* production of *A. pallipes*, whilst exploring effective techniques to monitor released individuals, so that their activity patterns and long-term survival could be assessed. A series of hatchery experiments were conducted investigating stocking density, grading and dietary regimes for rearing juveniles. Key findings were that young-of-the-year *A. pallipes* can be reared at high densities (300/m^2) without compromising survival; however, the optimal stocking density that maximised growth and health was 100/m^2. Juveniles exhibit sexual dimorphism as early as six-months of age, and sex, rather than size, was the main factor that led to dominance hierarchies and growth suppression in juveniles. Maintaining juvenile *A. pallipes* in single-sex groups was optimal. Live food was optimal for high survival and growth in hatchlings, and a plankton diet produced increased growth in juveniles than a pellet diet.

Key findings for the tagging and tracking studies were that *A. pallipes* with a carapace length of 21 mm and above can be safely injected with passive integrated transponder (PIT) tags without survival or growth being compromised. Detection of PIT-tagged crayfish is limited to 150 mm, which is reduced to a 45 mm detection range when crayfish are within refuges. Long-term tracking using acoustic telemetry can provide useful data on activity patterns*; A. pallipes* are strongly nocturnal and are most active in spring and autumn. However, the methodology may be limited when used in still-water sites possibly due to interference from surfaces or other sound waves.

The findings from this thesis have influenced the aquaculture methodologies now used at the Bristol Zoological Society's crayfish hatcheries. This has led to increased productivity and more wild releases, which are significantly contributing to the conservation effort for this species, helping to halt its decline in south west England.

Foreword

This PhD study has been funded by the Bristol Zoological Society and Mohammed Bin Zayed Conservation Grant. Throughout the course of my studies I continued working in my role as UK Conservation Manager at the Bristol Zoological Society, overseeing the crayfish hatcheries at Bristol Zoo Gardens, Priddy and Ubley, whilst delivering practical crayfish conservation within my role as a lead partner of the South West Crayfish Partnership Programme. I was also part of several research collaborations including co-supervising a MSc, investigating terrestrial movements of *A. pallipes* and assiting with *ex-situ* and ark site detection of *A. pallipes* using environmental DNA.

In total, I have led the writing and analysis for four scientific papers published during my PhD, and a fifth paper has been submitted and is currently under review. A sixth paper is currently being written up for publication.

Scientific publications:

1. Nightingale, J., Stebbing, P., Sibley, P., Brown, O., Rushbrook, B., & Jones G. (2017). The use of ark sites and associated conservation measures to secure the long-term survival of white-clawed crayfish in the UK. *International Zoo Year Journal, 51*, 50-68.

2. Nightingale, J., Stebbing, P., Taylor, N., McCabe G., & Jones, G. (2018). Determining an effective density regime for rearing juvenile *Austropotamobius pallipes* in a small-scale closed system hatchery. *Aquaculture Research, 49,* 3055-3062.

3. Nightingale, J., Stebbing, P., Taylor, N., McCabe G., & Jones, G. (2018). The effect of size-grading for rearing young-of-the-year white-clawed crayfish *Austropotamobius pallipes. Aquaculture Research, 49,* 3116-3122.

4. Nightingale, J., Stebbing, P., Taylor, N., McCabe G., & Jones, G. (2018). The long-term effects and detection ranges of passive integrated transponders in white-clawed crayfish *Austropotamobius pallipes. Knowledge and Management of Ecosystems, 419,* 20-28.

Dedication and acknowledgments

I would like to dedicate this thesis to my family. To my mother Iris Nightingale and my father, 'Pops 88', who encouraged me so much and patiently listened to my incessant ramblings, at every stage of my studies; forever enthusiastic and willing to impart guidance. I am so sorry and sad that you did not witness me reaching the end of this journey, but thank you for helping me to get here; I know how proud you would both be to witness this moment! To my sisters Claire and Sarah for believing in me and showing kindness and support all the way through this journey. To Timmo, my amazing best friend and husband; it was you who told me I could (and should) do this, and who gifted me a shed to make it so. Thank you for your plumbing skills, your patience, your love and support, for helping us maintain some kind of 'normality', amongst all the crazy busy. I am not sure this would have been possible without you, by my side, every step of the way! To Maya and Wilf, our amazing little people, who have been so positive about every new graph and every new crayfish arrival at our house; you are the most incredible human beings and I hope I have inspired you to be curious about all the marvels of our natural world, to question and explore, rather than put you off science completely! You have been so kind and supportive to your mummy over all these years and I am so looking forward to all the awesome adventures that are waiting patiently for us. Let's go and make some more magical memories, all four of us together…!

Thank you to my supervisors, Gareth, Gráinne and Paul. Thank you Gareth for agreeing to supervise me for a second time, many years on, for allowing me the freedom to create my own set of studies and for taking a chance on a crustacean rather than a mammal! Thank you Gráinne, for having the daunting task of being both my supervisor and manager throughout this journey; your guidance and support has been amazing! Thank you Paul, for all your crayfish expertise along the way and for your complete faith in my abilities! Thank you to all three of you for taking me on and indulging my crayfish passion. You have all been fantastic and so supportive throughout this journey. It certainly has had its ups and downs and has been such a huge emotional roller coaster for me. All three of you have been brilliant all the way through and, perhaps more importantly, you have made me a much better scientist! Thanks also to Nick Taylor for your patience, wisdom and help in steering my statistics. To Marian and Christos, thank you for the annual meetings and your sound advice, throughout my studies. To the Bristol Zoological Society thank you for allowing me to return to academia, yet again, to further my understanding of this small crustacean! For the autonomy you have granted me and for all the financial support that you have given me; you are a great organisation to work for! Thanks to the Mohamed Bin Zayed Species Conservation Fund, for funding my Crayfish Research Unit and research equipment, it made

a huge difference to this project, very early on and allowed a small idea to turn into a large reality, very quickly. Thanks to the Centre for Environment, Fisheries and Aquaculture Science (Cefas) for giving me your scientific advice and health screening expertise and to Bristol Water for allowing me to borrow your trout tanks at Ubley Hatchery for crayfish and providing additional financial support.

Special thanks to Jonathan Williams and Timothy Clements for assisting with husbandry care at the CRU, never once complaining no matter how cold and dark the nights were, or how fiddly the feeds! To Holly Thompson, my extremely dedicated and highly skilled BZS crayfish hatchery manager; thank you for your tireless commitment to the crayfish cause, your hard work, dedication and for allowing me to talk about crayfish endlessly!

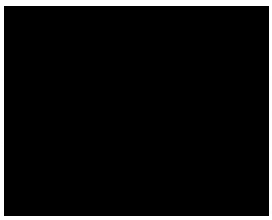
Thanks to Nathan Edmonds from Lowestoft Cefas for assistance with the PIT-tagging work and for helping me get started on that journey. Thanks to Jose Carral, John Hollows and Damian Rigg for allowing me to trial your crayfish diets. Thanks to Amy and Martin Stanton for allowing me to set up the telemetry study at Vobster Quay Inland Diving. Thank you to the Telemetry team - Clare Fitzsimmons, Kirsty Lees, Kiernan Hatton, Rachael Priest, Howard Hugo Angel, Duncan Roy, Tim Clements, Maxwell Fisher, Marcus Blatchford and Markilan Abrahams, it was great to have a go at this technique to see if we could make it work with little crayfish!

A final thank you goes to my Priddy friends, for all your laughter, love and kindness and for understanding those many months (years!) of hibernation – you never know, you might see me in the Queen Vic., once again!

Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University’s Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Jennifer Anne Nightingale

SIGNED: 

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List of Abbreviations & acronymns

ARTs	Artificial refuge traps
CABI	Centre for Agriculture & Bioscience International
Cefas	Centre for Environment, Fisheries and Aquaculture Science
CL	Carapace length
EA	Environment Agency
GBNNSS	Great Britain Non-Native Species Secretariat
GISD	Global invasive species database
GLM	Generalised linear model
HPE	Horizontal positioning error
IAS	Invasive alien species
IPM	Integrated pest management
INNS	Invasive non-native species
ISSG	Invasive Species Specialist Group – IUCN
IUCN	International Union for the Conservation of Nature
MBZCF	Mohammed Bin Zayed Conservation Fund
NE	Natural England
PIT	Passive integrated transponder
SAC	Special Area for Conservation
SSC	Species Survival Commission
SSG	Species Specialist Group
SWCP	South West Crayfish Partnership
WSSV	White spot syndrome virus

CHAPTER 1

General Introduction

Part of this introduction has been adapted from a manuscript published in the International Zoo Year Journal (2017):

Nightingale J., Stebbing P., Sibley, P., Brown O., Rushbrook B., & Jones G. (2017). The use of ark sites and associated conservation measures to secure the long-term survival of white-clawed crayfish in the UK. *International Zoo Year Journal, 51*, 50-68. (Appendix I).

Author contributions

JAN initiated the paper carried out the background research and produced the first draft. Oliver Brown contributed to the Welsh captive-breeding section and other authors contributed critically to the draft manuscript.

1.1. Crayfish distribution globally

There are an estimated 650 species of freshwater crayfish, which inhabit all continents, except the Indian sub-continent and Antarctic, and which are found in 60 countries globally (Figure 1.1); 98% of all crayfish species are endemic to a specific country (Richman et al. 2015). Freshwater crayfish live within a wide variety of habitats including rivers, still-water systems, riparian, estuarine and even terrestrial habitats (Reynolds et al. 2013). Crayfish are decapod crustaceans that belong to two superfamilies, the Parastacoidea in the Southern Hemisphere and the Astacoidea in the Northern Hemisphere. The Parastacoidea contains one family, Parastacoideae, which are restricted to South America, Australasia and Madagascar. Astacoidea contains two families, Cambaridae, (the most species-rich family), found in Eastern Asia and Eastern North America, and the Astacidae found in western North America and western Eurasia. Within Europe there are five indigenous crayfish species (Crandall & Buhay, 2008). Crayfish can range in size from the tiny lake yabby *Gramastacus lacus,* that lives in coastal lakes and swamps in New South Wales, Australia and is 12-18 mm long, weighing less than 7 g, to the Tasmanian giant freshwater crayfish *Astacopsis gouldi*, which can live up to 60 years, grow up to 80 cm in length, and weigh up to 6 kg. All crayfish species are similar in anatomy with a body that comprises a cephalothorax and an abdomen (Vogt, 2002). Crayfish are an important element of freshwater ecosystems, being keystone benthic invertebrates, important in energy transfer between trophic levels and they are also used as food, bait or pets (Gherardi et al. 2011).

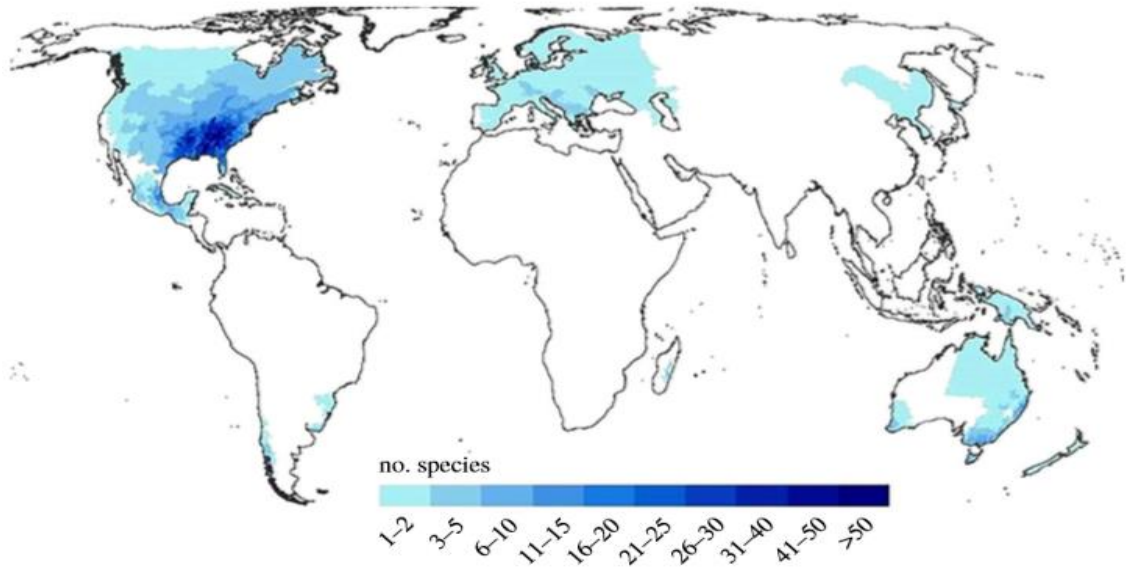

Figure. 1.1. DistributFigure 1.1. Distribution of all crayfish species (from Richman et al. 2015).

1.1.1. Crayfish in Britain and Ireland

The white-clawed crayfish *Austropotamobius pallipes,* is found throughout Europe (Figure 1.2) and it is the only species of crayfish recognised as native to the United Kingdom. For a species to be considered indigenous, within Britain, there need to be records of its existence before 1500 AD (IUCN 2003 as referenced in Holdich et al. 2009). For England, several historical records exist that provide evidence that *A. pallipes* was present in England in the 10th century. No information exists for Wales prior to 1500s; however, there is documentation of monastic establishments associated with *A. pallipes* (Holdich et al. 2009).

In Scotland, there are no native crayfish species, although *A. pallipes* populations have been established there. In Northern Ireland and the Republic of Ireland, *A. pallipes* is widespread within lowland limestone lakes and river catchments (Reynolds et al. 2002). The first *A. pallipes* populations in Ireland were reported in 1680; however, it is still uncertain as to where they originated from (Lucey 1999). Up until recently, Ireland was considered to be a natural ark site (a safe refuge site for threatened crayfish populations), having no known non-native crayfish species present (Reynolds, 1998); however, the common yabby *Cherax destructor,* has recently been discovered within an Irish lake.

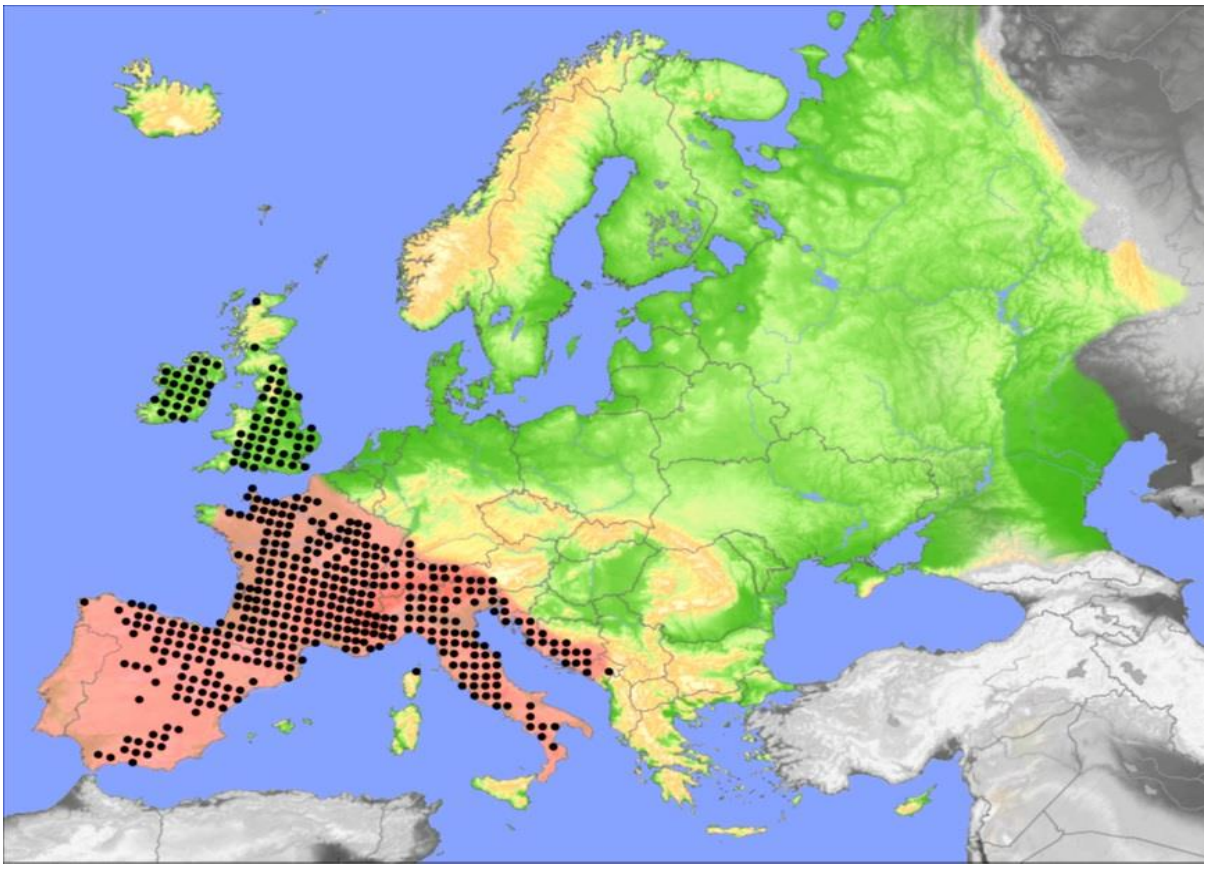

Figure 1.2. Distribution of *A. pallipes* in Europe, the presumed native range within mainland Europe is highlighted; black dot represents a population (from Kouba et al. 2014).

1.1.2. *Austropotamobius pallipes* life history

The white-clawed crayfish *Austropotamobius pallipes,* is a decapod freshwater crustacean, one of the largest indigenous freshwater invertebrates in Britain and Ireland and is our only native freshwater crayfish. It is a slow-growing, K-selected species, taking 3-4 years to reach sexual maturity (in the wild). Crayfish mate in late autumn, once day-length and water temperatures drop, typically to below 12 °C. External fertilisation occurs and the male delivers spermatophores, via gonopods (modified pleopods), to the females' ventral abdominal surface. These spermatophores are broken down by the glair secretion produced by the females, and fertilise the eggs as they are laid underneath the female's tail. Egg-laying typically occurs within a few days of mating and females will be berried (with eggs) by mid to end of November. The eggs are held, under the abdomen, throughout winter and then hatch in late spring / early summer.

The hatchlings will leave the females after approximately 2-3 weeks, once they have fully-formed mouth parts and can begin feeding independently. During the first year of life crayfish may moult every few weeks and this growth slows down as they mature. By the time they are sexually mature they will only moult 1-2 times per year in spring and autumn; berried females may only moult once per year as they cannot moult when carrying eggs. The moulting process (ecdysis) is a process whereby the crayfish sheds its hard exoskeleton and the soft cuticle underneath can then take up to 48 hours to fully harden. Mortality is greatest in the first year; survivors who reach sexual maturity, typically live 4-7 years, with maximum estimates of up to 8-12 years. Depending on the rate of growth, they reach a maximum size of approximately 12cm (Reynolds, 2012).

Austropotamobius pallipes is a keystone species in aquatic habitats (Matthews et al. 1993). It starts life as a carnivore, feeding on aquatic invertebrates, and then gradually becomes more omnivorous; by the time it is an adult it is primarily a detritivore and therefore important for ecosystem health. It is a slow-growing, long-lived species, which has a degree of phenotypic plasticity, being able to adapt morphologically to novel environmental conditions, making it resilient to translocation. For example, within a four-month growing period the carapace and aerola widths had increased when *A. pallipes* moved from a lotic (river) to lentic (still-water) environment (Haddaway et al. 2012).

1.2. Threats to crayfish

Approximately one-third of all crayfish species are threatened with extinction globally, including all species in Europe. Key threats include habitat fragmentation, pollution, invasive crayfish species and associated disease (Richman et al. 2015). *Austropotamobius pallipes* is classified as endangered by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Füreder et al. 2010), and listed under Annexes II and V of the EU Habitats Directive (Council Directive, 1992) and protected by UK legislation (Joint Nature Conservancy Council, 2017). In the last 20 years this species is suspected to have undergone a global decline of between 50-80% following the spread of the American signal crayfish *Pacifastacus leniusculus* and the red swamp crayfish *Procambarus clarkii* and the associated crayfish plague. The disease is caused by the oomycete water mould *Aphanomyces astaci* (Schultz & Schultz, 2004), in addition to habitat fragmentation, degradation and pollution events (Füreder et al. 2010).

In many regions of the UK, there have been dramatic declines in *A. pallipes* populations. In a recent assessment by Natural England investigating changes in distribution between 2013 - 2017, 23 new populations of signal crayfish were found and 23 populations of *A. pallipes* were lost (Chadwick, 2018; Figure 1.3). In the south west of England, there has been a 70% decrease in the number of occupied sites since the 1970s due to the introduction of *P. leniusculus* into Somerset in 1976 for farming (Sibley et al. 2011) (Figure 1.4). In Wales, a few remnant *A. pallipes* populations remain, which are restricted to streams in the south east and a small number of streams in the Wye catchment in Powys (Dyson, 2008). Within the Republic of Ireland, *A. pallipes* is still widespread (Demers et al. 2005). In Northern Ireland, *A. pallipes* is restricted to river catchments with suitable water quality; however, *A. astaci* outbreaks have occurred over the past twenty years in seven river catchments, causing the collapse of several local *A. pallipes* populations. This is despite there being no known populations of signal crayfish species within Northern Ireland or the Republic of Ireland (Reynolds et al. 2000).

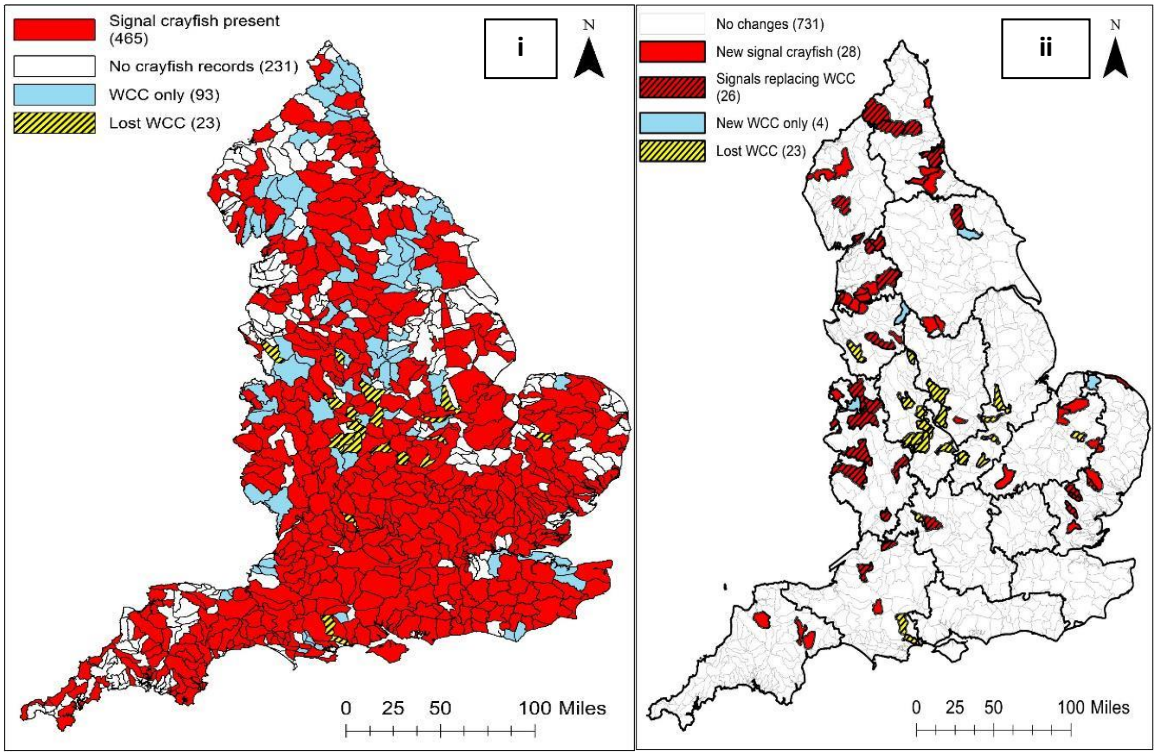

Figure 1.3.i) Distribution of both *A. pallipes* and *P. leniusculus* in 2017, in England; ii) changes in population distribution of *A. pallipes* and *P. leniusculus*, between 2013-2017; WCC is white-clawed crayfish (from Chadwick, 2018).

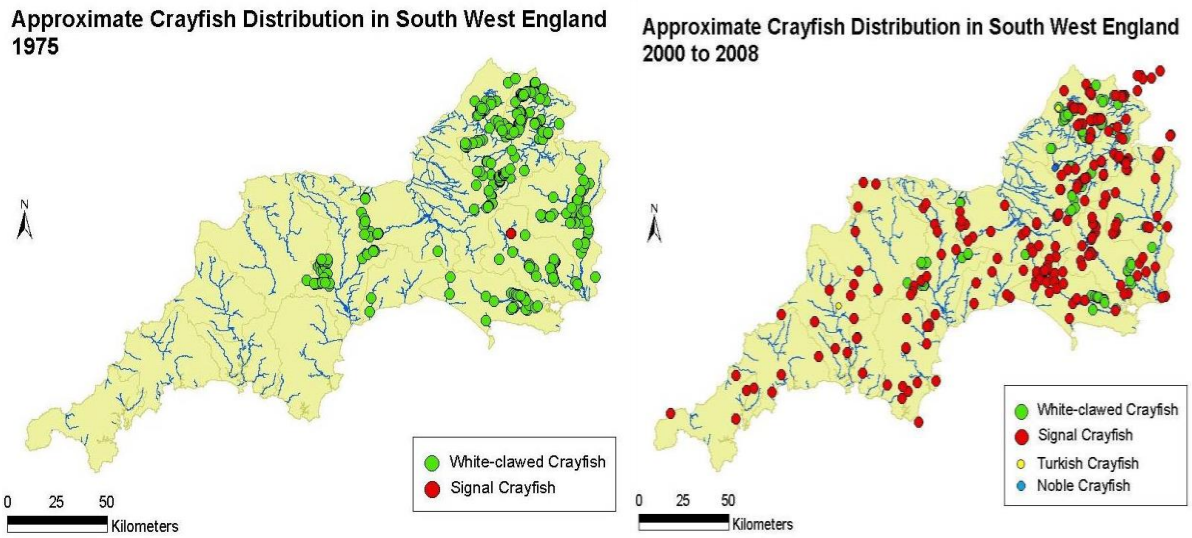

Figure 1.4. Distribution of *A. pallipes* and invasive crayfish species in south west England in 1975 and 2008 (from Sibley et al. 2011).

1.2.1. Invasive species

Invasive non-native species (INNS) are species that exist outside their natural geographic range that cause damage to the environment, economy or human health (Ehrenfeld, 2010*).* INNS differ from non-native or non-indigenous species that have been introduced but do not cause harm. Invasive species characteristics often include short life cycles, rapid growth rates, adaptive reproductive traits and a tolerance of a wide range of habitat and environmental conditions (Mooney & Cleland, 2001; Pyšek et al. 2020). Once introduced into novel habitats, INNS are often freed from their natural predators and pathogens, which may have controlled the species in their native range. Therefore, the mechanisms of population control are lost and they can rapidly increase in numbers and cause problems to native taxa. This is the Enemy Release Hypothesis, which predicts that a species will be successful in a novel environment if its natural predators are not present (Keane & Crawley, 2002). Habitat destruction further exacerbates species invasions because disturbed land can be easier to invade and colonise (Chytrý et al. 2008).

Invasive species are considered one of the greatest threats to global biodiversity, the largest being habitat loss and fragmentation (Millenium Environment Agency, 2005). They can be introduced through a variety of ways such as shipping vessels, via living produce, through the horticultural and aquatics trade, or illegally smuggled. Any species taken out of its natural habitat, that causes harm, can potentially become invasive and examples of INNS can be found in all taxonomic groups including animals, plants, fungi, parasites and viruses. Some of the most serious examples include species such as the mosquito *Anopheles quadrimaculatus*, which is the main vector of malaria in North America; the parasitic chytrid fungus *Batrachochytrium dendrobatidis* that kills millions of amphibians globally each year and the brown rat *Rattus rattus*, native to India, which has spread world-wide causing localised extinctions of species and which spreads disease (Lowe et al. 2000).

The IUCN, through their Species Survival Commission (SSC), Global Species Programme and Red List partners, carry out global ecosystem assessments regarding the status of native and invasive species, and produce a Red List of Threatened Species. In Europe, there are currently an estimated 12,000 alien (non-native) species of which 15% are invasive. These invasive species are the third largest threat to threatened native species (Figure 1.5). In Europe, there are currently an estimated 354 threatened species (229 animals, 124 plants and 1 fungus) that are affected by invasive alien species; this is approximately 19% of all known threatened European species (Genovesi, 2015).

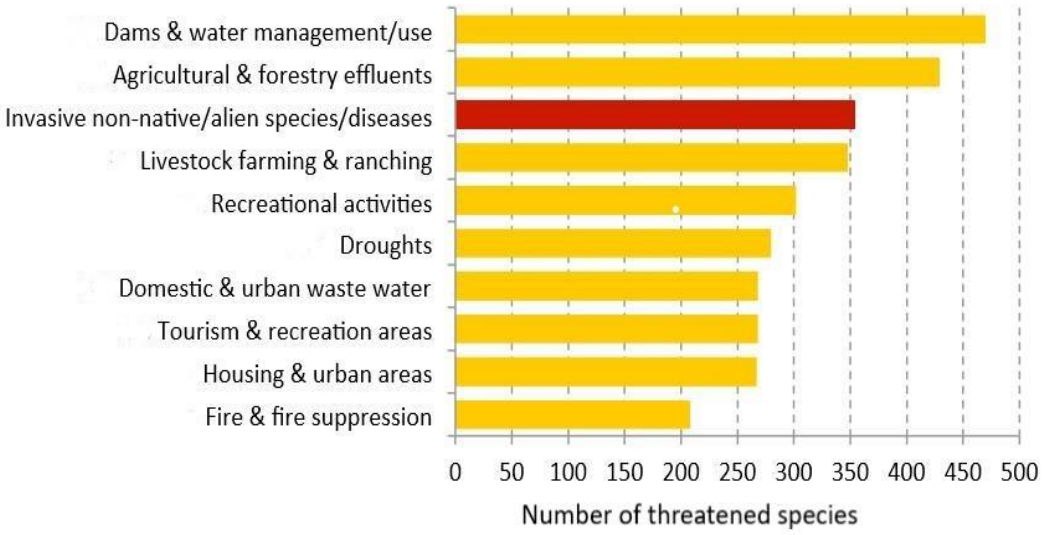

Figure 1.5 Ranked list of threats to the IUCN Red List assessed species in Europe (adapted from Genovesi et al. 2015).

1.2.2. Invasive species in the UK

There are over 2,000 known invasive species in the UK, which cost the economy approximately £1.7 billion annually (Williams et al. 2010). Invasions have come from every continent in the world except Antarctica. Examples include invasive plant species such as Himalayan balsam *Impatiens glandulifera*, native to the Himalayas, introduced into the UK in 1839 and Japanese knotweed *Fallopia japonica*, native to Japan, China and Korea, and brought into the UK in 1850 by Kew Gardens. Both are popular ornamental species (Bailey & Conolly, 2000). These plants will rapidly form monocultures and out-compete native species. *Fallopia japonica* has prolific roots that cause severe structural damage to buildings and is extremely difficult to eradicate.

The grey squirrel *Sciurus carolinensis* and American mink *Neovison vison*, are examples of mammalian species that have caused significant declines of our native species, such as the red squirrel *Sciurus vulgaris* and water vole *Arvicola amphibius,* and these invasives are now very well-established (Manchester & Bullock, 2000). The topmouth gudgeon *Pseudorasbora parva*, a small freshwater cyprinid Asia, was introduced into lakes in the UK in the mid-1990s. It has outcompeted native fish species in lakes within England and Wales; some of of these lakes have direct connection to river catchments (Britton et al. 2007).

1.2.3. Invasive species control programmes

The IUCN Invasive Specialist Group (ISSG) has developed the Global Invasive Species Database (GISD) that provides detailed assessments of all ecosystems globally. The overarching aims of these groups are to raise awareness of invasive species and promote control, prevention and management techniques. Integrated Pest Management (IPM) is regarded as the most effective way of controlling invasive species. This method assesses all the available control methods and then choses a combination of the most suitable applications for the specific situation (Kogan, 1998). This practice is used worldwide particularly for growing valuable crops such as rice, tomatoes, wheat and apples. The cassava *Manihot esculenta*, is an important staple food in Africa; however, it is susceptible to cassava mealybug *Phenacoccus manihoti*, which has previously devastated crops and affected the livelihood of millions of Africans. A South American species of parasitic wasp *Anagyrus pseudococci* was identified that fed on mealy bugs and was introduced into Africa. Within 10 years, it had reduced the mealy bug population by 95% (Daane et al. 2004).

In 2006, the Great Britain Non-Native Species Secretariat (GBNNSS) was established, which is a government-led forum for delivering invasive species control. From this, the 2008 GB Non-Native Species Strategy was written, which was revised in 2015 (Defra, 2015). These strategies link into the ISSG to ensure that global and national strategies are in line, delivering a combination of approaches to INNS management. Within the UK, IPM is delivered by the English branch of Centre for Agriculture and Biosciences International (CABI) established in 1992, which has produced bio-control agents for invasive plant species within the UK. Examples include the species-specific rust fungus *Puccinia* spp. to help control species of *I. glandulifera* and the psyllid *Aphalara itadori* to help control *F. japonica*. The trials of these bio-controls are still in their infancy; however, preliminary results are encouraging (Varia et al. 2016).

1.2.4. Crayfish invasions globally

Invasive species are more prevalent in freshwater systems than in terrestrial habitats. Crayfish have been widely introduced for both the food trade and in the ornamental fish pet trade. Of particular significance are *P. clarkii*, native to northeastern Mexico and South-Central America and *C. destructor* from Australia, which have been introduced to Asia, Africa, Europe and other parts of the Americas mainly for food production harvesting. These are highly adaptable and resilient species that can withstand desiccation, temperature extremes and are much larger and more aggressive than the majority of freshwater crayfish. Both species have successfully established in wild areas, within their non-native ranges, and pose a significant threat to freshwater ecosystems. In Europe, there are ten known invasive

species, of which currently *P. clarkii* and *P. leniusculus* are the greatest threat to the native crayfish species (Gherardi et al. 2011; Kouba et al. 2014; Manfrin et al. 2019).

The parthenogenetic marbled crayfish *Procambarus fallax* forma *virginalis* is also of concern. All hatchlings are female and genetically identical to the parent. This species is available through the aquarium trade and is a popular hobbyist animal. It has been released into the wild in three continents and occurs in Japan, Madagascar and in several European countries (Martin et al. 2010; Patoka et al. 2016). If the species was to become widespread, the consequences could be severe because even one released animal could produce a viable population; there is now an EU ban on the importation of this species into Europe (Gherardi et al. 2011). Until recently, *Procambarus fallax* forma *virginalis* was the only known species of crayfish to reproduce by parthenogenesis; however, *P. clarkii* is also now known to have the ability to produce clones (Yue et al. 2008).

1.2.5. Crayfish invasions in the UK

In England, there are now seven known invasive species of crayfish that have become established (Ellis, 2014). The first introduction was in the mid-1970s, when *P. leniusculus* was imported from Sweden to be farmed for the food industry; ironically it was mainly shipped to Scandinavian markets. Crayfish farms were set up throughout England and the crayfish escaped into the adjacent waterways and spread rapidly (Holdich & Reeve, 1991). Since the 1970s, six more crayfish species have been introduced, with the most recent being the white river crayfish *Procambarus acutus*, introduced in 2012 (Ellis, 2014; Table 1.1). Under the Prohibition of Keeping Live Fish (Crayfish) Order 1996 (as amended), created under the Import of Live Fish (England and Wales) Act 1980, it is illegal to hold any species of non-native crayfish without a licence. However, there are some exemption areas that allow *P. leniusculus* to be kept without a licence; i.e. in regions where it is already widespread in the wild. *Cherax destructor*, has also been found recently within Ireland; however, there are no known populations of this species in England.

Within the UK, the only non-native species that is allowed within the aquarium hobbyist trade is the red-claw crayfish *Cherax quadricarinatus*, from Australia, which is a tropical species and therefore should not be able to breed in the wild in the UK because it is not adapted to our aquatic conditions (Gherardi et al. 2011). In Ireland, the importation of non-native crayfish species is banned; however, there are several species available through the pet trade. It is thought that the pet trade is one of the major causes of non-native crayfish being released into the wild (Faulkes, 2017).

Table 1.1. Range of invasive crayfish species in UK (adapted from Ellis, 2014).

Species	**Introduction**	**Distribution**	**Characteristics**	**Crayfish plague**
Signal crayfish *Pacifastacus leniusculus*	From Northern America for aquaculture in 1970s; escaped &/or deliberately introduced to waterways.	Widely across England, Wales & Scotland.	Aggressive, fecund.	Vector
Turkish crayfish *Astacus leptodactylus*	From Eastern Europe, for food trade in 1970s; escaped &/or deliberately introduced to waterways.	Declining due to susceptibility to crayfish plague, e.g. several populations in Greater London disappeared in the 2000s.	Aggressive, fecund, fast-growing.	Susceptible
Noble crayfish *Astacus astacus*	From Europe, introduced for aquaculture in 1980s.	Originally found in a small number of sites in south-west England; current distribution unknown but unlikely to spread further due to susceptibility to crayfish plague.	K-selected species.	Susceptible
Spiny-cheek crayfish *Orconectes limosus*	Native to North America. Arrived early 2000s.	First population in Warwickshire in 2001; subsequently at 2 sites in Lincolnshire and Nottinghamshire (site connected to River Trent so likely to be present in main river).	Smaller and less aggressive than other species but attains high population densities.	Vector
Virile crayfish *Orconectes virilis*	From North America and Southern Canada.	First found in Southern England in small pond adjacent to River Lee. Spread quickly through catchment and now co-exists with signal crayfish. Currently confined to River Lee and adjacent gravel pits (although linked to Thames, Regent's Canal/Lower Grand Union Canal).	Fecund - early sexual maturity allows rapid establishment (e.g. within 7 years). Most northerly range of any crayfish and able to withstand very cold conditions. Also thrives in warm waters.	Vector
Red swamp crayfish *Procambarus clarkii*	From northern Mexico and southern US. Arrived early 1900s.	First found in Hampstead Heath Ponds, North London. Now in Regent's Canal and lower section of the Grand Union Canal. Probably in other waterbodies in London.	Can survive in burrows in seasonal wetlands. Able to quickly cover large distances including over land. Easily adapts to cool climates but likely to benefit from climate warming.	Vector
White-river crayfish *Procambarus acutus*	Native to USA. Introduced in 2012.	First found in Southern England in 2012. Distribution unknown.	Little known. Relatively small. Naturally suited to temperate climates.	Likely carrier

1.3. Crayfish conservation measures in the UK

In the UK, crayfish conservation is led by the Environment Agency, which has developed a national strategy that can then tie into regional strategies (Marshall, 2019). In an attempt to safeguard *A. pallipes* within south west England, a partnership of organizations, The South West Crayfish Partnership (SWCP), has developed a strategic, landscape-scale, conservation project. The project consists of five main strands: (i) mapping of catchments, to understand the range and extent of crayfish species present; (ii) establishing ark sites (safe refuges) to maintain threatened populations and genetic integrity for both wild-caught translocations and captive-born introductions of *A. pallipes*; (iii) a captive-breeding programme developed and maintained at Bristol Zoo Gardens, UK, providing plague-free *A. pallipes* for wild-release and brood-stock for other organisations; (iv) an outreach programme targeting key audiences including waterway users, restaurants, students, school children and zoo visitors; and (v) the development of a variety of techniques to control non-native crayfish species (see Nightingale et al. 2009; Robbins et al. 2013; Nightingale et al. 2019).

In Wales, a similar initiative was undertaken, The South East Wales Crayfish Project. This was primarily looking to identify potential donor and ark sites (Smith et al. 2009). This project, carried out by Cardiff University, highlighted the need to investigate the feasibility of developing a captive-rearing / breeding programme to provide donor stock for ark sites. This same period saw the inception of the Irfon Special Area of Conservation (ISAC) Project, funded by the EU Life+ programme, and led by the Wye and Usk Foundation (WUF). A proportion of this project focused on the conservation of *A. pallipes* in the Irfon catchment, a large tributary of the river Wye. This work focused on targeted survey effort, habitat management and enhancement, assessment of donor and receptor sites, and captive-rearing at Natural Resources Wales' Cynrig Hatchery (WUF, 2014).

1.3.1. Invasive crayfish control

Historically, the main method of crayfish control was physical removal using trapping, hand searches, netting and electrofishing, which can be fairly ineffective especially in river sites. De-watering plus physical removal has also been utilised. Chemical eradication using biocides, such as rotenone and pyrethrum has been employed since the early 2000s. Rotenone is lethal to many other species and therefore its use is controversial. Pyrethrum was successfully used within still-water sites in Scotland for the eradication of *P. leniusculus* (Peay et al. 2006). Biocides have an effect on the entire ecosystem and therefore are more appropriate for still-water, rather than river sites. More recently, bait-specific matrices are

being developed that are target-specific autocidal treatments, which should not affect the wider ecosystem; however, they still need to be tested in field trials (P. Stebbing, pers. comm. 2017). Male sterilisation has been trialled where the larger adult males are caught and their gonopodia are removed, or the crayfish are sterilised using X-ray radiation (Manfrin et al. 2019). This means that they cannot mate effectively but they still continue to dominate and therefore suppress any subordinate males in the area. Male sterilisation is being trialled in Exmoor (Green et al. 2010). Biological control methods are also employed such as the addition of predatory fish species, which when combined with trapping may prove an effective control mechanism. Recent research is now looking into other control techniques such as sexual attractants, establishing single-sex populations and RNA interference, i.e. gene silencing, to disrupt the insulin-like androgenic gland hormones, which affect reproduction and growth (Manfrin et al. 2019).

Where a crayfish invasion is noted early, physical removal can be effective; however, in cases where an invasive species becomes established, then a long-term approach to population reduction, rather than eradication, is the most feasible solution. Currently there is no recognised standard control technique. It is likely that a combined control programme using a variety of methods will be the most effective for long-term management.

1.3.2. Captive breeding for conservation measures

Captive breeding programmes for conservation translocations have existed since the 1960s when the first breeding programme for the Arabian oryx *Oryx leucoryx*, began at Phoenix Zoo, Arizona. Oryx were distributed to many zoos worldwide and the programme resulted in populations being reintroduced in Oman, Saudi Arabia and Israel (Abu-Jafar & Hays-Shahin, 1988). Over the past four decades, there have been many successful reintroduction and conservation translocations covering a wide range of taxa, with mammal programmes being the most prevalent. It is now estimated that more than 1,300 conservation translocations have taken place globally, with varying levels of success (Figure 1.6).

Within the UK, there have been successful conservation translocations of mammal species such as water voles *Arvicola amphibus* (Nightingale et al. 2002), pine martin *Martes martes*, wild boar *Sus scofra*, and most recently beaver *Castor fiber* (Jones & Campbell-Palmer, 2014). Bird species, such as the red kite *Milvus milvus* (Evans et al. 1997) and the white-tailed eagle *Haliaeetus albicilla* (Evans et al. 2009) have also been reintroduced successfully and are now thriving in the UK. There are also several examples of successful invertebrate reintroductions such as the fen raft spider *Dolomedes plantarius* and the large blue butterfly *Phengaris arion* (www.dolomedes.org.uk/conservation/translocation.html). The IUCN

Conservation Translocation SSG have produced comprehensive conservation translocation guidelines, which should form the basis of any reintroduction programme and much of its success will be down to accurate planning, thorough risk assessments, disease risk analysis and adherence to best practice (IUCN, 2013).

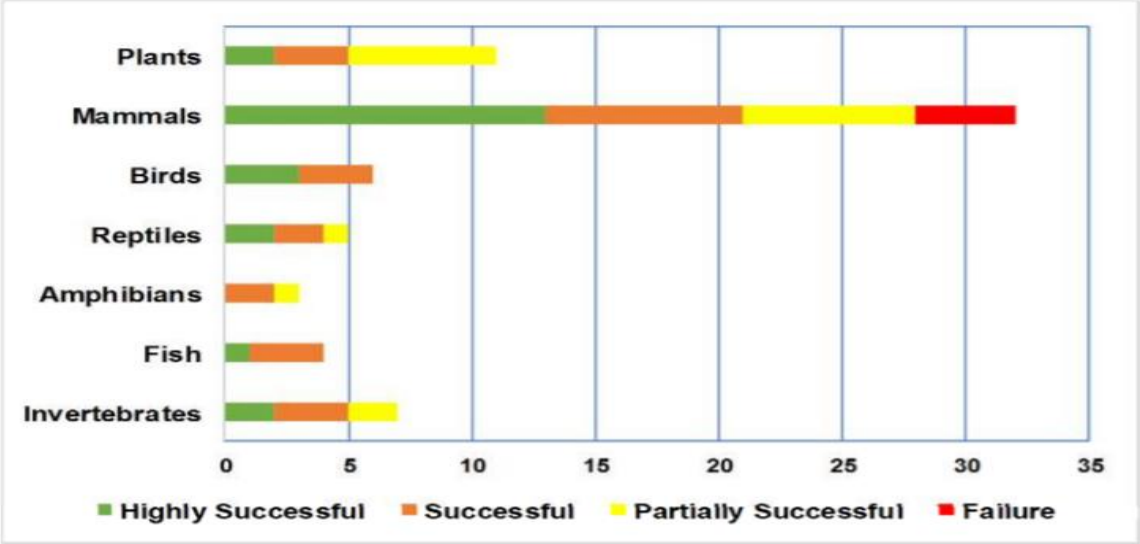

Figure 1.6. 2018 IUCN assessment of 59 global reintroductions (from Soorae et al. 2018).

1.3.3. Crayfish captive breeding

Captive-rearing (hatching and rearing juveniles from females mated in the wild), and captive-breeding (hatching and rearing juveniles from females mated in captivity) of *A. pallipes* is becoming a valuable resource for supplementation of wild populations and ark site establishment, both in the UK and in mainland Europe. In some countries, such as Finland and Sweden, wild harvesting is not allowed due to major population declines, and therefore crayfish aquaculture is the only method for reintroduction (i.e. to sites known to have previously supported native crayfish) and introductions (i.e. to sites not known to have previously supported native crayfish). In Spain, wild populations of native crayfish are now at such low levels that wild harvesting is simply not an option (Souty-Grosset & Reynolds, 2009). The same situation exists for several areas of southern England and Wales, where *A. pallipes* populations have either become locally extinct or have been reduced to either very low numbers or a few isolated populations (Sibley, 2003). In these cases, captive-breeding / rearing of crayfish is an effective tool for the preservation of these populations, and an essential part of a long-term conservation management programme.

It is estimated that less than 10% of a brood of *A. pallipes* will survive to reproduction in the wild (Ulikowski, 1996; Ulikowski et al. 2006; Neveu, 2007). In contrast, in captivity, up to 90% of a brood can be successfully reared to sexual maturity, and crayfish can mature as early as 16 months and typically by their second year (Policar et al. 2010; J. Nightingale pers. obs. 2014). By bringing ovigerous (egg-laden) females into captivity in spring for hatching and subsequent rearing of juveniles, survival can be greatly increased and thus produce large numbers of crayfish plague-free progeny for supplementation of wild populations, or for establishment of ark sites. If wild-caught females and captive-born juveniles are retained in hatcheries for breeding, this will reduce the need for removal of further crayfish from sensitive populations outside the recommended survey season. In the case where wild populations have declined to very low numbers this conservation strategy has many advantages. By establishing captive unrelated brood-stock, genetic diversity is preserved and maintained in successive generations. A key element for successful crayfish aquaculture is to ensure that the donor population is appropriate and robust to ensure that the brood-stock has as much genetic diversity as possible (Fetzner & Crandall, 2001).

1.3.4. Crayfish hatcheries within the United Kingdom

The first *A. pallipes* crayfish hatchery was established In Northern Ireland at Moneycarragh fish farm in 2006; however, no restocking with captive-born juveniles from this hatchery has taken place (Policar et al. 2008). In 2009, *A. pallipes* crayfish hatcheries were established at Bristol Zoo Gardens, England at the Natural Resources Wales' Cynrig Hatchery. Both these hatcheries have the key objective of enhancing local *A. pallipes* populations by captive-rearing and breeding for wild supplementations and ark site establishment. The hatchery at Bristol Zoo Gardens has four, fully closed circuits; discrete systems that function as separate bio-secure units allowing brood-stock from different wild-caught populations to be held separately and simultaneously, (see Nightingale & Rudd, 2011; Nightingale, 2012 for further detail). Bristol Zoo Gardens brings in wild-caught ovigerous females and also retains brood stock for closed-cycle, captive breeding of both wild-caught donor stock and captive-born individuals. Females mate in autumn and hold their eggs over winter, which then hatch in the following spring.

To date the Bristol Zoo Gardens hatchery has produced over 5,500 crayfish for ark site establishment, wild supplementations, hatchery brood-stock and to create crayfish collections at other British and Irish Association of Zoos and Aquaria (BIAZA) institutions. Regular health screening of a proportion of the population to be released is essential, to safeguard the potential introduction and spread of diseases not previously found in the receiving water body. Work at the Natural Resources Wales Cynrig Hatchery started in

2008/09 and over time it has developed, with Cynrig presently running three recirculation systems with a total volume of 5,300 L and a culture area of 13.7 m^2. Each system is discreet to allow rearing of crayfish from different rivers or catchments while limiting biosecurity risk. Each is borehole-fed and isolated from the rest of the fish farm and ambient river water to further reduce the chance of disease or pathogen transmission. Up to three donor populations may be targeted in each particular year depending on the location of receptor sites. At the Cynrig hatchery, wild-caught ovigerous females are brought in during the spring for egg hatching and for rearing juveniles. The juveniles are reared and released at 10-11 months of age, prior to the next batch of ovigerous females being collected in the following spring (see Brown, 2012; WUF, 2014 for further detail). More recently, juveniles have been retained and grown on for captive breeding. To date, Cynrig hatchery has produced over 6,000 juveniles, which have been released predominantly into Welsh rivers (O. Brown, pers. comm. 2019).

1.4. Crayfish translocations

Crayfish translocations involve moving parts of a wild population to a safe area. Reintroductions and introductions usually entail the production of captive-reared or bred juveniles (collectively referred to as captive-born) for release (Taugbol & Peay, 2004).

1.4.1. Crayfish translocations in Europe

In mainland Europe, reintroductions for the endangered crayfish species *A. pallipes* and noble crayfish *Astacus astacus*, have been taking place for several decades in Austria, the Czech Republic, France, Italy, Poland, Portugal, Russia, Spain, Sweden and Switzerland. There has been an extensive restocking programme of *A. astacus* (in countries such as Finland, Sweden and Norway) where populations have been wiped out previously by crayfish plague, and where there is no evidence of other crayfish species being present (Westman, 1992; Taugbøl & Skurdal, 1993). However, these programmes have a commercial value as the populations are maintained for harvesting, usually with strict controls on the size limit allowed to be taken (Westman et al. 1990). In Britain, *A. pallipes* is not harvested for the food industry and therefore translocations (including reintroductions) are solely for conservation purposes.

1.4.2. Crayfish translocations in the UK

In the absence of fail-proof options to prevent the introduction and spread of non-native crayfish species and diseases, measures to safeguard and expand the current distribution provide the most feasible hope for securing the survival of *A. pallipes* in the UK. Over the last decade one such measure, involving the translocation of *A. pallipes* from threatened donor sites to low-risk refuge or ark sites, has been employed at multiple locations across the UK with some success. Ark sites are 'discrete water bodies with optimal water quality that should remain free from invasion by non-native species for the foreseeable future' (P. Bradley, pers. comm. 2010). The stocking of still-water sites and rivers with *A. pallipe*s within Britain has occurred since at least the 18th century (Foster, 1998).

In Wales, the earliest recorded introductions were into the River Irfon in the 1800s (Jones, 1805; Slater, 2002). In Scotland, the first *A. pallipes* population was introduced in the early 20th century to Loch Croispol, Sutherland, in the Durness region of northern Scotland. More recently a second introduction occurred into the Whitemoss Reservoir in Renfrewshire (Maitland et al. 2001). It is only since the 1980s that *A. pallipes* conservation translocations, within the UK, have been occurring specifically as a conservation measure to combat the decline of this species due to the introduction of non-native *P. leniusculus* to British waterways. The majority of these have been translocations of individuals from threatened wild populations into isolated ark sites.

In Britain, ark sites can be either lentic or lotic sites; however, if using river systems, hydrological connectivity to non-native crayfish species must be considered. In south west England, nearly all river catchments are compromised with non-native crayfish species, primarily *P. leniusculus* (Sibley et al. 2002). Therefore, where river sites are used as ark sites, a natural or man-made barrier such as a weir or culvert should exist to limit up-stream invasion of non-native crayfish species. Discrete, isolated still-water sites, such as disused aggregate and mineral extraction sites, can provide ideal, long-term safe refuge options. Extraction operations often produce permanent water-filled sites that are suitable for *A. pallipes* with no further modification required. These quarry sites provide not only effective crayfish ark sites, but are also natural reserves for a plethora of species (Whitehouse et al. 2009).

The short-term measure of success for any newly established ark site will be to determine whether breeding has taken place over the first few years following translocation. Since the 1980s, within the UK and Ireland, there have been a variety of ark sites established and river supplementations into headwaters. In all the cases cited, the ark sites were established by translocating wild-caught individuals from threatened populations rather than setting up

captive-rearing and breeding programmes. Prior to the inception of the SWCP, there had only been two known captive-reared *A. pallipes* reintroduction within England. In Derbyshire, an *A. pallipes* population in the River Lathkill was wiped out by crayfish plague in 1993, and both captive-reared juveniles and wild-caught crayfish were reintroduced from 2000 over several years (Rogers & Watson, 2011). In Yorkshire, a small scale captive-breeding programme at Settle has been producing *A. pallipes* for over 12 years and in 2013, the first reintroduction was performed by releasing 20 captive-born individuals into a stream in the Yorkshire Dales headwaters (P. Bradley pers. comm.*)*.

1.4.3. Ark site establishment in south west England

Over the past decade, the SWCP has established 17 ark sites in six counties: Cornwall, Devon, Dorset, Gloucestershire, Hampshire and Somerset and one captive-bred river supplementation in Hampshire. The ark sites have been established either with wild-caught translocated *A. pallipes* from local, threatened populations, or with captive-reared or bred juveniles. Specifically, eight of the south west England ark sites have been established at river and eight at still-water sites, (Figure 1.7). Over 3,800 wild-caught *A. pallipes* have been moved from eight, highly threatened, natural populations into 13 ark sites. More than 2,300 captive-born crayfish have been released into four ark sites, and one ark site has been set up with both wild-caught and captive-reared and bred individuals. Bristol Zoological Society is attempting to safeguard these populations by hatching juveniles for wild supplementation and maintaining brood-stock groups to be held *ex-situ* within the hatchery.

Figure 1.7. Releasing *A. pallipes* into the Candover Stream, River Itchen, Hampshire, as part of an on-going river supplementation programme © J. Nightingale.

1.4.4. Post-release monitoring

An important element of both river supplementation and ark site establishment is to ensure a long-term monitoring programme is in place. Determination of presence and absence is the only possible form of monitoring in the early stages of ark site establishment; population estimates are not attempted. This is because it would be very disruptive, to the crayfish, to try to disturb all potential habitat areas, within a release site, to assess catch per unit effort (CPUE) and from this attempt to extrapolate population abundance. Even if detection is unlikely in the year following introduction, most ark sites are at least visited, but not necessarily surveyed, to ensure that habitat conditions still remain favourable. This is important so that, in the event of failure, there may be a record as to why establishment was unsuccessful. All wild-caught translocations were with crayfish with a carapace length greater than 15 mm; if crayfish are smaller than this they are more prone to predation when released. Therefore, if juveniles are found in subsequent years during monitoring this indicates that the population is now breeding. When captive-born introductions occur this usually takes place with young-of-the-year juveniles (crayfish that have hatched that year), due to space being at a premium to allow for further ovigerous females to be housed. However, in the past few years at Bristol Zoo Gardens, juveniles have been reared within hatcheries for longer, so that they are at breeding size when released.

Of the 18 sites (17 ark sites and one wild supplementation) that have been set up by the SWCP since 2008, 16 have been surveyed and, out of those 16, 12 (75%) showed presence of *A. pallipes* as recently as 2019. In five of them (31%), juveniles have been found, suggesting that the populations are now established and breeding. The average time before crayfish were detected was four years; however, in some cases crayfish were found the year following the introduction and in others it took up to five years to detect them (J. Nightingale, pers. obs.).

A variety of monitoring techniques are employed such as baited trapping, artificial refuge traps, stone turning, torch surveys and diver surveys. Advances in the use of environmental DNA (eDNA), where the habitat is sampled for crayfish DNA, which has been released from the animals, has great potential and could soon be a reliable technique for monitoring presence at ark sites and in wild populations (Treguier et al. 2014; Geerts et al. 2018; Mauvisseau et al. 2018; Strand et al. 2019).

1.5. Research gaps

1.5.1. Aquaculture

The aquaculture industry is fast-growing and currently provides approximately 50% of all food produced globally (Wang et al. 2015). More than 11,000 tonnes of freshwater crayfish are produced annually, with the majority from four key continents: 56% from China, America, 40% from America, 3.6% from Europe and less than 1% from Australia. The main species that is farmed is *P. clarkii,* accounting for 70-80% of the crayfish produced commercially (Huner, 1994; McClain & Romaire, 2004; Ackerfors 2000*).*

This is because this species has high fecundity and is tolerant to a range of environmental conditions. Consequently, crayfish aquaculture research has mainly focussed on the species with high commercial value and numerous studies exist regarding *P. clarkii* and its sustainable farming solutions (Jin et al. 2019a), reproduction, growth and survival (Jin et al. 2019b). In Europe, the main commercial focus is on native noble crayfish *Astacus astacus*, for both food production and for restocking threatened wild populations (Harlioğlu & Harlioğlu, 2004). There are also commercial fisheries for the production of invasive *P. leniusculus* for food (Gherardi et al. 2011).

Without a commercial value, there have been considerably fewer studies on *A. pallipes* and historically this species had a reputation for being a difficult crustacean to breed and rear successfully in captivity. Over the past three decades, there were studies on several key elements of its life cycle and associated husbandry. These included studies investigations on the stages of embryonic development (Celada et al. 1991) and studies on breeding (Woodlock & Reynolds, 1988; Reynolds et al. 1992; Carral et al. 1994; Pérez et al. 1998; Policar et al. 2010; Caprioli, 2014); artificial incubation (Pérez et al. 1999; Carral et al. 2003). Additionally, studies on nutrition (Gherardi et al. 2004); rearing temperature (Paglianti & Gherardi, 2004); behaviour and refuge use (Ghia et al. 2009; Tricarico & Gherardi, 2010). There was a marked improvement in young-of-the-year astacid crayfish studies, when it was recognised that there was a critical life-stage, post-hatching when the crayfish move from using their egg-yolk stores, to having fully-formed mouth parts for exogenous feeding. Therefore studies could be flawed by the quality of the first foods given, typically commercial fish farm fry feeds, which were not sufficient for promoting high survival rates (Gonázalez et al. 2010). Live food was tested on hatchling *P. leniusculus* and high survival rates (> 80%) were achieved (González et al. 2008). After realising that survival rates were significantly increased by feeding live food rather than fish pellets, it was then apparent that the results of previous studies may have been skewed by a lack of adequate nutrition. Survival may have

been reduced due to poor nutrition rather than being caused by the treatment being tested, such as refuge or density (Gonzáez et al. 2011; Celada et al. 2012).

There still remains a wide difference in opinion as to suitable densities for *A. pallipes* young-of-the-year rearing, feeding regimes and grading, and there is a need for more research into the aquaculture techniques for this crayfish species, in order to optimise production.

1.5.2. Monitoring

With the increase in ark sites being established and wild population translocations, the need for robust, long-term ark-site monitoring is pivotal to their success. This monitoring will help assess success and failures and feedback into how future translocations are carried out. To facilitate the monitoring, an effective way of permanently tagging crayfish so that they can be individually identified is required. The benefits of permanently marking crayfish pre-release are clear, they allow status of the reintroduction to be more accurately assessed. Tagging helps to show migration, survival and population recruitment and therefore it can be an important element when trying to understand the success of an ark site. Marking crayfish with Passive Integrated Transponders (PIT) tags has been trialled on captive adult *P. leniusculus* (Bubb et al. 2006) and *A. pallipes* in the wild (Bubb et al. 2008; Louca et al. 2014; Stead et al. 2015); however, there are no long-term *ex-situ* trials to determine the impact of tags on survival or growth, or to confirm whether sub-adult *A. pallipes* can be safely tagged. Tracking PIT-tagged crayfish remotely in the wild has had limited success with hand-held antenna, individual crayfish will only be detected intermittently over a survey period (Stead et al. 2015). Using either active tracking or in-stream antenna to detect PIT-tagged aquatic species can have relatively poor results when smaller species are tagged; the smaller size PIT-tag will reduce its detection ability (Burnet et al. 2013). Within aquaculture facilities, space is always at a premium and therefore there is a desire to release young-of-the-year crayfish to free up space in tanks for breeding, and before juvenile aggression causes survival and welfare issues. If young-of-the-year crayfish are released, they are more prone to predation from apex predators, such as dragonfly larvae and newt efts (juveniles), especially in still-water sites (Gydemo et al. 1990; O'Neill et al. 2011). Such sites are often favoured as arks as they offer more potential protection from invasion; however, without fish species present, efts and Odonata larvae populations can rapidly increase (Gydemo et al. 1990). This means that crayfish need to be released when they are larger i.e. at size > 20 mm carapace length. Crayfish are usually tagged, prior to release, to enable more information to be gathered regarding their movements and survival. Therefore there was a need to establish the minimum size that a crayfish could be tagged and how PIT-tagging affects *A. pallipes* long-term. It was also important to assess whether PIT-

tagged crayfish could be reliably detected, once released, using remote antenna. The use of radio tracking (active telemetry) with astacid crayfish species has been explored; with *P. leniusculus* (Anastácio et al. 2015; Bubb et al. 2006); and *A. astacus* (Bohl, 1999; Schiltze et al. 1999; Daněk et al. 2018). Radio tracking studies provide snapshots into crayfish activity and there have also been studies investigating *A. pallipes* using this technique (Robinson et al. 2000; Bubb et al. 2006). Acoustic telemetry has been used with many aquatic species over the past five decades and in more recent years it has been tested with lobsters (Macarthur et al. 2008; Moland et al. 2011; Skerritt et al. 2014). Passive acoustic telemetry allows data to be collected continually over long time periods.

1.6. Research aims, objectives and thesis outline

Crustacean aquaculture is a commercially valuable industry and there have been many studies looking at aquaculture techniques; however, this is typically for the larger Cambaridae species that have a high commercial value. *Austropotamobius pallipes* has only been bred for conservation purposes and therefore there are only a few published papers regarding its aquaculture.

The overall aims of the thesis are: (i) to improve aquaculture techniques, to increase the production of captive-bred *A. pallipes* for wild-release or captive-breeding programmes; (ii) to explore how these animals can be tagged, prior to release, so that their long-time survival, and success of the ark-sites can be ascertained; and (iii) to investigate how crayfish utilise an ark site, once released, to help inform future restocking programmes.

Chapter 2 provides details on the animals utilised for the experiments, the aquaculture facilities used and the associated methodologies and husbandry techniques utilised for each of the studies.

Chapter 3 investigates the optimal density for *ex-situ* breeding and rearing of *A. pallipes* hatchlings through the first year of life, prior to wild-release. The hypothesis that young-of-the year *A. pallipes* can be reared at high density without survival and growth being compromised is tested. This study identified a need to explore whether there were optimal size-ratios or if single-sex groups would be a more effective way of rearing young-of-the-year *A. pallipes.*

Chapter 4 builds upon the results of chapter three and explores whether it is beneficial to keep young-of-the-year *A. pallipes* in single-sex groups and / or size-graded groups. The hypothesis that juvenile *A. pallipes* exhibit sexual dimorphism as early as six months of age

and young-of-the-year male *A. pallipes* are more aggressive and dominant than the females is tested.

Chapter 5 investigates whether live food is a necessary component for hatchling *A. pallipes* for optimal survival and growth during the critical first few weeks of life or if other diets, which have been effective in other commercially bred crayfish species, can have good survival rates. Can commercially manufactured crayfish pellet diets produce equally good survival and growth in three-month-old juveniles, once the critical feeding stage is over? The hypothesis that live food and plankton, followed by just plankton, will produce the highest growth and survival rates in young-of-the-year *A. pallipes*, is tested.

Chapter 6 investigates whether a permanent marking system using passive integrated transponders (PIT) tags is viable with sub-adult and adult *A. pallipes*. The hypothesis that PIT-tagging will not affect survival and growth and determine is tested and the minimum safe size for PIT-tagging is ascertained. In the second part of the study, the minimum detection level of PIT-tagged crayfish *ex-situ* is investigated, to determine if there are benefits of tagging crayfish, using larger PIT tags.

Chapter 7 investigates how crayfish utilise ark sites by using acoustic telemetry. Is acoustic telemetry an effective tool for monitoring crayfish activity long-term and can it be used to investigate crayfish activity patterns and refuge fidelity? The hypothesis that crayfish will return to their territories if released away from them is tested.

In Chapter 8, key findings from this thesis, are discussed, addressing the primary questions of the dissertation, leading on to the broader implications of the results, and suggesting appropriate conservation measures and directions for future studies.

1.7. Ethics and licensing

All experiments were approved by the University of Bristol's Animal Welfare and Ethical Review Board (AWERB) and the Bristol Zoological Society's Conservation, Ethics and Sustainability Committee (CESC). Wild collection of animals was carried out under Natural England crayfish survey licence and an Environment Agency trapping licence. Crayfish were maintained in captivity under a Natural England scientific handling licence. The Crayfish Research Unit was inspected and certified as a hatchery facility by the Centre for the Environment, Fisheries and Aquaculture Science (Cefas). The still-water ark sites were inspected and approved by the Environment Agency and registered as fisheries by Cefas.

CHAPTER 2

General Methodology

Author contributions

JAN designed the Crayfish Research Unit (CRU) in Priddy, Somerset, which was then constructed by Tim Clements. JAN based all the crayfish husbandry methods on pilot trials carried out at the Bristol Zoo Gardens hatchery in liaison with Holly Thompson her crayfish aquarist, or from pilot studies that were carried out in the CRU. All collection and survey methods were designed based on best practice guidelines for crayfish or with guidance from fellow practitioners.

2.1. Introduction

This chapter introduces the methods that are common to all the data chapters, including information regarding the study areas, the survey and collection methods for the wild-caught crayfish in the tagging experiments and the females that were sourced to provide the juveniles for the experiments. It also provides details about the aquaculture facility and general husbandry methods that were common to all the *ex-situ* hatchery experiments.

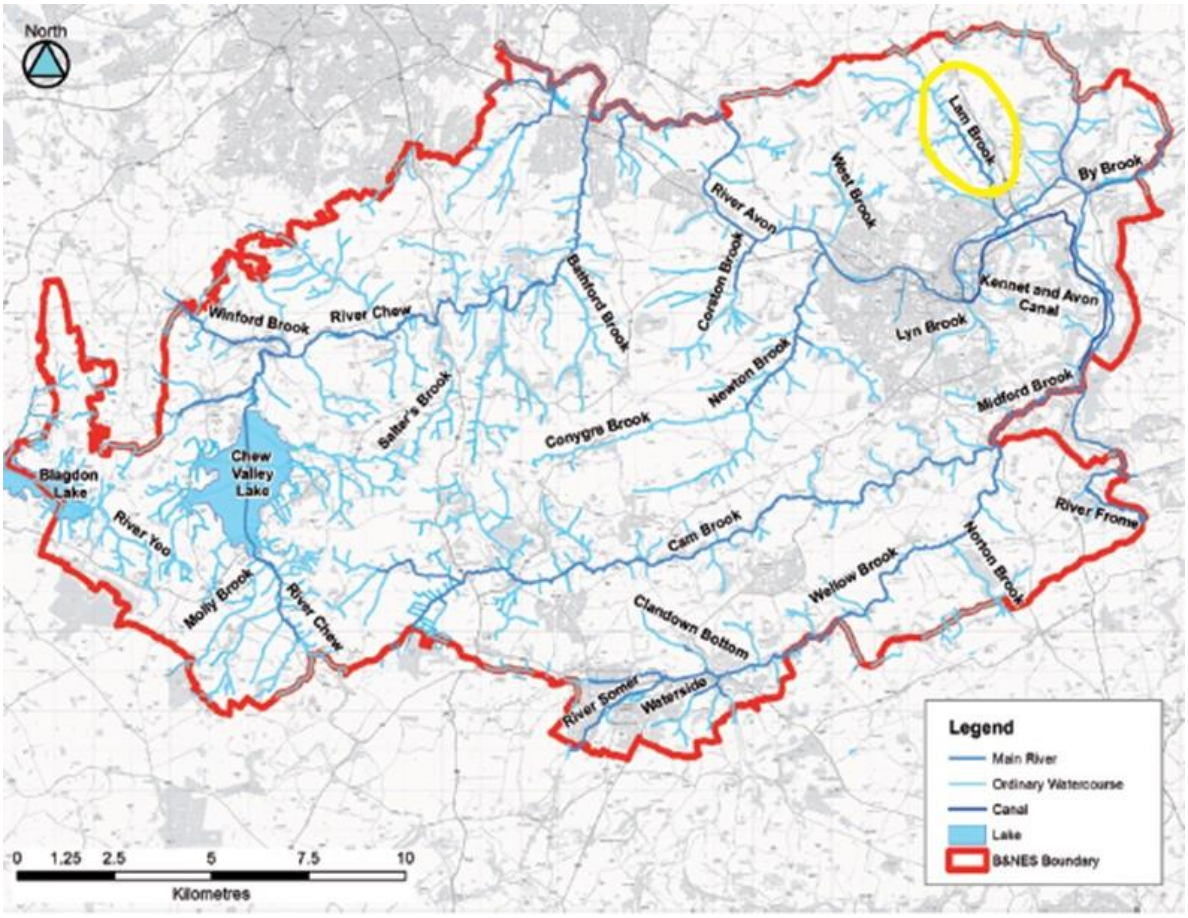

Figure 2.1. Location of Lam Brook, Bath & North East Somerset, circled in yellow (from www.bathnes.gov.uk/site).

2.2. Study area

The white-clawed crayfish *Austropotamobius pallipes* caught and bred for the research experiments were all from one donor stock of wild-caught animals collected from Lam Brook, a river within the Bristol Avon river catchment (the river catchment that contains rivers and

streams within counties around the city of Bristol). Lam Brook is approximately 4 km long and flows in a southerly direction through two English counties. It rises at several springs at Lansdown, Toghill and Cold Ashton, at the southern end of the Cotswold Hills in the county of South Gloucestershire, and flows to Lambridge, Bath, in the county of Bath & North East Somerset, where it joins the River Avon (Bristol Avon) (Figure 2.1). The river population is one of the only remaining abundant and thriving *A. pallipes* populations in southwest England and it is under threat from potential pollution events plus invasive signal crayfish, *Pacifastacus leniusculus*, which are present in the Bristol Avon. There are physical barriers such as water mills to prevent signal crayfish moving upstream into the Lam Brook, which may be a reason for the health of the population of *A. pallipes*.

2.3. Collection & production of *A. pallipes*

2.3.1. Collection of wild-caught *A. pallipes* for the Crayfish Research Unit

All aquaculture research that took place at the Crayfish Research Unit (CRU) in Priddy, Somerset was with first generation captive-born *A. pallipes,* born from wild-caught donor stock from Lam Brook. The standard survey season for crayfish is between July and October; however, a Natural England scientific licence permitted capture of ovigerous *A. pallipes* females in spring, between March and May, outside the typical survey season. The survey team involved in this process was the author plus other team members and volunteers from the Bristol Zoological Society's UK Conservation Team. Ovigerous female crayfish, (females with fertile eggs attached; Figure 2.2.iv), were collected from Lam Brook during March and April of 2013, 2014 and 2015 by hand searches and the use of artificial refuge traps (ARTs). The ARTs were positioned within river banks for four weeks prior to checking. The ARTs are a series of PVC pipes of varying diameters (32-63 mm) attached to a metal base plate, (Figure 2.3.ii), that are placed in a water body, on the bedrock, under banks, or within tree roots, perpendicular to the water flow. The ARTs were secured in place by tying them to a nearby structure such as a tree or root and then weighting it down with a large stone or brick. When an ovigerous female was captured, the animal was placed into a pot, which was a modified 250 mL lidded bait box, drilled to allow for water exchange. Grass and river water were added before the female was retained in the pot within a make-shift corral, (constructed with river stones), at the river bank until the required number of females were found (Figure 2.2.iii). The animals were then transported, within their individual pots, contained in a larger polystyrene box with grass and water packed around them, to minimise movement. During transportation, air conditioning was used, to minimise temperature

fluctuations and to ensure that the water temperature remained similar (± 2 °C) to the river temperature at the time of collection. At the aquaculture facility, crayfish were acclimated to the new system by slowly adding hatchery water to the polystyrene box containing pots over a 10-20 minute period. When water temperature was the same within the collection pots and the hatchery tanks, each female, in her individual pot, was added to one of the system's glass tanks and then carefully removed from their pot and placed into a plastic pipe, which acted as a refuge. The females were then closely monitored and checked regularly to assess the stage of egg development, to ensure that the time of hatching for each female was noted and recorded. Once the juveniles had hatched and were free-living, the females were retained in captivity for a further 2-3 months, and then released within a local ark-site (a safe site that is free from invasive crayfish species), once they had moulted and were feeding well. A proportion of crayfish (5%) were health screened by Centre for Environment, Fisheries and Aquaculture Science (Cefas, Weymouth, UK), to ensure no novel diseases were introduced back into the rivers, in line with the International Union for the Conservation on Nature, (IUCN) reintroduction guidelines (IUCN, 2013). Of particular concern was white spot syndrome virus (WSSV) that is found within prawn farms. The first epidemic of this virus was in Taiwan in the 1990s; however, there are no known cases in the UK (Wu et al. 2005). Crayfish were also screened for porcelain disease caused by the microsporidian parasite *Thelohania contejeani* and for crayfish plague *Aphanomyces astaci,* an oomycete fungus or water mould.

2.3.2. Collection of ark-site crayfish for acoustic telemetry study

For the acoustic telemetry study (chapter 7), *A. pallipes* were captured from the still-water ark-site where the research took place, Vobster Quay Inland Diving, Somerset, a public dive site within a 55 ha freshwater quarry, (grid reference: ST 705 498). In this case, plastic mesh crayfish funnel traps were used (Figure 2.2.i). The traps measured 595 x 300 mm, with a 12 mm mesh width and an entrance of < 90 mm (to prevent otters being trapped). This type of trap was used rather than the ARTs because the site was a deep-water site (maximum depth 25 m) and therefore the ARTs were not the most effective method of trapping. This is because the ARTs would have to be attached to very long lines and thrown in rather than positioned manually, which would mean that they might not land flat on the bottom, which might prevent the crayfish from entering them. The funnel traps were baited with sprats and left in the quarry overnight, tied to bank-side trees.

2.3.3. Production of captive-bred *A. pallipes* for *ex-situ* experiments

Once the juveniles had hatched, the females were monitored daily to prevent cannibalism of their youngsters. When the eggs hatch, the females produce a brood hormone that inhibits their feeding. This hormone is recognised by the juveniles as 'safe' (Little, 1976). Stimulation by juveniles on the mother's pleopods may reinforce the feeding inhibition. The hatchling crayfish undergo two moults whilst on the female. At stage-1 (Figure 2.3.i) they lack a fully-developed tail and remain on the female's pleopods at all times. As the juveniles become more independent at stage-2, (when they are fully formed; Figure 2.3.ii, iii & iv), the lack of mechanical stimulation of the hatchlings and the reduced weight on the pleopods may cause the females to revert to normal feeding and therefore begin to cannibalise their young (Little, 1976). The youngsters utilise their egg yolk as food for the first two weeks post-hatching, and then start foraging once their mouth parts develop. Once at stage-2, the juveniles were either used in the experiments or maintained within their brood group, until they were required for an experiment.

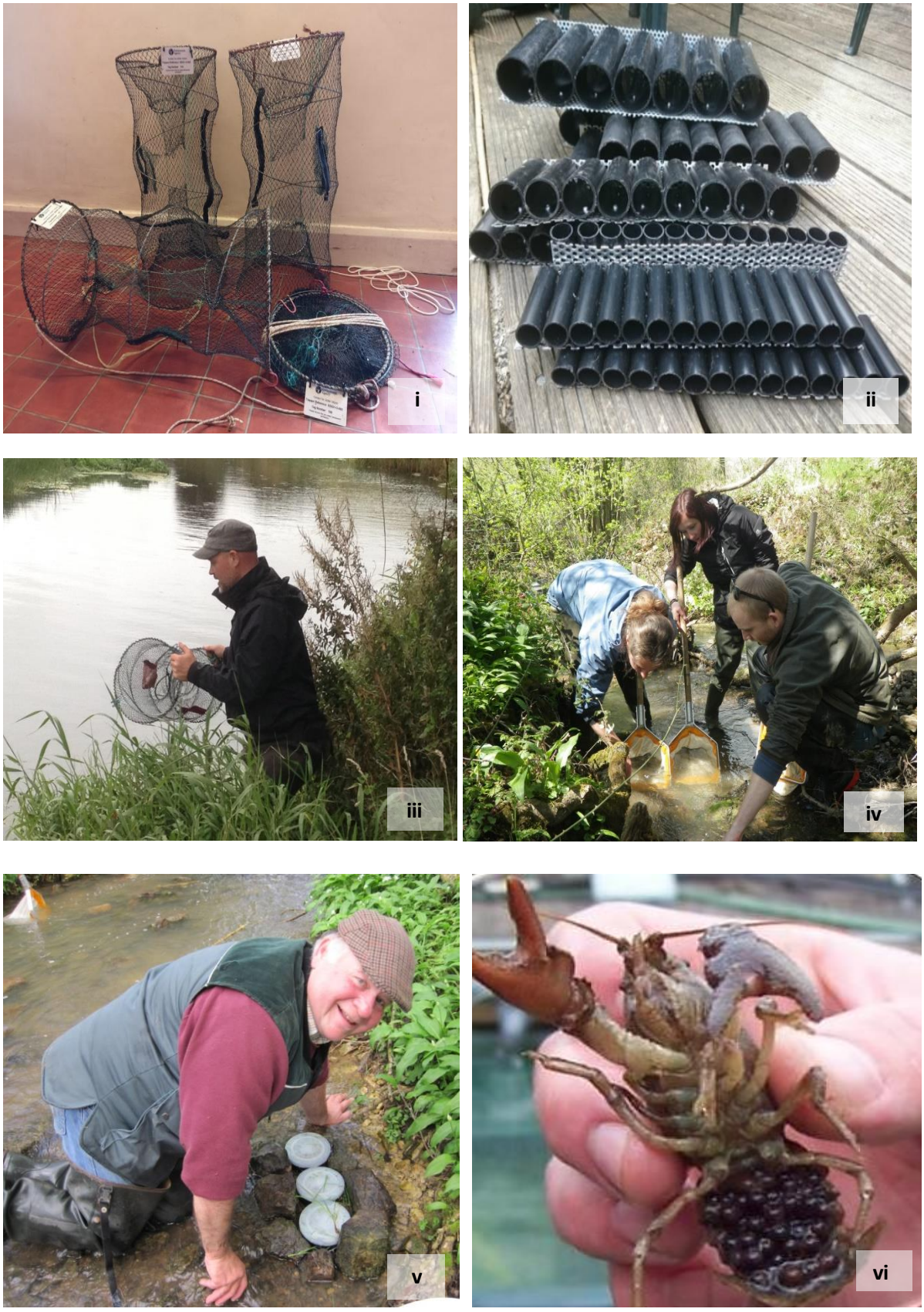

Figure 2.2.i) Collapsible baited crayfish traps; ii) Artificial Refuge Traps (ARTs); iii) deploying a baited trap; iv) stone-turning searching for crayfish; v) corral of bait pots containing ovigerous females; vi) ovigerous female © M. Ivey.

Figure 2.3. *Austropotamobius pallipes*: i) female with spermatophores; ii) adult male; iii) stage-1 hatchlings; iv) stage-2 hatchlings; v) & vi) stage-2 free-living hatchlings © M. Ivey.

2.4. Aquaculture facilities

2.4.1. Crayfish Research Unit

The aquaculture experiments were conducted from July 2013 through to August 2018 within the indoor Crayfish Research Unit (CRU), in Priddy, Somerset, (grid reference ST 527 515). The CRU was built within a wooden shed (1.8 x 2.4 m). The system frame was constructed from recycled plastic wood (Kedel Ltd, Lancashire, UK) and was a small-scale closed system of 24 glass, 45 L tanks, that recirculated, via a Tropical Marine System 1000 reservoir-based filtration unit, including mechanical filters, a fluidised sand filter and a trickle tower filter (Tropical Marine Centre, Bristol, UK) (Figures 2.4; 2.5; Table 2.1). Total system volume was 1100 L, with a turnover rate of three times per hour. Temperature was kept within a maximum range of 5 °C per day using three heaters during the winter months and two chillers (Aquamedic Ltd, Leicestershire, UK) during the summer, run in tandem to build in a safety margin. Water replacement was from rainwater harvested within a 300 L polyurethane storage tank, collecting water from roof gutters. The system was remineralised with calcium, magnesium and iron sulphates, to ensure that there were adequate hardness and calcium levels within the system. Extra aeration was provided to the tanks via an air pump (Evolution Aqua Ltd, Greater Manchester, UK) and additional sponge filters were added to each of the tanks to provide additional filtration and to ensure that there was enough oxygen within each. No artificial light was added to the building, which had a large window to allow lighting by natural daylight.

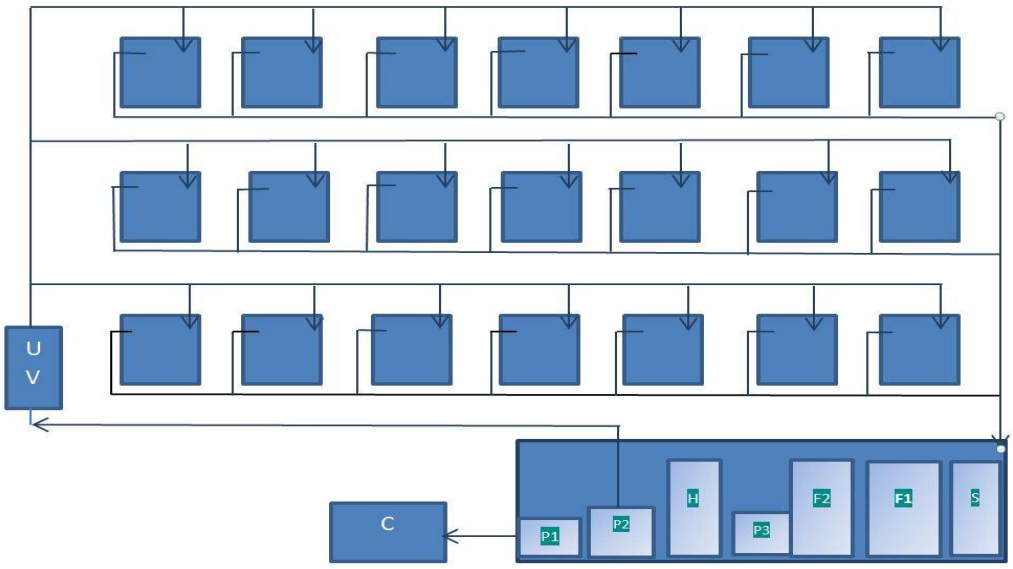

Figure 2.4. Schematic diagram of the Crayfish Research Unit, Priddy, Somerset; UV is ultraviolet filter; C is chiller; P1, P2 & P3 are submersible pumps; H is heater; F1 & F2 are filters; S is filter bag.

Table 2.1. Crayfish Research Unit technical specification of equipment items used.

Item	Detail	Size/rate
System volume	Total including tanks, reservoir and tank	1,100 L
24 x Seashell Aquariums Ltd	Glass lidded tanks	45 L; 300x450x300 mm (0.12 m^2 bottom area)
PVC ring-main	Pipe ring-main from sump to filter & UV, surface supply to tanks and returning to sump via 5 mm meshed outlets that drain into wastepipe to sump	50 mm ring-main pipe 25 mm tank inlets 32 mm tank outlets 90 mm waste pipe
Reservoir	HDPE	250 L
UV - TMC P2 110	Ultra violet steriliser	110 w; 3 x per hour
C - Aquamedic Titan 500	2 x chillers run in tandem in summer	150 w
P1 - TMC V2 Power 1300	Submersible sump pump to run chiller	25.5 w
P2 - TMC V2 Power 5500	Submersible sump pump to run system	105 w; 3 x per hour turnover rate
H - XiLong heaters	3 run in tandem to achieve a seasonal range between 5 - 22 °C	1 x 500 w 2 x 300 w
P3 - TMC V2 Power 800	Submersible sump pump to run fluidised sand-filter	13.5 w
F1 - TMC System 1000	Trickle filter tower	1000 L
F1 - TMC bio-rings	Polypropylene filter media for tower	30 mm
F2 - TMC V2 – Bio 1500F	Fluidised sand filter	1500 L
F2 - TMC aragonite sand	Filter media for fluidised sand filter	2 kg; surface area 10,000 m^2/m^3
S - TMC filter bags	2 x polyester felt filter to trap waste particles from waste pipes returning from tanks	50 micron mesh
Evolution Aqua Airtech130	Air compressor to supply air to sponge filters and reservoir – *not shown*	130 L via 8 mm airline
Boyu SF-100	24 x sponge filters in tanks – *not shown*	50 L capacity

Figure 2.5. The Crayfish Research Unit, Somerset: i) the 24-glass tiered system; ii) the filtration reservoir; iii) the shed © J. Nightingale.

2.4.2. Ubley Hatchery

The pit-tagging experiment (chapter 6) took place from January 2015 through to January 2016, at a local fishery hatchery in Ubley, Somerset, owned by Bristol Water, a local water company (Figure 2.6). This system consists of seven fibreglass tanks (1850 x 1850 x 500 mm; 3.42 m^2 bottom area), on a flow-through system using water from one of the local water reservoirs. Temperature therefore fluctuated naturally and was dependent on the local reservoir temperature. Water flowed out of the tank via a 2 mm meshed downpipe. All tanks were lidded and water level was kept low to avoid escapes and predation. No water changing took place due to the system being on continuous flow-through and therefore total water was replaced five times per hour. The crayfish were not provided with supplementary food because a constant supply of live food, leaf litter and algal matter entered the tanks.

Figure 2.6. Ubley hatchery experimental vats © J. Nightingale.

2.5. General crayfish husbandry

2.5.1 Water changing, tank cleaning and water quality

Crayfish are susceptible to water pollutants such as nitrates, phosphates and heavy metals (Holdich & Reeve, 1991). Therefore, it was essential to ensure that the water quality in the hatchery was optimal for crayfish growth and breeding (Table 2.2). Crayfish exoskeletons are made out of calcium carbonate and therefore the crayfish require a level of water hardness for successful growth. During the moulting process, crayfish absorb cuticular

calcium from their exoskeletons into the gastroliths in the foregut wall of their digestive tract (Greenaway, 1985). However, the gastroliths can only store a limited amount of calcium and therefore the exoskeletons were also left in the tank so that the crayfish can consume more of the shell should they require extra calcium than what is present within their gastroliths to help with the high demand for calcium post-moulting. Crayfish may also bury or cache their moults (excuviae), to avoid attracting attention from potential predators as to their vulnerable body state. They also save their excuviae, so that they can eat them when their mouth parts have hardened (Buřič, 2016). Water changes were carried out on a weekly basis and up to 25% of the system water was replaced each time. Water quality tests were carried out on a weekly basis, to ensure that the quality was maintained.

Table 2.2. Optimal and maximum water quality parameters for breeding and rearing *A. pallipes* within a closed system, *ex-situ* hatchery.

Parameter	**Optimal value/range**	**Minimum - maximum tolerance**
	mg/L	
Dissolved Oxygen	8.5	3.4 - 20
pH	7.9	6.9 - 9.0
Ammonia	< 0.1	0.1 - 0.74
Nitrite	< 0.1	0.1 - 25
Nitrate	< 25	0.1 - 50
Calcium	> 20	1.0 - 100
Phosphate	< 0.5	< 0.1 - 2.5
General hardness	150	100 - 300
Carbonate hardness	150	100 - 300

Water quality chemical levels remained consistent throughout the experiments: ammonia < 0.1 mg/L, nitrite < 0.1 mg/L, nitrate < 15 mg/L, phosphate < 0.2 mg, pH 7.8, calcium ≥ 35 mg/L, general hardness 150 mg/L, carbonate hardness 120 mg/L and a level of dissolved oxygen > 90%.

2.5.2. Tank theming

Crayfish require refuges and will spend the majority of time within the refuge, only leaving when foraging, during the breeding season or moulting. Crayfish prefer to utilise refuges that are only slightly bigger than themselves; therefore, as they grow they require larger refuges (Lodge & Hill, 1994). Crayfish will defend their refuges from tank mates (Tricarico & Gherardi, 2010). Therefore it is important to provide several appropriately-sized refuges for each animal (Figure 2.7; Table 2.3).

Figure 2.7. Refuges for crayfish: i) 3-hole engineered brick; ii) 10-hole engineered brick; iii) plastic pipes and twin wall plastic © J. Nightingale.

2.5.3. Temperature regime

Temperature was manipulated using both aquarium heaters and chillers, to maintain a steady temperature in the tanks. This is then manipulated during the year to mimic, as much as possible, natural river temperature (Table 2.4).

2.5.4. Lighting regime

The Crayfish Research Unit had one large window and no artificial lights had been added to the crayfish tanks. Therefore the photoperiod for all the experiments was natural and average values were 12 h light and 12 h dark.

Table 2.3. Refuges and substrate required for rearing and breeding *A. pallipes* within hatchery tanks.

	Crayfish age-classes					
AGE CLASS:	**0+**	**6-months**	**1-year**	**18 -months**	**Adult**	**Breeding**
***REFUGE:**	Twin-wall plastic; sand/fine gravel	16 mm pipes glued together	20 mm pipes glued together; engineered bricks: 8-10 hole	32 mm pipes glued together; engineered bricks: 3-4 plus 8-10 hole	40 mm pipes; glued together; engineered bricks: 4-8 holes double layer	Double layer of engineered bricks: 4-8 holes; mating tubes
Coral sand / gravel (0.4-1 mm) for all age-classes; acts as a buffer increasing calcium carbonate						
*Ensure 2-3 refuges per crayfish for each age group						

Table 2.4. Annual temperature regime for breeding and rearing *A. pallipes* within a closed-system, *ex-situ* hatchery; 0+ are young-of the year; 1+ are one-year-old crayfish.

Month	***Temperature (°C)**					
	0+	**1+**	**Sub-adults**	**adults**	**Brood-stock**	**Ovigerous females**
January	10-12	10-12	10-12	10-12	5	5-6
February	11-13	11-13	11-13	11-13	9	8-9
March	12-14	12-14	12-14	12-14	9	9-10
April	13-15	13-15	13-15	13-15	15	11-15
May	14-16	14-16	14-16	14-16	15	14-15
June	15-17	15-17	15-17	15-17	17	15-17
July	16-19	16-19	16-19	16-19	17	16-19
August	15-19	15-19	15-19	15-19	17	16-19
September	14-17	14-17	14-17	14-17	15	14-17
October	12-14	12-14	12-14	12-14	10	12-10
November	11-13	11-13	11-13	11-13	9	9-10
December	10-12	10-12	10-12	10-12	5	5-6

*Maximum temperature range per 24-hour time period = 4 °C

2.5.5. Feeding regime

The crayfish were fed daily at 19:00 hours, on a carefully selected diet, with a proven track record of high survival and growth. Hatchling crayfish were fed live food plus defrosted enriched plankton. After six weeks the live food was gradually removed and defrosted enriched plankton plus a vegetable mix was offered, at a rate of three parts plankton to one part vegetable mix (Table 2.5). The diet included defrosted, gamma-irradiated plankton mix of bloodworm, *Daphnia*, *Cyclops*, *Mysis*, krill and rotifers (Tropical Marine Centre, Kent, UK). This was enriched with trace elements (Dennerle Vital Elixir; ProShrimp, Mansfield, UK) and New Era frozen food enrichment: a liposome-based product containing vitamins and antioxidants (World Feeds Limited, Thorne, UK). NatuRose, a natural source of the caratenoid Astaxanthin derived from the microalgae *Haematococcus pluvaili* (Dr T&T Health UK Ltd, Northamptonshire, UK), was added to the feed twice per week. A lack of caretenoid can result in *A. pallipes* developing blue hues; a similar finding has been seen in black tiger prawn *Penaeus monodon fabricus* (Menasveta et al. 1933).

The amount and type of food varied according to life stage of the crayfish (Table 2.6) and enough food was offered, to ensure that there was some uneaten food left over, which was then siphoned of the tank, to prevent water quality deterioration. Diets were reduced by 20% when temperatures were below 10 °C and the adult females were not fed when the eggs had hatched as their appetite was inhibited at this stage. They resumed feeding again once the last stage-2 juvenile had become free-living.

The live food was hatched from freeze-dried *Artemia franciscana* eggs (Ocean Nutrition, Essen, Belgium) in salt water of specific gravity 1.022. The salt water was prepared using artificial sea salt Tropic Marin (Tropical Marine Centre, UK), added to rainwater and aerated and heated to a temperature of 27 °C. Approximately 35 g of salt was added to 1 L of rainwater to make up the desired salinity. Three 2 L clean drinks bottles were inverted with an airline through the screw top. Fully saline water was added to all three bottles and 50 w stick heaters were added, to ensure a temperature of approximately 28 °C. Approximately 7 g of *Artemia* eggs was added to the first bottle and left for 24-hours to hatch. On day-2, a new bottle of eggs was set up. On day-3, the first bottle of *Artemia,* now hatched, was rinsed by straining the *Artemia* through a 200 micron mesh strainer and rinsing with warm, clean saline water. The *Aretmia* was then gut-loaded with 0.1 mL of *Nannochloropsis* spp., (Zebrafish Management Systems Ltd, Berkshire, UK) per 1 L of culture and enriched with 0.5 mL of highly unsaturated fatty acids (HUFA), per 1 L of culture. After 12 hours the *Artemia* was strained again through the mesh into sump water, mixed with enriched, defrosted plankton and added, via syringe, into the tanks of hatchling crayfish. This process was

repeated daily for six weeks until all hatchlings were feeding completely on defrosted plankton.

Table 2.5. Feeding regime for hatchery *A. pallipes* at different age-classes.

Age-class	**Stage-2 hatchlings**	**1-6 months**	**7-12 months**	**Sub-adults & adults**
Food items offered daily	Gut-loaded, enriched live *Artemia* nauplii plus enriched freeze-dried plankton: rotifers, copepods and *Daphnia*	Enriched freeze-dried plankton: *Artemia*, *Daphnia*, *Mysis*, bloodworm	Enriched freeze-dried plankton: *Artemia, Daphnia*, *Mysis*, bloodworm. Vegetables: peas, kale, spinach, chard	Enriched freeze-dried plankton: *Artemia, Daphnia*, *Mysis*, bloodworm Vegetables: peas, kale, spinach, chard, carrot
Percentage per body weight of food given daily	4	10	15	Sub-adult (20); adults (5); brood-stock (10)

CHAPTER 3

Determining effective density regimes for rearing juvenile *Austropotamobius pallipes*

An adapted version of this chapter has been published in:

Nightingale, J., Stebbing, P., Taylor, N., McCabe G., & Jones, G. (2018). Determining an effective density regime for rearing juvenile *Austropotamobius pallipes* in a small-scale closed system hatchery. *Aquaculture Research, 49,* 3055-3062. (Appendix 2).

Author contributions

JAN designed the study and collected and analysed the data. NT checked through the statistical analysis. JAN produced the manuscript draft and GJ, GM and PS provided support and guidance with the experimental design and contributed criticially to the manuscript and chapter drafts.

Abstract

With recent advances in aquaculture techniques, captive-breeding of the endangered white-clawed crayfish *Austropotamobius pallipes* for restocking is becoming a widespread conservation method. Establishing optimal stocking densities for aquaculture is essential in maximising productivity, and increases the likelihood of crayfish survival when released. A 240-day experiment took place using two-month-old juvenile, captive-born, *A. pallipes,* within a small-scale, closed-circuit hatchery to investigate survival, growth and aggression at three treatment densities, low (100/m^2), medium (200/m^2) and high (300/m^2). Crayfish were counted and measured every 60 days, between August 2015 - April 2016. Mean survival rates were high across all three densities (87.7 ± 2.8%). Carapace length was significantly longer at low density than at medium and high densities. While growth was not significantly different between treatments, it was significantly higher in the first two months, across all three treatments (47.1 ± 6.6%) than in subsequent periods (14.1 ± 5.8%). Size variation within groups increased with density, suggesting that social dominance hierarchies are established with increasing stocking density: dominant individuals are larger and competitively exclude smaller individuals from food resources. Males were significantly larger than females from six-months of age, (when they could be reliably sexed), in all three treatments. The larger male size suggests that sexual dimorphism begins prior to sexual maturity, with males growing faster and being more dominant and aggressive than females. In conclusion, young-of-the-year *A. pallipes* can be reared at high densities without compromising survival; however the optimal stocking density that maximises growth and health is 100/m^2.

3.1. Introduction

Crayfish aquaculture, is an important component of the food industry in some countries, i.e. the American red swamp crayfish *Procambarus clarkii*, in both Asia and North America (Ackefors, 2000; Romano & Zeng, 2017). Determining optimal rearing densities is one of the key elements in crayfish aquaculture and it is well established that stocking density is directly related to growth and survival of crayfish species (Savolainen et al. 2004; Romano & Zeng, 2017). The effects of stocking density on juveniles have been well studied in some crayfish species with economic value. These include Australian crayfish species *Cherax* spp., (Jones & Ruscoe, 2000; Naranjo-Páramo et al. 2004; Rodgers et al. 2006); American red swamp crayfish (Figureiel & Miller, 1995; McClain, 1995); plus astacid species such as the signal crayfish *Pacisfastacus leniusculus*, (Ahvenharju et al. 2005; Ulikowski et al. 2006;

Harlioğu 2009; González et al. 2011a), and noble crayfish *Astacus astacus*, (Pursiainen et al. 1983; Keller, 1988). For juvenile astacid crayfish species, a range of rearing densities from 50/m^2 up to 1,200/m^2, have been investigated, and show that survival is significantly compromised with increasing density, due to intraspecific competition for food and space (Savolainen et al. 2004; Harlioğlu, 2009; González et al. 2010).

Aquaculture of crayfish species for conservation and restocking is a relatively new concept. As with all crayfish aquaculture, it is important to maximise productivity and to produce large, robust crayfish that will have an increased chance of survival when released into the wild. In captivity, *A. pallipes* grows more quickly than in the wild. It is estimated that *in-situ A. pallipes* matures in the third or fourth year of life (Reynolds et al. 1992). In contrast, in captivity, the fastest growing males and females can reproduce in the second year of life (Policar et al. 2010). Captive-born *A. pallipes* introductions typically occur with young-of-the-year crayfish in order to free up hatchery space for the next hatching attempt, (Nightingale et al. 2017). Therefore, if their growth potential in captivity is maximised, this has important recruitment implications, increasing the chances of reproduction in the release year. This in turn should increase the chance of establishment and the success of the wild supplementation. Therefore, a key component for *A. pallipes* aquaculture is to establish an optimal density regime that will produce crayfish of a large enough size that can potentially breed in the second year of life. Chelae autotomy, where crayfish lose one or both of their claws, can occur when crayfish are housed communally and is an indication that there have been aggressive encounters between individuals (Figureiel & Miller, 1995). A lack of chelae can lead to a reduction in survival because the crayfish have injury trauma and may experience an increase of agonistic encounters and reduce their potential to feed optimally. This increased aggression can cause a reduction in fitness, which in turn can lead to increased mortality (Figureiel & Miller, 1995; Sáez-Royuela et al. 2001).

There are no known experiments investigating the effect of stocking density on growth and survival of juvenile *A. pallipes*. Previous laboratory experiments, investigating shelter, feeding and temperature regimes when rearing stage-2 juvenile *A. pallipes* employed a wide variation in stocking densities in their research; between 50/m^2 (Sáez-Royuela et al. 2001) and 500/m^2 (Policar et al. 2010). The aim of this research was to examine survival, growth and aggression rates of juvenile *A. pallipes*, maintained at differing stocking densities, to establish the optimal stocking density for young-of-the-year crayfish held within a closed-circuit hatchery facility.

3.2. Materials and Methods

For a detailed description of the wild collection, hatchery details and husbandry routines see Chapter 2 "General Methodology".

3.2.1. Source of experimental animals

The juvenile *A. pallipes* were hatched from 20 wild-caught, ovigerous females, which were collected from a local river population and brought into the hatchery two months prior to the experiment commencing. The females were removed once the juveniles were at stage-2; i.e. had undergone two moults and were free-living. The juveniles were reared for a further two months, which is the most critical survival period, prior to the experiment commencing.

3.2.2. Experimental set up and design

The experiment took place, in an indoor closed-circuit aquaculture facility, in Somerset, England. The study subjects were 432, juvenile white-clawed crayfish *Austropotamobius pallipes*, which were 60 ± 7 days old, with a mean carapace length (mm ± SD) of 7.24 ± 0.33 mm. The crayfish were randomly assigned into three different treatments: 72 were maintained at low density (100 crayfish/m^2), 144 were maintained at medium density (200 crayfish/m^2) and 216 were maintained at high density (300 crayfish/m^2). The experiment took place over 240 days between August 2015 and April 2016.

3.2.3. Experimental procedure

Every 60 days the percentage of surviving individuals in each tank was calculated and each crayfish was examined. Measurements were not taken more frequently to avoid the effects of human handling on the survival, growth and condition of the animals.

Table 3.1. Treatment categories showing density and number of crayfish per tank, n=6.

Treatment	Density category	Crayfish / tank	Density equivalent /m^2
1	low	12	100
2	medium	24	200
3	high	36	300

Figure 3.1. Measuring techniques for hatchling *A. pallipes*: (i) using callipers and (ii) using photography and ImageJ software © J. Nightingale.

3.2.4. Data collection and analysis

From day-60, carapace length, was measured from the anterior edge of the rostrum to the posterior edge of the cephalothorax to the nearest 0.1 mm using Vernier 1500 mm callipers (Moore and Wright, Sheffield). For day-1, carapace length was calculated by taking a photograph of each individual on a known calibrated scale and then using the computer software programme ImageJ (Schneider et al. 2012), to determine the length. This reduced stress to the crayfish by minimising handling time and ensured accurate measurements. Variation in each group size was recorded and percentage growth along the time series was calculated by subtracting the new mean carapace length from the previous mean carapace length and dividing this figure by the previous mean carapace length. The percentage of missing chelae, a standard measurement of crayfish aggression (Figureiel & Miller, 1995), was also recorded. Five data sets were collected at days 1, 60, 120, 180 and 240. From day-120 the crayfish could be sexed. Average sex-ratios in the three density treatments were 42:58 (100/m^2), 43:57 (200/m^2) and 51:49 (300/m^2). To determine if there were any differences in growth, survival or aggression between the three treatment densities, data were examined by using an ANOVA, where variables were tested at the tank level and generalized linear mixed models (GLMMs) (function *glmer*, R package *lme4* Bates et al. 2015), where variables were tested at an individual level. Goodness-of-fit to normal distributions was checked by running the Shapiro - Wilk test on residuals and prior to using an ANOVA data were log transformed, to stabilise the variance. Treatment density (low,

medium or high) and sex of crayfish were considered as fixed effects, whilst tank number was considered a random effect. Interactions between fixed effects were tested within each model; however, all interactions were not significant and therefore dropped from the final models. Only variables that had a significant effect were retained within the models. Statistical analyses were performed using R 3.0.1 (R Core Team, 2016).

3.3. Results

3.3.1. Survival

There was no significant difference in survival ($F_{2,333}$ = 0.13, p = 0.33) in the present study, across all three treatments. Average survival rate across all three treatments was 87.7 ± 2.8% (Figure 3.2).

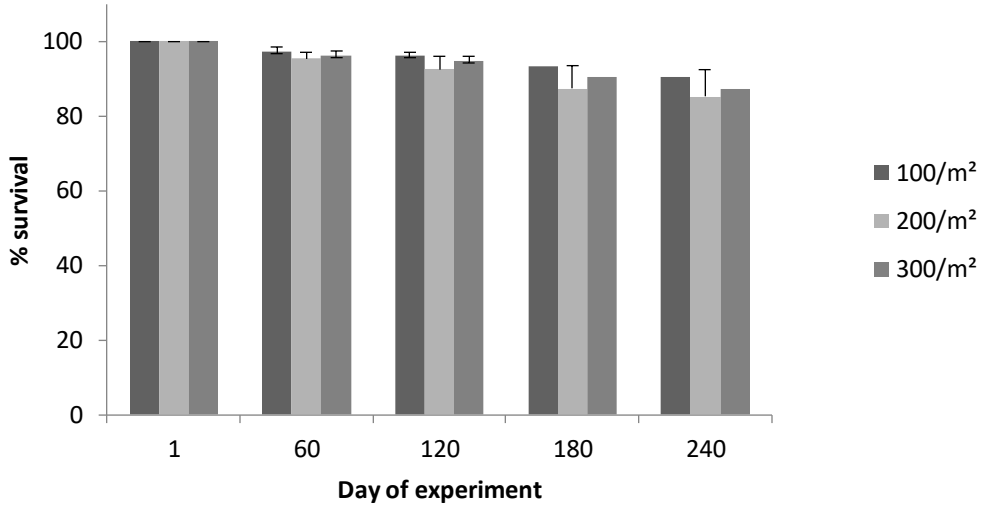

Figure 3.2. Percentage survival of *A. pallipes* at different treatment densities: low (100/m^2), medium (200/m^2) and high (300/m^2), over the time series, day-1 to day-240. Error bars represent 95% confidence intervals.

3.3.2. Carapace length and growth

At day 240, mean carapace length at low density was significantly longer than the mean carapace length at high density ($F_{2,372}$ = 8.12, p < 0.001). There was no significant difference between growth at medium and high density or between low and medium densities (Figure 3.3.a). Males (16.8 ± 0.51 mm) were overall significantly larger (11.3%)

than females (14.9 ± 0.58 mm) at all treatments, ($F_{2,372}$ = 130.94, p < 0.001) (Figure 3.3.b); however, the interaction between sex and treatment on carapace length was not significant.

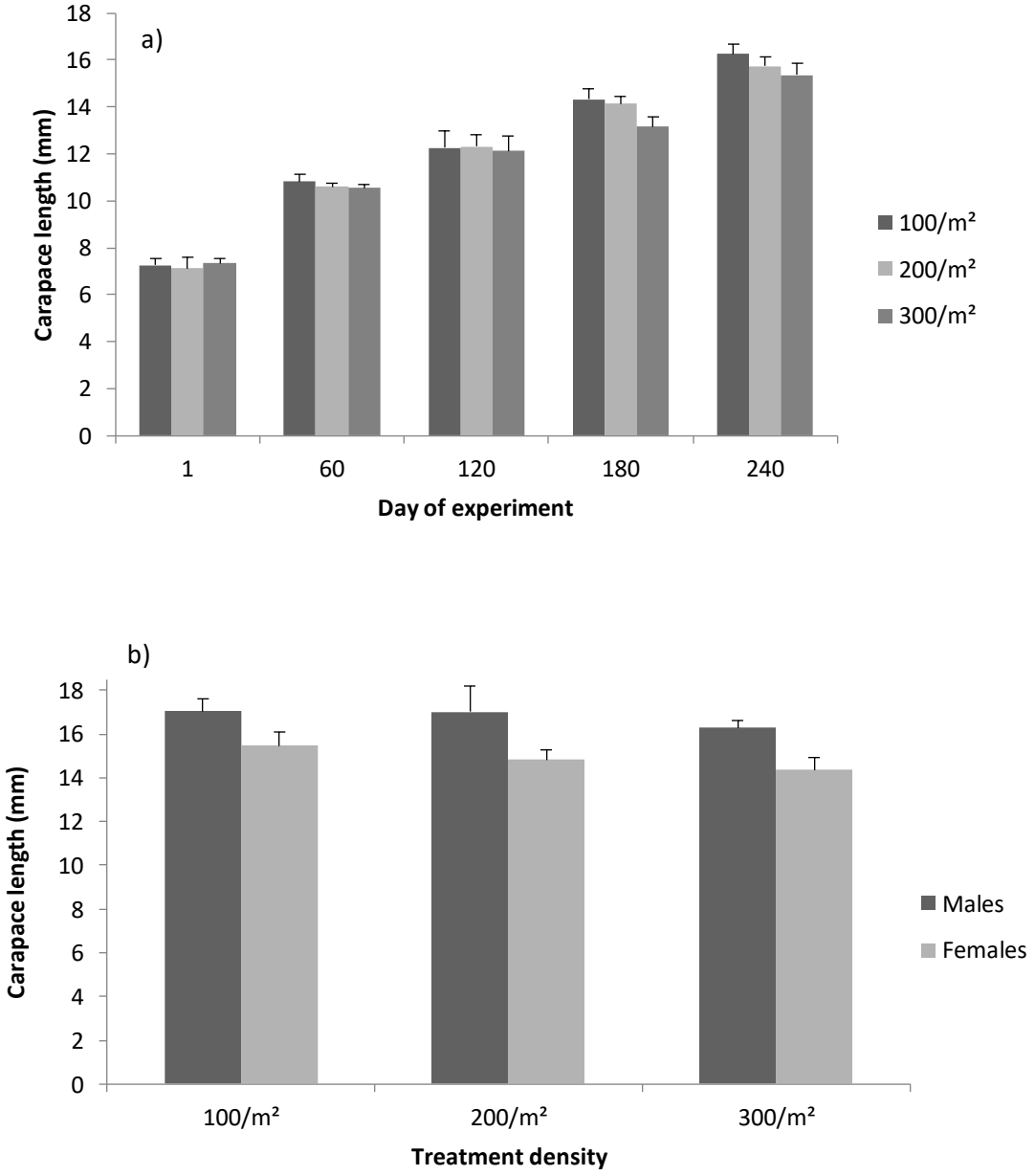

Figure 3.3. Carapace length of *A. pallipes* at different treatment densities: low (100/m^2), medium (200/m^2) and high (300/m^2) over the time series, from day-1 to day-240; a) with males and females combined; b) with males and females separate. Error bars represent standard deviations.

There was no significant difference between overall mean growth of the three different treatments ($F_{2,69}$ = 0.1, p = 0.9). However, the mean growth at the beginning of the time series was significantly higher (47.1 ± 6.6%) between day-1 and day-60 than over each 60-day time period (14.1 ± 5.8%) throughout the rest of the time series ($F_{5,66}$ = 174.2, p <

0.001). For the two data sets (day-180 and day-240), where comparisons of growth of males and females could be made, growth was significantly greater (on average 6.8%) in males than females at all densities ($F_{2,370}$ = 132.1, p < 0.001), (Table 3.2). There was no significant, interaction between sex and treatment on growth.

Table 3.2. Percentage growth at each density treatment, shown throughout the time series from day-60 to day-240. Mean ± standard deviations shown.

Density /m^2	Day 1-60	Day 60-120	Day 120-180			Day 180-240		
	All	All	All	Male	Female	All	Male	Female
100	48.8 ± 6.2	13.3 ± 6.8	17.2 ± 4.7	21.2 ± 3.9	14.3 ± 4.9	13.5 ± 3.5	13.0 ± 3.8	12.3 ± 4.1
200	49.0 ± 9.4	16.2 ± 4.9	15.0 ± 6.0	16.0 ± 5.6	12.8 ± 6.2	11.3 ± 1.9	13.6 ± 3.7	9.8 ± 1.0
300	43.5 ± 4.1	14.7 ± 6.7	13.5 ± 6.3	13.5 ± 6.5	12.2 ± 5.8	11.8 ± 1.2	13.3 ± 0.8	10 ± 2.4

3.3.3. Size variation

Size variation (2.14 ± 0.15 mm), between individuals within treatment groups on day-1, was not significantly different. As the experiment progressed, the size variation, across all groups significantly increased ($F_{3,66}$ = 41.67, p < 0.001). Size variation was greater in the higher density groups; at day 240, size variation across groups was significantly lower at low density (5.2 ± 0.7 mm) than at high density (7.6 ± 1.2 mm) ($F_{2,69}$ = 3.46, p = 0.03). There was no significant difference in size variation between low and medium or medium and high density (Figure 3.4.a). There was no significant size variation between males and females ($F_{1,106}$ = 2.38, p = 0.13), (Figure 3.4.b).

3.3.4. Chelae autotomy

The percentage of individuals showing chelae autotomy increased in the medium and high density treatment groups as the experiment progressed; however, due to some of the crayfish starting with missing chelae on day-1 of the experiment, the relative amount of chelae autotomy within the low density treatment group fell to zero by day-120 and then increased up until the end of the experiment (Figure 3.5.a). The percentage of individuals

showing chelae autotomy was greatest in the medium density group, trending towards being significantly higher than in the low density group (z_{370} = -1.901, p = 0.057). At day-240, relative chelae autotomy (% ± SD) was significantly higher in females (29.6 ± 18.3%) than males (16.2 ± 13.7%) (z_{370} = 2.84, p = 0.004). Males on average experienced 8.9% less chelae autotomy than females; however, the interaction between sex and treatment on relative chelae autotomy was not significant (Figure 3.5.b).

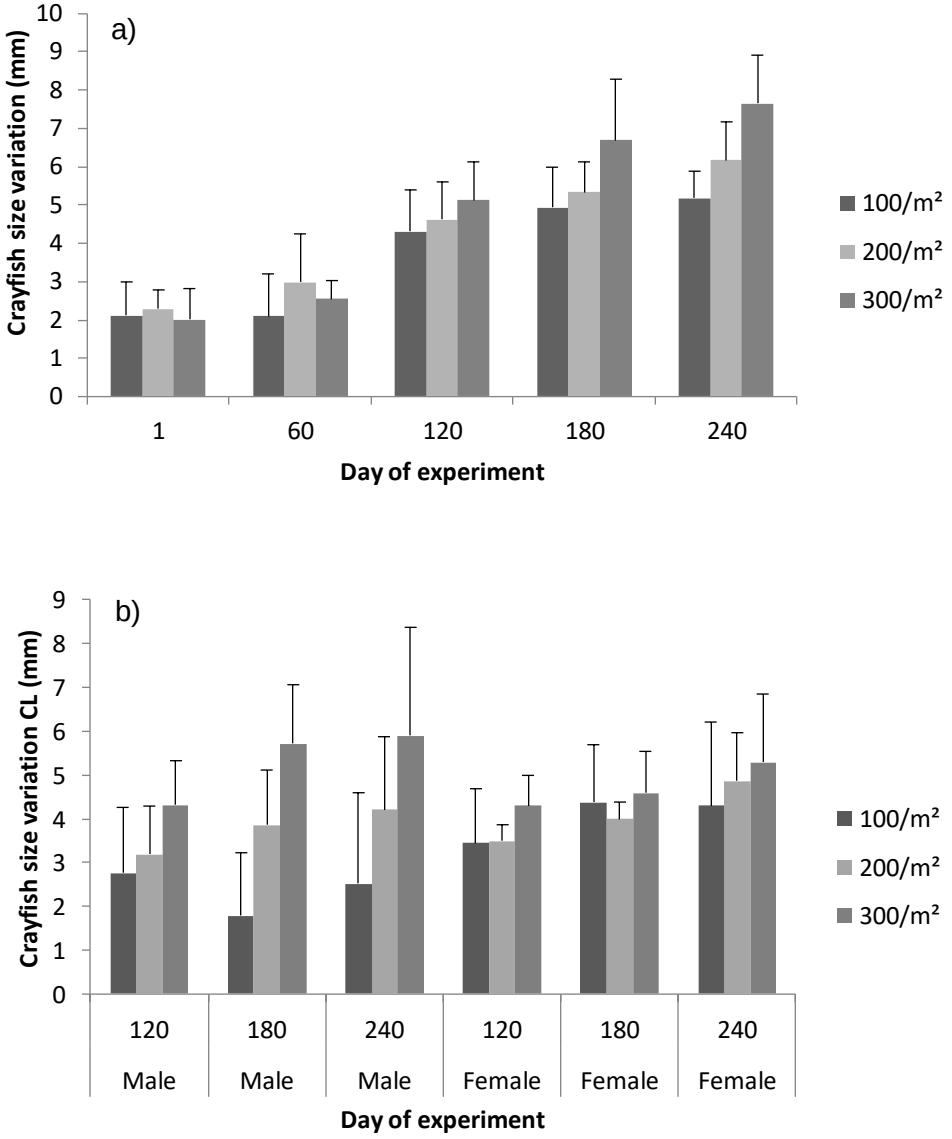

Figure 3.4. Size variation of *A. pallipes* at different treatment densities: low (100/m^2), medium (200/m^2) and high (300/m^2) over the time series; a) from day-1 to day-240, with males and females combined; b) separate males and females at the last three time series points: day-120, day-180 and day-240. Error bars represent standard deviations.

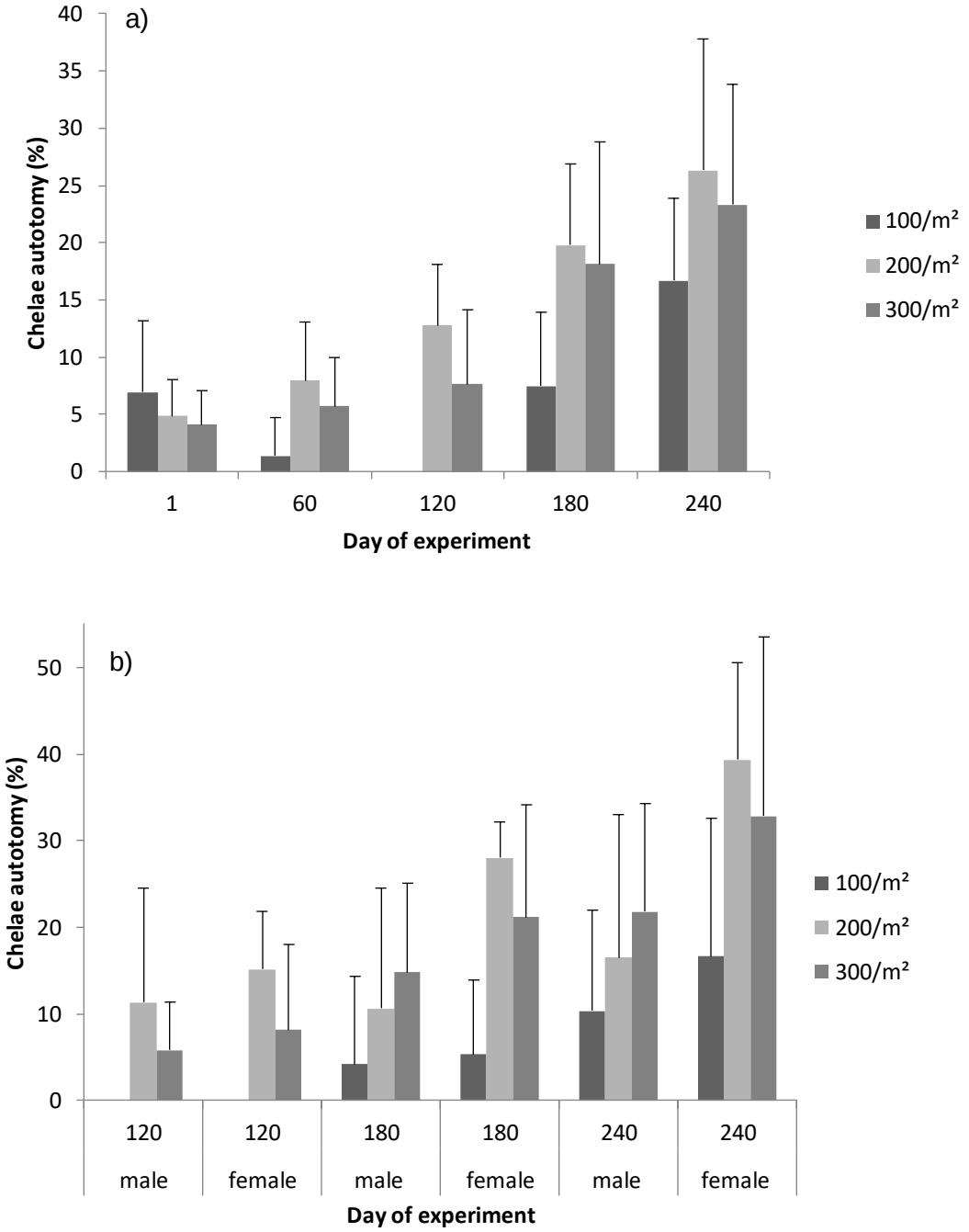

Figure 3.5. Percentage of *A. pallipes* experiencing chelae autotomy at different treatment densities: low (100/m^2), medium (200/m^2) and high (300/m^2) over the time series; a) from day-1 to day-240, with males and females combined; b) separate male and female data for the last three time series points: day-120, day-180 and day-240. Low density groups are not shown on day-120 as there was no chelae autotomy within these groups at this time point. Error bars represent standard deviations.

3.4. Discussion

There have been several analyses of density-related effects in astacid crayfish that have demonstrated that increasing density results in a reduction in survival and growth, greater size variation and more aggressive encounters (Naranjo-Páramo et al. 2004; Savolainen et al. 2004; Harlioğlu, 2009; González et al. 2010). However, there are large disparities between studies as to an acceptable rearing density for juvenile crayfish. What is acknowledged is that there is a critical survival period for juvenile crayfish during the first few weeks of life, (Sáez-Royuela et al. 2001; González et al. 2010). Supplying the correct type of food during this time period may be more important than the density at which the animals are maintained. Historically, many of the experiments investigating juvenile astacid rearing densities, used commercially available dry fish diets as a first feed for the crayfish. These diets yield poor survival and growth in hatchling *A. pallipes* (Sáez-Royuela et al. 2001) and *P. leniusculus* (Ulikowski et al. 2006; Sáez-Royuela et al. 2007). Consequently, some research may be skewed by mortality and growth deficiencies due to inadequate nutrition rather than being caused by the treatments applied and the lack of standard methodology in crustacean studies can make results difficult to interpret (González et al. 2010; Carral et al. 2011).

In the current study, the experiment began when the juveniles were two months of age, after the critical feeding period had ended, and the crayfish were fed to excess, to ensure that the results would be density-related rather than confounded by other husbandry elements. As the objective was to maintain high survival rates, the densities selected for this experiment were based on previous pilot studies and were significantly lower than in some other astacid crayfish experiments where the driver was for commercial rather than conservation purposes, therefore the threshold density for *A. pallipes* may not have been reached.

3.4.1. Survival

Density did not significantly impact the survival of *A. pallipes* in this study; high survival rates were achieved at all three treatment densities. It is therefore encouraging to note that up to a relatively high density of 300/m^2, even at ten months of age, crayfish survival was not compromised. This finding is consistent with research by Policar et al. (2010), where survival rates of up to 80% were achieved when rearing *A. pallipes* hatchlings at an initial density of 500/m^2 and then reducing the density to 115/m^2 after 100 days. In studies with *P. lenisuculus* and *A. astacus,* stage-2 hatchlings were reared at densities of up to 1200/m^2, with significantly greater survival achieved at densities of up to 400/m^2, suggesting that is the threshold density (Savolainen et al. 2004; Ulikowski et al. 2006; González et al. 2010).

3.4.2. Carapace length and growth

In the present study, there was a significant difference in carapace length between the different density treatments; there was an inverse relationship between length of carapace and increasing treatment density. This result is consistent with previous studies, which found that growth diminishes with increased density (Jones & Ruscoe, 2000; Ulikowski et al. 2006). This is likely to be due to increased encounters with other crayfish whilst foraging, which could reduce the amount of time feeding, even when food is offered in excess. Similar growth was recorded in *Cherax quadricarinatus*, a comparatively non-aggressive crayfish species; the number of encounters, even when non-aggressive, increased energy expenditure and reduced foraging opportunities consequently reducing growth. Growth was most rapid at the beginning of the experiment presented here, with a mean growth of 47.6% across all densities, after which the growth remained at a steady average increase of approximately 14.1% every two months (Jones & Ruscoe, 2000). In general smaller crayfish grow at a higher proportional rate than larger crayfish (Evans & Jussila, 1997; Jones & Ruscoe, 2000). An unexpected finding was that males were significantly larger than females throughout the time series, even from day-120 of the experiment; i.e. at the age of six months when sex could be reliably determined. Growth in males was significantly higher than in females; this was particularly marked within the low density group, possibly because the males were more dominant in the smaller groups, and therefore suppressed the females' growth to a greater extent. Hierarchical dominance is well known in crayfish species; larger animals will rapidly form hierarchies and defend resources from their subordinates, which will supress the growth of the smaller individuals (Goesmann et al. 2000; Tricarico et al. 2005; Alonso & Martinez, 2006; Harrison et al. 2006; Herberholz et al. 2007; Tricarico & Gherardi, 2010). González et al. (2011b) suggested that the dominant crayfish stress the smaller crayfish and prevent them from feeding at an optimal rate, even when food is supplied in excess. Studies with juvenile *P.clarkii* have demonstrated that dominant / subordinate relationships and social hierarchies can form very soon after hatching (Issa, 1999; Sato & Nagayama, 2012).

It is widely accepted that at sexual maturity, sexual dimorphism occurs and male crayfish grow larger chelae and females grow wider tails (Scalici & Gibertini, 2009a; Wang et al. 2011). A study by Franke et al. (2013), with sub-adult *A. astacus* showed that males grew faster than females; however, there are no known published studies that have found interspecific sex differences in dominance hierarchies or growth within juvenile, young-of-the-year crayfish. The observed size difference in the current experiment could be the start of early sexual dimorphism, prior to reaching sexual maturity.

3.4.3. Size variation

Size variation was significantly greater in the high density group than in the medium and low groups. The larger variation in sizes in the higher density treatments may be due to the effects of social dominance being more pronounced when there are an increased number of crayfish present. Size variation was greater in females than males in the low and medium density groups, throughout the time series, but was similar to those of males in the high density group. This could be because the hierarchical dominance of males over the females was more prevalent in the low and medium densities and therefore growth suppression of the females was more noticeable, and so there was a wider range in sizes. Growth was faster in the males at all densities, throughout the time series. This increased growth occurred even at six months of age and therefore the larger animals were predominantly male and consequently suppressed the growth of the smaller animals. The majority of the smaller crayfish within each group were female. However, because some crayfish appear to have an intrinsic capacity to grow faster (González et al. 2011b), there will be some females that will grow more quickly than individuals of both sexes. This may therefore explain why there was a wider size variation within the females of each group.

3.4.4. Chelae autotomy

Chelae autotomy in crustacean species is accepted as an indicator of agonistic aggression (Figureiel & Miller, 1995). In captive environments, there is an increasing occurrence of chelae injuries/loss with increasing density as the frequency of attack by conspecifics increases (Savolainen et al. 2004; Harlioğlu, 2009). In my study, rates of chelae autotomy increased as the experiment progressed; as the crayfish grew in size, resource competition increased. Relative chelae autotomy was greater in the medium density group, across the time series, than in the other two density treatments, which suggests that, at medium densities, there were elevated levels of agonistic aggression. The reason for this might be because at high density the chances of winning an encounter was reduced as resource competition is higher and therefore less dominant animals do not try and defend resources. Whereas at medium density investing energy in competing for resources was worth the potential cost and therefore less dominant crayfish had more agonistic encounters. Females showed significantly more chelae autotomy than males across the time series. This may be due to males being more aggressive and having greater social dominance over the females than over other males. If the females were more subordinate to the males, there is a higher chance of males winning an aggressive encounter, than one with another male, and therefore more reason to attack a female rather than risk losing an agonistic encounter with

another male. Similarly, larger females may be more likely to attack other females rather than the more dominant males.

3.5. Conclusions

This present study illustrates that young-of-the-year *A. pallipes* can be maintained for the first ten months of life at densities up to 300/m^2 without survival being compromised. However, at the higher densities of 200-300/m^2, there will be a wider variation in sizes, an increasing amount of chelae autotomy, and a reduction in mean carapace length. By six months of age, there was a significant difference in carapace length and chelae autotomy between the sexes; males were larger than the females and more aggressive, suggesting they were more dominant. Therefore, sexual dimorphism in *A. pallipes* may start when juvenile and as early as six months of age and it may be appropriate to rear the crayfish in single-sex groups from that age. When breeding *A. pallipes* for wild-release or for *ex-situ* brood-stock, maximising survival and growth of young-of-the-year crayfish is paramount; therefore the recommendation from this study would be to rear juvenile *A. pallipes* at a maximum density of 100/m^2.

In the next chapter, I explore the theory that *A. pallipes* males are more intrinsically aggressive than females, even when juveniles, and whether size-grading and single-sex culturing of this species is important for optimising growth and survival of young-of-the-year crayfish.

CHAPTER 4

Assessing the effect of size-grading for rearing young-of-the-year *Austropotamobius pallipes*

An adapted version of this chapter has been published in:

Nightingale, J., Stebbing, P., Taylor, N., McCabe G., & Jones, G. (2018). The effect of size-grading for rearing young-of-the-year white-clawed crayfish *Austropotamobius pallipes. Aquaculture Research, 49,* 3116-3122. (Appendix III).

Author contribution

JAN designed the study and collected and analysed the data. NT checked through the statistical analysis. JAN produced the intial draft manuscript and GJ, PS and GM provided support and guidance with the experimental design and contributed criticially to the manuscript and chapter drafts.

Abstract

Crayfish growth can vary considerably among individuals from the same brood, and social dominance hierarchies in crustacean species occur frequently. These hierarchies can reduce growth and survival when rearing communal groups. Size-grading and single-sex culturing are methods used to combat this. A 160-day experiment took place on 288 young-of-the-year captive-born *Austropotamobius pallipes,* within a closed-circuit, indoor aquaculture facility. Crayfish were reared in three treatments i) equal numbers of large males + small females (LMSF); ii) equal numbers of small males + large females (SMLF); iii) individuals of the same size, equal sex-ratio; plus two control groups of single-sex, same sized individuals. Female survival in the LMSF was significantly reduced (mean ± SD) (52.8 ± 20.7%), whereas overall survival in all other groups was high (83.1 ± 15.1%). Male growth (6.3 ± 0.6 mm) was greater than female growth (4.9 ± 0.9 mm) over 160 days, across all groups. Chelae autotomy was significantly greater (8.8%) in males (26.7 ± 14.1%) than females (18.0 ± 17.8%). This study suggests that young-of-the-year juvenile male *A. pallipes* grow faster and are more aggressive than females. Large males will suppress and reduce survival in smaller females whereas small males, when housed with larger females, will still grow faster than the females. We suggest that it is sex not size that is the main factor that causes dominance hierarchies and growth suppression within juvenile *A. pallipes*. Maintaining juvenile *A. pallipes* in single-sex groups is optimal to ensure high survival and growth.

4.1. Introduction

High survival rates can be achieved when raising young-of-the-year *A. pallipes*; however, even at low densities, there can be a large variation in size and health of crayfish juveniles within a single brood, even as early as six months of age (Ahvenharju et al. 2005; J. Nightingale pers. obs. 2014).

Hierarchical dominance structures can develop in fish and crustacean species, and in crayfish intraspecific hierarchies can establish very quickly even without the influence of resource competition (Issa et al. 1999; Goessmann et al. 2000; Tricario et al. 2005; Harrison et al. 2006; Sato & Nagayama, 2011). Dominance hierarchies can result in larger animals suppressing the growth of smaller individuals, when they competitively exclude them from resources such as food and shelter (Bergman & Moore, 2003; Herberholz et al. 2007; Tricarico & Gherardi, 2010). Size-grading, where animals are put into same-sized groups,

can help to reduce these effects. The practice of size-grading is well established within the aquaculture industry where commercial fish farms size-grade the fish to increase survival and growth (Gunnes, 1976; Wallet et al. 2005). Size-grading in the prawn industry is also a standardized procedure and can produce improved growth and feeding efficiency when prawns are reared with same-sized individuals (Daniels & D'Abramo, 1994; Tidewell et al. 2004). Most crayfish grading experiments have occurred in large, commercially farmed, species in the family Cambaridae, such as the blue pearl crayfish *Cherax albidus* (Lawrence et al. 2000), the redclaw crayfish *Cherax quadricarinatus* (Curtis & Jones, 1995; Jones & Roscoe, 2000; Parnes & Sagi, 2002; Rodgers et al. 2006) and the hairy marron crayfish *Cherax tenuimanus* (Qin et al. 2001). Historically, the aquaculture of astacid crayfish species, such as *A. pallipes and* the signal crayfish *Pacifastacus leniusculus* had limited success due to high mortality in the critical post-hatching phase, (at stage-2 when exogenous feeding has begun), due to inadequate nutrition (Sáez-Royuela et al. 2001; González et al. 2009a).

In recent years, feeding regimes for *A. pallipes* have improved, with the use of live *Artemia* being offered in the critical first few weeks post-hatching (J. Nightingale pers. obs. 2012) and therefore research into the effects of size-grading stage-2 hatchlings has been possible. There are two known grading experiments that have taken place on juvenile *P. leniusculus* (Ahvenharju et al. 2005; González et al. 2011). However, results from size-grading experiments in fish and crayfish species have been varied, and there is no known published research on the effect of size-grading on growth and survival in *A. pallipes*. Therefore this study had two key objectives: (i) to establish if size-grading and or same-sex culturing would be beneficial during rearing of juvenile *A. pallipes* and (ii) to establish what the optimal size and sex-ratio for rearing young-of–the year *A. pallipes* is without compromising survival and growth. Both these objectives should assist in the main aim of producing consistently large, young-of-the-year robust crayfish, for wild-release or *ex-situ* brood-stock for subsequent captive-breeding programmes.

4.2. Materials and methods

For a detailed description of the wild collection, hatchery details and husbandry routines see Chapter 2 "General Methodology".

4.2.1. Source of experimental animals

The juveniles were hatched from 12 wild-caught, ovigerous females, collected from a local river population, 1.5 months prior to the experiment and kept in the hatchery until the eggs hatched and the juveniles were free-living. The juveniles were then reared for a further two months, which is the most critical survival period, prior to the experiment commencing.

4.2.2. Experimental set up and design

A 160-day experiment, with 288 five-month-old, captive-born *A. pallipes*, was carried out, within an indoor closed-circuit aquaculture facility in Somerset, England. The crayfish tanks, within the system, were randomly assigned to the treatment and control groups. These included three treatments, i) equal numbers of large males + small females (LMSF); ii) equal numbers of small males + large females (SMLF); iii) individuals of the same size, equal sex-ratio, plus two control groups of single-sex, same sized individuals, (Table 4.1). Twelve crayfish were added to each tank (at an equivalent density of 100 crayfish/m²). The crayfish were fed 0.2 g of food, per crayfish, per day, i.e. fed to excess, at approximately 14:00 hours. The experiment ran from November 2016 to April 2017.

Table 4.1. Treatment groups for *A. pallipes* with starting mean carapace length (CL) +/- standard deviation; 12 crayfish per tank at an equivalent 100/m^2 density.

Treatment	Male CL (mm)	Female CL (mm)	Sex-ratio	Treatment replicates
Large male, small female LMSF	13.2 ± 0.7	9.4 ± 0.7	6:6	6
Small male, large female SMLF	10.1 ± 0.3	12.8 ± 0.6	6:6	6
Equal sized male / female	11.5 ± 0.4	11.5 ± 0.5	6:6	5
All-male control	12.1 ± 0.2	-	-	2
All-female control	-	11.2 ± 0.3	-	5

4.2.3. Experimental procedure

Carapace length was measured from the anterior edge of the rostrum to the posterior edge of the cephalothorax to the nearest 0.1 mm using Vernier 1500 mm callipers (Moore and Wright, Sheffield, UK). The crayfish were counted, measured, examined and their biometric

data recorded at day-1 and day-160. Missing chelae, a standard measure of aggression (Figureiel & Miller, 1995), and stage in moulting cycle were also recorded.

4.2.4. Data analysis

Variation in size within each group was recorded and growth along the time series was calculated by subtracting the mean end carapace length from the mean start carapace length for each treatment group. To determine if there were any differences between the survival to the three treatments and controls, data were log-transformed and examined by using nested binomial generalized linear models (function *glmer,* R package *lme4*). To determine if there was any difference in carapace length, growth and chelae autotomy among the different treatments and the control groups, data were examined using linear mixed models (function *lmer*, R package *lme4,* Bates et al. 2015). The treatments, plus sex, were considered as fixed effects, and tank was considered a random effect. The alpha level was set at $p<0.05$. Only variables that had a significant effect were retained in the model. Statistical analyses were performed using R 3.2.5 (R Core Team, 2016).

4.3. Results

4.3.1. Survival

Survival was highest in the all-female control group (90%, SD=7.0%) and lowest in the LMSF group (70.8%, SD = 21.1%), (Figure 4.1). Survival of LMSF group was significantly lower than all other treatments (z_{251} = -3.1, p < 0.002). Survival (% ± SD) between other treatments was not significantly different and was on average 83.1 ± 15.1%. Survival between males and females were exactly the same in the equal-sized group (86.8 ± 13.9%). Survival of the males in the LMSF group was significantly higher (88.9 ± 13.6%) than female survival (52.8 ± 20.7%) i.e. 31.1% greater (z_{71} = 3.32, p < 0.001). In contrast survival in the SMLF groups was not significantly different between males (80.5 ± 13.6%) and females (88.9 ± 6.8%) (z_{71} = -0.97, p = 0.33). Survival in the control groups was considerably lower in the all-male (70.8 ± 29.4%) than the all-female groups (90 ± 7.0%), but not significantly so (p=0.08, df=41, z=-1.75); however, there were only two male control groups with considerable variation between them. Survival between the two all-male control groups was significantly different (z_{23} = -2.0, p = 0.04); however survival between the five all-female control groups was not significantly different (z_{59} = 0.76, p = 0.45).

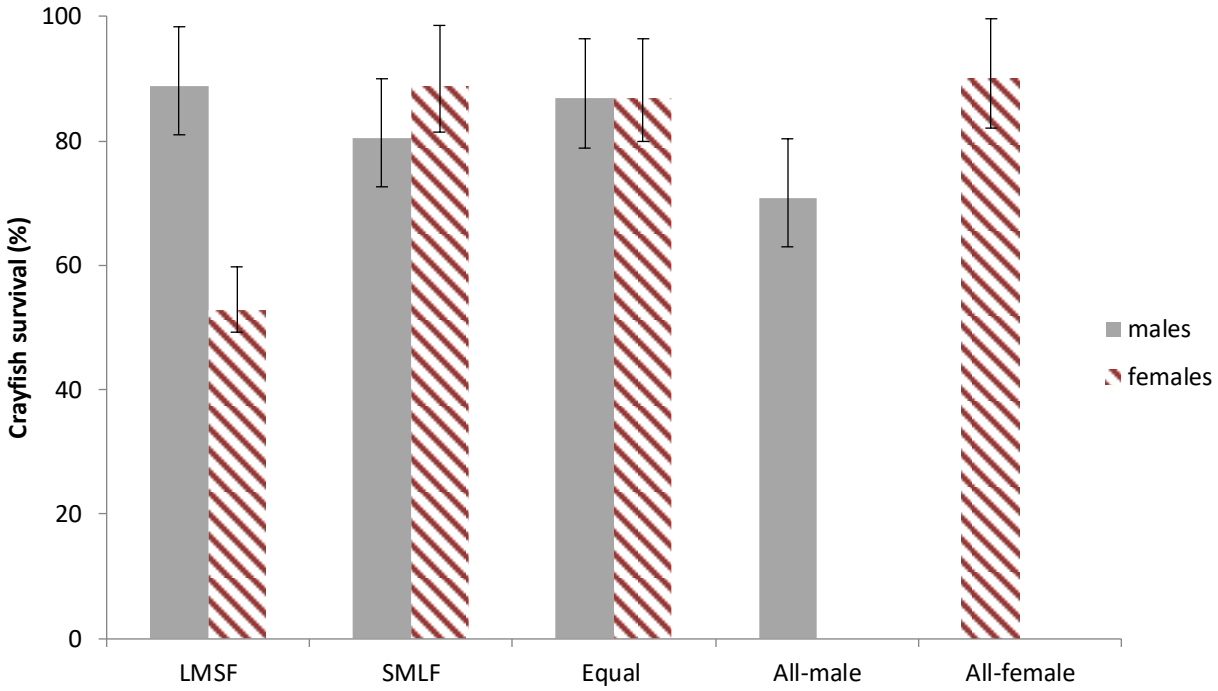

Figure 4.1. Mean percentage survival of male and female *A. pallipes* at day-160, within the different treatment and control groups: large male, small female (LMSF); small male, large female (SMLF); equally sized, even sex-ratio (Equal); male control group (All-male); female control group (All-female). Error bars represent 95% confidence intervals.

4.3.2. Carapace length

Carapace lengths (mean ± SD) at day-1 were not significantly different between males (11.7 ± 1.1 mm) and females (11.1 ± 1.2 mm). At the end of the experiment, at day-160, males (18.1 ± 1.9 mm) were significantly larger ($F_{1,201}$ = 19.35, p < 0.001), than females (16.0 ± 2.1 mm). The LMSF group contained the largest males, with a mean carapace length of 20.1 ± 0.7 mm by day-160, and the SMLF group had the largest females (mean carapace length 18.3 ± 0.9 mm). At the end of the experiment, mean carapace lengths were not significantly different between treatments ($F_{4,198}$ = 1.74, p = 0.14) (Figure 4.2.).

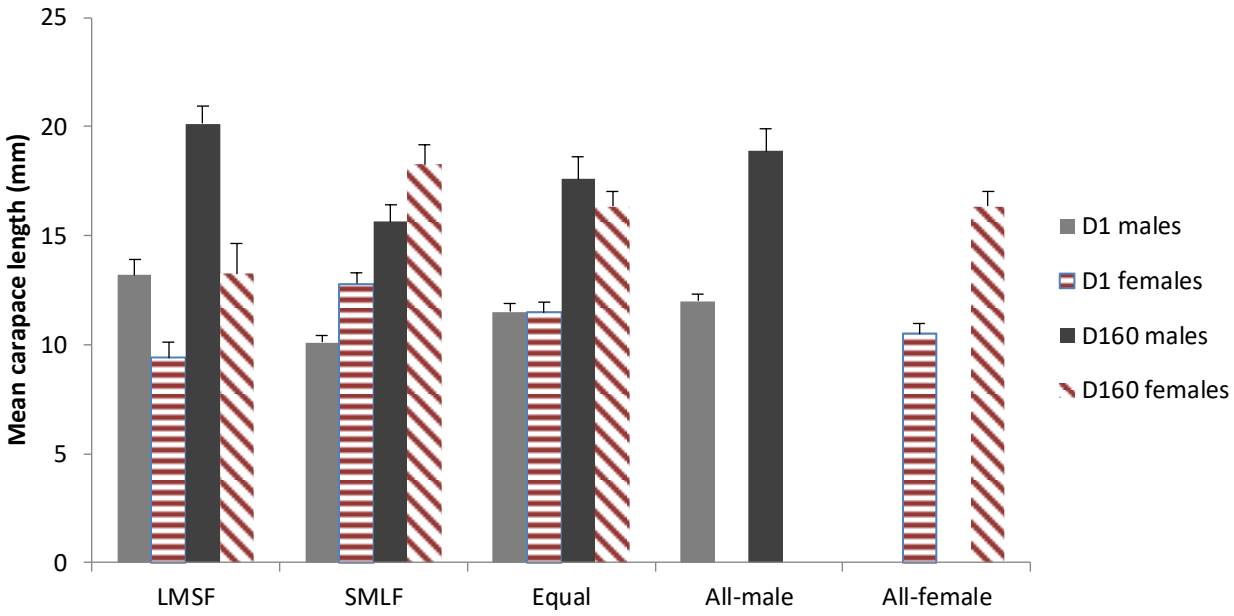

Figure 4.2. Mean carapace lengths of *A. pallipes* at day-1 of the experiment (D1), to day-160 (D160), shown for the different treatment and control groups: large male, small female (LMSF); small male, large female (SMLF); equally sized, even sex-ratio (Equal); male control group (All-male); female control group (All-female). Error bars represent standard deviations.

4.3.3. Growth

Growth calculations (i.e. subtracting the starting mean carapace length from the final mean carapace length), showed that the mean growth (mean ± SD) across all groups (5.6 ± 1.0 mm) was not significantly different. However, male growth (6.3 ± 0.6 mm) was greater than female growth (4.9 ± 0.9 mm) across all treatment groups and was significantly greater than female growth in the LMSF, equal and single-sex groups ($F_{1,223} = 93.68$, $p < 0.001$) (Figure 4.3).

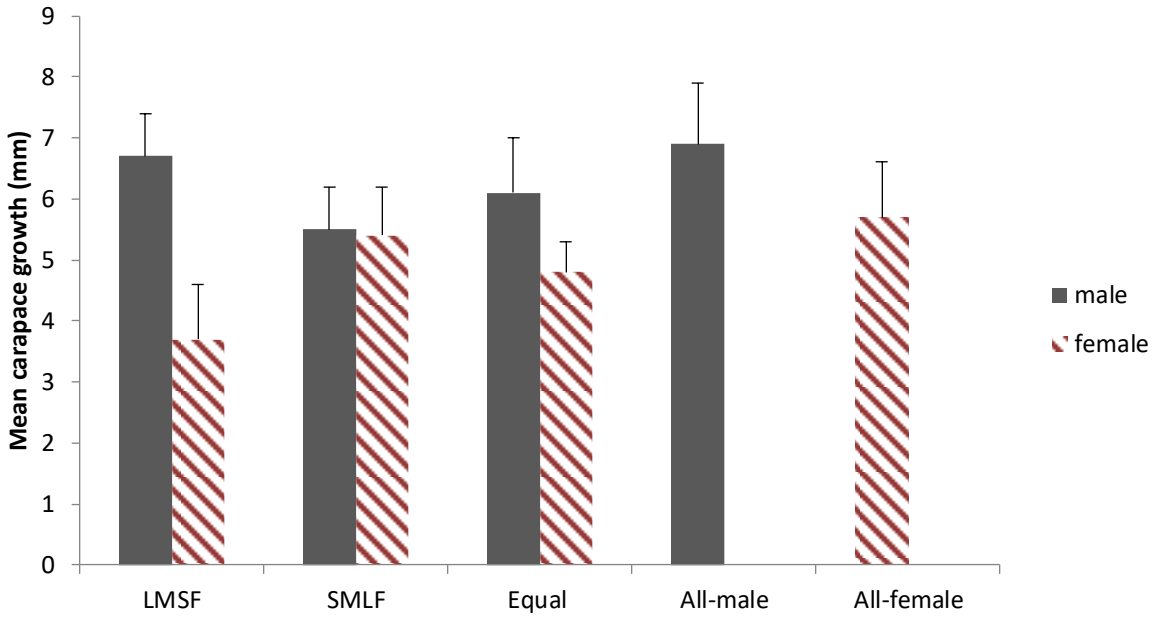

Figure 4.3. Mean carapace growth (mm) of male and female *A. pallipes,* throughout the 160-day experiment within the different treatment and control groups: large male, small female (LMSF); small male, large female (SMLF); equally sized, even sex-ratio (Equal); male control group (All-male); female control group (All-female). Error bars represent standard deviations.

4.3.4. Chelae autotomy

Mean chelae autotomy (% ± SD) was 22.3 ± 15.9% over all the treatments (sexes combined) and was not significantly different between treatments. The all-female control group had the least chelae autotomy (14.6 ± 9.8%) and the SMLF group had the highest (26.5 ± 23.4%). When the sexes were looked at separately, chelae autotomy in males (26.7 ± 14.1%) was significantly greater (by 8.7%) than in females (18.0 ± 17.8%) (t_{203} = -2.1, p = 0.04), throughout all treatments except the equal-sized treatment group, where chelae autotomy was the same in both sexes (20.7 ± 18.2%), (Figure 4.4). Crayfish with both chelae present were significantly (t_{200} = 4.5, p < 0.001) larger than those with missing chelae.

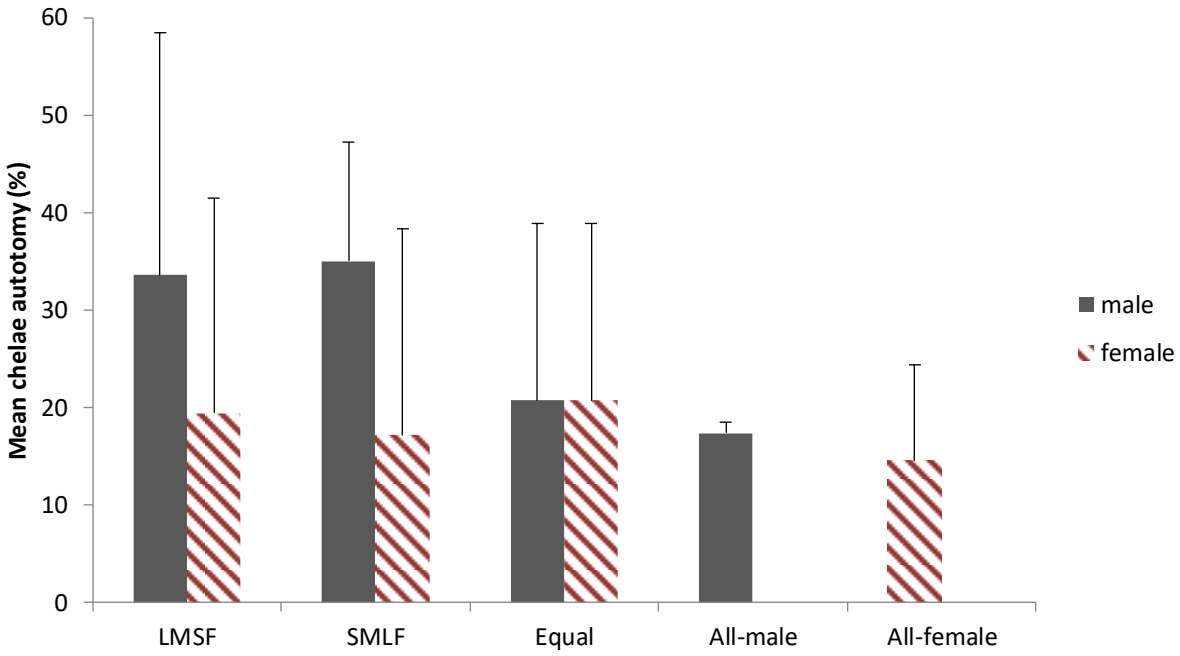

Figure 4.4. Mean percentage of chelae autotomy for male and female *A. pallipes* at day-160 within the different treatment and control groups: large male, small female (LMSF); small male, large female (SMLF); equally sized, even sex-ratio (Equal); male control group (All-male); female control group (All-female). Error bars represent standard deviations.

4.4. Discussion

4.4.1. Survival

There was no significant difference in survival between the equal-sized, SMLF and all-female control group, which all had high survival; however, there was a significantly higher mortality of females in the LMSF, compared to the other two treatment groups and female control group (Fig. 4.1). This supports previous astacid crayfish studies, which graded juvenile, young-of-the-year *P. leniusculus* into small and large sizes and found that survival was significantly higher in the graded rather than the non-graded groups (Ahvenharju et al. 2005; González et al. 2011). Within the LMSF group, where female survival was significantly reduced, larger males may dominate and kill the females. Figureler et al. (2005), demonstrated that adult male *Procambarus clarkii* were more likely to attack non-maternal females rather than males or maternal females. In a similar manner, larger male *A. pallipes* may attack the females rather than the other males in the group. When large females were housed with small males, the survival of the males was not reduced suggesting that aggressive attacks and hierarchical dominance is predominantly sex rather than size-related. Although the survival in the male control group was lower than the

average survival, this result needs to be viewed with caution as there were only two replicates for this group in contrast with five or six replicates for each other treatment group (due to the small number of crayfish available). There was also considerable variation in survival (91.6% and 50.0%) between the two different all-male control tanks. The all-female control groups had the highest survival than any of the other females within the other treatments. They also showed the least variability in survival rates between the five different control tanks (survival 90 ± 7.0%). This is supported by single-sex experiments in *C. albidus* (Lawrence et al. 2000) and *C. quadricarinatus* (Rodgers et al. 2006); these studies found that there was no significant reduction in male survival when reared in all-male groups.

4.4.2. Growth

Males grew faster than females in all treatments, even when males and females were matched for sex and size, suggesting that juvenile *A. pallipes* exhibit sex differences in growth. This is in contrast to *C. albidus* where crayfish grow at the same rate until sexual maturity (Woodland, 1967). The all-male *A. pallipes* group had the fastest growth and the lowest male survival suggesting that some males grew large and out-competed the smaller males for resources. The males that were in the LMSF group did not significantly benefit from the fact that they were suppressing the smaller individuals, and the males within the all-male group actually grew faster. This is in contrast to the situation in *P. leniusculus* where larger juveniles benefited from being with smaller individuals, and grew faster than when housed separately (Ahvenharju et al. 2005). The all-female control groups on average grew faster than the females in the other treatment group, suggesting that all-female groups are optimal for growth and that in mixed-sex groups, the females' growth may be supressed by the more dominant males. Both the male and female single-sex groups grew faster than mixed-sex groups as determined by Lawrence et al. (2000), and Rodgers et al. (2006), who found that males and females *C. albidus* and *C. quadricarinatus* grew faster in single-sex rather than mixed-sex groups. However, these studies were with larger species and in the case of Rodgers et al. (2006), the study was with adults rather than juveniles. When juvenile *P. clarkii* were raised in single-sex or mixed-sex groups, there were no differences in growth (Figureiel et al. 1991; Wang et al. 2014).

Growth of the females in the LMSF was significantly reduced, suggesting that the larger males dominate the smaller females and supress their growth. Other studies on crayfish have shown that larger individuals dominate smaller animals and will compete for resources (Tricarico et al. 2005; Herberholz et al. 2007). However, in this study, within the LFSM group, male growth was still faster than in females, suggesting that the larger females do not

dominate and suppress the smaller males. Some studies on crayfish species such as *C. tenuimanus* (Qin et al. 2001) found that size-grading did not affect growth. This has also been the case for fish species (Sunde et al. 1998). This indicates that social structure cannot be generalized across species and therefore size-grading has to be assessed at species level. Therefore it was important to carry out this research rather than assume that size-grading would have similar effects for *A. pallipes* as in the astacid species *P. leniusculus* (Ahvenharju et al. 2005; González et al. 2011).

4.4.3. Chelae autotomy

In all the treatments and control groups, males suffered significantly more chelae autotomy than females, suggesting that males are more likely to fight each other in competition for resources; this is supported by the female control groups having the lowest amount of chelae autotomy, suggesting that they are less aggressive when kept in single-sex groups. This study implies that males are more aggressive than females, even before sexual maturity, which is supported by the study of Figureiel & Miller (1995) on juvenile *P. clarkii,* who found that the percentage of chelae loss in males (15.8%) was higher than in females (10.5%). In crayfish species such as *P. clarkii* (Figureler et al. 2005) adult crayfish males are more aggressive and will dominate and out-compete females; however, this behaviour has not been reported in juvenile crayfish. A study in juvenile *P. clarkii* reported social dominance behaviour occurring; however, differences between sexes was not investigated (Herberholz et al. 2007). Crayfish with missing chelae were significantly smaller ($p < 0.001$) than those with both chelae present, perhaps because they were the subordinate within the social hierarchies and consequently were not efficient at competing for resources such as food. Crayfish with missing chelae will expend energy regenerating a new limb and this may also account for their slower growth (Figureiel & Miller, 1995; Kouba et al. 2011).

4.5. Conclusions

This current study found that social hierarchies and dominance in *A. pallipes* start within six-months of hatching. We suggest that sex, rather than size, plays a more important role in hierarchical dominance, which can lead to a reduction in female survival and growth. In an endangered species, which is being produced for both wild-release and for brood-stock, production of large-sized females, with high survival rates, is paramount. The study indicates that size-grading of juvenile *A. pallipes* is beneficial and can increase survival and growth, with all-female groups achieving the best results. The optimal size and sex-ratio for rearing young-of-the-year *A. pallipes* would be to split the crayfish into single-sex groups at

six months of age, when they can be reliably sexed. These groups should be size-graded, with a carapace size differential of no more than 2 mm between individuals, and held at a maximum density equivalent of 300/m^2 up until ten months of age when the crayfish should then be size-graded again and the density reduced to 50/m^2 (J. Nightingale unpublished data). Crayfish can then be maintained at this density, within these size-graded groups, up until their release, which ideally should be during their second year, prior to the breeding season (J. Nightingale pers. obs. 2014). This size-grading, single-sex grouping strategy would be advantageous, producing larger females that will be ready to breed in their second year. Where brood-stock groups are being maintained for *ex-situ* breeding-programmes then larger females should be housed with smaller males, to ensure that males do not supress female growth or compromise their survival. If equally-sized males and females are housed together the faster male growth will result in the males rapidly becoming larger than the females, which will cause them to out-compete the females for tank resources, such as food and shelter, which in turn may increase female mortality.

Following on from ascertaining density and grading regimes for effective *A. pallipes* production, another important factor of crayfish aquaculture is nutrition. In the next chapter I explore dietary regimes for rearing crayfish from hatchlings to release, to maximise growth and survival in preparation for wild release.

CHAPTER 5

Effects of different diet types on growth and survival of juvenile *Austropotamobius pallipes*

An adapted version of this chapter has been submitted for publication in *Frontiers in Ecology and Evolution*:
Nightingale, J., Jones, G., McCabe, M & Stebbing, P. (2020). Effects of diet types on growth and survival of *Austropotamobius pallipes*.

Author contributions

JAN designed the study and collected and analysed the data set. Jose Carral, John Hollows and Damian Riggs allowed their specific crayfish pellet diets to be used in the experiment. Cefas provided information on the agar gel diets ingredients. JAN produced the draft manuscript and GJ, PS and GM provided support and guidance with the experimental design and contributed criticially to the manuscript and chapter drafts.

Abstract

Developing an optimal diet for rearing endangered white-clawed crayfish *Austropotamobius pallipes* is important for captive breeding programmes prior to introduction into the wild. Four *ex-situ*, 40-day experiments assessed survival and growth of crayfish fed different treatment diets. Two experiments (A & B) were undertaken with stage-2 hatchlings to determine if live food was an essential dietary component during the first few weeks after hatching. The second set of experiments (C & D) were undertaken with 60-day-old *A. pallipes,* to determine an optimal diet after the initial critical feeding stage.

In experiment A, we fed hatchlings i) live *Artemia* nauplii + defrosted plankton (Live+P); ii) decapsulated *Artemia* cysts + defrosted plankton (Cyst+P) or iii) decapsulated *Artemia* cysts + defrosted plankton encapsulated in agar gel (Gel+CP). Survival and growth was significantly greater with Live+P than with the other two diets. In Experiment B we compared four different treatment diets: i) live *Artemia* nauplii + Australian pellet (Live+Aus); ii) live *Artemia* nauplii plus New Zealand pellet (Live+NZ); iii) live *Artemia* nauplii + plankton (Live+P); (iv) practical Spanish crayfish pellet diet, which contained *Artemia* cysts (Spain). In both experiments (A & B), mean crayfish survival and growth (91.7 ± 6.4%; 2.4 ± 0.1 mm), were significantly higher with *Artemia* + plankton (Live+P) than with *Artemia* cysts in pellet (Spain) (80.2 ± 6.1%; 2.17 ± 0.06 mm) or *Artemia* cysts + plankton with (Gel+CP) or without agar (Cyst+P); (43.7 ± 16.0%; 2.0 ± 0.2 mm). Growth was also significantly greater with *Artemia* + plankton (Live+P) than with *Artemia* + pellets (Live+Aus and Live+NZ), (2.27 ± 0.03 mm).

In experiment C, 60-day-old juvenile *A. pallipes* were fed i) defrosted plankton plus vegetables (Standard) or (ii) defrosted plankton plus vegetables encapsulated in agar gel (Gel+PV). Survival was not significantly different between the diets; however, growth was significantly greater with the Standard diet rather than Gel+PV. In experiment D, *A. pallipes* were fed four different diets: i) Australian pellet (Australia); ii) New Zealand pellet (New Zealand); iii) plankton and vegetables (Standard); or iv) practical Spanish diet (Spain). Survival was significantly lower in crayfish fed the New Zealand diet. Crayfish growth was significantly greater with the Standard diet (2.8 ± 0.4 mm) than all three pellet diets: Australia, New Zealand and Spain: 1.7 ± 0.6 mm). Our results showed that live food is optimal for high survival and growth in *A. pallipes* hatchlings for the first 40-days, and a plankton plus vegetable diet produces better growth in older juveniles compared to pellet diets. Time and cost constraints may make the pellet diet a more realistic solution for large-scale *A. pallipes* production; however, further research is required to ascertain long-term effects of a pellet-only diet on this species.

5.1. Introduction

The white-clawed crayfish *Austropotamobius pallipes* is endangered throughout its native range in the UK and mainland Europe (Sibley et al. 2011). The loss of this species is attributed to the spread of the invasive American signal crayfish *Pacifastacus leniusculus* and associated crayfish plague, caused by the pathogen / oomycete *Aphanomyces astaci*, along with habitat degradation and pollution (Sibley et al. 2011). In response to this decline, white-clawed crayfish aquaculture is increasing in the UK and mainland Europe, to aid in the rehabilitation of the species through conservation action restocking and stock enhancement. Several captive-breeding hatcheries have been established, for example, at Bristol Zoo Gardens, England, which maintains *A. pallipes* brood-stock from highly threatened populations, with the aim of producing juveniles for restocking to preserve and enhance *in-situ* populations (Nightingale et al. 2017). A major issue with the mass-rearing of animals in captivity is the provision of a well-balanced and nutritional diet to ensure good survival / growth and development, which is particularly important for juvenile life stages. With crayfish, a critical period of survival occurs during the first few weeks, post-hatching, when high mortality rates can occur due to a lack of adequate nutrition (Gonzalez et al. 2011; Celada et al. 2012). When crayfish hatch they initially feed on their egg yolk and remain attached to the females's pleopods. When they have undergone two moults and are at stage-2 free-living, their exogenous mouth parts have formed and feeding begins (Reynolds, 2002). Observations from the wild can provide key information on providing a suitable diet for captive-bred animals. Wild *A. pallipes* are opportunistic omnivores, feeding on invertebrates, carrion, vegetable matter, and organic and inorganic detritus (Gherardi et al. 2004). Scalici & Gibertini, (2007) found stomach contents of wild-caught *A. pallipes* differing with age and sex; insect larvae were found to be a key component of juvenile and adult female diets. In contrast, adult males mainly fed on vegetable matter.

Several studies have examined feeding and nutrition requirements of captive-bred crayfish, including *P. leniusculus* (Carral et al. 2011; Gonzalez et al. 2012), red swamp crayfish *Procambarus clarkii* (Hua et al. 2015) and common yabby *Cherax destructor* (Austin et al. 1997). Commercially available fish-feed pellets were historically fed to all age-classes resulting in low survival rates of hatchlings in both *A. pallipes* (Sáez-Royuela et al. 2001) and *P. leniusculus* (Ulikowski et al. 2006; Sáez-Royuela et al. 2007). *Artemia* nauplli have also been used as feed for captive-bred juvenile crayfish, as these are a readily available and easily produced substitute for insect larvae found in the diet of wild juvenile crayfish. *Artemia* nauplii are a popular first feed within aquaculture as they are high in protein and lipids and contain proteolytic enzymes, which can aid the digestive abilities of young animals

(Bengtson et al. 1991). However, *Artemia* spp. is deficient in some nutrients, such as poly-unsaturated fatty acids and therefore it is enriched prior to feeding larvae.

When hatchling *P. leniusculus* were reared using live *Artemia* nauplli, high survival rates of up to 80% were achieved (González et al. 2008). After day 20, however, there was no significant difference in survival between *P. leniusculus* fed a pellet diet and ones fed live *Artemia* nauplii, although growth was significantly greater if live *Artemia* were fed up to day 50 (González et al. 2011). Following on from this research, live *Artemia* nauplii were replaced with *Artemia* cysts and fed to stage-2 (free-living) hatchling *P. lenisuculus*, also resulting in high survival rates (81%), (González et al. 2009). Subsequently, a practical pellet feed, incorporating decapsulated *Artemia* cysts, was developed and high survival rates (86%) were achieved with hatchling *P. leniusculus* (Carral et al. 2011). *Artemia* will also readily feed on a wide variety of food items and therefore provides a useful vessel for enrichment products, such as lipids and algae (Léger et al. 1986).

Crayfish graze periodically; therefore, if their food source is encapsulated within a gel, it should not degrade as quickly in water and should retain both its palatability and nutritional value for longer. This is supported by a study investigating survival and growth in juvenile (10 g) white yabby *Cherax albidus*. The crayfish were either fed fish and potatoes or this fresh food was encapsulated within pectin, alginate, agar or chitosan. There was a significant increase in growth when using the gel diets in comparison to a fresh food diet without gel (Coccia et al. 2010).

This study presents results from a series of experiments testing different diet formulations on the growth and survival of hatchling and juvenile *A. pallipes.* Four experiments (A, B, C & D) were conducted, over two years, to ascertain optimal dietary regimes for rearing *A. pallipes*. The initial two experiments (A & B) were undertaken with stage-2 hatchlings to determine if: i) live food (plus a plankton mixture of cyclops, *Daphnia* and rotifers) was an essential dietary component during the first 40 days after the stage-2 hatchlings start feeding or ii) whether decapsulated *A. fransicana* cysts (plus a plankton mixture of bloodworm, *Mysis*, krill and *Daphnia*), iii) cysts and plankton in agar gel, or iv) the practical pellet crayfish diet could provide equally favourable outcomes. The second set of experiments (C & D) were undertaken with 60-day-old *A. pallipes* to determine what is an optimal diet for growth and survival, after the initial critical feeding stage was over. The treatment diets tested were a selection of complete pellet diets: i) a 'standard' diet of defrosted enriched plankton and vegetable matter; ii) the 'standard' diet encapsulated within agar gel; iii) the practical crayfish pellet diet formulated in Spain; and iv) a crayfish aquaculture pellet formulated for a research programme in Australia and; v) a New Zealand crayfish aquaculture pellet diet. We predict

that crayfish hatchlings will grow better on a live plankton diet rather than a pellet diet and a gel diet might increase growth in juveniles.

5.2. Materials and Methods

For a detailed description of the wild collection, hatchery details and husbandry routines see Chapter 2 "General Methodology".

5.2.1. Source of experimental animals

The crayfish used within the experiments were captive-born juvenile *A. pallipes*, hatched from 21, wild-caught, ovigerous females (collected from a local river population in South Gloucestershire, England, under Natural England licence). The females were brought into an indoor, closed-circuit, aquaculture facility (in Somerset, England), two-months prior to the experiment commencing. They were removed once the hatchlings were at stage-2; i.e. had undergone two moults and were free-living. The experiments took place within the same aquaculture facility.

5.2.2. Experimental set up and design

Four feeding experiments (A - D) took place over two breeding seasons. Experiments A & B were with juvenile stage-2 hatchling (20-day old) *A. pallipes* with an initial mean carapace length (mm ± SD) of 5.3 ± 0.14 mm. Experiments C & D used 60-day-old, juvenile crayfish, with an initial mean carapace length of 7.9 ± 0.23 mm. For each experiment, the juvenile *A. pallipes* were randomly selected and put into different treatment tanks, with six replicates of each, at varying densities for each food treatment to be trialled (Table 5.1). All experiments ran for 40 days from July 2016 to August 2017.

Experiment C used juvenile crayfish that had not been used in experimental trials and had been fed an enriched diet of live *A. fransicana* nauplii and defrosted plankton since hatching. In contrast, experiment D used the same experimental crayfish that were in B as an extension of this experiment; i.e. to ascertain the longer-term effects of using a solely pellet-based diet, compared to a pellet diet with an initial live food component. Therefore, in experiment D, the live food component was removed after day-40 for treatments i, ii and iii, whereas treatment iv remained the same throughout both experiments B and D as no live component was added initially. (The live food component is only required for the more critical first 4-6 weeks, post-hatching (González et al. 2011).

In both stage-2 hatchling experiments, plankton or pellet was offered, in addition to the live food element, recognising that a combination diet may be important for crayfish growth and survival.

Table 5.1. Four feeding experiments A, B, C & D, on *A. pallipes* including dietary treatments, density equivalent, treatment replicates, duration of experiment and age-class of animals.

Experiment	Treatments	Date	Crayfish / tank (/m^2)	Treatment replicates	Age-class
A	(i): Live *Artemia* + plankton[1] (Live+P) (ii): *Artemia* cysts + plankton[1] (Cyst+P) (iii): *Artemia* cysts + plankton[1] in gel (Gel+CP)	Jul-Aug'16	12 (100)	6	hatchlings
B	(i): Live *Artemia* + Australian pellet (Live+Aus) (ii): Live *Artemia* + pellet (Live+NZ) (iii): Live *Artemia* + plankton[1] (Live+P) (iv): *Artemia* cysts within pellet (Spain)	Jun-Jul'17	16 (150)	6	hatchlings
C	(i): Plankton[2] + vegetable[3] (Standard) (ii): Plankton[2] + vegetable[3] in gel (Gel+PV)	Aug-Sep'16	12 (100)	6	60-day
D	(i): Australian pellet (Australia) (ii): New Zealand pellet (New Zealand) (iii): Plankton[2] + vegetable[3] (Standard) (iv): *Artemia* cysts within pellet (Spain)	Jul-Aug'17	16 (150)	6	60-day

[1]Experiments A & B – defrosted plankton = equal proportions cyclops, *Daphnia* and rotifers
[2]Experiments C & D – defrosted plankton = equal proportions of bloodworm, *Mysis*, krill and *Daphnia*
[3]Vegetable – equal proportions of spinach, chard, peas, carrot and kale bl**e**nded and frozen

All diets, (except the pellet-only treatment), were enriched with 1 mL multivitamins, 1 g *Spirulina*, 1 mL lipids, plus 1 g of the carotenoid Astaxanthin, which was added to prevent the crayfish turning blue (Menasveta et al. 1993; Lorenz & Cysewski, 2000). The defrosted plankton used in all four experiments was gamma-irradiated, prior to freezing. The *Artemia* nauplii were hatched from *A. fransicana* eggs.

The gel diet (for experiments A and C) was made by mixing 1.5 g of potato dextrose agar powder and 0.8 g of locust bean gum, which was added to help binding. To this mixture, 50 mL of fresh water was added and then boiled for 1 minute. The mixture was allowed to cool to 45 °C and other food items were added before being put in a fridge to set. For experiment A, 9 g of enriched plankton and 2 g of enriched decapsulated *A. fransicana* cysts were added to the gel. For experiment C, 8 g of enriched plankton and 3 g of vegetable mix, plus 1g of *Spirulina* were added to the gel, (Table S5.2). The pellet diets were crayfish-specific pellets (Table S5.3). For experiment B and D, the crayfish the standard diets were made up at a ratio of three parts plankton to one part vegetable mix. All crayfish were fed to excess,

at a rate of approximately 4% of bodyweight of food per individual, presented at 18:00 daily. For hatchlings (experiment A & B), live *A. fransicana* or decapsulated *A. fransicana* cysts were fed at a rate of 500 /crayfish/day; plankton, pellet or gel diets were fed at a rate of 0.01 g/crayfish/day. For juveniles (experiment C & D), crayfish were fed at a rate of 0.02 g of food per animal per day.

5.2.3. Data collection and analysis

All crayfish were counted and individually measured on day-1 and day-40 (mm ± SD) of the experiments. The carapace length was measured from the anterior edge of the rostrum to the posterior edge of the cephalothorax to the nearest 0.1 mm using Vernier callipers (Moore and Wright, Sheffield). Crayfish growth in each treatment group was calculated by subtracting the starting average carapace length from the final average carapace length. To determine if there were any differences between the survival (% ± SD) with the different dietary treatments, data were log-transformed and examined using nested binomial generalized linear mixed models (function *glmer*, R package *lme4*) (Bates et al. 2015). Goodness-of-fit to normal distributions was checked by running the Shapiro - Wilk test on residuals (Shapiro & Wilk, 1965). To determine if there were differences in growth among the treatments, data were log-transformed and examined with linear mixed models (function *lmer*, R package *lme4*) (Bates et al. 2015) or ANOVA if tested at tank level. The treatments were considered as fixed effects, and tanks were considered a random effect. The alpha level was set at $p < 0.05$. Only variables that had a significant effect were retained in models. Statistical analyses were performed using R 3.2.5 (R Core Team, 2016).

5.3. Results

5.3.1. Experiment A

Stage-2 hatchling *A. pallipes* fed on three different treatment diets: i) live *Artemia* nauplii plus plankton (Live+P); ii) *Artemia* cysts plus plankton (Cyst+P); or iii) *Artemia* cysts plus plankton incorporated into agar gel (Gel+CP).

5.3.1.1. Survival

Survival for those fed with the Live+P diet (91.7 ± 6.4%) was significantly higher than for those fed the Cyst+P (43.7 ± 23.2%) or Gel+CP (43.7 ± 8.9%) diets ($z_{285} = 6.28$, $p < 0.001$). There was no significant difference in crayfish survival between the Gel+CP and Cyst+P diet ($z_{285} = 0.06$, $p = 0.95$) (Figure 5.1).

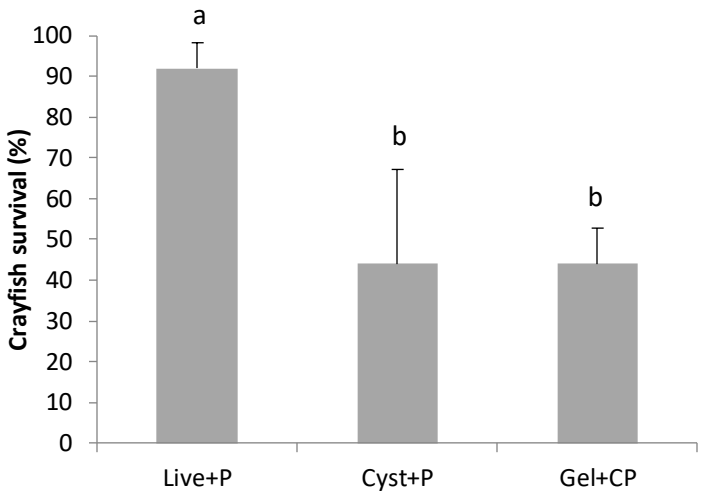

Figure 5.1. Percentage survival of hatchling *A. palliipes*, within the different treatment diets: live *Artemia* nauplii plus plankton (Live+P); *Artemia* cysts plus plankton (Cyst+P); or *Artemia* cysts plus plankton incorporated into agar gel (Gel+CP), at day-40. A different letter denotes significance between treatments. Error bars represent standard deviations.

5.3.1.2. Growth

Crayfish growth with the Live+P was significantly greater (2.4 ± 0.1 mm), than with the Cyst+P (2.0 ± 0.3 mm), or Gel+CP (2.0 ± 0.2 mm) diets, ($F_{2,169}$ = 14.94, p < 0.001). There was no significant difference in growth between the Gel+CP and Cyst+P diet (Figure 5.2).

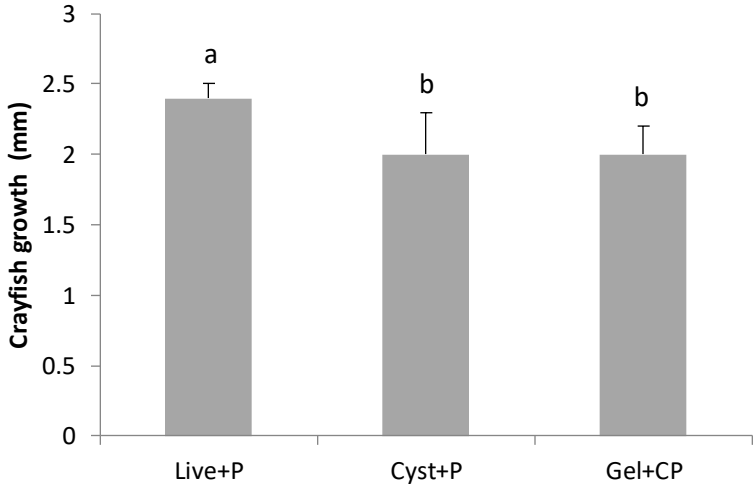

Figure 5.2. Mean growth (final carapace length – start carapace length mm) of hatchling *A. pallipes*, within the different treatment diets: live *Artemia* nauplii plus plankton (Live+P); *Artemia* cysts plus plankton (Cyst+P); or *Artemia* cysts plus plankton incorporated into agar gel (Gel+CP), at day-40. A different letter above a treatment denotes significance. Error bars represent standard deviations.

5.3.2. Experiment B

Hatchling crayfish fed four different treatment diets: i) live *Artemia* nauplii plus Australian pellet (Live+Aus); ii) live *Artemia* nauplii plus New Zealand pellet (Live+NZ); iii) live *Artemia* nauplii plus plankton (Live+P); and iv) the Spanish practical crayfish pellet diet (Spain).

5.3.2.1. Survival

Hatchling crayfish survival from day-1 to day-40 was significantly higher (z_{383} = 2.3, p = 0.02) with the Live+P diet (95.8 ± 5.1%) than with the Live+Aus (85.4 ± 12.3%) and Spain treatment diets (80.2 ± 6.1%) (z_{383} = -3.0, p = 0.002). Crayfish survival with the Live+NZ diet (91.7 ± 7.6%) was significantly higher than with the Spain diet (z_{383} = 5.5, p = 0.03) but was not significantly different from the Live+Aus diet (Figure 5.3).

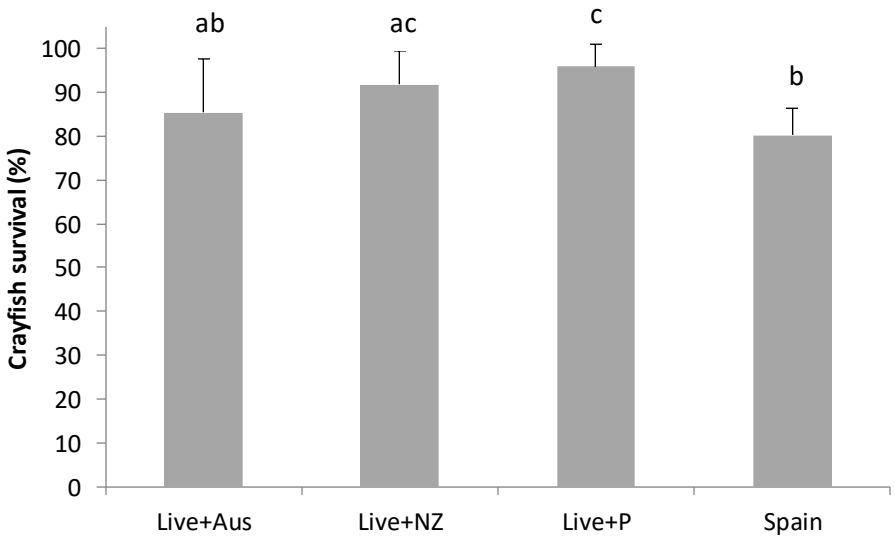

Figure 5.3. Percentage survival of hatchling *A. pallipes* for the four different treatment diets: Live *Artemia* nauplii plus Australian pellet (Live+Aus); Live *Artemia* nauplii plus plankton (Live+P); Live *Artemia* nauplii plus New Zealand pellet (Live+NZ) and the Spanish practical crayfish pellet diet (Spain), from day-1 to day-40. A different letter above a treatment denotes significance. Error bars represent standard deviations.

5.3.2.2. Growth

From day-1 to day-40, crayfish growth was significantly greater with the Live+P treatment diet (2.5 ± 0.1 mm) than with the Live+NZ and Spain diet ($F_{3,335}$ = 7.1, p < 0.001). There was no significant difference between growth with the other three diet treatments (mean 2.24 ± 0.06 mm) (Figure 5.4).

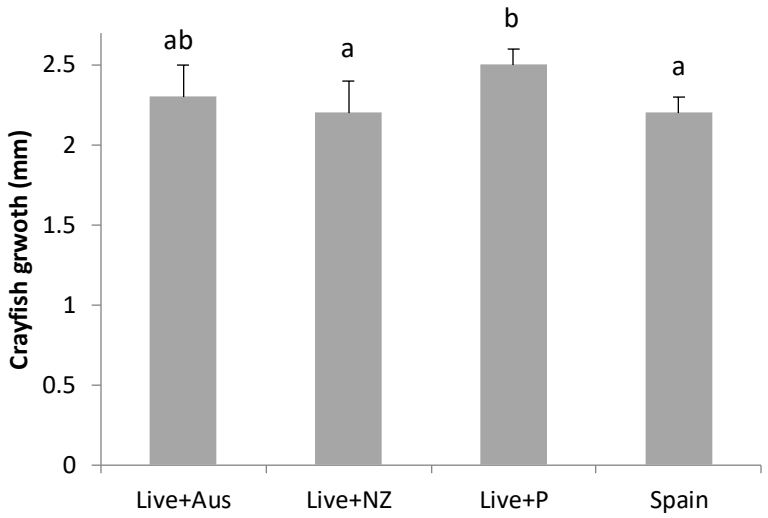

Figure 5.4. Mean growth, (final CL – start CL mm) of hatchling *A. pallipes* for the four different treatment diets: Live *Artemia* nauplii plus Australian pellet (Live+Aus); Live *Artemia* nauplii plus plankton (Live+P); Live *Artemia* nauplii plus New Zealand pellet (Live+NZ) and the Spanish practical crayfish pellet diet (Spain), from day-1 to day-40. A different letter above a treatment denotes significance. Error bars represent standard deviations.

5.3.3. Experiment C

Juvenile, 60-day-old crayfish fed on either: i) plankton plus vegetables (Standard), or ii) plankton plus vegetables incorporated into agar gel (Gel+PV).

5.3.3.1. Survival

For the juvenile *A. pallipes*, there was no significant difference in crayfish survival between Standard (97.9 ± 5.9%) and Gel+PV diet (96.9 ± 6.2%) (t_{187} = 0.45, p = 0.65) (Figure 5.5).

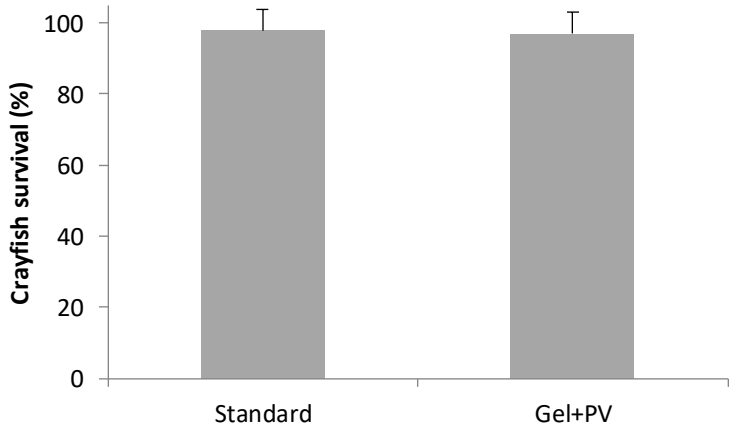

Figure 5.5. Percentage survival of juvenile *A. pallipes*, within the different treatment diet, plankton plus vegetable (Standard) or plankton plus vegetables incorporated into agar gel (Gel+PV), between day-1 to day-40. Error bars represent standard deviations.

5.3.3.2. Growth

Crayfish growth (mm ± SD) on the Standard diet (3.1 ± 0.3 mm) was significantly greater than those on the Gel+PV diet (2.4 ± 0.2 mm), (t_{187} = 4.38, p < 0.001) (Figure 5.6).

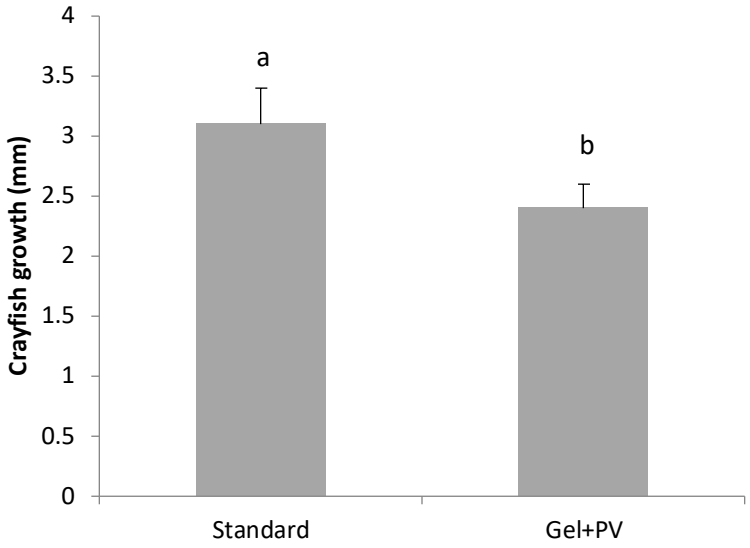

Figure 5.6. Mean growth, (final CL- start CL mm) of juvenile *A. pallipes*, within the different treatment diets, plankton plus vegetable (Standard) or plankton plus vegetable incorporated into agar gel (Gel+PV), between day-1 to day-40. A different letter above a treatment denotes significance. Error bars represent standard deviations.

5.3.4. Experiment D

Juvenile, 60-day-old crayfish fed four different treatment diets: i) Australian pellet (Australia); ii) New Zealand pellet (New Zealand); iii) plankton plus vegetables (Standard); and iv) the Spanish practical crayfish pellet diet (Spain).

5.3.4.1. Survival

For the juvenile crayfish, survival from day-40 to day-80 was significantly lower in the New Zealand treatment diet (78.4 ± 10.3%) than all the other three treatments: Australia (97.4 ± 4.1%); (z_{383} = 3.2, p < 0.001), Standard (92.5 ± 11.6%); (z_{383} = 2.2, p = 0.03) and Spain (96.3 ± 4.0 %); (z_{383} = 3.2, p < 0.001). Crayfish survival was not significantly different between the other three treatment diets (Figure 5.7).

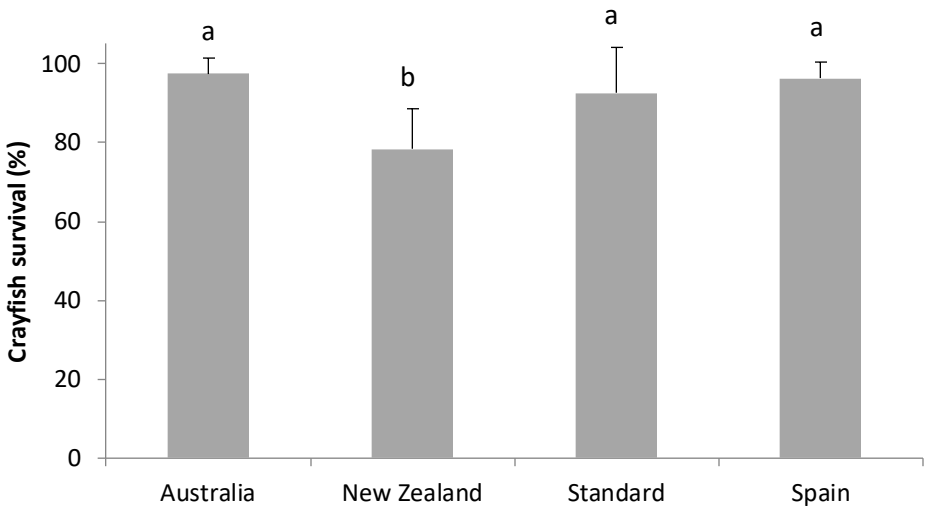

Figure 5.7. Percentage survival of juvenile *A. pallipes* for the four different treatment diets: Australian pellet (Australia); New Zealand pellet (New Zealand); plankton plus vegetable (Standard); and the Spanish practical crayfish diet (Spain); from day-40 to day-80. A different letter above a treatment denotes significance. Error bars represent standard deviations.

5.3.4.2. Growth

From day-40 to day-80, crayfish growth was significantly greater within the Standard treatment diet (2.5 ± 0.4 mm) than with all three other treatment diets: Australian (1.4 ± 0.3 mm, $p < 0.001$); New Zealand (2.0 ± 0.2 mm, $p = 0.01$); and Spanish diets (1.1 ± 0.3 mm, $F_{3,20} = 28.52$, $p < 0.001$). Crayfish growth with the New Zealand pellet was significantly greater than with the Australian pellet ($p = 0.01$) and Spanish practical diet ($F_{3,20} = 28.52$, $p < 0.001$) (Figure 5.8).

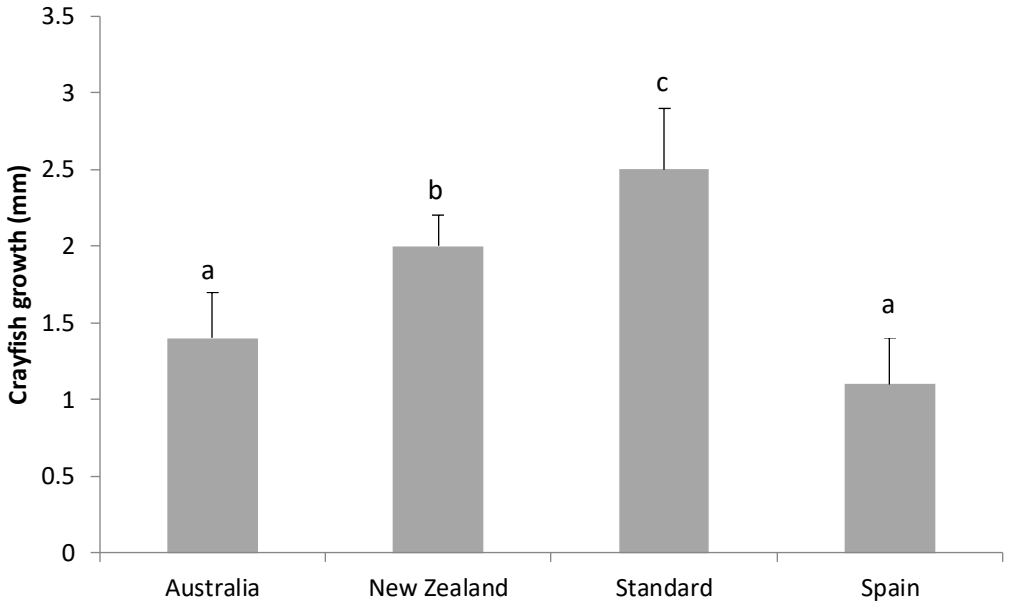

Figure 5.8. Mean growth, (final CL – start CL mm) of juvenile *A. pallipes* for the four different treatment diets: Australian pellet (Australia); New Zealand pellet (New Zealand); plankton plus vegetables (Standard); and the Spanish practical crayfish diet (Spain), from day-40 to day-80. A different letter above a treatment denotes significance. Error bars represent standard deviations.

5.3.5. Experiments B & D – total survival and growth

Survival and growth of the juvenile crayfish was then examined by analysing the results from experiments B & D together and redefining these crayfish into groups 1, 2, 3 and Spain. This was so that survival could be assessed when feeding crayfish with the Spanish practical crayfish diet over a longer-time period in comparison to feeding other diets.

Group 1 (G1): stage-2 hatchlings fed live *Artemia* nauplii plus Australian pellet (Live+Aus) from day 1-40 and Australian pellet (Australia) only from day 40-80.

Group 2 (G2): stage-2 hatchlings fed live *Artemia* nauplii plus New Zealand pellet (Live+NZ) from day 1-40 and New Zealand pellet (New Zealand) only from day 40-80.

Group 3 (G3): stage-2 hatchlings fed live *Artemia* nauplii from day 1-40 (Live+P) and plankton plus vegetables (Standard) from day 40-80.

Spain: stage-2 hatchlings fed a practical Spanish crayfish pellet diet throughout both experiments i.e. from day 1-80.

5.3.5.1. Total survival

Overall total survival from day-1 to day-80 of crayfish fed on with Live+P/Standard (G3) diets (88.5 ± 12.1%) was significantly greater (z_{383} = 2.53, p = 0.01) than with the Live+NZ/New Zealand (G2) diet (75 ± 18.1%) and Spanish practical (Spain) diet (77.1 ± 3.2%) (z_{383} = -2.07, p = 0.04). There was no significant difference between survival rates with the Live+Aus/Australia (G1) diet (83.3 ± 13.5%) and the other treatment diets (Figure 5.9).

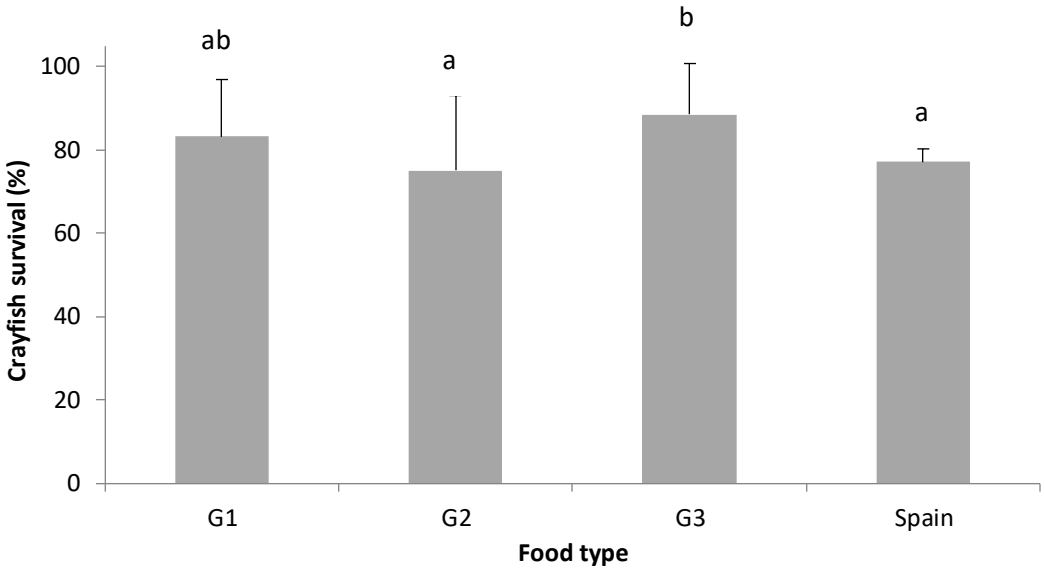

Figure 5.9. Percentage survival of *A. pallipes* from experiment C & D, day-1 to day-80. G1: stage-2 hatchings fed live *Artemia* nauplii plus Australain pellet (Live+Aus) from day 1-40 and Australian pellet (Australia) only from day 40-80. G2: stage-2 hatchlings fed live *Artemia* nauplii plus New Zealand pellet (Live+NZ) from day 1-40 and New Zealand pellet (New Zealand) from day 40-80. G3: stage-2 hatchlings fed live *Artemia* nauplii and plankton (Live+P) from day 1-40 and plankton plus vegetables (Standard) from day 40-80. Spain: stage-2 hatchlings fed a practical Spanish crayfish pellet diet from day 1-80. A different letter above a treatment denotes significance. Error bars represent standard deviations.

5.3.5.2. Total growth

Overall total crayfish growth (final carapace length - start carapace length) from day-1 to day-80 was significantly greater with the G3 (Live+P/Standard) diets (5.0 ± 1.4 mm) than with the G1 (Live+Aus/Australia) diet (3.7 ± 0.2 mm) and the Spain diet (3.3 ± 0.3 mm) ($F_{3,20}$ = 38.95, p < 0.001). There was no significant difference in crayfish growth between any of the other diets (Figure 5.10).

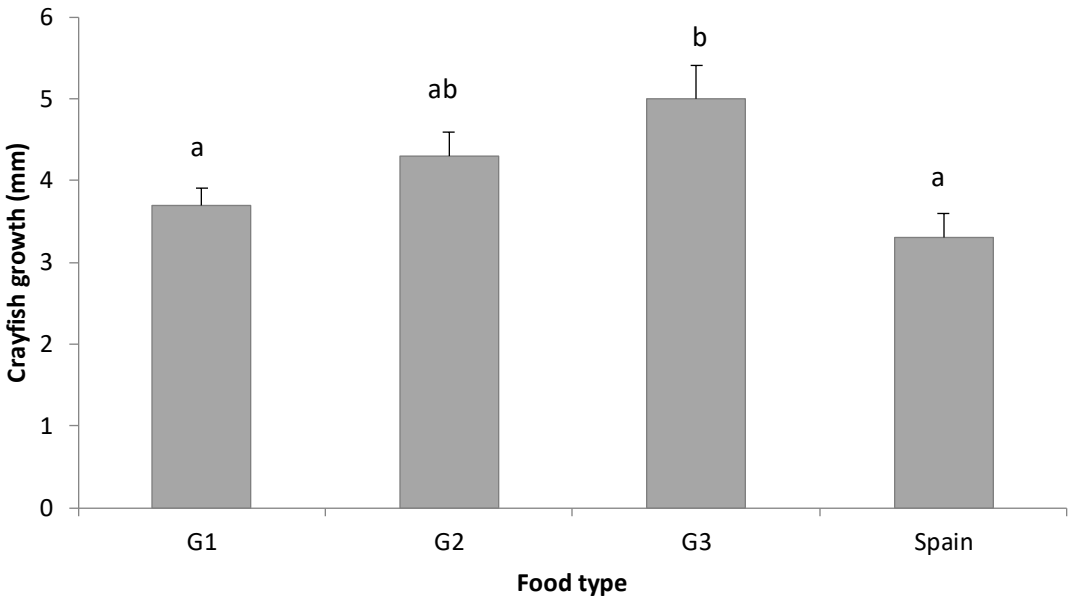

Figure 5.10. Juvenile *A. pallipes* mean growth, (final carapace length – start carapace length mm). Total growth and survival of *A. pallipes* from experiment C & D, day 1-80. G1: stage-2 hatchlings fed live *Artemia* nauplii plus Australian pellet (Live+Aus) from day 1-40 and Australian pellet (Australia) only from day 40-80. G2: stage-2 hatchlings fed live *Artemia* nauplii plus New Zealand pellet (Live+NZ) from day 1 - 40 and New Zealand pellet (New Zealand) from day 40-80. G3: stage-2 hatchlings fed live *Artemia* nauplii plus plankton (Live+P) from day1-40 and plankton plus vegetables (Standard) from day 40-80. Spain: stage-2 hatchlings fed a practical Spanish crayfish pellet diet from day 1-80. A different letter above a treatment denotes significance. Error bars represent standard deviations.

5.4. Discussion

In both experiments with stage-2 hatchling *A. pallipes* (A & B), consistently high survival rates (> 85%) were achieved with crayfish fed with enriched live *A. fransicana* (with additional food sources). This is likely to be due to its nutritional components, having a high protein content (> 50%) and good levels of lipids and fatty acids (Treece, 2000); however, the essential nutritional elements that hatchling crayfish require still remain unknown (González et al. 2011). González et al. (2012), demonstrated that a level of 55% protein was optimal for survival and growth of hatchling *P. leniusculus*; however, levels of over 33% would not compromise survival. During the first 4-6 weeks of life, the levels of digestive enzymes steadily increase as the hepatopancreas matures (Hammer et al. 2000). The proteolytic enzymes that *Artemia* nauplii contain may make digestion of dietary items easier for crayfish hatchlings and contribute to the maturation process of the hepatopancreas (Léger et al. 1986; Bengston et al. 1991).

Lower survival rates occurred with *Artemia* cysts plus plankton (experiment A), than with live *Artemia*. This suggests that the nutritional content was not optimal and live *Artemia* nauplii are a more suitable first food item than *Artemia* cysts. This is potentially because prey motility is important for *A. pallipes* hatchlings; the movement of the food may stimulate them to feed and this is provided by the nauplii but not the cysts. This is in contrast to a study on stage-2 hatchling *P. leniusculus*, where no significant difference in survival between *Artemia* cysts and live *Artemia* nauplii was observed (González et al. 2009).

When four age classes of yabby *Cherax destructor* were tested with live plankton versus pellet food, they spent 85% of their time feeding on live food and 15% feeding on the inert pellets (Meakin et al. 2008), demonstrating a preference for live food items. This was also observed in a study where juvenile hairy marron *Cherax tenuimanus*, when presented with both live *Daphnia* and pellet, showed a significant preference for feeding on the live food (Meakin et al. 2009). While observations on feeding preferences were not made as part of this study, a preference for live food items may be a result of their greater nutritional value. A stronger feeding response might have also been triggered by the presence of the live *A. fransicana* and therefore more food was ingested by the hatchlings in comparison to alternative diets. The survival and growth of hatchling *A. pallipes* fed live food is consistent with previous studies on *P. leniusculus* (Saez-Royuela et al. 2007) and *C. destructor* (Austin et al. 1997). In both cases growth and survival of hatchling crayfish was significantly higher when fed live food rather than other treatment diets. This finding is also supported by *in-situ* analysis of the gut contents of *A. pallipes* juveniles, which were shown to be feeding predominantly on aquatic invertebrates (Paglianti & Gherardi, 2004).

Growth of the hatchlings (experiment A) was also significantly greater when fed live *Artemia* nauplii plus plankton diet (Live+P) in comparison to decapsulated *Artemia* cysts plus plankton (Cyst+P). This was despite significantly lower numbers of crayfish within the cyst treatment groups during this time period due to mortalities. Previous studies have shown there is an increase in growth with a reduction in crayfish density (Nightingale et al. 2018a), which was not observed in this case, and therefore suggests that diet was a limiting factor.

The practical Spanish diet (experiment B), which contained *Artemia* cysts, within the pellet mix, achieved higher survival rates in hatchling crayfish than the rehydrated, decapsulated *Artemia* cysts, with or without agar gel (experiment A). The higher survival rate suggests there were some nutritional elements lacking from the non-live treatment diets in experiment A, which could be due to the quality of the original food elements (such as the cysts) or the preparation. In a study by Kouba et al. (2011), industrially decapsulated *Artemia* cysts

produced lower growth and survival rates than freshly decapsulated cysts fed to six-month-old noble crayfish *Astacus astacus*.

Crayfish within the *Artemia* nauplii plus plankton treatment (Live+P diet) were significantly larger than the crayfish in all three pellet treatment diets (Live+NZ; Live+Aus and Spain (containing *Artemia* cysts)), which indicates the plankton was providing the crayfish with more nutrition than the pellets. When *P. clarkii* were tested with a variety of "dead" foods versus live fish, defrosted zooplankton was the most stimulatory prey item (Kreider & Watts, 1998). Therefore, in this current study, *A. pallipes* may be eating more of the plankton than the pellet diets offered as it elicits a stronger feeding response, suggesting that palatability is a key element of their dietary requirements.

When sixty-day-old crayfish were fed different treatment diets, there was no significant difference in survival with the plankton or plankton plus agar gel diets (experiment C), indicating that the animals received enough nutritional content at the correct quantity to survive. When these diets were fed to stage-2 hatchlings, there was a significant reduction in survival; however, this same diet, fed to older juveniles, did not result in decreased survival. Therefore this provides evidence of the importance of the diet of crayfish during the first 40-days, after *A. pallipes* hatchlings are free-living, where correct nutrition is important for high survival and growth. A possible explanation is an ontogenetic diet shift; the digestive enzymes within the hepatopancreas alter as crayfish mature and this corresponds to changes in diets (Hammer et al. 2000; Figueiredo & Anderson, 2003).

In the case of both the agar-encapsulated (experiments A & C) and the pellet diets (experiments B & D), versus the plankton diets (experiments A, B, C & D), the plankton may be more palatable to the crayfish, in terms of both taste and texture, and therefore more is ingested, which increases growth. Studies on the palatability of diets have shown that crustaceans will increase their feeding when particular stimulants are added to their diet (Harpaz et al. 1987; Hua et al. 2015); therefore, there may be particular amino acids within the plankton diet making it more desirable to the crayfish. Crustacean species within aquaculture have preferences for specific textures and softness of food items (Cox & Johnston, 2003) and *A. pallipes*, may also prefer the texture of the plankton over the pellet or agar.

The fact that hatchling survival and growth was improved with live food and plankton is supported by a study of *A. pallipes* analysing gut contents, which found that juvenile crayfish ingest a high proportion of invertebrates in their diet (Gherardi et al. 2004). It has been suggested that as crayfish species mature they become predominantly detritivores (Paglianti & Gherardi, 2004); however, isotopic analysis of tissue suggests that aquatic invertebrates

form a significant part of the diet throughout all age classes as found in koura *Paranephrops zealandicus* (Hollows et al. 2002) and *P. leniusculus* (Stenroth et al. 2006). A recent study investigated reproductive ability and growth in *P. clarkii* and showed that there was a significant increase in both fecundity and specific growth when zooplankton diets were offered rather than a commercial pellet feed. It indicates that the importance of zooplankton within crayfish diets at all age classes should not be underestimated (Sonsupharp & Dahms, 2017). This could explain why even in the older crayfish growth was still improved with the standard diet, which was still predominantly consisting of plankton.

The aquaculture facility that was used was a closed-circuit recirculating system and therefore any chemical attractants contained within the specific diets could possibly end up in diluted amounts in other tanks. These chemical attractants could make the diets more palatable (Hua et al. 2015). The water from each tank returned into the filtration sump, via very fine micron-mesh filters, which helped to remove and dilute any attractants. If there was any affect it is likely to have been minor and all the tanks would have received this in the same amount and so therefore it should have had a similar effect on all. As this study was looking specifically at growth and survival from the nutritional qualities of the diets offered, the authors considered this to be an acceptable factor and having a closed circuit system ensured that there were no differences in water quality or temperature that might mask the direct effects of the treatments.

Significantly higher growth was achieved with plankton diets in both experiments C & D, suggesting that the other treatment diets (Gel+PV and the pellet diets), were less nutritionally optimal. In the case of the agar treatment diets (experiments A & C), although this has been found to produce good survival and growth in larger juvenile white yabby crayfish *C. albidus* (Coccia et al. 2010), the agar may not be suitable as an early food item for younger juveniles. Crayfish may be consuming too much agar and not enough of the other dietary, protein-based items at a life-stage where protein is an important dietary component. High survival rates were achieved with the Australian, Standard and Spanish pellet diets, indicating that the diets were of sufficient quality for the age age-class. However, crayfish fed the New Zealand pellet diet had significantly lower survival rates than the three other diets, suggesting there may be some nutritional element lacking within this particular diet for *A. pallipes*. When the live food was removed (at day-40) there was a reduction in survival in the NZ pellet group, supporting the theory that the diet was lacking a nutritional element. There are lower levels of protein in the NZ diet, compared to the other pellet feeds, which may result in lower survival rates during this critical life-stage. Mortalities were often observed during the moulting stage, i.e. moult death syndrome where a lack of adequate nutrition can cause crayfish to die whilst moulting due to a lack of sufficient energy

(Bowser & Rosemark, 1981). In contrast to survival rates, the New Zealand pellet diet elicited higher growth when compared to the Australian and Spanish diets; although this may have been due to cannibalism in the New Zealand pellet trial tanks, as this corresponded to a reduction in crayfish numbers within the tanks and not all the mortalities were accounted for. The crayfish may have been attacking moulting crayfish due to a nutritional deficiency within the diet (synchronised moulting was not occurring and therefore some crayfish were susceptible to cannibalism at this time). Alternatively, the reduced numbers of crayfish in the treatment tanks, due to the lower survival rate, caused an increase in growth of the remaining individuals (Savolainen et al. 2004; Nightingale et al. 2018a).

5.5. Conclusions

Enriched live diets are important for high survival and growth of *A. pallipes* from when the hatchlings are free-living, up until day-40, after which time the hepatopancreas and associated proteolytic enzymes are more mature. The hatchling may be using the protetolytic enzymes from the *A. fransicanca* to aid digestion at this early life stage. The enriched plankton diet consistently produced high rates of survival and growth in *A. pallipes* in both the hatchling and juvenile experiments (A, B, C & D), in comparison to all other diets offered, which suggests that nutritional quality and palatability is optimal. This is supported by studies of wild-caught *A. pallipes* juveniles, which consume a diet of predominantly aquatic invertebrates, (Scalici & Gibertini, 2007). Therefore it is possible that pellets do not give optimal nutrition in comparison to a more natural diet and the *A. pallipes* juveniles are showing a natural tendency to consume plankton rather than the artificial feeds offered. Evidence suggests that zooplankton may be important within crayfish diets at all age classes Hollows et al. 2002; Reynolds & O'Keeffe, 2005; Sonsupharp & Dahms, 2017; Stenroth et al. 2017). Therefore further research is required, to assess the long-term effects on growth, survival and fecundity of *A. pallipes* offered a solely pellet-based diet, rather than more natural food items.

Pellet diets are a cheaper and more convenient option than producing bio-secure live food or gamma-irradiated frozen plankton. Where time and financial constraints are not an issue, feeding live *Artemia* plus enriched plankton for the first 40-days and then moving on to an enriched plankton and vegetable diet is optimal for high survival and growth in *A. pallipes*. However, if *A. pallipes* are to be produced on a larger-scale, it may be more efficient to offer the practical crayfish pellet diet for all life-stages, but growth, survival and potentially fecundity may be compromised. All the diets trialled in these experiments have been

specifically designed for particular species of freshwater crayfish, which may have different nutritional requirements, and therefore there may not be a specific diet that will work for all the species that are bred and reared. Therefore, for juvenile *A. pallipes* it is suggested that an enriched plankton and vegetable diet, with a live food element during the first weeks, will produce the best survival and growth.

In the first three data chapters (section one of my thesis) I explored the key aquaculture elements to increase *A. pallipes* captive-breeding productivity. In the second section of my thesis I explore their wild release. In the next chapter I look at the efficacy of using passive integrated transponder tags as a permanent way of marking crayfish, to allow their long-term survival to be monitored once released into ark sites.

5.6. Supplementary material

Table S5.2. Composition and details of enrichment and feed items used in experiments A-D.

Product	Company address	Composition
Vitazin	Waterlife Research Ltd, Middlesex, UK	vitamins: A, B1, B2, B6, B12; nicotinic acid; D3, E, K & panthotenic acid
Spirulina	Zebrafish Management Systems Ltd, Hampshire, UK	protein: 65%; fats: 10%; carbohydrates: 15%; ash: 10%
Vitalis frozen food enrichment	World Feeds Limited, Thorne, UK	oils; fats; proteins; canthaxanthin 12.5 mg/kg; vitamin A 15,000 iu/kg; vitamin D3 2,000 iu/kg; vitamin E 200 mg/kg
Astaxanthin	Dr T&T Health UK Ltd, Northamptonshire, UK	naturose derived from the microalgae *Haematococcus pluvaili*
Artemia eggs	Zebrafish Management Systems Ltd, Hampshire, UK	100% eggs for hatching prior to feeding
Nannochloropsis spp. (for gut-loading live *Artemia* spp.)	Zebrafish Management Systems Ltd, Hampshire, UK	liquid microalgae (68B cells/mL)
HUFA live *Artemia* enrichment	Zebrafish Management Systems Ltd, Hampshire, UK	lipids: 32%; crude ash: 2%; DHA/EPA ratio: 9:1, sum w3; HUFA 150 mg/g; vitamin A 110,000 iu/kg; vitamin D3 10,000 iu/kg; vitamin E 5,400 mg/kg; vitamin C 8,000 mg/kg
decapsulated *Artemia* cysts	Aquarama, Maidstone, Kent, UK	100% decapsulated cysts for feeding: protein 60%; crude oil & fat 12%; Crude fibre 1%; crude ash 2%
Plankton	Tropical Marine Centre, Hertfordshire, UK	gamma-irradiated: rotifers; copepods; bloodworm; *Daphnia*; *Mysis* & krill
potato dextrose agar	Oxoid Ltd, Hampshire, UK	potato extract 4.0; dextrose 20.0; agar 15.0
locust bean	Special Ingredients Ltd, Derbyshire, UK.	E410 locust bean: 100%

Table S5.3. Composition of pellets and the practical pellet diet used in experiments B and D.

Australian CISRO	**New Zealand pellet**	**Spanish practical diet**
	Ingredients %	
Soybean	Dry matter 90.1	Soy lecithin[1] 0.1
Fish meal	Organic matter 94.2	Fish meal[2] 61.48
Canola meal	Nitrogen 4.5	Corn meal[3] 13.0
wheat gluten		Dried decapsulated *Artemia* cysts[4] 15.0
Dicalcium phosphate		Cod-liver oil[5] 3
Bentonite		Cholesterol
Mineral premix		Mineral premix[6] 2.0
Vitamin premix		Vitamin premix[7] 0.28
Bio mos		Astaxanthin[8] 0.1
Krill hydrolysate (attractant)		Ascorbyl monophosphate[9] 0.04
		Choline chloride[9] 0.5
		calcium phosphate[9] 1.0
		Cholesterol[10] 0.5
		Carboxymethylcellulose[11] 3.0
Composition %		
Crude protein 49	Crude protein 28.3	Crude protein 55.5
Lipid 10	Non-structural carbohydrates 54.8	Carbohydrates 12.33
	Starch 33.5	Moisture 8.3
	Soluble sugars 9.5	
	Neutral detergent fibre 8.5	Fibre 3.78
	Acid detergent fibre 3.8	Lipids 12.14
	Crude fat 2.6	
	Ash 5.8	Ash
Gross energy (kJ·g−1)		
19	14.5	19.33

1 BIOVER NV/SA Brujas, Belgium.
2 BIOMAR Iberia / PROAQUA Nutrición, Dueñas (Palencia), Spain.
3 ADPAN, Siero-Asturias, Spain.
4 *Artemia* cysts INVE Aquaculture Nutrition, High HUFA 430 µm, Dendermonde, Belgium.
5 ACOFARMA, Terrassa (Barcelona), Spain.
6 (mg·100 g^{-1} premix): $CoCl_2$ 4; $CuSO_4$-$5H_2O$ 250; $FeSO_4$ 4000; $MgSO_4$-$7H_2O$ 28 398; $MnSO_4$-H_2O
7 (mg·100 g^{-1} premix): Thiamine 2142.9; Riboflavin 1892.9; Niacin 7142.9 ; Pyridoxine 1785.7; Pantothenic acid 3785.7; Biotin 35.7; Folic acid 571.4; Cyanocobalamin 7.1; Myoinositol 14 285.7; Retinol 53.7; α-tocopherol 2382.1; Cholecalciferol 392.86; Naphthoquinone 312.43; Ethoxyquin 3571.43.650; KI 67; Na_2SeO_3 10; $ZnSO_4$-$7H_2O$ 13 193.
8 BIOMAR Iberia / PROAQUA Nutrición, Dueñas (Palencia), Spain.
9 NUTRAL SA, Madrid, Spain.
10 Sigma-Aldrich Chemie GMBH, Steinheim, Germany.
11 HELM IBERICA SA, Madrid, Spain.

CHAPTER 6

The long-term effects and detection ranges of passive integrated transponders with *Austropotamobius pallipes*

An adapted version of this chapter has been published in:

Nightingale, J., Stebbing, P., Taylor, N., McCabe G., & Jones, G. (2018). The long-term effects and detection ranges of passive integrated transponders in white-clawed crayfish *Austropotamobius pallipes. Knowledge and Management of Ecosystems, 419, 20-28.* (Appendix IV).

Author contributions

JAN designed the two studies and collected and analysed the data sets. NT checked the statistical analysis. Nathan Edmonds from Cefas demonstrated the tagging procedure and tagged the crayfish for the first experiment. JAN tagged the crayfish for the second experiment. Nick Taylor checked my statistical workings. JAN produced the manuscript draft and GJ, PS and GM provided support and guidance with the experimental design and contributed criticially to the manuscript and chapter drafts.

Abstract

Individual identification of the endangered white-clawed crayfish (*Austropotamobius pallipes*) can provide valuable information when assessing long-term survival of animals released into the wild; currently the most effective method is the use of passive integrated transponders (PIT) tags.

A 360 day *ex-situ* experiment was undertaken on 20-month-old, captive-born *A. pallipes* of carapace length (CL): 22-31 mm, to assess growth and survival after PIT-tagging. Thirty crayfish, matched for sex and size, were PIT-tagged, with 30 untagged crayfish as a control. All crayfish survived for the first 60 days post-tagging, indicating that there was no short-term survival effect of the procedure, in controlled conditions. There was no significant difference in survival or growth over the year between tagged and untagged crayfish, indicating that *A. pallipes* (≥ 22 mm CL) can be PIT-tagged safely.

A second *ex-situ* experiment investigated the detection range of adult, wild-caught, PIT-tagged *A. pallipes*. Eighteen *A. pallipes* were tagged with either 8 mm or 12 mm tags and added to different treatments (bare tank, tank with substrate, brick refuge, pipe refuge, pipe refuge plus slate), and the distance to detection was measured. Throughout all treatments the *A. pallipes* tagged with 12 mm PIT tags were detected significantly further away (35.6 ± 3.8 mm) than the 8 mm PIT-tagged crayfish.

6.1. Introduction

One of the recognised conservation techniques, to help safeguard the endangered white-clawed crayfish *Austropotamobius pallipes,* is to establish ark sites (safe refuges) into which wild populations can be translocated, or captive-born animals introduced (Souty-Grosset & Reynolds, 2009; Nightingale et al. 2017). Evaluation of the long-term success of these ark sites and the levels of recruitment can be greatly assisted by permanent marking and subsequent monitoring of the crayfish being released. If released crayfish individuals can be identified and tracked over an extended time period, within an ark site, this will provide valuable information on the status, long-term viability, and health of these populations.

The standard marking techniques for tracking crayfish have historically included cauterization, hole-punching, marking with correction fluid or oil-based pens/paints and radio-tracking (Abrahamsson, 1965; Guan, 1997; Ramalho et al. 2000; Haddaway et al. 2010; Robinson et al. 2010; Louca et al. 2014). However, none of these methods provides a

permanent method of marking that is retained during moulting and some methods, such as cauterization, have been shown to reduce growth (Guan, 1997).

There are several options for permanent marking of crayfish. Visible implant elastomer (VIE) is a liquid elastomer that is injected under the skin, allowing identification of a limited number of individuals or groups by colour combinations or implant location. Visible implant alpha tags (VI Alpha) are small, fluorescent tags with an alphanumeric code; VIE and VI Alpha are both designed to remain visible after they have been implanted within the animal (Gotteland, 2013). Both methods have limitations in terms of unique identification, retention rate, long-term readability and, because the tagged animals cannot be detected remotely, they have to be recaptured to be identified (Buřič et al. 2008; Haddaway et al. 2010). Coded micro-wire tags (CWT) are widely used in the fisheries industry. They are very small (1.1 x 0.25 mm), and therefore can be implanted without survival being compromised. The tags can be detected using hand-held readers; however, individual identification of live animals is difficult because the tag usually needs to be removed to be read (McMahan et al. 2012). One of the most successful methods of permanent tagging, with easy individual identification, is the use of passive integrated transponders (PIT) tags.

PIT tags are electronic chips, encased within glass, ranging in size from approximately 7 mm to 32 mm in length. They remain passive, i.e. dormant, until they are activated by a reader, which emits a close-range electromagnetic field, causing the PIT tag to transmit its unique code. PIT-tagging provides a permanent method to uniquely identify animals (Gibbons & Andrews, 2004). The use of PIT tags for marking animals began in the 1980s, when it was trialled with salmonids in the fisheries industry (Prentice & Park, 1983). With the development of PIT tag readers with extendable antenna, the tags can be read remotely, hence allowing animals to be identified *in-situ* without having to be captured or seen. This can potentially provide a very useful tool for monitoring populations of species long-term and validating survival rates when captive-born animals are released into the wild, without the need to physically trap or handle the animals to identify individuals.

The use of PIT tags on fish species is a recognised practice (Prentice and Park, 1983; Roussel et al. 2000). In recent years, this technique has been used on crayfish species and there have been several *in-situ* and *ex-situ* studies published. Field experiments include studies on *A. pallipes* (Bubb et al. 2010; Louca et al. 2014; Stead et al. 2015), slender crayfish *Orconectes compressus,* (Black et al. 2010), giant Tasmanian crayfish *Astacopsis gouldi* (Shepherd et al. 2011) and signal crayfish *Pacifastacus leniusculus* (Stead, 2015). Laboratory experiments include studies on *P. leniusculus* (Bubb et al. 2008; Wiles & Guan, 1993), spiny-cheek crayfish *Orconectes limosus* (Buřič et al. 2008), *O. compressus* (Black et

al. 2010), and woodland crayfish *Orconectes hylas* (Westhoff & Sievert, 2013). High survival rates and good growth have been reported in most cases, when tagging crayfish > 25 mm carapace length. However, previous laboratory studies were relatively short-term, typically less than 60 days in length, with one longer six-month study reported (Bubb et al. 2002).

There have been no known published laboratory trials, to assess survival and growth of PIT-tagged *A. pallipes.* There are a few published field studies, which have shown that PIT-tagged *A. pallipes* survived when returned to the wild (Bubb et al. 2008; Louca et al. 2014; Stead et al. 2015). However, in all of these studies, only a proportion of the released tagged crayfish were detected and therefore percentage survival and growth of *A. pallipes*, post-tagging, could not be confirmed. PIT tags are available in a range of sizes and there have been several studies looking at the detection range of fish species that have been tagged with different sized PIT tags (Morhardt et al. 2000; Burnett et al. 2013). However, there are no known published studies looking at the difference in detection range of crayfish tagged with different sized PIT tags. Bubb et al. (2002) and Burnett et al. (2013) assessed the specific range at which different sized PIT tags could be detected when placed in a river, but these crayfish were not internally PIT-tagged.

The objectives of the present study were two-fold: (i) to investigate the long-term effects of PIT-tagging on survival and growth of captive-born *A. pallipes,* within the laboratory, and to establish a minimum size at which *A. pallipes* can be safely tagged; and (ii) to investigate the detection range of PIT-tagged captive-born *A. pallipes* with 8 mm versus 12 mm PIT tags, within a variety of laboratory conditions. Both experiments should help to inform the efficacy of using PIT tags for long-term monitoring of *A. pallipes in-situ* and the safe minimum size at which *A. pallipes* can be PIT-tagged.

6.2. Materials and methods

6.2.1. Investigating the effect of pit-tagging on growth and survival *of A. pallipes ex-situ*

For a detailed description of the wild collection, hatchery details and husbandry routines see Chapter 2 "General Methodology".

6.2.1.1. Source of experimental animals

The *A. pallipes* used in this experiment were captive-born crayfish that were hatched from 20 wild-caught, ovigerous females, collected from a local river population. The crayfish were

hatched and reared for the first year in the Crayfish Research Unit an indoor, closed-circuit aquaculture facility. They were then moved to the outdoor Ubley Hatchery aquaculture facility and reared in large, outdoor, flow-through tanks for another ten-months prior to the experiment commencing.

6.2.1.2. Experimental set up and design

A year-long experiment was conducted at Ubley Hatchery. Sixty, captive-born, 20-month-old *A. pallipes* were used in the experiment: 30 males and 30 females. The crayfish were split into three groups, 20 crayfish in each, with an equal sex-ratio and, within each sex, an equal mean carapace length and weight. Each tank group was then divided into tagged (treatment) and control sub-groups, with equal sex-ratio (Table 6.1).

Table 6.1. Mean carapace lengths (CL mm) +/- standard deviation, of *A. pallipes* within the treatment and control groups for each of the replicate tanks (Tank 1-Tank 3); n=30.

Treatment/control	**Tank 1**	**Tank 2**	**Tank 3**
	CL (mm)	**CL (mm)**	**CL (mm)**
male tag	27.4 ± 4.0	27.5 ± 4.5	25.6 ± 3.3
male untagged	28.1 ± 7.1	27.3 ± 1.2	26.1 ± 3.6
female tag	25.6 ± 1.1	26.1 ± 2.6	24.8 ± 2.7
female untagged	24.4 ± 1.7	25.0 ± 3.5	24.6 ± 3.0

Crayfish in the treatment sub-groups were tagged with Trovan ID100A/1.4, (RFID Systems LTD, East Yorkshire, UK), 8 x 1.4 mm PIT tags and animals in the control group were left untagged. For the tagging procedure, the crayfish were held around the cephalothorax and an incision made, with a sterile 2 mm-gauge, hypodermic needle, through the cuticle and abdominal muscle of the third ventral abdominal segment (i.e. between the second and third set of pleopods). The tag was then injected into the abdominal muscle and the crayfish was scanned with a Trovan LID-55 midrange reader (RFID Systems LTD, East Yorkshire, UK) and the tag code recorded (Figure 6.1).

Each group of 20 crayfish was then randomly assigned to one of three experiment tanks (0.9 m^2 bottom area), on continuous flow-through from a local water reservoir and with an excess

of refuges, (engineered bricks and PVC pipes, no substrate). Water temperature was allowed to fluctuate naturally with the incoming water and was recorded hourly with an aquatic TinyTag data logger (Gemini Data Loggers Ltd, West Sussex, UK). Temperature varied seasonally between 5.8-22.5 °C, with no more than 2.8 °C variation over a 24 h period. Water quality was monitored monthly and chemical levels remained constant throughout the experiment: ammonia < 0.1 mg/L, nitrite < 0.1 mg/L, nitrate 15 mg/l, phosphate < 0.1 mg/L, pH 8.0, calcium 35 mg/L, general hardness 120 mg/L, carbonate hardness 100 mg/L and a level of dissolved oxygen > 80%. The photoperiod was natural and therefore fluctuated with season; average values were 12 h light and 12 h dark. No supplementary feeding occurred during the experiment; the crayfish foraged on live invertebrates and plant matter, existing within the tanks. This naturally occurring food supply was in constant supply from the incoming water.

6.2.1.3. Experimental procedure

The experiment ran for 12 months, from the end of January 2016 until beginning of January 2017. The crayfish were measured and weighed every month. At the end of the experiment, all surviving tagged crayfish were X-rayed with a Roentgen 703 machine (C&G Medical, West Midlands, UK) and processed with a Direct Digitizer Regius model 110 (Konica Minolta, Essex, UK), to assess the internal position of the PIT tags.

The crayfish were counted every 60 days; i.e. on days 1, 60, 120, 180, 240, 300 and 360. On each counting day, crayfish were scanned and biometric data recorded. Carapace length was measured from the anterior edge of the rostrum to the posterior edge of the cephalothorax to the nearest 0.1 mm using Vernier callipers (Moore and Wright, Sheffield, UK). The crayfish were dried with paper towel and weighed to the nearest 0.1 g using a digital weighing scale (Smart Weigh SWS600, Mosbacher Ltd, London, UK). Missing chelae, a standard measure of aggression, (Figureiel & Miller, 1995) and stage in moulting cycle, (i.e. whether inter-moult, pre, or post-moult), were also recorded. During the breeding season reproductive status was recorded; i.e. if females were in reproductive glair (development of glair glands on the ventral abdomen of the female), egg production or had spermatophores (deposited by males) present.

Figure 6.1.i) Passive integrated tags; ii) hypodermic needle showing ventral abdominal surface where PIT-tag is inserted; iii) crayfish being weighed; iv) crayfish being x-rayed © J. Nightingale.

6.2.1.4. Data collection and analysis

To determine if there was any difference in the survival of the tagged treatment and the untagged control, data were log transformed and examined by using nested binomial

generalized linear mixed models (function *glmer*, R package *lme4,* Bates et al. 2015). To determine if there was any difference in growth of the tagged and untagged group, data were examined using linear mixed models (function *lmer*, R package *lme4*, Bates et al. 2015). The treatment, control groups and sex were considered as fixed effects, and tank was considered as a random effect. The alpha level was set at $p < 0.05$. Only variables that had a significant effect were retained in the model, and the most appropriate model was identified by using the Akaike's Information Criterion corrected for small sample sizes (AICc). Where models were considered equivalent; i.e. AICc < 2, the model with the fewest parameters was chosen (Burnham et al. 2011). Statistical analyses were performed using R 3.2.5 (R Core Team, 2016).

6.2.2. Investigating the detection rates of tagged *A. pallipes ex-situ*

6.2.2.1. Source of experimental animals

Eighteen, wild-caught adult *A. pallipes* crayfish (collected from the River Itchen, Hampshire), which had been maintained in an outdoor aquaculture facility for 9-13 months.

6.2.2.2. Experimental set up and design

The crayfish were PIT-tagged one week prior to the experiment commencing, using the technique described in method 2.1 (Table 6.2) and the study took place in May 2017.

Table 6.2. Mean carapace lengths (CL mm) +/- standard deviation, of *A. pallipes* within the two PIT-tagged treatment groups: 8 mm and 12 mm tags; n=8.

Treatment	Male CL (mm)	Female CL (mm)	Male: female
8 x 1.4 mm PIT-tag	34.5 ± 1.5	35.1 ± 3.2	5:4
12 x 2.1 mm PIT-tag	40.9 ± 3.2	38.9 ± 1.7	4:5

An experimental tank (2000 x 500 x 750 mm) was used and water from the crayfish aquaculture system was added to a depth of 650 mm. The tank was set up in one of five different treatments: (i) bare tank; (ii) tank with gravel substrate (to a depth of 20 mm); (iii) gravel substrate plus pipe refuge (50 mm diameter); (iv) gravel substrate plus brick refuge (50 mm diameter); (v) gravel substrate plus pipe refuge with slate (15 mm thickness) over pipe.

6.2.2.3. Experimental procedure

The PIT-tagged crayfish were added, one-by-one, to the experimental tank and the range of detection of each crayfish in each of the different treatments was measured using a single-coil waterproof (IP54 rated) Trovan ANT-610F square antenna attached to a Trovan LID-65 decoder box (RFID Systems LTD, East Yorkshire, UK; Figure 6.2). The same crayfish was subjected to all five treatments sequentially and then removed and the next crayfish tested. For treatments iii, iv and v, refuges were added and the crayfish was put inside the refuge before the detection range was measured. The antenna was placed in the tank under the surface of the water and moved towards the crayfish until the decoder box produced an audible noise, signifying that the PIT tag had been detected. The distance between the crayfish and the antenna was then measured to an accuracy of 1 mm using a 300 mm rule. The process was repeated three times for each crayfish and the mean value calculated.

Figure 6.2. Trovan antennae and PIT-tag reader plus car battery power pack © J. Nightingale.

6.2.3.4. Data analysis

To determine if there were any differences between the PIT tags of variable size and the distance to detection in the five different treatments, data were examined by using linear models (function *lm*, R package *lme4*, Bates et al. 2015) with normal distribution. The five different treatments, sex and tag size were considered as fixed effects. Model selection was as outlined in 6.2.4a.

6.3. Results

6.3.1. Investigating the effect of PIT-tagging on growth and survival of *A. pallipes ex-situ*

6.3.1.1. Survival

All crayfish survived up until day-60. From day-60 to 360, several crayfish went missing and were therefore presumed dead, although their bodies / gastroliths were not recovered; the crayfish could not have escaped as the outflows were meshed and the tanks lidded. Eight tagged crayfish died in total, with carapace lengths (CL) at the start of the experiment ranging from 22-32 mm: males 22-32 mm and females 25-27 mm. Two tagged crayfish died prior to each of the May (day-120), July (day-180) and September (day-240) counts and one tagged crayfish died prior to each of the November (day-300) and final January (day-360) count. Five of the eight crayfish (CL: 25-32 mm) that died had moulted successfully at least once. The other three crayfish (CL: 22-27 mm) died before moulting could be confirmed. From day-180, three untagged crayfish died in total, with starting CL: 22-29 mm.

All untagged crayfish had undergone two successful moults prior to death. The two males (CL: 26 mm and 29 mm) died between August (day-180) and November (day-300) and the only untagged female to die (initial CL=22 mm), was found dead at the end of the experiment, CL=33.4 mm. Although only one untagged female died in comparison with 5 tagged males, there were no significant effects of sex (Z_{56} = -1.0, p = 0.31) or tagging (pooled sex: Z_{56} = 1.63, p = 0.1) on survival rates (Figure 6.3).

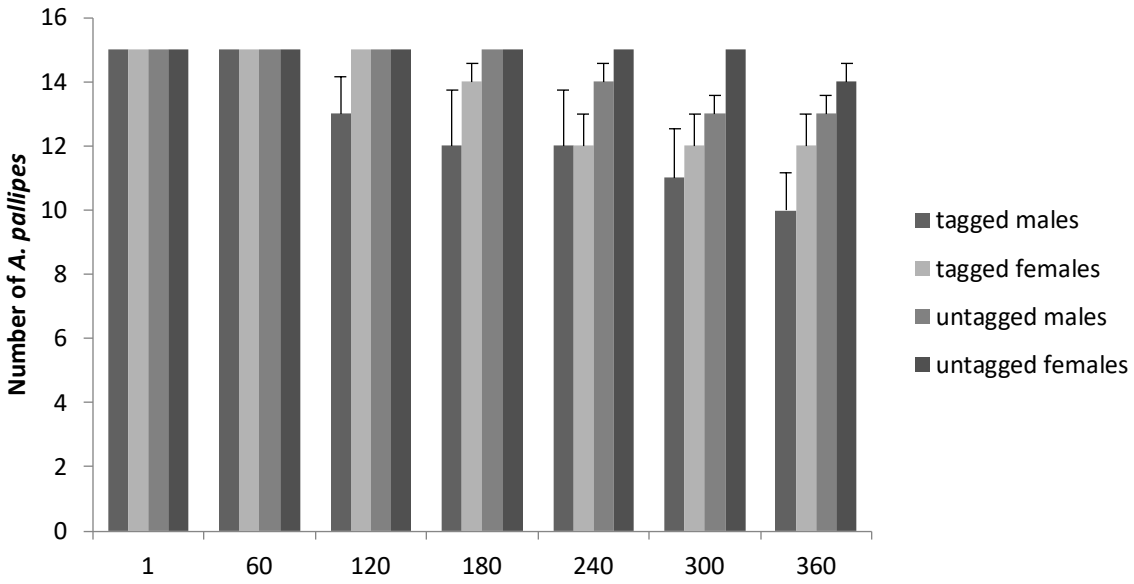

Figure 6.3. Survival of PIT-tagged male and female *A. pallipes* compared with untagged males and females, shown throughout time series from day-1 to day-360 of experiment, n=30. Error bars represent standard deviations.

6.3.1.2. Growth

Carapace length at the start of the experiment were not significantly different between tanks ($F_{2,56}$ = 1.97, p = 0.16) or treatments ($F_{1,56}$ = 0.24, p = 0.63) but males were significantly larger (mean ± SD) (27.0 ± 2.6 mm) than females (25.0 ± 2.1 mm); ($F_{1,44}$ = 44.88, p < 0.001). After adjusting for the differences in starting length for males and females, the model showed that there was no significant effect of treatment (t_{48} = -0.27, p = 0.61) or tank on final carapace length (t_{46} = -0.71, p = 0.48).

There was a significant effect of sex; over the course of the experiment, on average the males' carapace length increased by 8.6 ± 1.1 mm and the females increased by 7.5 ± 1.5 mm, showing that the males grow faster than the females (t_{46} = 3.41, p < 0.001), (Figure 6.4).

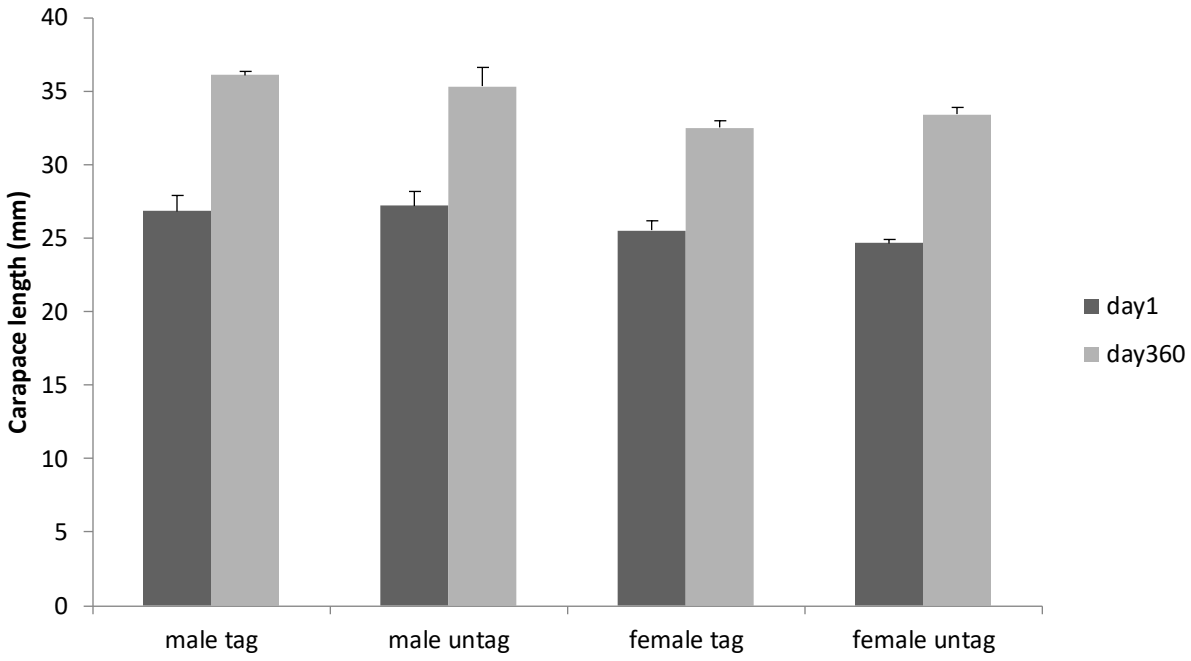

Figure 6.4. Carapace length (mm) of PIT- tagged male and female *A. pallipes* compared with untagged males and females, at the beginning (day-1) and end of the experiment (day-360) n=30. Error bars represent standard deviations.

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6.3.1.3. Moulting events

All moulting events occurred between the end of March (day-60) and the end of September (day-240). First moults had all occurred between day-60 and day-120. Of the tagged crayfish, 90% moulted successfully and it was unconfirmed if the three tagged crayfish that died between counts had already moulted because their bodies were not recovered. Males with CL < 29 mm moulted three times, and males with CL > 29 mm moulted twice. Females with CL < 26 mm moulted three times, and females with CL > 26 mm moulted twice. There was an inverse relationship between moult increments; i.e. the increase in size of crayfish between moults, and carapace length and the percentage moult increment decreased as the crayfish grew. Moult increments decreased with an increase in size of crayfish from a maximum of 5.2 mm CL increase (18.8% moult increment) down to a 2 mm CL increase (5.9% moult increment). Females on average grew slower than males at all sizes.

The crayfish were X-rayed at the end of the experiment. In 25% of the crayfish, the PIT-tag had remained within the abdomen, and in 75% of the crayfish, the PIT tag had moved into the cephalothorax and was positioned close to the dorsal cuticle, adjacent to the hepatopancreas (Figure 6.5).

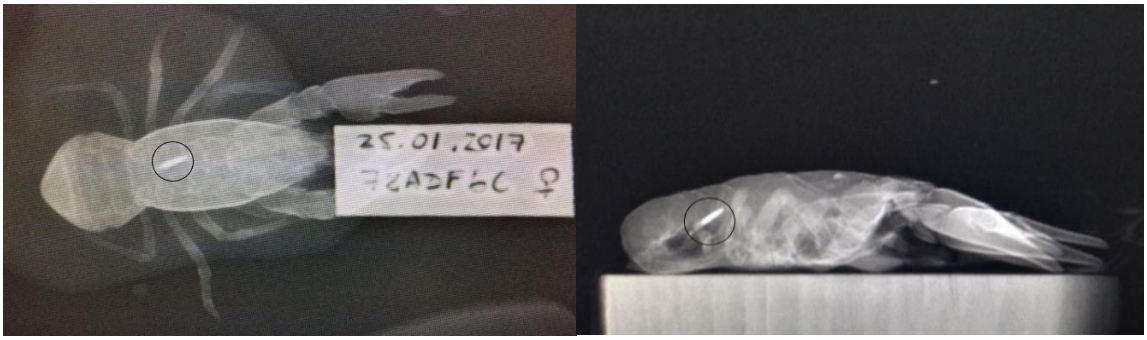

Figure 6.5. X-rays of PIT-tagged *A. pallipes* taken one-year post-tagging, with a black circle denoting the position of the PIT-tags © M. Barrows.

6.3.1.4. Fecundity

All tagged female *A. pallipes* came into reproductive glair and 80% of the untagged females came into glair, which was visible at the September count (day-240). At the November count (day-300), three tagged and one untagged female had spermatophores present and one tagged and one untagged female had a single fertile egg attached to their abdomens. Both eggs were still present and viable at the end of the experiment (day-360).

6.3.2. Investigating the detection rates of tagged *A. pallipes ex-situ*

There was a significant difference in detection range between crayfish tagged with 8 mm tags to crayfish tagged with 12 mm tags in all five treatments (t_{79} = 9.4, p < 0.001). Crayfish tagged with 12 mm tags were detected by the PIT tag antenna on average 35.6 mm (SD=3.8 mm) further away than the crayfish tagged with 8 mm PIT tags. There was no significant difference in distance detection rates with bare tanks and tanks with a gravel substrate (t_{79} = 0.59, p = 0.55). There was a significant difference between detection range of crayfish in a bare / substrate tank versus within bricks (t_{79} = -11.90, p < 0.001), pipe (t_{79} = -10.47, p < 0.001) or pipe plus slate (t_{79} = -10.37, p < 0.001). There was no significant difference between detection rate of tagged crayfish within bricks versus pipes (t_{79} = 1.45, p = 0.16) or bricks versus pipe plus slate (t_{79} = 1.53, p = 0.13). There was also no significant difference between pipes versus pipes plus slate (t_{79} = 0.10, p = 0.92) (Figure 6.6). There was no significant difference in detection rate between males and females (t_{83} = -0.53, p = 0.59), or crayfish of different sizes (t_{77} = 1.57, p = 012) (Figure 6.6).

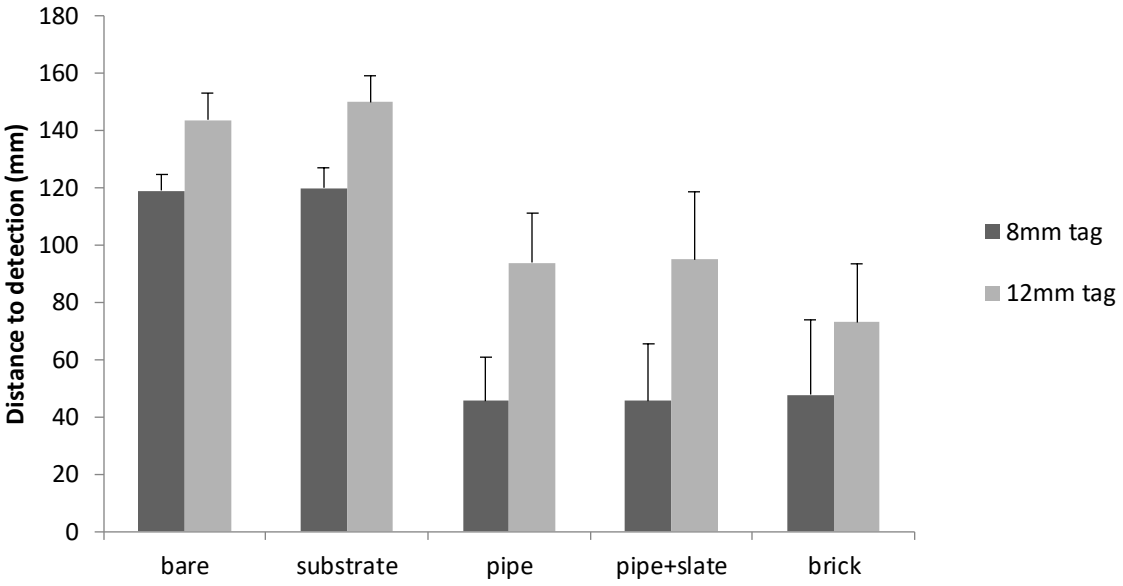

Figure 6.6. Comparison of detection ranges of *A. pallipes* tagged with 8 mm or 12 mm passive integrated transponders, within five different tank treatments, n=8. Error bars represent standard deviations.

6.4. Discussion

The current study found that there was no significant difference in survival, growth or fecundity between *A. pallipes* injected with 8 mm passive integrated transponders and the untagged control animals. All of the crayfish survived for at least the first 60 days of the experiment, and there was a 100% retention rate of PIT tags. This experiment was conducted with a fairly small number of animals due to the endangered status of the species. It would not have been ethical to tag a large number in case survival was significantly compromised by the procedure.

These experimental findings are supported by previous laboratory trials tagging other crayfish species. In a 50-day experiment undertaken by Wiles & Guan (1993), survival and growth of *P. leniusculus* tagged with 12 mm tags was not significantly different to untagged crayfish if CL > 26 mm. Bubb et al. (2008), undertook a 182-day experiment and found that survival and growth of *P. leniusculus,* CL: 33.7-61.4 mm, tagged with 12 mm tags, was not significantly different to untagged crayfish, (93.3 and 96.7% survival rate respectively). In a laboratory experiment with *O. hylas*, Westhoff & Seivert (2013) randomly tagged 96 crayfish CL: 15.3-33.3 mm, (44 were untagged as a control). They found that mortality dropped

below 20% if crayfish CL > 23 mm and CL > 26 mm were tagged with 8.5 mm and 12.5 mm tags, respectively. However, they also experienced a high mortality of untagged crayfish (20%), which may suggest there were underlying husbandry issues that might have masked the results of the experiment. Black et al. (2010), experienced high mortality when *O. compressus* with a carapace length of < 23 mm were tagged with 8 mm tags.

In a laboratory-based experiment with *P. leniusculus*, Wiles & Guan (1993), used 13 mm tags and injected the tags either at the base of the fourth pereopod or at the first pleopod ventrally into the cephalothorax, close to the internal organs. They observed unusual behaviour after tag insertion with crayfish stretching out legs and or chelae after the procedure and in crayfish CL < 25 mm, 47.6% died within ten days. They concluded that the PIT tags caused internal organ damage in the smaller crayfish and temporary reduction in leg movement in the some of the larger specimens. In this current experiment, the PIT-tags were injected lower down the crayfish, into the muscle of the third segment of the abdomen, to reduce the risk of damaging internal organs whilst the tag was being inserted (as in Buřič et al. 2008). The difference in tagging procedure may explain why smaller crayfish could be tagged without survival being compromised, and no temporary reduction in crayfish mobility was observed. The X-rays of the tagged *A. pallipes* taken on day-360 showed that most of the tags had only moved slightly from where they were injected into the abdomen and were positioned either just into the cephalothorax (next to the hepatopancreas) or still just slightly further up the abdomen (Figure 6.5).

All of the *A. pallipes* in this study survived the first 60 days indicating that tagging does not cause mortality in the short-term, under controlled conditions, and, up to day-180, there were only three deaths (10%) of PIT-tagged individuals. After day-180, similar numbers of tagged (4) and untagged (3) crayfish died, suggesting that these mortality events were not connected to the tagging event. This is supported by Wiles & Guan (1993), who concluded that crayfish mortalities occurring a few weeks after the tagging procedure could not be attributed to the physical tagging procedure because control animals also died.

Mortality events in the current experiment could be linked to moulting. The crayfish were held at a density equivalent of 5.8/m^2 and therefore, during moulting, they could be susceptible to antagonistic encounters from tank-mates. There was no supplementary feeding during the experiment, which might also have had an effect. Bubb et al. (2008), and Wiles & Guan (1993), housed the experimental crayfish individually and therefore removed any potentially negative effects of tank-mates or competition for resources such as food and shelter.

This study is the first known laboratory trial of PIT-tagging *A. pallipes*. There are several published field studies on PIT-tagged *A. pallipes* released into rivers, which indicate that a large proportion of tagged crayfish survive at least short-term. These field studies did not investigate survival or growth of individual crayfish over time or minimal size of crayfish that could be safely tagged, (Bubb et al. 2008; Louca et al. 2014; Stead et al. 2015). Therefore, the current study is the first to illustrate that growth and survival of both sub-adults and adult *A. pallipes* is not compromised by the PIT-tagging procedure.

During this experiment, both tagged and untagged crayfish successfully moulted up to three times from spring through to autumn, and growth was not significantly different between tagged and untagged crayfish. Laboratory studies on other crayfish species also found that growth was not affected by PIT-tagging (Wiles & Guan, 1993; Westhoff & Seivert, 2013). Bubb et al. (2006), found that although growth was not significantly different, the tagged crayfish were 10% smaller than untagged individuals by the end of the experiment; tagged male crayfish were 4.7% larger than untagged males, whereas untagged females were 3.9% larger than tagged females.

In this current experiment, the captive-born crayfish were only 2.5 years old at the beginning of the breeding season, and therefore, were potentially too immature to successfully produce large clutches of eggs. This has also been seen in other groups of 2+ year captive-born crayfish (J. Nightingale pers. obs.); however, it could be that keeping the crayfish with an equal ratio of males at a density of 5.8/m^2 caused other males to interrupt mating causing egg loss during egg-laying. It was encouraging that all the tagged females came into glair and that there were signs of males having mated successfully, with two females producing viable eggs. This is supported by Guan (1993), who tagged ovigerous *P. leniusculus*, which then carried their eggs full-term.

This study shows that *A. pallipes* with a carapace length of 22 mm could be PIT-tagged without survival or growth being compromised. When releasing captive-born crayfish, the individuals will rarely be larger than this size and therefore tagging with larger-sized PIT-tags is not a suitable option as this may well compromise survival. When the detection range of the 8 mm tagged crayfish was compared with 12 mm tagged crayfish the average difference in range was marginal (35.6 mm), which indicates that tagging with 8 mm tags is a good compromise. Using a single-coil antenna, the 8 mm tagged crayfish could be detected to a maximum distance of 120 mm when not within refuges, which was reduced to a maximum distance of 60 mm when the crayfish was inside a refuge. Although 60 mm is a fairly small range, it was encouraging to note that, even when the crayfish was within a brick refuge, the PIT tag antenna could still detect the animal. Larger, multiple-coil antennae are available,

which can increase the detection range; however, they are heavier and more expensive. In reality, it can be very difficult to reliably detect crayfish remotely *in-situ*, and recapture through trapping is the preferred method. Burnett et al. (2013) compared detection rates, plus range of detection, for tags of sizes 12, 23, and 32 mm. The tags were attached to rocks underwater and the range of detection was an average of 120, 202, and 290 mm, respectively. When tagged fish were tested, detection efficiency significantly increased with size of tag from 55% for 12 mm, 91% for 23 mm and 97% for 32 mm tags. However, 23 mm tags are too big for even the largest of *A. pallipes*, which have a maximum CL of up to 55 mm (Matthews & Reynolds, 1995). The smallest crayfish that were tagged with an 8 mm tag had a minimum CL of 22 mm; i.e. the tag was 36% of the size of the carapace. Working on this principle, if a 23 mm tag to be used, the crayfish would need to have a CL of 63 mm, minimum.

When captive-born crayfish are released into the wild, this is typically done with 1+ animals. The ideal minimum size for release is at a CL of 22 mm or above to make them less prone to predation and more likely to breed in the year of release. At this size, it also allows them to be safely PIT-tagged prior to release. However, passive integrated transponders are still relatively expensive (€1.1/tag); in comparison to other internal markers, such as visible implant elastomer (VIE) and coded micro-wire tags (CWT), which are considerably cheaper options (a few cents per tag). These other tagging options are suitable for crayfish < 22 mm CL, (due to their smaller size); however, they have their limitations. Haddaway et al. (2010) tested VIE with *A. pallipes* (CL: 9.5-31.1 mm) and found there was an 87.9% retention rate and a 36.0% tag migration rate over a 103-day period. They did not find a significant decrease in survival; however, survival in both the tagged (64.4%) and untagged (60.0%) groups was fairly low. Gotteland (2013) found no significant difference in survival when using VI Alpha, but experienced 33.0% mortality during a 60-day trial using VIEs with *A. pallipes*. In an experiment with the American lobster, *Homarus americanus*, McMahan et al. (2012), fitted CWTs and had an average 96% retention rate, CL: 12-30 mm. Where large groups of crayfish require a group identification system at low cost, VIE could potentially be a solution. However, with this technique, identification of individual live animals would be difficult, tag retention is not 100%, there is an issue with tag migration and therefore, long-term tag visibility.

6.5. Conclusions

Austropotamobius pallipes can be tagged with 8 mm passive integrated transponders at a minimum carapace length of 22 mm, without survival or growth being compromised. Care must be taken, to ensure that the tag is injected into the muscle of the second or third segment of the ventral abdomen so that there is no risk of damaging internal organs during the insertion process. Although 8 mm PIT tags, within *A. pallipes,* are detected at a shorter distance than 12 mm tagged crayfish, it is only an average of 35.6 mm difference. This detection difference is outweighed by the benefits of using a smaller tag, which allows captive-born, sub-adult crayfish to be tagged prior to release, and reduces the risk of internal organ damage to any size-class of crayfish. Although PIT-tagging is more expensive than other internal marking methods, it currently offers the only completely reliable method of permanently identifying individual animals and detecting them without having to recapture the individuals.

In reality, detecting the crayfish remotely, once released, by using antenna, is still fairly limited and therefore the crayfish need to be recaptured to give us information as to their movements and survival once released. Passive integrated transponder tagging does not provide information as to how they are utilising ark sites on a continuous basis but provides a snapshot in time. In the next chapter I explore the use of acoustic telemetry as a means of continuously tracking individual crayfish, to help inform us as to how they utilise a space and better inform us as to how to set up ark sites in the future.

CHAPTER 7

A year in the life of a crayfish:

investigating the use of acoustic telemetry for tracking *Austropotamobius pallipes* within an ark site

Author contributions

This study was undertaken in collaboration with Dr Clare Fitzsimmons and Kirsty Lees of University of Newcastle who assisted JAN with the design and implementation. Amy and Martin Stanton gave permission for the study to take place at Vobster Quay Inland Diving Centre. The hydrophone receiver array was borrowed from Dr. Clare Fitzsimmons. Howard Hugo Angel, Tim Clements, Kieran Hatton and Rachel Priest helped to position the hydrophone receiver array and carried out dive surveys to collect the hydrophones when the data downloads were required. Kieran Hatton also provided diver support and photography. Kirsty Lees assisted with data downloading and interpretation. Maxwell Fisher provided professional GPS points and Marcus Blatchford provided an arthomosaic from drone survey to help with geo-referencing the hydrophone receiver array. Markilan Abrahams helped to geo-reference the hydrophone receiver array, produced the GIS maps and assisted with the initial data sorting in Excel. JAN trapped and tagged the crayfish, downloaded the hydrophone data sets and submitted them to Vemco. Vemco produced the data files, which JAN analysed. GJ, PS and GM provided support and guidance with the study design and contributed criticially to the drafts.

Abstract

A 14-month passive acoustic telemetry study took place with white-clawed crayfish *Austropotamobius pallipes* at a crayfish quarry ark site in Somerset, between June 2016 and August 2017. Twelve *A. pallipes* were tagged and their movements were monitored by an array of 12 underwater hydrophone receivers, to assess temporal and spatial patterns of activity. Eight crayfish were released at the site of capture and four crayfish were released 200 m from their original capture location. The hydrophone receiver synctags showed considerable spatial and temporal inaccuracies, possibly due to reflection issues. Despite this, the crayfish did not travel far from the original site where they were released. The four crayfish that were released 200 m from their capture site, remained *in-situ* and did not exhibit homing behaviour. All 12 crayfish were significantly nocturnal, being more active between 21:00 - 03:00 h than at any other times of day, and this was consistent over all seasons. The crayfish were on average most active in spring and least active in summer. Females were more active than males at all times of day. Females in spring were significantly more active than males in summer. This is the first study using acoustic localisation of telemetry signals on a freshwater crayfish species. Acoustic telemetry can provide useful insights into the long-term activity of *A. pallipes*; however, this data set may have been improved if test-runs with the array had been carried out prior to the study commencing.

7.1. Introduction

Acoustic telemetry is a system whereby acoustic waves are used to determine and record the location of animals that are fitted with transmitter tags, which emit unique sound pulses, at a frequency, typically 69 or 180 kHz (Lucas & Baras, 2000). Acoustic telemetry can either be passive or active. Active telemetry relies on the data being detected by a portable receiver that is picking up sound pulses, which are emitted by the transmitter tags. Passive telemetry relies on an underwater hydrophone receiver array, set up in a grid pattern, to detect and record acoustic signals, which are emitted from acoustic transmitter tags fitted to aquatic animals. Typically, with passive telemetry, if the sound pulses from a tagged animal are picked up by at least three receivers, then detection will be registered. The average time taken for the sound to reach the receivers will be calculated and triangulated to determine the location of the animal (Leclerq et al. 2018). Passive acoustic telemetry allows aquatic animals to be tracked for long periods of time because many of the underwater hydrophone receivers and synctags have long battery lifespans. Active telemetry is usually carried out

over periods of a few days or weeks and is limited by the operator, time spent in the field, range of detection and accessibility of sites (Lucas & Baras, 2000).

Passive acoustic telemetry has been utilised for over 50+ years, to track a range of aquatic species. Species that have been tracked using this technique include Atlantic cod *Gadus morhua* (Rillahan et al. 2009; Ward et al. 2012), Gulf sturgeon *Acipenser oxyrinchus desotoi* (Dorazio & Price, 2019), Atlantic salmon *Salmo salar* (Juell & Westerberg, 1993; Føre et al. 2011), abalone *Haliotis discus hannai* (Hwang & Shin, 2010), lingcod *Ophiodon elongates* (Andrews et al. 2011) and black tip sharks *Carcharhinus melanopterus* (Simpfendorfer et al. 2002). This type of telemetry was first tested on crustaceans, over 20 years ago, and is now a relatively well established technique for tracking lobsters (Macarthur et al. 2008; Moland et al. 2011; Skerritt et al. 2015). Acoustic telemetry has several advantages over radio telemetry for underwater tracking because the tags can be used in both marine and freshwater environments and it can be used at depth; radio telemetry is only possible in freshwater up to 35 m depth (DeCelles & Zemeckis, 2014).

Traditionally, activity patterns in crayfish would have been recorded by visually observing the crayfish (Gherardi et al. 1998; Barbaresi & Gherardi, 2001). More recently underwater video cameras, both *in-situ* and *ex-situ*, have provided useful data sets with noble crayfish *Astacus astacus,* spiny cheek crayfish *Orconectes limosus* (Musil et al. 2010) and rusty crayfish *Orconectes rusticus* (Davis & Huber, 2007). Other methods include periodic trapping of crayfish species such as *A. astacus* and red swamp crayfish *Procambarus clarkii* within the field (Gherardi et al. 2000; Hudina et al. 2008); magnetic field detection (Lozan, 2000) or by using actographs *ex-situ* (Barbaresi & Gherardi, 2001). A fairly familiar method of extrapolating patterns of movement is by using capture mark recapture methods (Gherardi et al. 1988).

The development of Passive Integrated Transponder (PIT)-tagging, has refined ways in which crayfish behaviour is studied in the wild. Animals can now be injected with permanent tags, which enable the crayfish to be uniquely identified, and allows for potentially longer-term studies (Bubb et al. 2002; Bubb et al. 2006; Johnson et al. 2014; Nightingale et al. 2018c). Active radio telemetry has been applied to the study of a broad range of crayfish species: white-clawed crayfish *Austropotamobius pallipes* (Robinson et al. 2000; Bubb et al. 2006); signal crayfish *Pacifastacus leniusculus* (Bubb et al. 2002; Anastácio et al. 2015); *A. astacus* (Bohl,1999; Schiltze et al. 1999; Daněk et al. 2018); Tasmanian crayfish *Astacopsis gouldi* (Webb & Richardson, 2004); *O. limosus* (Buřič et al. 2009a, Buřič et al. 2009b); *P. clarkii* (Gherardi et al. 2002; Barbaresi et al. 2004; Aquiloni et al. 2005; Anastácio et al. 2015); Murray River crayfish *Euastacus armatus* (Ryan et al. 2008); new river crayfish *Cambarus chasmodactylus* (Loughman et al. 2013); and stone crayfish *Austropotamobius*

torrentium (Daněk et al. 2018). In contrast, the application of passive acoustic telemetry in the study of crayfish has never been tested.

There are several unanswered questions concerning the *in-situ* behaviour of *A. pallipes*, during its daily and seasonal cycles. When are crayfish active and does activity vary temporally? Do crayfish have home ranges and if so, how are these utilised? How far do crayfish range? Do crayfish exhibit refuge fidelity? Is there a difference in activity between sexes? Direct observations of this species can be difficult given its secretive nature, making these questions difficult to resolve. *A. pallipes* is endangered throughout its range in the UK and mainland Europe (Füreder et al. 2010) and establishing safe ark sites is a recognised conservation strategy for this species (Nightingale et al. 2017). Being able to closely monitor crayfish movements over an extended time period can be a great asset to conservation efforts. Understanding habitat utilisation, for example, can aid in ark site selection and targeted environmental modifications, to improve population sustainability. Passive acoustic telemetry presents an opportunity for continuous data recording, over extended time periods, to potentially enable us to answer these questions. However, this technique has not been tested on smaller crustaceans with small-scale, home ranges and there are no known published studies on tracking crayfish by using passive acoustic telemetry. The aim of this present study was to examine the behaviour of tagged *A. pallipes* using fine-scale passive acoustic telemetry. Data were collected, to try and resolve the questions regarding their *in-situ* behaviour and activity patterns.

7.2. Material and Methods

7.2.1. Study site

A still-water *A. pallipes* ark site at Vobster Quay Inland Diving Centre in Somerset (National Grid Reference: ST 705 498) was selected for the experiment. The site is a disused limestone quarry filled with water approximately 14.5 ha in size with a depth of up to 21 m (Figure 7.1.i). The site was established in 2009 as a crayfish ark site. Adult *A. pallipes* from a local native population (in Bath and North East Somerset), under threat from *P. leniusculus* invasion, were translocated into the site.

7.2.2. Experimental design

In May 2016, an array of 12 VR2W single-channel, omnidirectional 69 kHz hydrophone receivers (Vemco, Nova Scotia, Canada), was set up in a grid pattern at the site, with approximately 15-40 m between each hydrophone. The array covered an area of

approximately 120 m^2 and was within the original location where the *A. pallipes* were released (Figure 7.1.ii).

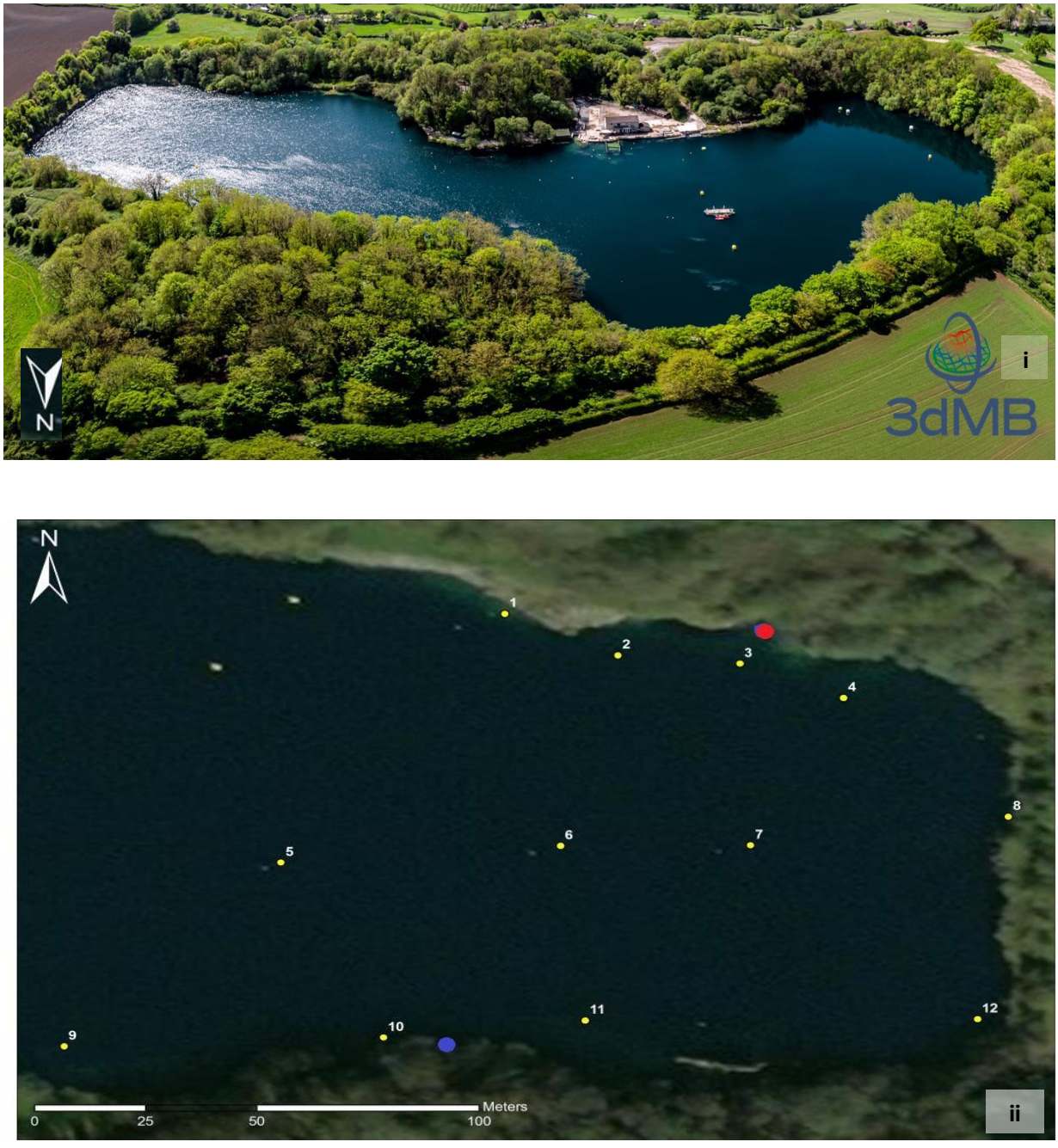

Figure 7.1. Photos of Vobster Quay Inland Diving; i) aerial drone view of quarry showing hydrophone area on left-hand side of photograph © M. Blatchford; ii) position of the 12 hydrophone receiver array (numbered yellow dots), plotted over a Google Earth basemap. The blue dot denotes the location where all 12 crayfish were captured and the eight of the crayfish (cray1 - cray8) were released when tagged. The red dot denotes the location of where the other four crayfish (cray9 - cray12) were released after tagging.

Each of the hydrophone receivers was moored to the bedrock by a rope, with a weight at the bottom. A V13-1x coded synchronisation tag (synctag), was paired to each receiver and attached to the lines, 1 m below the hydrophone receivers (Figure 7.2). The position of each hydrophone was marked by a surface buoy. This methodology was adapted from the methods used by Skerrit (2014). The synctags act as a control to help validate the data received from the tagged animals (Mathies et al. 2014) and to help with clock drift. Each hydrophone receiver has an internal clock, which can drift up to four seconds per day and this clock skew will be detected by the synctags (Andrew et al. 2011). The synctags were set to repeatedly send their acoustic signal with a random delay between 200-400 s. The animal tags were set to send a signal between 120-180 s to minimise signals / sound waves colliding and therefore not being detected. The hydrophone receivers varied in their depth depending on the depth of the quarry at their location. Hydrophone receivers 1, 2, 3, 4, 8, 9, 10, 11 & 12 were at 3 - 4 m depth and hydrophone receivers 5, 6 and 7 were at 21-22 m depth. In all cases, the synctags were attached 1 m below the hydrophone receivers. These depths were taken into account by Vemco when they carried out the data analysis. Vemco is an international company, based in Nova Scotia, Canada and is one of the world leaders in producing acoustic telemetry equipment and providing data analysis.

Tagged divers were deployed in the area to test detection levels of the receivers, to ensure that the array was in the correct position. The hydrophone locations were mapped, initially using a handheld Garmin Geographical Positioning System (GPS) unit, (Southampton, UK). This was then verified by using drone photographs and geo-referencing with GIS and using a professional GPS unit, a Topcon GPS 1 L1/L2 RTK, (Tamworth, UK). An average value was taken for each position and cross-checked with a satellite image from Google Earth. Surface water temperature was recorded every week, over the course of the experiment, using a digital thermometer (Figure 7.4).

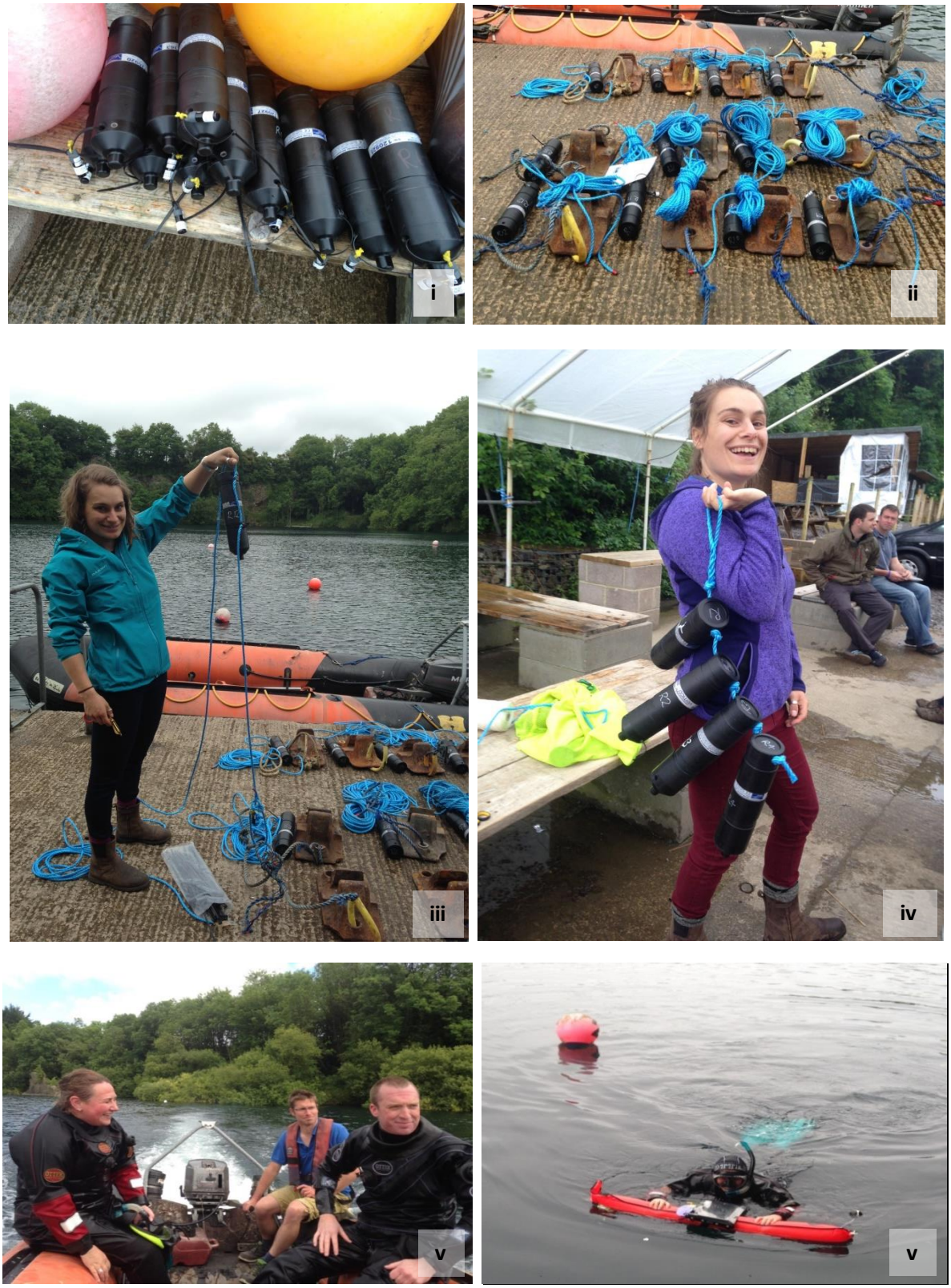

Figure 7.2. i) Hydrophone receivers; ii) hydrophones attached to weights; iii) hydrophone with synctag and weight attached; iv) diver deployment system; v) divers heading to hydrophone deployment site; vi) diver surveying hydrophones and recording GPS locations © J. Nightingale.

7.2.3. Pilot study

Prior to the experiment commencing, a wild-caught *A. pallipes* (from a local natural river population), which had been in captivity with two other crayfish and monitored for several months, was tagged and monitored. The tag was glued to the dorsal carapace and the crayfish was monitored for a further four weeks to assess if the tag was affecting it in any way (Figure 7.3.iv). The other two crayfish acted as controls. Monitoring included: activity patterns, movement, space utilisation, refuge selection, interaction with conspecifics and feeding frequency. After this pilot study there were concerns that this was not the most reliable method of attachment and a more robust technique would be to tie the tags to the chelipeds. After the pilot study a further test-run was carried out by tagging only three male crayfish and releasing them into the quarry and assessing data received over a four week period.

7.2.4. Crayfish collection

The crayfish were captured from the still-water Vobster Quay Inland Diving quarry ark-site where the research took place. Plastic mesh crayfish funnel traps and collapsible crayfish traps were used. The traps measured 595 x 300 mm, with a 12 mm mesh width and an entrance of < 90 mm, (to prevent otters being trapped). The traps were baited with sprats *Sprattus sprattus*, and left in the quarry overnight. Crayfish were caught, tagged and returned to the site between 20.06.16 and 20.07.16, with a total of 12 crayfish caught and tagged. A Vemco V7-4L 69 kHz transmitter tag of size 22.5 length x 7 mm diameter; 1.0 g weight in water (i.e. approximately 3% of crayfish bodyweight), was inserted into 6 mm airline (Figure 7.3.ii). This was then gently cable-tied around one of the two crayfish chelipeds, using the same technique that has been developed for lobster tagging (Moland et al. 2011; Skerritt, 2014), the exception was the first crayfish to be tagged for the pilot study (see section 7.2.3). Once tagged, the crayfish were monitored within a tank for approximately 30 minutes, to ensure that they were fully mobile, could right themselves correctly and could jack-knife effectively, prior to release. All 12 crayfish were in good visible health, with no physical deformities. Mean carapace length (± SD) was 47.45 ± 3.73 mm and mean weight (± SD) was 34.24 ± 9.67 g (Table 7.1). Eight crayfish were released from the locations where they were trapped. The other four crayfish (equal sex-ratio) were released on the other side of the quarry edge, approximately 200 m from where they were originally trapped, to establish if the crayfish would exhibit homing behaviour and return to the site where they were caught.

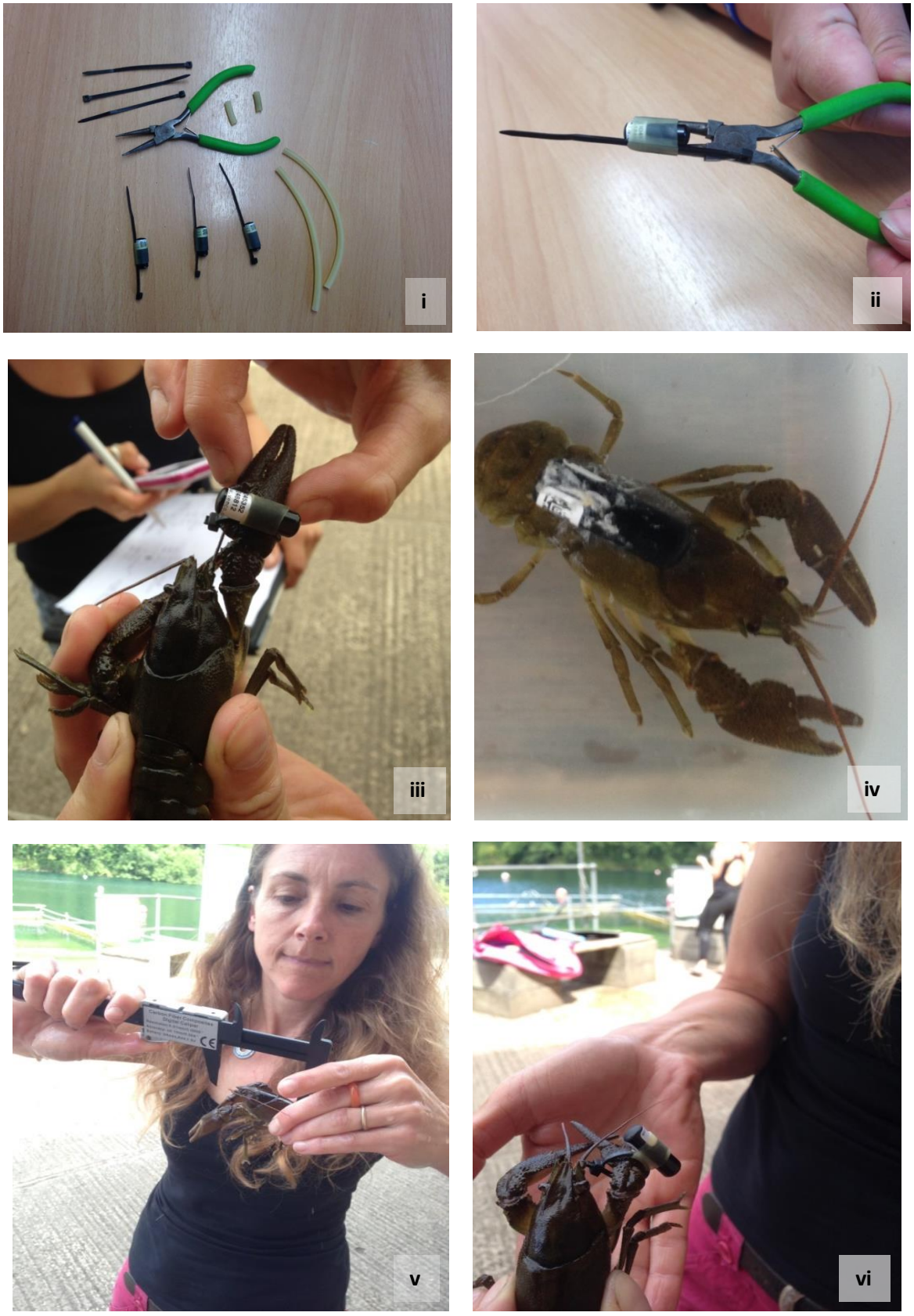

Figure 7.3. i) 8 mm PIT-tags; ii) inserting tag into airline for cable tie attachment © J. Nightingale; iii) attaching cable tie to crayfish cheliped; iv) pilot trial tag glued on to dorsal carapace; v) measuring carapace length; vi) tagged crayfish ready for release © C. Fitzsimmons.

Table 7.1. Data from individual crayfish (cray1 - cray12) including biometric information and carapace length (CL), plus tag attachment details, release date, release location, plus date of final tag transmission. M is male, F is female.

Crayfish	Sex	CL (mm)	Size (g)	Released	Release area	Attachment	Last transmission
Cray1	M	44.3	30.0	22/06/16	capture site (cs)	cheliped	16.07.16
Cray 2	M	43.2	25.0	22/06/16	capture site	cheliped	08.07.17
Cray3	M	49.4	42.8	22/06/16	capture site	cheliped	08.07.17
Cray4	F	31.0	9.1	04/07/16	wild-caught	carapace	01.07.17
Cray5	M	43.5	27.1	20/07/16	capture site	cheliped	31.07.16
Cray6	M	45.5	33.5	20/07/16	capture site	cheliped	04.08.17
Cray7	F	47.5	29.5	20/07/16	capture site	cheliped	04.08.17
Cray8	F	48.0	30.4	20/07/16	capture site	cheliped	31.08.16
Cray9	M	47.5	33.5	20/07/16	200 m from cs	cheliped	04.08.17
Cray10	M	56.0	59.0	20/07/16	200 m from cs	cheliped	02.11.16
Cray11	F	46.0	27.8	20/07/16	200 m from cs	cheliped	04.08.17
Cray12	F	51.0	38.1	20/07/16	200 m from cs	cheliped	22.06.17

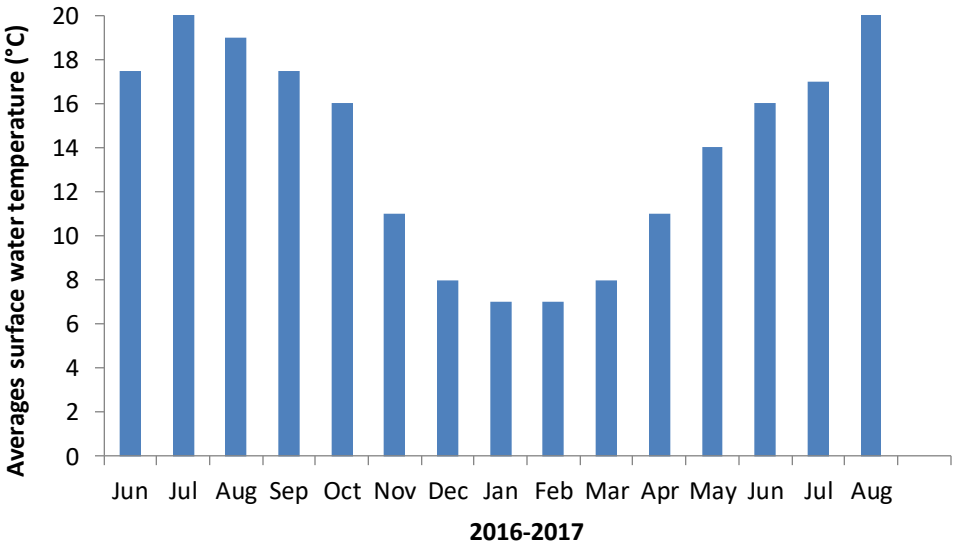

Figure 7.4. Average surface water temperature at Vobster Quay Inland Diving from June 2016 to August 2017.

7.2.5. Data collection

Data were collected by the hydrophone receivers every 180 seconds over a 12-month period, from all tagged crayfish. The hydrophone array was kept within the quarry for 16 months from June 2016 until September 2017 and the data collection period covered all four seasons, spanning June 22, 2016 to August 4, 2017. For data analysis the year was divided into four seasons: winter: December 1 – February 28; spring: March 1 – May 31; summer: June 1 to August 31 and autumn: September 1 to November 30. Over the course of the study period there were two summer periods, summer1 (2016) and summer2 (2017). The data were downloaded from the hydrophone receivers periodically throughout the year: August 2016, September 2016, May 2017 and September 2017. This was done by a team of divers who removed the hydrophone receivers from their marker buoys and weights and brought them ashore where the data could be retrieved. Once the data download was completed, the hydrophones were returned to the same location. The downloaded data were then checked using the VUE (VEMCO User Environment) software programme. This software allows the data from the hydrophone receivers to be uploaded into a central database. By uploading the data we could ensure data were still being received by the hydrophones and the receiver tags on the crayfish were active. In order to determine precise locations of the crayfish, the full data set was then analysed by Vemco using their Vemco Positioning System, to give high resolution tracks of individual crayfish.

7.2.6. Data analysis

Vemco calculated the position of each tag by calculating the average time difference of arrival of the tag to at least three hydrophone receivers. When all the raw data from all 12 crayfish tags were plotted, there was a great deal of error, with approximately 25% of the recordings seemingly corresponding to an area on land (Figure 7.5). Vemco use a system called horizontal positioning error (HPE), to validate the accuracy of the tag position. This system is based on several variables such as water temperature, hydrophone and synctag depth and the accuracy of the data received from the synctags on the receivers, which are at known locations. The HPE produces relative values, without units: the higher the value, the higher the error. For the synctags, this error is applied to metres (HPEm) as the synctags are at known locations (Smith et al. 2013).

Figure 7.5. Complete data set for all 12 tagged crayfish over the course of the study from June 2016 – August 2017.

When the raw data for the synctags were checked for accuracy, there was considerable variation (Figure 7.6). All data points on the synctags, where the HPEm was > 3 m, were removed, to ensure that the synctags were being recorded at the correct place. Out of 41,000 locations, 18,216 data points were accurate, within 3 m of their known location point (approximately 30%) (Figure 7.7.a). These inaccuracies in data locations were particularly prominent in the first month from June to July 2016, partly due to the fact that three of the hydrophones were repositioned before the other eight crayfish were released. Therefore the first month was removed from the data set. With the HPEm > 3 m rule applied, this removed 70% of the data set and provided a more accurate map in terms of the location of the hydrophone receiver synctags (Figure 7.7.b). When this data set was explored, the times when the synctag data points were accurate, were scattered throughout each day and over the entire year. For example, when a day (11.08.2016) was randomly selected and the data from all 12 synctags were compared, there were no set times in the day when all 12 synctag locations were accurate. The fact that there was no consistency as to when the synctags were recording accurately meant that there were no specific times and days that could be removed. If there had been certain times in the day or year when the location data for all 12 synctags were consistently accurate, then only the crayfish data from these same days and times would have been analysed, which may have produced more accurate maps.

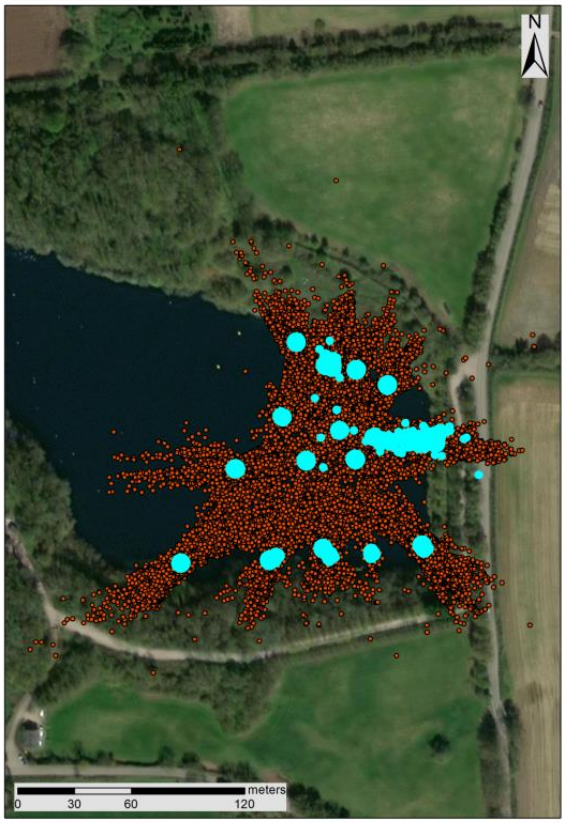

Figure 7.6. Raw data for all 12 x synctags, attached to the 12 hydrophone receivers, with all the data received over the full experiment from June 2016 – August 2017. The orange dots are the complete data set, the blue dots represent the data received from the 12 synctags once the HPEm > 3 m rule is applied.

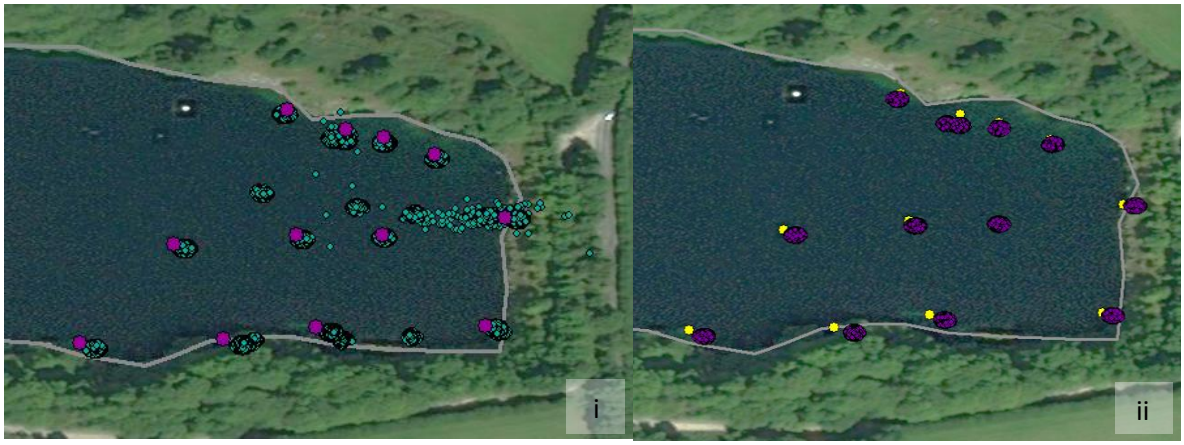

Figure 7.7.i) All data received from June 2016 – August 2017 from the 12 synctags attached to the 12 hydrophone receivers, after the HPEm > 3m rule was applied. Purple dots indicate the actual location of the hydrophone receivers; green dots are the synctag location data. ii) Data received from the 12 synctags from August 2016 after three hydrophone receivers were repositioned. Yellow dots indicate the actual location of the hydrophone receivers, purple dots are the synctag location data after the HPEm > 3 m rule was applied

The recommended HPE > 24, which is an arbitrary recommended value (Smith et al. 2013) was then applied to the crayfish data set, to see if the data set was any more accurate; however, this still resulted in obvious inaccuracies in crayfish location; i.e. crayfish data points were still on land (Figure 7.8). Although *A. pallipes* can move terrestrially on occasion, most of these terrestrial locations are probably inaccurate (Masefield, 2019).

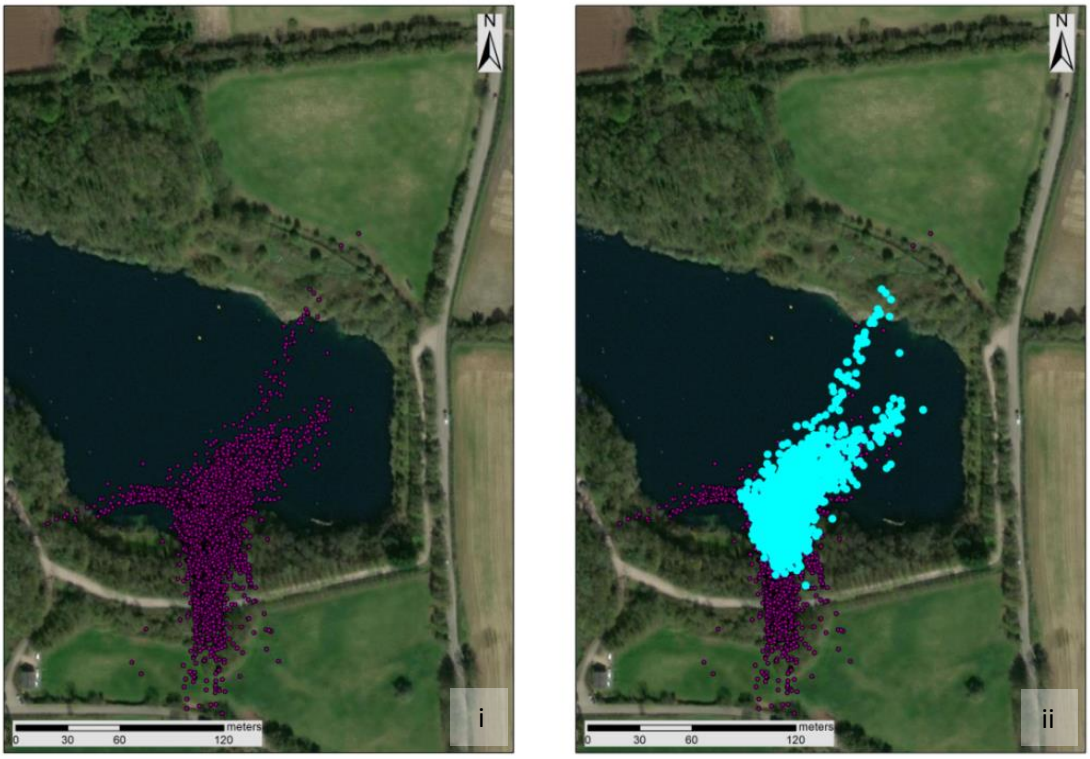

Figure 7.8. Revised data set for cray6 from June 2016 – August 2017 after the data from the inaccurate synctags were removed: i) raw data; ii) raw data with the blue dots showing the data set once the HPE > 24 error was applied.

The maps for each crayfish were examined, to assess the broad movement range of the animal. The only analysis that could reliably take place was on the activity patterns of the crayfish. The tags were set to transmit every 180 s and it was assumed that if a crayfish could be 'seen' by the hydrophone receivers then it was not hiding (within a refuge) and was assumed to be active. This assumption is supported by the findings of Skerrit et al. (2015): when lobsters were within their refuges the tags could not be detected by the hydrophones. The days were divided into eight, 3-hour time blocks and the number of times each crayfish was recorded was converted to a percentage for each time block. This was done by calculating the amount of possible times the crayfish could be seen and dividing it by the amount of actual time crayfish movement was detected. The data were analysed to assess activity levels over each day, season, sex and individuals. Activity levels differences were

analysed with ANOVA and data were log transformed to normalise variations. Tukey's post-hoc test was applied to assess pairwise differences in activity levels between days, seasons and sexes. The statistical analysis was carried out using the software package R 3.2.5 (R Core Team, 2016).

7.3. Results

7.3.1. Crayfish detection patterns

From the complete data set, there were 3,736,575 animal tag detections logged over the course of the data collection period. There were 12 unique animal tags detected; of these, total detections ranged from 2,034 (cray5) to 737,654 (cray11) and 58.5% of animal tag transmissions were detected on at least three receivers, and each animal tag transmission was detected 3.6 times on average. A total of 449,885 synctag positions and 472,477 animal tag positions were calculated by the Vemco Positioning System. Positions were calculated for 12 different animals; of these, yields ranged from 30 positions (cray5) to 104,695 positions (cray11).

Four crayfish (cray6, 7, 9 and 11), out of the 12, were assumed to still be carrying their tags at the end of the experiment. This was because the tags were still being detected by the hydrophones until the batteries ran out on 4.8.17, (when the transmissions stopped between 22:05-22:55). The other eight crayfish presumably died, dropped or lost their tags prior to the end of the study period. Length of detection varied from 24 days (cray1) to 381 days (cray6, 7, 9 & 11), Table 7.1. From the raw data for each of the crayfish, it can be seen that despite the obvious error in the data set, the crayfish remained near their release sites and their range did not cover the entire quarry area during the study period (Figure 7.9).

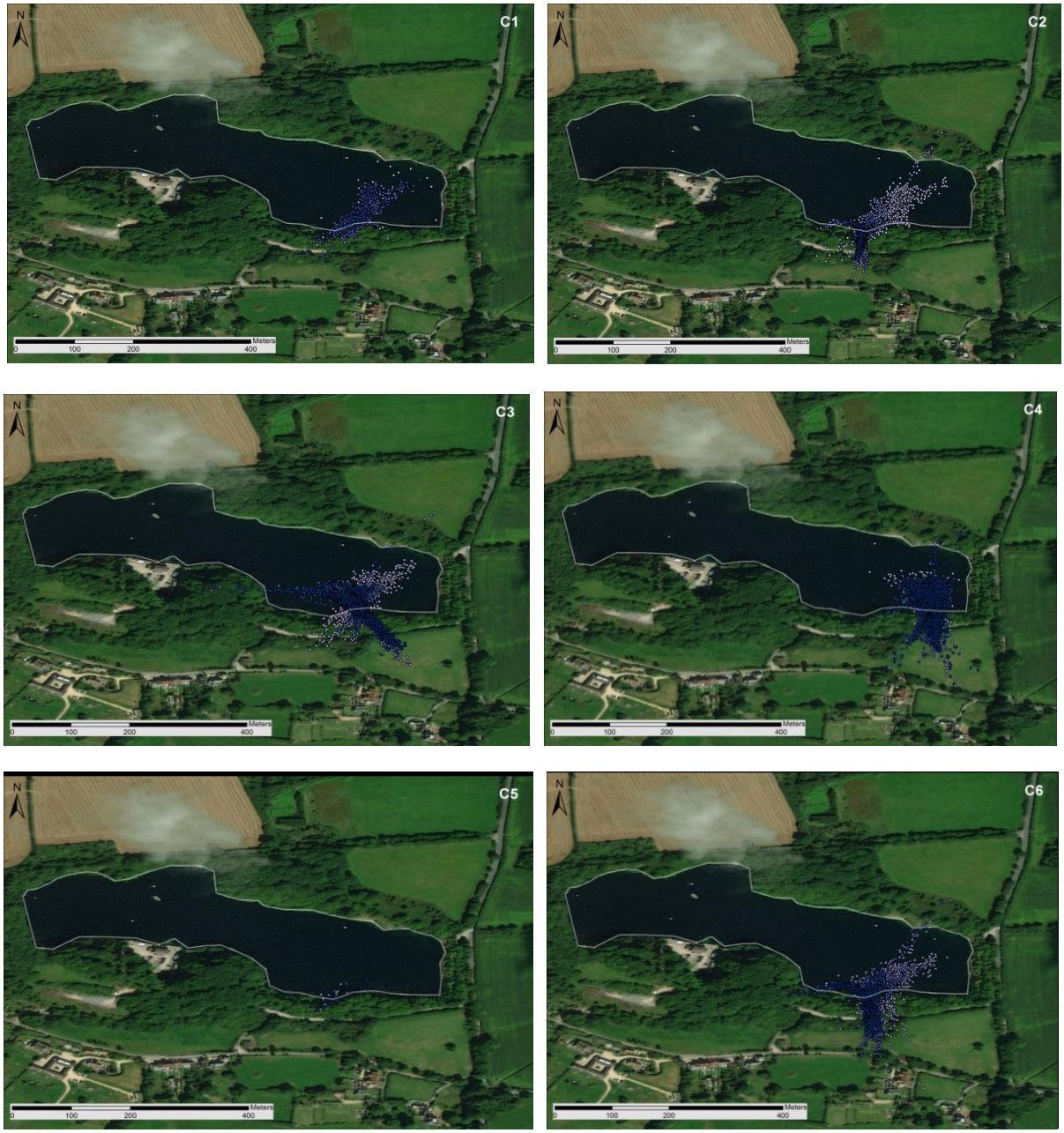

Figure 7.9. Location of the crayfish cray1 - cray8 (C1 - C8) at Vobster Quay Inland Diving, which were released at the same site from where they were originally captured. Recorded over the entire study period from June 2016 – August 2017, complete data set shown. Detection dots are coloured by date, with lighter shades indicating earlier dates.

7.3.2. Home range study

The four crayfish that were released at the opposite side of the quarry, to where they were trapped, remained near to the site of release (See Figure 7.1). They did not return to the location where they were originally captured (Figure 7.10).

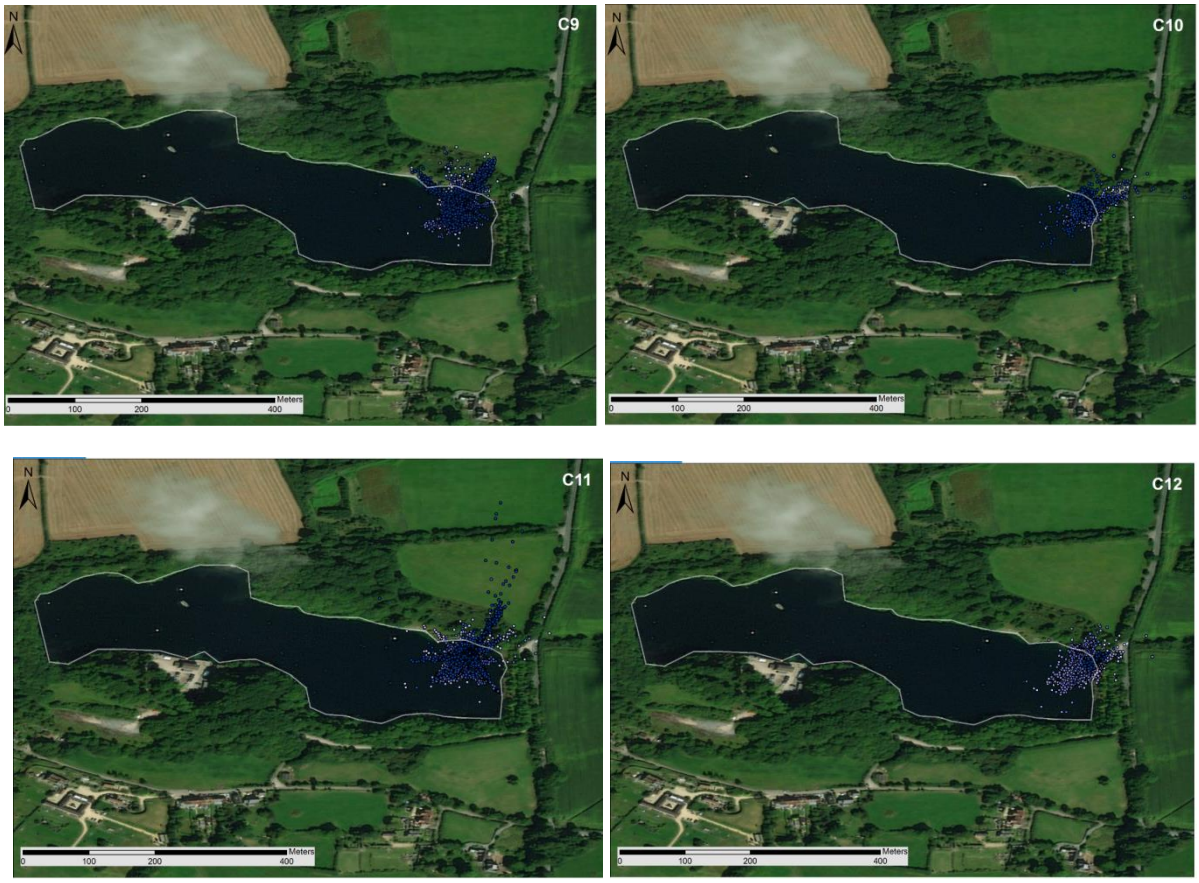

Figure 7.10. Locations of crayfish cray9 - cray12 (C9 - C12) at Vobster Quay Inland Diving, recorded over the entire study period from June 2016 – August 2017. Complete data set shown.

7.3.3. Activity patterns

Daily activity

Crayfish were significantly less active (13.9%) between 09:00-15:00 than at all other times of the day ($F_{7,80}$ = 3.77, p < 0.001). There was no significant difference between any other times within a 24-hour time period, when their activity varied between 25-40% (Figure 7.11.a). Females were on average more active than males at all time periods. Both sexes were significantly less active between 09:00-15:00 ($F_{1,176}$ = 4.89, p < 0.001) (Figure 7.11.b).

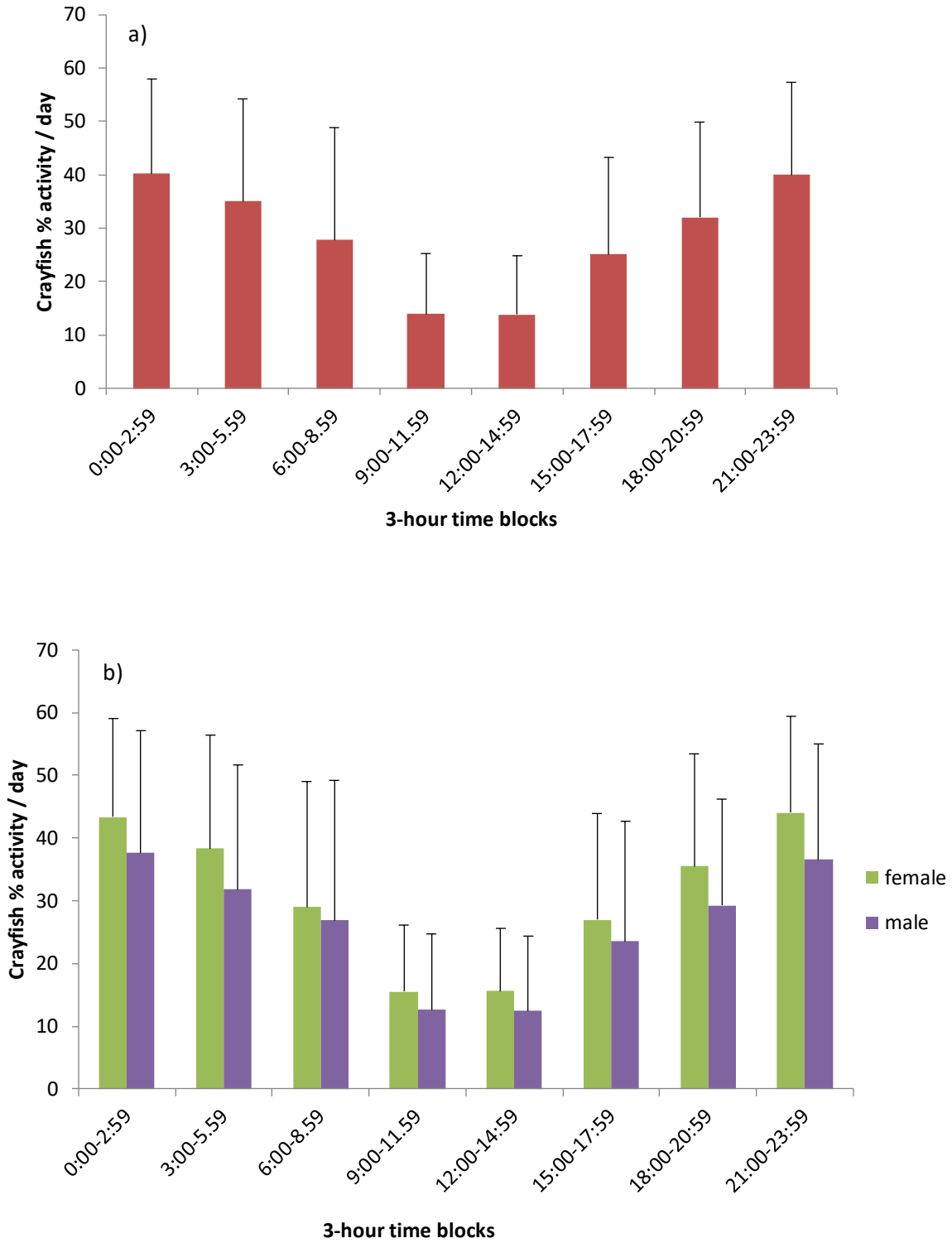

Figure 7.11. Average percentage of time that all 12 crayfish were active during a 24-hour period each season over the duration of the study from June 2016 – August 2017. Each day was split into 3-hour blocks: a) all crayfish together; b) with separate sexes shown. Error bars represent standard deviations.

Seasonal activity

The seasonal activity of the individual crayfish varied. On average (mean ± SD), they were most active in spring (35.5 ± 9.2%) and least active in both summers (26.1 ± 9.8%). Activity in spring was significantly greater than summer1 ($F_{4,355}$ = 2.73, p = 0.02) but not significantly different to any other season. There was no significant difference between summer, autumn and winter activity (Figure 7.12.a). There was a significant interaction between sex and season. Post-hoc Tukey analysis revealed that the activity of males in summer1 and summer 2 was less than the activity of females in spring ($F_{4,350}$ = 2.78, p = 0.03) (Figure 7.12.b).

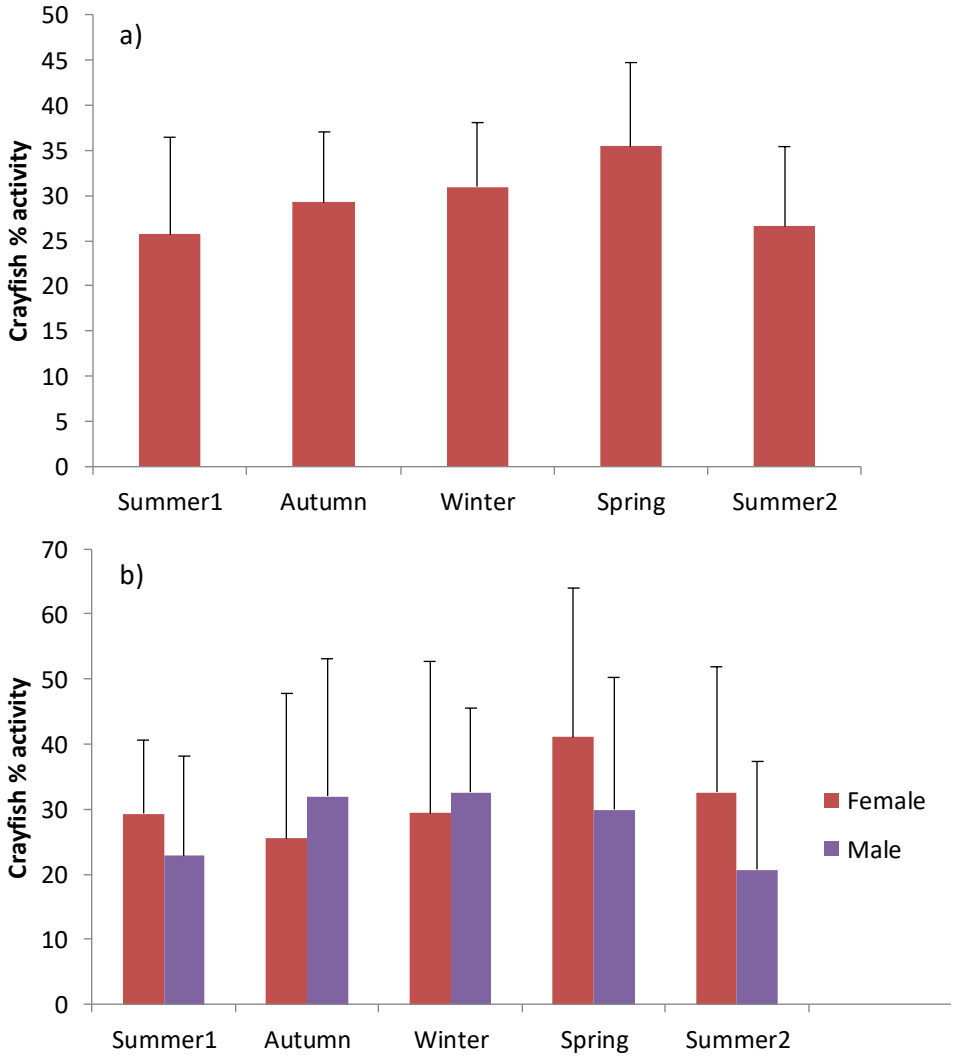

Figure 7.12. Average percentage of time that all 12 crayfish were active during each season over the duration of the study from June 2016 – August 2017; a) all crayfish together; b) with separate sexes shown. Error bars represent standard deviations.

Survivors

Four crayfish, cray6 (male); cray7 (female); cray9 (male) and cray11 (female), were still transmitting data up until the end of the study period, when the batteries within their tags stopped. When their activity patterns were analysed there was no significant difference in average seasonal activity ($F_{3,155}$ = 0.89, p = 0.47). However, there was considerable variation in seasonal activity between the four crayfish, over the entire study period. Three out of four (cray6, 9 and 11) were most active in the autumn and winter months. On average the female cray11 was the most active and the male cray6, the least (Figure 7.13). There was no significant difference between the activity of cray9 and cray11 and cray6 and 7; however, there was a significant difference between the activity rates when cray6 and cray7 were compared to cray9 and cray11 ($F_{3,156}$ = 37.13, p < 0.001).

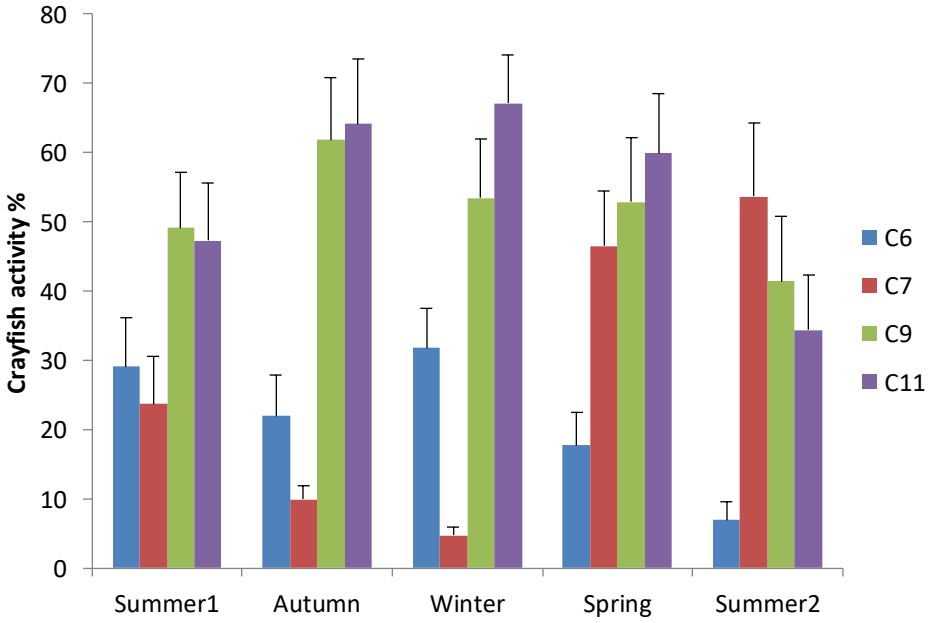

Figure 7.13. Average percentage of time that the remaining four crayfish (cray6, 7, 9 & 11) were active during each season throughout the study from June 2016 – August 2017. Error bars represent standard deviations.

7.4. Discussion

7.4.1. Telemetry tracking

From this study it is clear that *A. pallipes* can be successfully tagged either by attaching tags to their chelipeds or by glueing them on to their dorsal carapace. However, the tag will be lost when the crayfish moults, or if the crayfish loses the cheliped to which the tag is attached. Tags were lost, likely as a result of such events, for example, Cray5 probably died, moulted, or dropped its cheliped soon after the study began as the location data for its tag was only received for 24 days. In some cases, it is evident that there was a period of inactivity at the end of the data transmission probably due to a moult, which lies inactive for a while before being buried with sediment. In many cases, a fair amount of activity was noted right up until the point where the pulses stop, which may imply that the crayfish had been eaten by a predator and removed from the area, or that the animal entered a burrow to die or moult and the tag was not detected again. When the crayfish were being selected for the study, it was difficult to confidently assess whether the crayfish was going to moult. All crayfish selected were fully-grown, adult crayfish, which moult less frequently than smaller individuals. In the case of the crayfish where transmission was lost during summer and autumn, it was quite likely due to a moulting event especially with cray5, which stopped transmitting after less than one month. With cray10, where transmission stopped in late autumn, we can assume that this was not due to moulting as it was during a time when temperatures were too low for moulting (Scalici & Gibertini, 2009b). In this case it is likely that the crayfish was eaten by a predator, lost the cheliped to which the tag was attached, or the tag became detached during courtship activity.

In this study, the telemetry errors were high making spatial data unreliable and therefore impossible to inform us of the precise movements of the crayfish. The hydrophone array would not have picked up the sound waves emitted from the tags if the crayfish had been out of the water. Therefore we know that the crayfish were not moving terrestrially and we know, from the physical presence of the surface marker buoys attached to the hydrophones, where the synctags were located in the quarry. The large and numerous inaccuracies within the data set; i.e. data points of both the crayfish tags and synctags out of water, outside the perimeter of the quarry, could be due to reflected sound. The stone surface of the east side of the flooded quarry would reflect sound more effectively than other substrates causing spatial errors in detection. This may not have been an issue with the north side as it is made up of soft clay. The south side, although rocky, may not be as reflective as the east side (Kessel, 2015). In addition, with 12 crayfish frequently transmitting data in a relatively small area, the sound waves may have overlapped, i.e. tag collision, causing inaccuracies (Binder

et al. 2016), or there may have been issues with unknown factors such as thermoclines interfering with sound wave propagation.

7.4.2. Home ranges

Austropotamobius pallipes may have very small home ranges, and their precise movements and activity patterns may be too small and therefore the potential error in detection too large, to accurately detect with acoustic telemetry in its current form. In another acoustic telemetry study of trout within Lake Huron in North America analysed by Vemco, there was also considerable spatial and temporal variability with the synctag data being transmitted from the hydrophone array. This may have been due to the difference in depths of the hydrophone receivers, or due to the large number of fish present (Binder et al. 2016). Binder et al. (2016) acknowledged that there would be benefit in setting up a pilot study, with tags at known locations, so that the variability of data received could be tested during different times. This would enable the effect of different environmental effects to be assessed such as spawning, temperature changes, aquatic activity, visibility and weather (Binder et al. 2016). What can be seen from my data set is that, during the study period, all 12 crayfish generally stayed around the area where they were released and within the vicinity of the originally release site when the ark site was set up in 2009.

By looking at the data set of the four crayfish (cray9 - 12) released approximately 200 m from their original capture site, it is evident that they remained within their area of release. They did not exhibit homing behaviour and return back to the site where they were originally caught. This is consistent with the study by Robinson et al. (2000), who studied 18 tagged crayfish and tracked them for up to four weeks. They put eight crayfish into different novel locations: four males upstream and two males and two females downstream of where they were originally caught. None of the eight crayfish returned to their original site and showed no site fidelity or homing instinct.

7.4.3. Crayfish activity

Crayfish activity patterns significantly varied throughout each 24-hour period, with crayfish being most active (40%) nocturnally during 21:00-03:00 and least active diurnally between 9:00-15:00 (13%). Activity then steadily increased from 15:00 (25%) up until 21:00 and steadily decreased from 03:00-09:00. This was consistent for all crayfish both males and females. This was the same pattern throughout all four seasons, despite the fact that the timing of dawn and dusk varied throughout the year. Nocturnal activity is expected as it reduces potential predation from other aquatic species that use vision for hunting. The results are supported by a radio telemetry study where 18 *A. pallipes* were tagged and

tracked for up to four weeks during summer. The crayfish were significantly more active at dusk (21:00-00:00) than at dawn (06:00-09:00), morning or afternoon (Robinson et al. 2000). Barbaresi & Gherardi, (2001) carried out active counts of *A. pallipes* for two days in March, May, July, and December, within a 150 km stretch of stream, and found that large crayfish (carapace length > 35 m) were more active at night in spring and at dusk in autumn. Night time activity was highest in spring and autumn and lowest in winter. In the laboratory, *A. pallipes* activity increased with temperature. This nocturnal behaviour has been seen in other crayfish species such as *A. astacus* and *A. leptodactylus* (Lozan, 2000; Musil et al. 2010) and *P. clarkii* (Gherardi et al. 2000). However, laboratory studies have shown that *P. leniusculus* is fairly active diurnally. Musil et al. (2010), found that *O. limosus* was both diurnally and nocturnally active, which was supported in studies by Lozan, (2000) and Buřič et al. (2009b). Significant diurnal activity has also been seen in *A. gouldi* (Webb & Richardson, 2004).

Activity patterns of the *A. pallipes* changed throughout the day and seasonally and were variable between the sexes and crayfish individuals. Females showed more variability in their activity patterns both daily and seasonally and were on average more active than males over each time period of the day and significantly more active than males in springtime when compared to male summer activity levels. This is in contrast to a previous radio-telemetry study where no difference in activity levels between sexes in *A. pallipes* was found (Robinson et al. 2000). In other crayfish species, such as *A. astacus* (Musil et al. 2010), *P. leniusculus* (Bubb et al. 2002) and *O. rusticus* (Byron & Wilson, 2001), there was also no difference in male and female activity. However, in a laboratory study with *O. limosus,* females were more active than males (Musil et al. 2010). Females were, on average more active in spring than in autumn and summer1, (with no difference in activity between autumn and winter). Males were more active in autumn and winter than in summer (with no difference in activity levels between spring, autumn and winter). Spring activity may be relatively high in females due to increased activity after winter foraging in preparation for the eggs to hatch. When the eggs hatch they may leave their refuges more frequently in order to increase the aeration to the youngsters. When the hatchlings leave the females they will need to feed and moult and so will increase their feeding to compensate for the hatching period when feeding was inhibited. In summer, activity may be reduced in both sexes as there is no moulting activity and the hatchlings have left the female. Autumn activity was greater than summer activity probably because autumn is the breeding season when crayfish are looking for mates and then mating and laying eggs. Females are less active than males in autumn and winter. In autumn females may conceal themselves once they have mated and are berried, whereas males will continue to search for females to mate with.

In winter males are only slightly more active than females. In a previous study on *A. pallipes,* no differences in activity were found between sexes. Crayfish were more abundant in summer than in spring, autumn and winter and crayfish activity increased with temperature (Barbaresi & Gherardi, 2001).

The surprising finding was that crayfish were still relatively active during winter months, despite lower temperatures. This suggests that *A. pallipes* are not as dormant and inactive in winter and that they may still forage and move around. This is contrast to findings from a radio-tracking study with 20 *P. leniusculus*, where activity decreased in winter and this was attributed to the decrease in temperature at that time of year (Bubb et al. 2002).

7.5. Conclusions

Acoustic telemetry is possible with *A. pallipes*; however, there were problems using the system to determine spatial movements within a still-water quarry site possibly because it was conducted in too small an area with too many signals or because there was some other unknown interference happening. This technique can potentially provide a more continuous data set than with radio telemetry, which is intermittent over a shorter period of time. Despite the spatial inaccuracies it was evident that the four crayfish that were released 200 m away from their trapped location did not attempt to return and showed no homing behaviour. Assuming that signal loss is related to refuge use, I could infer activity patterns; as predicted, *A. pallipes* is strongly nocturnal but activity patterns vary considerably among individuals, seasons and between the sexes. Future studies should investigate the seasonal variation and activity rates in males and females in more depth and with a larger sample size.

CHAPTER 8

General Discussion

Captive breeding for wild-supplementation can be an effective tool to halt or reverse the decline of threatened species. The aim of this thesis was to explore how wild-supplementation can be applied to the native white-clawed crayfish *Austropotamobius pallipes,* identifying and addressing major knowledge gaps. These gaps fall into two main areas; i) rearing of sufficient number of animals successfully and ii) survival of those animals in the wild. The work focuses primarily on different aquaculture techniques, within an *ex-situ*, small-scale, closed-circuit aquaculture facility, to maximise production of captive-born *A. pallipes* for successful wild release. The research was based on five years of previous pilot studies and captive-breeding trials that came from the formation of the South West Crayfish Partnership (SWCP), which established *A. pallipes* captive-breeding as one of the key elements of its conservation strategy (Nightingale et al. 2019).

One of the key aims of my thesis was to determine baselines for key husbandry elements that need to be understood for successful *A. pallipes* aquaculture. In the first part of my thesis, I investigated optimising aquaculture techniques for *A. pallipes* by exploring density, grading and feeding regimes in order to maximise the amount of healthy robust crayfish produced. Prior to my studies, there was a fairly limited amount of research available on *A. pallipes* aquaculture and considerable variability in the literature for other astacid crayfish species. The first aquaculture element that I investigated was density. There were no known studies on optimal density regimes for *A. pallipes*; studies exploring feeding and refuge preference in juvenile *A. pallipes* used very variable densities for example between 500/m^2 (Policar et al. 2010) and 50/m^2 (Sáez-Royuela et al. 2001). Density studies for other astacid species show considerable variability in the accepted density for juvenile rearing (Savolainen et al. 2004; Harlioğlu, 2009; González et al. 2010). I proved that *A. pallipes* hatchlings can be reared at relatively high densities of 300/m^2 without growth and survival being compromised. However, it was evident from my density study, that there was a significant size variation in crayfish brood-stock groups reared under the same conditions and considerable interspecific growth differences.

Therefore the findings from my density study led on to my second aquaculture element, exploring size-grading in juveniles and rearing the crayfish in single-sex, size-graded groups to ascertain if this would be a more effective way of rearing young-of-the-year *A. pallipes.* There are no known published studies that have found interspecific sex differences in dominance hierarchies or growth within juvenile, young-of-the-year crayfish and no known studies investigating juvenile sexual dimorphism and social dominance within *A. pallipes* or other astacid species. Studies with the more aggressive, cambarid crayfish species *P. clarkii,* showed that dominance hierarchies formed in juveniles (Herberholz et al. 2000; Issa et al. 2000; Sato et al. 2012). However, there were discrepancies in studies exploring dominance hierarchies in adult *P. leniusculus*. In two studies the adult females were dominant over males (Momot & Leering, 1986; Sippel et al. 1995), whereas Nakata & Goshima, (2003) found the converse. Studies with adult cambarid crayfish were consistent in that males were dominant over females (Rodgers et al. 2006; Wang et al. 2014). What was very surprising and evident from my studies was that sexual dimorphism begins prior to sexual maturity, as early as six-months of age, and that males are more aggressive and dominant than the females, even as juveniles.

The third aquaculture element of my studies investigated, different dietary regimes for rearing hatchling and juvenile crayfish. I wanted to establish if there were alternatives to expensive and time-consuming live *Artemia* nauplii food production for hatchlings such as utilising decapsulated *Artemia* eggs or crayfish-specific pellet feeds. I was curious whether coating feeds in agar gel may help preserve the food for longer, which I thought may be important for an intermittent feeder. Feeding studies with *A. pallipes* were limited and this species had not been tested with crustacean-specific pellet feeds, plankton or gel diets. Studies with other astacid species suggested that crustacean-specific pellet diets can provide high survival rates even during the critical first few weeks, post-hatching (González et al. 2009). In contrast, my studies indicate that the best hatchling survival and growth is achieved with a live food and plankton diet, whilst for juvenile *A. pallipes* a plankton plus vegetable diet is beneficial. Juvenile growth and survival were greater than with any of the other diets trialled.

The aquaculture findings from my studies have had important implications for how we run crayfish hatcheries, and we have now altered our regimes at Bristol Zoo Gardens to maximise production. Space is at high premium in a small-scale hatchery facility and therefore it was imperative to understand how many crayfish could be reared together and at what point they would need to be thinned out before aggression from tank-mates became an issue. We now confidently rear our hatchlings at relatively high densities; however, we have altered our regime and sort the juveniles at six-months of age. The animals are put into

single-sex, size-graded groups; once the individuals can be reliably sexed and are more robust, after the early critical life stages, post-hatching. We have also improved our feeding regime. We continue to produce live *Artemia* nauplii for the hatchling crayfish but have also removed the pellet component of the diet, in later age-classes, focussing on a larger variety of enriched plankton and vegetable-based diets for all crayfish housed. Within the Bristol Zoo crayfish hatchery this has increased both survival rate and growth of all age-cohorts held. We can house more brood-stock and produce larger numbers of robust *A. pallipes* for wild release. This in turn has meant that captive-born females are now at a size when they can breed in the second year of life and approximately 70% of the 16-month females are mating and producing viable eggs. This is in contrast to wild populations where females mature at between 3-4 years of age (Reynolds, 2002).

The aim of the second part of my thesis was to evaluate what happens once the captive-bred crayfish are released. I wanted to explore the reintroduction process of crayfish conservation in a little more detail; does captive-breeding for release really work? One of the most effective ways to evaluate reintroduction success is to individually permanently tag animals, prior to release, so that they can be tracked long-term. This helps to inform us as to how quickly a population recruits and grows and how the species colonises and utilises available habitats. Being able to monitor released crayfish individuals has great potential in terms of assessing population structure and viability long-term. By assessing how *A. pallipes* faired over an entire year when injected with passive integrated transponder (PIT)-tags *ex-situ*, would help to justify if this species could be safely tagged, prior to release. There were again limited studies available. No long-term tagging studies existed for *A. pallipes,* which is a less-robust, more delicate species than many other crayfish species. There was also no literature available on the minimum size that *A. pallipes* could be safely tagged. In other astacid crayfish species, there were no long-term studies on the effects of PIT-tagging on survival and growth; the longest study was for 6-months with *P. leniusculus*, (Bubb et al. 2002). Studies on *P. lenisculus* suggest that the safe minimum size for tagging is 30 mm carapace length (Bubb et al. 2002). This was supported by a study with *O. compressus*, which found that there was significant mortality when tagging with 8 mm tags at a carapace below 23 mm (Black et al. 2010). My study demonstrated that sub-adult *A. pallipes* could be tagged, i.e. at a carapace length of 21 mm or above, without survival or growth being compromised. In the second part of the chapter, I explored the detection range of *A. pallipes* once tagged; there was no literature available on this element. When I investigated detection rates I found that even when larger tags (12 mm) were used, the crayfish were not easy to detect, when hidden within refuges such as bricks or under slate. It was apparent from my studies and from other practitioners (Stead, 2015), that detecting

PIT-tagged crayfish remotely, *in-situ*, using antennae was not a reliable tracking method and that animals needed to be recaptured again in order for them to be easily identified.

The PIT-tagging research has been pivotal to the wild release element of our crayfish conservation programme and how we manage and structure our releases and in turn how we structure the hatchery. It has meant that we can now confidently tag sub-adult crayfish, prior to release. Since this study, we have released over 700 PIT-tagged crayfish, into ark sites, which are now part of long-term monitoring programmes. The follow-on effect is that we also keep the crayfish in captivity for longer, until they have reached a taggable size. We have continued with hatchery trials and can now safely tag *A. pallipes* at a minimum size of 18 mm carapace length. Consequently, this means that they are now slightly larger than the size we previously released them at and subsequently they are less prone to predation.

Prior to this research we were releasing untagged young-of-the-year crayfish in the following spring after they hatched and at a size of < 18 mm. By keeping them until they are at a size large enough to be tagged, it means that they are released in two batches. The first release takes place with the larger young-of-the-year crayfish in spring and the second batch of one-year-olds are released in the autumn, just prior to the breeding season (late October). By releasing a second cohort, later in the year, the crayfish should still be in relatively close proximity to one another as the breeding season approaches. Therefore, they should be able to locate each other more easily as they will have had less time to disperse throughout the river or pond site. This new phased release model, which we have implemented at the Bristol Zoo crayfish hatchery, is proving very effective.

The fact that we could not reliably track crayfish using PIT tags and antennae led me on to the final investigation where I looked at trying to track crayfish remotely within an ark site using passive acoustic telemetry. Passive acoustic telemetry has the advantage over radio telemetry that has been used previously on this species (Bubb, 2002; Robinson et al. 2009) in that activity can be monitored continuously over an extended time period. There were also no previous studies using acoustic telemetry on his species; radio tracking and PIT-tagging only provide snapshots into their activity and behaviour. My telemetry study is the first continual, long-term activity data set for *A. pallipes* and has provided a useful insight into how they utilise an ark site. Tagging of the crayfish with acoustic telemetry tags proved successful, and four of the 12 crayfish were still transmitting data at the end of the year. However, there was a major issue with the system in the freshwater quarry and the location data was unreliable possibly due to reflective issues. I could determine that *A. pallipes* does not exhibit homing behaviours, and that they did not range very far within the release site

during the year. Monitoring activity proved more successful: I illustrated that *A. pallipes* are significantly nocturnal, and that they remain active over the entire year, despite lower temperatures in winter, and there are both seasonal and individual variations in activity levels.

My research has effectively changed our rearing and release programme methodology at Bristol Zoo Gardens and we have adopted a new model, which is proving very effective. We now release older, tagged crayfish into our ark sites, which in turn allows for long-term monitoring of the individuals. This has enabled us to assess survival and recruitment and recapture rate both within still-water and river sites. Small-sized, young-of-the-year crayfish are extremely vulnerable to predation, especially in still-water ark sites, which can be prolific breeding grounds for amphibians and predatory aquatic invertebrates such as Odonata larvae (Gydemo et al. 1990). By PIT-tagging all captive-born crayfish, prior to release, we ensure that they are of a larger-size prior to release, allowing us to closely monitor populations long-term. This should in turn provide a more in-depth knowledge of crayfish survival and fitness at ark sites longer-term. The acoustic telemetry study has also influenced our crayfish release strategy: we understand that during wild releases it is important to carefully select the release sites where there is optimal habitat and to release large enough groups together as the crayfish may not disperse far.

We understand that the crayfish show remarkable plasticity in response to the changing climate and there are reports of *A. pallipes* living within acidic tarns within Northumberland (I. Marshall pers. comm.) and changing its chelaphorax structure within a few moults when moved from a lotic to lentic site (Haddaway et al. 2014). We also know of a few populations of *A. pallipes* within Europe that are now resistant to crayfish plague (Martin-Torrijos et al. 2017). Therefore, with the advances in our knowledge and success of captive-breeding of this species, we may be able to select and breed for certain characteristics, to ensure its long-term survival and this may be an important element of our aquaculture studies in the future.

Natural populations of *A. pallipes* will not be saved from decline, and potential extinction, until invasive crayfish species (and their associated diseases) are controlled. Invasive crayfish species control is a fundamental part of *A. pallipes* conservation. Pilot control studies such as male sterilisation, genetic manipulation, development of a crustacean-specific bait-matrix, electrofishing and the introduction of predatory fish may all help to reduce the spread of invasion (Manfrin et al. 2019) and further research into this control work is a fundamental part of the *A. pallipes* conservation strategy. What is of paramount importance is that we preserve and safeguard the highly threatened *A. pallipes* populations,

whilst trying to control the invasive crayfish issues. Advances in *A. pallipes* aquaculture has made the use of captive-breeding for supplementation a realistic method to halt the decline of this species, especially where local populations are now too small to make wild harvesting a realistic option. Closed-circuit hatcheries have the added advantage of being able to produce plague-free, bio-secure captive born crayfish for release. Such individuals have been seen to be more adaptable and less likely to migrate from novel environments than their wild-caught translocated counterparts, leading to more successful ark site establishment and reduced emigration (Kozak et al. 2011).

Prior to my study, *A. pallipes* had a reputation for being notoriously difficult to breed and rear and several historic laboratory trials had been masked by other husbandry issues (Gonzalez et al. 2011). Having worked on the captive-breeding of *A. pallipes* for over ten years, many of the key aquaculture issues have been resolved and we have a much better understanding of how to effectively produce good quality numbers of robust crayfish for release. We can now achieve a > 80% survival rate from hatching through to release at high density. One of the overarching drivers for this research was so that it could culminate in producing an *A. pallipes* captive-breeding manual for other practitioners, which is now in progress. Establishing additional *A. pallipes* hatcheries is a realistic solution to helping safeguard this species whilst river catchment restoration work can progress. What is evident from our conservation efforts for this elusive, keystone species, is that the success of these programmes relies on a multi-faceted, coordinated, strategic, landscape-style approach. Although several important findings have been made during the course of this thesis there still remains much investigation to be done, (e.g. brood-stock diets; breeding regimes, plague resistance and refuge preference), to ensure that we optimise *A. pallipes* productivity and our long-term conservation strategy for this species. We are fortunate that there is an extensive international network of passionate crayfish-focussed conservationists that are working together, to try and safeguard this species so that future generations can witness and enjoy *A. pallipes* in our British rivers.

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Appendix I

International Zoo Yearbook

50 REINTRODUCTIONS AND OTHER CONSERVATION TRANSLOCATIONS

Int. Zoo Yb. (2017) **51:** 50–68
DOI:10.1111/izy.12161

A review of the use of ark sites and associated conservation measures to secure the long-term survival of White-clawed crayfish *Austropotamobius pallipes* in the United Kingdom and Ireland

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In response to the global decline of the White-clawed crayfish *Austropotamobius pallipes*, key conservation strategies have been developed in the United Kingdom and Ireland, including the supplementation of existing populations and establishment of new populations, using captive-breeding methods and/or translocations. The South West Crayfish Partnership (SWCP), a group of UK-based conservation organizations, oversees population-enhancement programmes in south-west England. Since 2006 the SWCP has established 16 ark sites (safe refuges) and conducted one river supplementation. In total, 17 sites have been stocked with over 5000 translocated and captive-hatched *A. pallipes*, increasing the number of discrete *in situ* populations in the region by at least 75%. A similar programme in southern Wales, led by Natural Resources Wales, has restocked three river catchments and one English still-water site with a total of over 4700 captive-reared juvenile *A. pallipes*. Although many of these ark sites are newly established, preliminary monitoring results are encouraging; at least 75% of ark sites in south-west England are currently viable and the three Welsh sites that have been monitored so far suggest continued presence of White-clawed crayfish.

Key-words: ark site; captive breeding; captive rearing; introduction; non-native species; reintroduction; translocation; white-clawed crayfish.

INTRODUCTION

The White-clawed crayfish *Austropotamobius pallipes* (Lereboullet 1858) is classified as Endangered by the International Union for Conservation of Nature (IUCN) Red List (IUCN, 2016), is listed under Annexes II and V of the EU Habitats Directive (Council Directive, 1992) and is protected by UK legislation in the form of the Wildlife and Countryside Act 1981. In the last 10 years this species is suspected to have undergone a global decline of between 50% and 80% as a result of the spread of the American Signal crayfish *Pacifastacus leniusculus* (Dana 1852), the Red swamp crayfish *Procambarus clarkii* (Girard 1852), the associated crayfish plague [caused by the oomycete water mould *Aphanomyces astaci* (Schikora 1906)], habitat degradation and pollution events (Füreder *et al.*, 2010). *Austropotamobius pallipes* is the only species of crayfish native to the UK, is one of

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the largest indigenous freshwater invertebrates in the country and is a keystone species in aquatic habitats (Matthews *et al.*, 1993) (Plate 1). In south-west England, *A. pallipes* populations have experienced a dramatic 70% decline since the 1970s (Sibley *et al.*, 2011). In Wales a few remnant populations of the White-clawed crayfish remain but these are restricted to streams in the south-east and a small number of streams in the Wye catchment within the county of Powys. Even the River Wye, designated as a Special Area of Conservation under the Habitats Directive because of the presence of *A. pallipes* (i.e. an 'Annex II species that is a primary reason for selection of this site'), was found to be in 'Unfavourable Condition' (Dyson, 2008). Furthermore, the results of surveys conducted in 2014/2015 suggest populations of White-clawed crayfish have declined further (Howells & Slater, 2004; Rogers & Watson, 2016).

Several historical records exist that provide evidence that *A. pallipes* was present in England in the 1400s and historical references refer to crayfish being eaten in monasteries during the 10th century (Holdich *et al.*, 2009). No specific information exists for Wales prior to the 1500s, although monastic establishments there are also believed to have been associated with *A. pallipes* (Holdich *et al.*, 2009). In Scotland there are no native crayfish species, although there have been at least two *A. pallipes* introductions (Jay & Holdich, 1981; Maitland *et al.*, 2001; Holdich *et al.*, 2009; Kouba *et al.*, 2014). In Northern Ireland and the Republic of Ireland, the first *A. pallipes* populations may have been introduced possibly from western France or England. Ireland does not have any known non-native crayfish species present and, therefore, water bodies there are considered natural ark sites for *A. pallipes* (Reynolds, 1998). Ark sites are discrete water bodies with optimal water quality that should remain free from invasion by non-native species for the foreseeable future (P. Bradley, pers. comm.).

Within Europe there are five indigenous crayfish species all of which are under threat from non-native crayfish species, disease and habitat fragmentation (Holdich *et al.*, 2009). Translocations, reintroductions and introductions are widely used methods to enhance existing populations and to create new safe native populations in England, Wales, Northern Ireland and the Republic of Ireland (Souty-Grosset & Reynolds, 2009). Crayfish translocations involve moving part of a wild population to a safe area. Reintroductions (i.e. to sites known to have previously supported native crayfish) and introductions (i.e. to sites not known to have previously supported native crayfish) usually entail the production of captive-reared or bred juveniles (collectively referred to as 'captive hatched') for release; all of these methods are widely used in mainland Europe (Schulz *et al.*, 2002). In the absence of fail-proof options to prevent the introduction and spread of non-native crayfish species and diseases, measures to safeguard and expand the current distribution provide the most feasible hope for securing the survival of *A. pallipes* in the UK and Ireland. Over the last decade one such measure, involving the translocation of White-clawed crayfish from threatened donor sites to low-risk refuge or ark sites, has been employed at multiple locations across the UK and Ireland with some success.

Plate 1. White-clawed crayfish *Austropotamobius pallipes. Kenrina Maidment (all rights reserved).*

Captive rearing (hatching and rearing juveniles from females mated in the wild) and captive breeding (hatching and rearing juveniles from females mated in captivity) of *A. pallipes* are becoming valuable resources for supplementation of wild populations (i.e. the addition of individuals to an existing population) and establishment of ark sites, both within the UK and Ireland, and in mainland Europe. In some countries, such as Finland and Sweden, wild harvesting is not allowed because of major population declines; therefore, crayfish aquaculture is the only method available for reintroduction and introductions. In Spain, wild populations of native crayfish are now at such low levels that some controlled wild harvesting does occur but not for restocking purposes (Souty-Grosset & Reynolds, 2009). The same situation exists for several areas of southern England and Wales, where *A. pallipes* populations have either become locally extinct, or have been reduced to very low numbers or a few isolated populations (Sibley, 2003). In these cases, captive breeding/rearing of crayfish is an effective tool for the preservation of these populations, and an essential part of a long-term conservation-management programme.

Austropotamobius pallipes is a K-selected species, being a slow-growing invertebrate with late maturation at around 3–4 years of age, and only one breeding cycle per year with relatively few young produced (Reynolds *et al.*, 1992). In the wild it is estimated that fewer than 10% of a crayfish brood will survive to reproduction (Ulikowski, 1996; Ulikowski *et al.*, 2006; Neveu, 2007). In contrast, in captivity up to 90% of a brood can be successfully reared to sexual maturity, and crayfish can mature as early as 16 months of age and typically by their second year (Policar *et al.*, 2010; J. Nightingale, pers. obs, 2015). By bringing ovigerous (egg-laden) females into captivity in spring for hatching and subsequent rearing of juveniles, survival can be greatly increased and thus large numbers of plague-free crayfish progeny can be produced to supplement wild populations or to establish ark sites. If wild-caught females and captive-hatched juveniles are retained in hatcheries for breeding this will reduce the need to remove more crayfish from sensitive populations outside the recommended survey season. When wild populations have declined to very low numbers this conservation strategy has many advantages. By establishing a population of unrelated brood stock, genetic diversity is preserved and maintained in successive generations. A key element for successful crayfish aquaculture is to ensure that the donor population is appropriate and robust, maintaining as much genetic diversity as possible in the brood stock. All females are collected under the appropriate licence, because the species is protected by law.

In an attempt to safeguard *A. pallipes* within south-west England, a partnership of organizations was formed – The South West Crayfish Partnership (SWCP) – and a strategic landscape-scale conservation project was developed. The project comprises four main strands: (1) establishing ark sites (safe refuges) to maintain threatened populations and genetic integrity for both wild-caught translocations and captive-hatched introductions of *A. pallipes*; (2) developing and maintaining a captive-breeding programme at Bristol Zoo Gardens, UK, to provide plague-free *A. pallipes* for wild release and brood stock for other organizations; (3) creating an outreach programme to target key audiences, including waterway users, restaurants, students, schoolchildren, and zoo and aquarium visitors; (4) developing various techniques to control non-native crayfish species (see Nightingale *et al.*, 2009; Robbins *et al.*, 2013). In Wales, a similar initiative was undertaken by The South East Wales Crayfish Project, primarily looking to identify potential donor and ark sites (Smith *et al.*, 2009). The study, carried out by Cardiff University, highlighted the need to investigate the feasibility of developing a captive-rearing/breeding programme to provide donor stock for ark

sites. This same period saw the inception of the Irfon Special Area of Conservation (ISAC) Project, funded by the EU Life+ programme, and led by the Wye and Usk Foundation (WUF). A proportion of this project focused on the conservation of *A. pallipes* in the Irfon catchment, a large tributary of the River Wye. This work included targeted survey efforts, habitat management and enhancement, assessment of donor and receptor sites, and captive rearing at the Cynrig hatchery of National Resources Wales (Wye and Usk Foundation, 2014).

The work reported here reviews key processes by which *A. pallipes* conservation is being delivered in the UK. Specifically, to provide a preliminary assessment of success and/or effectiveness, we examine: (1) captive-rearing and breeding programmes being delivered in south-west England and in Wales, (2) the process and delivery of supplementation of wild populations and ark-site establishment in the UK, and (3) ark-site establishment and river supplementation by the SWCP and Natural Resources Wales (formerly through Environment Agency Wales) over the past decade.

BREEDING AND CAPTIVE REARING OF WHITE-CLAWED CRAYFISH

In 2009, in response to local *A. pallipes* population declines, crayfish hatcheries were established at Bristol Zoo Gardens and at the Cynrig Hatchery of Natural Resources Wales, with a key objective of enhancing local White-clawed crayfish populations by captive rearing and breeding for wild supplementations and ark-site establishment.

Bristol Zoo Gardens hatchery

The hatchery at Bristol Zoo Gardens has three fully closed circuits; discrete systems that function as separate biosecure units allowing brood stock from different wild-caught populations to be held separately and simultaneously. The largest system is an outdoor 3000 litre recirculating arrangement, containing 13 polyurethane holding tanks (each with a bottom area of 0·46 m^2). There are also two recirculating 1000 litre indoor systems, each containing tiered units of nine lidded glass tanks (each with a bottom area of 0·29 m^2). All three systems have reservoir sumps where the water is mechanically and biologically filtered, and treated with ultraviolet radiation. Temperature is controlled using water chillers and heaters to ensure a constant thermal regime that will vary seasonally between 5 and 20°C to mimic local *in situ* water temperatures. A 25% weekly water change is performed on each system with treated mains water (for further details see Nightingale & Rudd, 2011; Nightingale, 2012).

Bristol Zoo Gardens brings in wild-caught ovigerous females and also retains brood stock for closed-cycle captive breeding of both wild-caught donor stock and captive-hatched individuals. Females mate in autumn and hold their eggs over winter before they hatch the following spring. To date the Bristol Zoo hatchery has produced over 4000 crayfish for ark-site establishment, wild supplementations, hatchery brood stock, and to create crayfish collections at other British and Irish Association of Zoos and Aquariums (BIAZA) institutions. Regular health screening of a proportion of the population selected for any release is essential to safeguard the potential introduction and prevent the spread of diseases not previously found in the receiving water body. On completion of maternal brooding the majority of the females are returned to their point of capture.

Cynrig hatchery

Work at the Cynrig hatchery started in 2008/2009 and, at the time of writing, runs three recirculation systems, with a total volume of 5300 litres, and a culture area of 13·7 m^2. Each system is discrete to allow rearing of crayfish from different rivers or catchments while limiting biosecurity risks. Each system is borehole-fed and isolated

from the rest of the fish farm and ambient river water to reduce further the chance of unintended transmission of disease or pathogens. Ultraviolet and biological filtration maintains water quality while incorporation of a heater/chiller (with backup capability) maintains water temperature at 11–13°C. Less than 5% of system volume is exchanged daily with borehole-water top up. Two systems consist of standard 1–1·5 m glass-reinforced plastic (grp) tanks (Wye and Usk Foundation, 2014), while the most recently constructed system uses 2 m grp Canadian-style troughs. To date, the troughs have proven to be easier to maintain and also allow a greater proportion of the available space to be used than with standard circular tanks.

Up to three donor populations may be targeted in each particular year, depending on the location of receptor sites. At the Cynrig hatchery, wild-caught ovigerous females are brought in during the spring for egg hatching and rearing of juveniles. On completion of maternal brooding the females are returned to their point of capture. The young crayfish are released at 10–11 months of age, prior to the next batch of ovigerous females being collected the following spring (for further details see Brown, 2012; Wye and Usk Foundation, 2014). More recently juveniles have also been retained and grown-on for captive breeding. To date Cynrig hatchery has produced over 4700 juveniles, which have been released predominantly into Welsh rivers.

THE SELECTION AND ESTABLISHMENT OF ARK SITES IN THE UK

In mainland Europe, reintroductions for *A. pallipes* and the Noble crayfish *Astacus astacus* (Linnaeus 1758) have taken place for several decades in Austria, the Czech Republic, France, Italy, Poland, Portugal, Russia, Spain, Sweden and Switzerland. There has been an extensive restocking programme of *A. astacus* (in Finland, Sweden and Norway) where populations have been wiped out previously by crayfish plague, and where there is no evidence of crayfish species being present (Westman, 1992; Taugbøl & Skurdal, 1993). However, these programmes have a commercial value as the populations are maintained for harvesting, usually with strict controls over the size limit allowed to be taken (Westman *et al.*, 1990). In the UK, *A. pallipes* is not harvested for the food industry and, therefore, all reintroductions, introductions and supplementations are solely for conservation purposes.

The stocking of still-water sites and rivers with *A. pallipes* within the UK has occurred since at least the 18th century (Foster, 1998). In Wales the earliest recorded introductions were into the River Irfon in the 1800s (Jones, 1805; Slater, 2002). In northern Scotland the first *A. pallipes* population was introduced in the early 20th century to Loch Croispol, Sutherland, in the Durness region. More recently a second introduction occurred into the Whitemoss Reservoir in Renfrewshire (Maitland *et al.*, 2001).

It is only since the 1980s that *A. pallipes* introductions, reintroductions and supplementations within the UK and Ireland have been carried out specifically as a conservation measure to combat the decline of this species following the introduction of non-native *P. leniusculus* to British waterways. The majority of these conservation actions have been translocations of individuals from threatened wild populations into isolated ark sites. In UK, ark sites can be either lentic (still-water) or lotic (river) sites; however, if using river systems, hydrological connectivity to non-native crayfish species must be considered and assessed for risk. In south-west England, nearly all river catchments are compromised with non-native crayfish species, primarily *P. leniusculus* (Sibley *et al.*, 2002). Therefore, where river sites are used as ark sites, a natural or artificial barrier, such as a weir or culvert, should exist or be put in place to limit upstream invasion of non-native crayfish species.

Discrete, isolated still-water locations, such as disused aggregate and mineral-extraction sites, can provide ideal, long-term safe refuges. Extraction operations often produce permanent water-filled sites that are suitable for *A. pallipes* with no further modification required. These disused quarries provide not only effective crayfish ark sites but also are nature reserves for other species (Whitehouse *et al.*, 2009). When attempting to establish ark sites, an assessment of environmental quality and current crayfish status, including information about non-native species, should be supplemented with historic records and anecdotal reports. These layers of evidence make it possible to build a more complete understanding of the level of threat to any extant *A. pallipes* populations in a given catchment area. Simultaneously, this evidence can be used to identify potentially suitable ark sites, perhaps in discrete catchments, remote headwaters or isolated still waters. Once it has been agreed that a surviving or threatened *A. pallipes* population would benefit from a translocation attempt, the screening process progresses to a more detailed level of investigation. Potential sites (with agreeable landowners) must be subjected to a process of feasibility assessment. This helps to determine the likely impact of the addition of *A. pallipes* on the ecology of the proposed site as well as the suitability of the site for the threatened species. This information will be required by the regulatory authorities prior to the approval of any licence for a translocation (Robbins, 2011). Experience has shown that the establishment of self-sustaining crayfish populations at new ark sites can benefit from the creation or improvement of habitat features. Artificial refuges, in the form of clay pipes, rocks or stick bundles, can markedly improve shelter options for newly introduced crayfish as well as diversifying the habitat for a range of other species, including potential prey items, such as snails, insect larvae or small fish. Crayfish are omnivorous, and will eat plants, live animals, carcasses and other crayfish. Once a site has been assessed, and provided that all the steps and licences are in place for a translocation attempt to proceed, protocols for the removal of crayfish from the donor site, and their transport and introduction to the ark site can be employed. Local stakeholders should be involved in all aspects of the project, including agreeing the actions and objectives, to ensure its long-term success (Taugbøl & Peay, 2004). The short-term measure of success for any newly established ark site will be to determine whether breeding has taken place over the first few years following translocation.

RIVER SUPPLEMENTATION AND ARK-SITE ESTABLISHMENT

River supplementations typically take place upstream of naturally occurring *A. pallipes* populations. In northern parts of England, such as Yorkshire, where strongholds of *A. pallipes* populations still exist without non-native crayfish in the river catchments, reintroducing crayfish into headwaters where populations exist downstream can be an effective conservation strategy (Kemp *et al.*, 2003). River supplementations can also be carried out within a river catchment where there are non-native crayfish species; however, the non-native species must always be downstream of the supplementation site, usually separated by a natural or artificial barrier to prevent upstream migration.

In the Republic of Ireland, Northern Ireland and England, the earliest records of *A. pallipes* introductions for conservation are from the 1980s (Table 1). In south-west England, one of the first translocations to a specific ark site was undertaken in 2006, where *A. pallipes* were translocated from a Bristol river population under threat following invasion by *P. leniusclus,* to a Somerset river that was free from non-native species (Table 2; Plate 2). The group that carried out this translocation went on to form the SWCP (Sibley *et al.*, 2007). Their first experiences, beginning with the identification of potential ark sites, provided

COUNTRY/COUNTY	STOCKING YEAR(S)	TYPE OF 'ARK'/UPSTREAM SUPPLEMENTATION	NUMBER STOCKED	YEAR LAST SEEN	REFERENCES
SCOTLAND					
Sutherland	20th century	lentic: Croispol Loch	N/A	2010	Maitland *et al.* (2001), Gladman (2012)
Renfrewshire	*c.* 30 years	lentic: Whitemoss Reservoir	N/A	2010	Maitland *et al.* (2001), Gladman (2012)
IRELAND					
County Clare	1980s	lentic: from Borrisokane, Tipperary	N/A	N/A	Reynolds *et al.* (2002)
County Westmeath	1989/1991	lentic: Lough Lene, Westmeath	110	1996	Reynolds *et al.* (2002)
County Westmeath	1999–2001	lentic: White Lake, Westmeath	450	2001	Reynolds *et al.* (2002)
County Tyrone	2006	lentic: Evishanoran Lake*, from Ballinderry River	150	2010	Horton (2009)
WALES					
Powys	1987/1988	lentic: pond	40	N/A	Slater *et al.* (1992), Slater (2013)
Powys	1987/1988	lentic: upland lake	100	N/A	Slater *et al.* (1992), Slater (2013)
Powys	1987/1988	lentic: pond	60	N/A	Slater *et al.* (1992), Slater (2013)
Powys	1987/1988	lentic: pond	20	N/A	Slater *et al.* (1992), Slater (2013)
Powys	1987/1988	lentic: pond	55	N/A	Slater *et al.* (1992), Slater (2013)
Powys	1987/1988	lentic: pond	30	N/A	Slater *et al.* (1992), Slater (2013)
Powys	1987/1988	lotic: Wye tributary	60	N/A	Slater *et al.* (1992), Slater (2013)
Powys	1987/1988	lotic: Wye tributary	65	N/A	Slater *et al.* (1992), Slater (2013)
Powys	1987/1988	lotic: Usk tributary	45	N/A	Slater *et al.* (1992), Slater (2013)
Powys	1989	lotic: Wye tributary	100	1999	Slater *et al.* (1992), Slater (2013)
Merthyr Tydfil	1989	lotic: Taff	100	2004 (at Llwyn-on)	Slater *et al.* (1992), Slater (2013)
Rhondda Cynon Taff	1989	lotic: Neath	100	N/A	Slater *et al.* (1992), Slater (2013)
Powys	1989	lotic: Neath	100	N/A	Slater *et al.* (1992), Slater, (2013)
Neath Port Talbot	1989	lotic: Neath	100	N/A	Slater *et al.* (1992), Slater (2013)
ENGLAND					
Wiltshire	1982/1986 & 1994	lotic: Sherston Avon, from Sherston tributary	363	2007 (plague)	Spink & Frayling (2000)
Wiltshire	1987	lotic: Sherston Tetbury, from Mells River	276	2007 (plague)	Spink & Frayling (2000)
Derbyshire	2000–2009	lotic: River Lathkill, from Bestwood ponds	2500	2010 (plague)	Rogers & Watson (2011)
Derbyshire	2003	lotic: Carsington Water, from Nanpantan	450	N/A	LDBP (2007)
Derbyshire	unk	lentic: Linacre Reservoirs, Holmebrook Country Park	N/A	N/A	LDBP (2011)
Somerset	2006	lotic: Butcombe	250	2016	Sibley *et al.* (2007)
Yorkshire	2006	lentic: Roundhay Park, Leeds, upper lake: habitat improvements to increase natural range: from local becks	N/A	N/A	I. Marshall, pers. comm.

Table 1. (continued)

COUNTRY/COUNTY	STOCKING YEAR(S)	TYPE OF 'ARK'/UPSTREAM SUPPLEMENTATION	NUMBER STOCKED	YEAR LAST SEEN	REFERENCES
Yorkshire	2006	lotic: Meanwood Beck, Leeds: pollution control to increase natural range	N/A	N/A	I. Marshall, pers. comm.
Yorkshire	unk	lotic: Wyke Beck, Leeds – habitat restoration to increase natural range.	N/A	N/A	I. Marshall, pers. comm.
Yorkshire	unk	lotic: Pudsey Beck, Leeds reintroduction	N/A	N/A	I. Marshall, pers. comm.
North Yorkshire	unk	lentic: old quarry	N/A	N/A	Yorkshire Wildlife Trust (2016)
Yorkshire	2008	lentic: Ribblesdale, from Gill Beck	500	N/A	Peay & Guthrie (2008)
South Yorkshire	2009	lotic: upstream at Porter Brook	420	N/A	Dangerfield (2011)
South Yorkshire	2009	lotic: Sheffield site, from Porter Brook	186	N/A	Dangerfield (2011)
Kent	2009	lentic: Brown Bridge Mill, from Buxford Mill River Stour	133	N/A	Friend (2010)
North Yorkshire	2010	lotic: River Rye, moved upstream (drought)	500	N/A	Hirst (undated)
South Yorkshire	2011/2012	lotic: Sheffield: Limb Brook, from Porter Brook	100	N/A	I. Marshall, pers. comm.
Norfolk	2011–2014	lentic: River Stiffkey lotic: Gunthorpe stream lentic: Glaven Lake lotic: upstream River Glaven, from River Glaven	various	2013	Pugh (2014)
Staffordshire	2012–14	lotic: 7 sites at Cannock Chase's forest streams	80–150	2013–2016	Mott (2013)
Staffordshire	unk	lotic: headwater Upper Penk	N/A	N/A	Mott (2015)
Essex	2011	lentic: moved from River Chelmer	212	N/A	Essex Rivers Hub (undated)
Essex, Norfolk, Suffolk	unk	lentic & lotic: 7 sites	N/A	N/A	Essex Biodiversity Project (2015)
Yorkshire	2013	lotic: Yorkshire Dales headwaters	20	N/A	P. Bradley, pers. comm.
Yorkshire	2015	lotic: Wharfe catchment: upstream supplementation	N/A	N/A	I. Marshall, pers. comm.

Table 1. Summary of ark sites established [both lotic (river) and lentic (still-water) sites] and river supplementations for White-clawed crayfish *Austropotamobius pallipes* within the United Kingdom and the Republic of Ireland from the 1980s through to the present day, excluding all the South West Crayfish Partnership (SWCP) sites and the Natural Resources Wales (NRW) sites (see Table 2): N/A, information not available; unk, unknown; *artificial gravel pit.

ARK SITE	STOCKING YEAR(S)	TYPE OF ARK/RIVER SUPPLEMENTATION	WILD CAUGHT OR CAPTIVE REARED	CRAYFISH STOCKED	YEAR FIRST DETECTED	BREEDING POPULATION?
ENGLAND						
South-west, ark/river supplementation sites*						
Somerset	2006/2008	river translocation	wild caught	302	2011	yes
Somerset	2008	river translocation	wild caught	160	2012	unconfirmed
Somerset	2009	river translocation	wild caught	111	not detected	unconfirmed
Somerset	2009	still-water introduction	wild caught (376); captive reared (347)	723	2009	yes
Somerset	2009	river translocation	wild caught	259	2011	yes
Somerset	2010	river translocation	wild caught	300	2015	unconfirmed
Somerset	2010	river translocation	wild caught	258	2011	yes
Devon	2010/2011	still-water introduction	wild caught	594	not detected	unconfirmed
Gloucestershire	2010/2011	still-water introduction	wild caught	178	2014	unconfirmed
Devon	2010/2011	still-water introduction	wild caught	335	not detected	unconfirmed
Cornwall	2011	still-water introduction	captive reared	200	not detected	unconfirmed
Devon	2011	still-water introduction	wild caught	35	2013	yes
Dorset	2013	river translocation	wild caught	350?	2014	unconfirmed
Somerset	2013	river translocation	wild caught	289	2015	unconfirmed
Hampshire	2014/2015/2016	river supplementation	captive reared/bred	559	2015	unconfirmed
Somerset	2015/2016	still-water introduction	captive reared	342	2016	unconfirmed
Hampshire	2016	still-water introduction	captive reared/bred	94	no survey	unconfirmed
TOTAL				5089		
WALES						
Ark/river supplementation sites						
Rhymney tributary	2011	river supplementation	captive reared	45	no survey	unconfirmed
Irfon tributary 1 (upper)	2012/2013	river introduction	captive reared	786	2013	unconfirmed
Irfon tributary 1 (lower)	2013/2014	river introduction	captive reared	907	2015	unconfirmed
Irfon tributary 2	2014	river introduction	captive reared	841	2015	unconfirmed
Ennig tributary	2015/2016	pollution mitigation	captive reared	1478	no survey	unconfirmed
Talybont tributary	2016	river introduction	captive reared	540	no survey	unconfirmed
TOTAL				4597		

Table 2. Summary of ark sites established and river supplementations for White-clawed crayfish *Austropotamobius pallipes* within south-west England and Wales from 2006 to 2016: *precise locations are not given to ensure location confidentiality for biosecurity purposes.

Plate 2. White-clawed crayfish *Austropotamobius pallipes* **within an ark site in Somerset, United Kingdom.** *Kenrina Maidment (all rights reserved).*

invaluable knowledge that has contributed to the best-practice guidance now available to crayfish conservationists (Peay, 2009; Souty-Grosset & Reynolds, 2009; Robbins, 2011). Since the 1980s, within the UK and Ireland, there have been various ark sites established and river supplementations into headwaters (Table 1).

In all the early attempts, the arks were established by translocating wild-caught individuals from threatened populations rather than setting up captive-rearing and breeding programmes. Prior to the inception of the SWCP, there had only been one known captive-reared *A. pallipes* reintroduction within the UK. In Derbyshire an *A. pallipes* population in the River Lathkill was wiped out by crayfish plague in 1993, and both captive-reared juveniles and wild-caught crayfish were reintroduced from the year 2000 over several years (Rogers & Watson, 2011).

In Yorkshire, a small-scale captive-breeding programme has been producing *A. pallipes* for over 12 years and in 2013 carried out the first reintroduction in the region, using captive-hatched crayfish, by releasing 20 individuals into a stream in the Yorkshire Dales headwaters (P. Bradley, pers. comm.). Captive breeding of *A. pallipes* in Northern Ireland (at Moneycarragh fish farm) was established in 2006; however, no restocking with captive-hatched juveniles has taken place to date (Policar *et al.*, 2008).

South-west England

Over the past decade, the SWCP has established 16 ark sites in six counties: Cornwall, Devon, Dorset, Gloucestershire, Hampshire and Somerset (Table 2). The ark sites have been established either with wild-caught translocated *A. pallipes* from local, threatened populations, or with captive-hatched juveniles. Specifically, eight of the ark sites in south-west England have been established at river (lotic) and eight at still-water (lentic) sites. Over 3400 adult *A. pallipes* have been moved from eight, highly threatened, natural populations into 13 ark sites. More than 1500 captive-hatched crayfish have been released into four ark sites, and one ark site has been set up with both wild-caught and captive-hatched individuals (Table 2).

Since 2006 the SWCP has also delivered one river supplementation. There are only two known remaining *A. pallipes* populations in Hampshire; one within the upper tributaries of the River Itchen and a recently rediscovered relic population on a tributary of the River Test (Rushbrook, 2016). A river supplementation of captive-hatched *A. pallipes* to the River Itchen population was a logical initial step. Between 2014 and 2016 more than 550 juvenile crayfish of age classes 0+–2+ years have been reared at Bristol Zoo and released upstream of the upper limit of distribution of the existing population, in an attempt to consolidate and expand its range. During 2016 a still-water ark site was also established in Hampshire, to assist with the long-term survival of this population (Table 2). A similar situation exists within the county of Devon with only two known populations of *A. pallipes* remaining. Historically, the species occurred on the Rivers Creedy/Yeo, Culm, Clyst, Creedy and Otter in Devon. Populations are now restricted to the Culm and Creedy/Yeo. On the Culm, *A. pallipes* are found in small pockets of very low density over a *c.* 8 km stretch of river and are mixing with introduced *P. leniusculus* at their upstream limit. On the

Creedy/Yeo *A. pallipes* occurred at low density over at least 12·8 km of river in the early 2000s; however, since that time it is estimated that 50% of the population has been replaced by Signal crayfish advancing downstream (N. Green, pers. comm.). Bristol Zoo Gardens is attempting to safeguard these populations by hatching juveniles for wild supplementation and maintaining groups of brood stock within the hatchery.

South Wales

The spread of *P. leniusculus* makes it increasingly difficult to find ark sites sufficiently distant from populations of this invasive species. A study carried out in 2009, focusing on south-east Wales, found only two potential ark sites more than 50 km from known *P. leniusculus* populations. River supplementations and introductions were carried out between 2012 and 2016 within the Irfon catchment. To date, reintroductions led by Natural Resources Wales have used entirely captive-reared stock and focused mainly on the Wye catchment. These have been a combination of releases to compensate for pollution incidents and introductions to stream ark sites (Table 2). More recently *A. pallipes* have been introduced to the Usk catchment with plans to release into the Monnow and Lugg/Arrow (England) in 2017.

POST-RELEASE MONITORING

An important element of both river supplementation and ark-site establishment is to ensure a long-term monitoring programme is in place. Existing recommendations for ark-site monitoring are that surveys should be conducted annually or biennially for a minimum of 5 years (Peay, 2003; Souty-Grosset & Reynolds, 2009). In south-west England the SWCP is committed to longer-term monitoring, recognizing that some populations may not be apparent for many years following introduction. This is supported by Hiley (2003), who suggests that it may take up to 10 years before smaller introductions grow to population sizes that become detectable by conventional survey methods. Schulz *et al.* (2002) recommended waiting 3–5 years after ark-site establishment before commencing monitoring. The SWCP monitors its ark sites in the second or third year post introduction, depending on the site, and then periodically for at least 10 years and in most cases longer.

Presence and absence are the only possible forms of monitoring in the early stages of ark-site establishment; population estimates are not attempted. Despite the fact that detection is unlikely in the year following introduction, and thus these sites are not necessarily surveyed, most ark sites are at least visited to ensure that habitat conditions remain favourable. This is important so that, in the event of failure, there will be a record of factors that may have affected ark-site establishment. All wild-caught translocations were carried out with crayfish with a carapace length greater than 15 mm. Therefore, if juveniles are found in subsequent years during monitoring this indicates that the population is breeding. Introductions of captive-hatched stock usually take place with young-of-the-year juveniles because space at the hatchery is always at a premium to allow for further ovigerous females to be housed. However, in the past few years at Bristol Zoo Gardens, juveniles have been reared within hatcheries for longer, so that they are at breeding size when released.

South-west England

Of the 17 sites (16 ark sites and one river supplementation) that have been set up by the SWCP since 2008, 16 have been surveyed and 12 (75%) show presence of *A. pallipes*. In five (31%) of the surveyed sites juveniles have been found, suggesting that the populations are now established and breeding. The remaining ark site was established in November 2016 and has not yet been surveyed. The average time before crayfish were detected was 4 years; however, in some cases crayfish were found the year following the introduction and in

others it took up to 5 years to detect them (Table 2).

Various monitoring techniques are employed, such as baited traps left overnight, stone turning, torch surveys, diver surveys and the use of artificial refuges. Artificial refuges comprise up to six plastic tubes of various diameters (from 32 to 63 mm) anchored to a 1·5 mm perforated aluminium base plate with a 2 m rope attached (Green, 2009). This method is an inconspicuous way of monitoring; the refuges are placed in the water for up to 4 weeks prior to the survey and then checked regularly. The refuges will catch crayfish from all age classes, including the juveniles that are often undetected when using other sampling methods.

Advances in the use of environmental DNA (eDNA) have great potential and this could soon be a reliable technique for monitoring 'presence' at ark sites and in wild populations (Treguier *et al.*, 2014; Troth, 2015). Several of the ark sites in the south-west are now being used to assist in the development of this tool. All captive-hatched crayfish that are released in the south-west are genetically sampled and their DNA stored. The use of Passive Integrated Transponder (PIT) tags has been tested in crayfish with promising results (Bubb *et al.*, 2002; Stead *et al.*, 2015) and in the most recent still-water ark site established, all crayfish that were large enough (i.e. a minimum of 22 mm carapace length) were PIT tagged (*c.* 7% of the individuals released) to assess long-term post-release survival. Tagging a proportion of the crayfish released will make it possible to assess the long-term survival of captive-hatched individuals within ark sites and give some indication as to the rate of recruitment within populations. Both the genetic sampling and PIT tagging should help to understand population dynamics over time.

South Wales

To date the only sites that have been monitored are those within the Irfon catchment. Initial surveys in June/July 2013 carried out by Cardiff University did not locate any crayfish at the release sites, although this was not an unexpected result as introductions had only occurred earlier that year. Routine salmonid electro-fishing surveys in August 2013 recorded two crayfish 15 months after introduction at the upper 'Irfon tributary 1' site [Wye and Usk Foundation, 2014; Andrew Gott (Natural Resources Wales), pers. comm.]. In late June 2015 a preliminary stone-turning survey on the lower river of the 'Irfon tributary 1' site found four animals in 2 hours, including three individuals within 14 minutes in good habitat (O. Brown, pers. obs, 2015). Based on size, these were considered to be one individual stocked in 2013 and three from 2014. A survey on the 'Irfon tributary 2' also found one individual considered to be 2014 stock in 1·5 hours of stone turning (O. Brown, pers. obs, 2015). These early results from the Irfon ark sites are very encouraging; however, a more robust measure of success will be the discovery of first-generation wild-bred offspring from these stocked individuals (Table 2). Those sites stocked in 2015/2016 will be surveyed in 2017.

DISCUSSION

There have been variable success rates with the ark sites established in mainland Europe. A review of 59 crayfish reintroduction and introduction case studies within Europe (France, Ireland, UK, Spain, Italy and Austria) revealed that only 26 (44%) were successful and a wide range of methodologies was used. Successful reintroductions were found to be those that that had been more thoroughly planned (Souty-Grosset & Reynolds, 2009). The sites in UK, Ireland and France generally had better success rates. One of the main reasons for failure was where ark sites were selected in areas where there had been previous outbreaks of crayfish plague (Souty-Grosset & Reynolds, 2009). The UK ark sites that were documented as having failed included Sherston

Avon, Tetbury Avon and the River Lathkill; in all these sites *A. pallipes* had been reintroduced after a plague outbreak and the site failed following recurrence of the disease. More research is required to establish why there may be a higher incidence of a second plague outbreak, even after the site is left without crayfish for many years. The situation is complex and poorly understood; in particular, it is not clear how (or if) plague lingers for years without detection. It is recommended that ark sites where there have been previous plague incidents should not be selected for reintroduction projects (Rogers & Watson, 2011).

It is clear that practitioners need to understand fully the importance of following the reintroduction guidelines that are readily available and, in turn, these guidelines should be regularly updated to reflect the experiences gained as more reintroductions and ark sites are established. Restocking in Europe has in the past used donor animals from distant stocks: for example, *A. pallipes* became extinct in Portugal and sites were restocked from Spain (Souty-Grosset & Reynolds, 2009). Stefani *et al.* (2011) discovered unexplained genetic similarities between crayfish populations in Italy and France, which are separated by the Alps, suggesting that human translocations had occurred. Indeed there have been many studies examining the genetic similarities and differences among and within crayfish populations throughout Europe, and their findings should be taken into consideration when translocating populations, to ensure that genetic distinctiveness and potential inbreeding are evaluated. Peay (2011) recommended that, where possible, ark-site animals should be selected from as close to the ark as possible, ideally within the same river catchment or river basin. Ensuring genetic integrity is important if arks are to be established as a conservation measure; however, where there is limited genetic diversity and restocking for harvesting is the priority, it may be beneficial to mix populations to increase genetic fitness and improve chances of survival (Taugbøl & Peay, 2004). With a species so close to the brink of extinction, such as *A. pallipes,* the relative importance of maintaining genetic integrity compared with losing the whole species needs to be discussed. Within south-west England, all ark sites established by the SWCP are from single populations, to ensure not only that genetic integrity is maintained but also that there is no cross-contamination of diseases.

Ark sites and river supplementations have proven to be effective methods for addressing the decline of *A. pallipes* in both south-west England and south Wales. River catchments within south-west England are rapidly becoming invaded by non-native crayfish species, increasing the risks to *A. pallipes* both in terms of crayfish plague and competitive exclusion. Therefore, within the region, discrete lentic sites, rather than lotic sites, are becoming the preferred option for arks. In south-west England 50% of ark sites are river sites (lotic), the other 50% are still-water sites (lentic) (Table 2: not including a river supplementation in Hampshire), and these sites have increased discrete *in situ A. pallipes* crayfish populations by over 75%. At the time of writing only 16 known natural *A. pallipes* populations remain in south-west England. At least 12 of the 16 ark sites are viable, which means that there are at least 28 *A. pallipes* populations now present in south-west England. If the introductions prove successful in the Irfon catchment in Wales, the number of streams known to hold *A. pallipes* will have increased from three to five.

Ark-site monitoring begins within 3 years of all sites being established and the crayfish will usually be detected within 1–5 years of being introduced. In south-west England *A. pallipes* have been detected on average at between 2 and 3 years, although at four of the sites (one river site and three still-water sites) crayfish have still not been detected for up to 7 years. The river site has variable water levels and flows, so the crayfish may have migrated or been washed downstream,

where habitat and water quality have remained optimal, so further investigation is required. All three still-water sites are large and, therefore, *A. pallipes* numbers may still be too low for detection. In the case of still, deep-water sites, surveying can prove especially problematic. Two of these sites are not only large but also fairly inaccessible and secluded, with large amounts of complex habitat, such as overhanging trees and considerable root systems penetrating the water. This causes issues for both diver and trapping surveys, and it is recognized that these sites may require much longer-term monitoring to allow the crayfish to establish population sizes that are detectable. Although crayfish within these sites may not be readily detected and monitoring presents several major challenges, there are some real advantages to having discrete, elusive sites that should be safe from interference and subsequent invasion of non-native crayfish species.

The river reintroductions in Wales are still in the relatively early stages of establishment. Although individuals were detected at 1–2 years post introduction, densities were low and required extensive searching. Stocking with juveniles approaching 1 year post hatch probably limits detection likelihood in the early stages because of the small size of the animals, and will also delay when introduced populations will start to breed.

In all of these ark sites where crayfish remain undetected, there have been no apparent negative environmental factors that may have caused localized population extinction. It is therefore perhaps premature to conclude that these ark sites have failed. Perhaps the crayfish migrated downstream or populations are still too small for detection. Indeed, crayfish density may need to exceed 0·2 animals m^{-2} before populations can be detected using stone-turning methods (Peay & Hirst, 2002).

It is vital that long-term monitoring of all ark sites is carried out to inform future conservation measures for this species. There are many variables that will affect the time to establishment and detection of the animals in an ark site, such as size of the site, number of animals stocked and extent of suitable habitat. With advances in monitoring techniques and the subsequent potential for using less-invasive and labour-saving monitoring methods, such as eDNA, it should be possible to monitor many ark sites more regularly and for much longer than the 5 year minimum timescales recommended in monitoring guidelines at the time of writing. Furthermore, the authors suggest that the reintroduction-monitoring guidelines (Peay, 2003; Souty-Grosset & Reynolds, 2009) should be updated to represent more accurately the time it can take for an ark-site population to establish properly and start recruiting. For example, in 1987–1989, between 20 and 100 crayfish were introduced to receptor sites in Wales (Slater *et al.*, 1992). Subsequent monitoring failed to find any sign of the introduced crayfish until between 6 and 15 years post introduction (Slater, 2013).

In recent years, advances in *A. pallipes* aquaculture (i.e. captive rearing and breeding) have made the use of ark sites a viable, realistic method for supplementation of wild populations, introduction and reintroduction, as is well illustrated in south-west England and south Wales. Crayfish aquaculture is the only solution where populations are at a level that renders wild harvesting impracticable, such as Wales and in the English counties of Hampshire and Devon. In some European countries it is the only legal option available. The use of captive-hatched individuals also has the added advantage of providing biosecure, plague-free brood stock. Additionally, captive-hatched juveniles may not roam as far as wild-caught translocated adults and, therefore, post-release migration may not be such an issue (Taugbøl, 2004; Kozák *et al.*, 2011). In addition, captive-hatched juveniles are more adaptable to a novel environment than wild-caught translocated individuals (Kozák *et al.*, 2011). Although in the UK captive-hatched *A. pallipes* have only been used for ark-site establishment

and river supplementations since 2009, the initial post-establishment monitoring results are very encouraging. One important decision to make is the age at which the captive-hatched crayfish should be released. Intuition (along with a wealth of evidence from other species) would suggest that the older/larger the crayfish, the higher the probability of post-release survival. This needs to be balanced against the production capacity of the rearing facility. If 1 year-old juveniles are kept for another year, although maintenance costs and efforts are lower because of the robustness of this size class, valuable tank space is taken up that could be utilized to establish another production cycle.

Future work will need to be conducted to compare the survival and reproductive success of 1 year-old and 2 year-old crayfish released into the wild, to inform optimal release age. In all cases, it can take time for populations to establish and recruit at ark sites. This is especially relevant for introductions involving captive-hatched young-of-the-year, which may take a year or more to start breeding; whereas a translocation with sexually mature crayfish can, in theory, start recruiting that same year. With captive-hatched individuals now being permanently tagged and genetically sampled prior to release, this should provide a much more informed picture of the long-term survival potential and genetic fitness of these individuals in the wild. Genetic profiling and PIT tagging potentially allow for more detailed tracking and monitoring of populations, and should provide a much better understanding of how crayfish populations are surviving over time and the levels of recruitment.

The restoration of a species to its previous natural range cannot realistically be achieved until the pressures resulting in the need for the application of conservation measures have been adequately removed. In the case of *A. pallipes*, the introduction of *P. leniusculus* and other non-native crayfish species (e.g. *P. clarkii*), along with their associated diseases, has had a deleterious effect. Therefore, removal of *P. leniusculus,* or at least a reduction in the threat posed by this species, is an essential part of the conservation efforts for *A. pallipes*. Considerable discussions and research have taken place about the effective control and/or elimination of non-native crayfish populations, and this research is ongoing (e.g. Hein *et al.*, 2006; Stebbing *et al.*, 2014; Green *et al.*, 2016; and references therein). The long-term goal is to either eradicate or control at a manageable level invasive crayfish populations to allow indigenous and non-native species to coexist within the same river catchments. Once this has been achieved, proportions of populations that have been held within lentic ark sites might be able to be reintroduced back into their rivers of origin.

CONCLUSION

Much of the success of the South West Crayfish Partnership is down to a strategic approach drawing on expertise from many practitioners, and the effective dissemination of knowledge and adherence to best-practice guidelines. By employing a multi-faceted approach to crayfish conservation the decline of White-clawed crayfish in south-west England has been reversed. Control of non-native species and raising awareness are as important as the direct conservation of a species through reintroductions, introductions and translocations. It is also vital that the details of all arks sites established, including relevant guidelines, techniques and monitoring results, are available within the professional domain, to ensure that fellow practitioners can learn and be guided by experiences. Further research is required to examine in detail on a case-by-case basis the long-term successes and failures of all the ark sites that have been established within the UK, to make it possible to develop a national conservation strategy, and an effective communication and support system. In Europe it has also been recognized that there is a need for a conceptual model to be

developed in order to preserve and protect the indigenous crayfish species (Kozák *et al.*, 2011).

Sceptics may argue that discrete, still-water sites, are unnatural as the crayfish do not have the opportunity to migrate and the populations are likely to remain permanently isolated. However, these sites have a major benefit of preserving a specific population of crayfish for the long term, without the pressures or competition experienced within a river habitat or the risk of invasion to contend with. Haddaway (2012) demonstrated that *A. pallipes* showed morphological plasticity when they were translocated from lotic to lentic sites, with their carapaces becoming wider, demonstrating their adaptability to novel environments. Although some may view still-water ark sites as not true wild restocking, with the advances being made in the methods that can be used to control non-native crayfish, in future it may be possible to use these isolated populations for restocking river catchments. Still-water ark sites can provide nature reserves for a multitude of species, preserving biodiversity and providing a long-term refuge for some of the most threatened *A. pallipes* populations. These lentic sites are possibly the safest long-term solution to prevent the extinction of this iconic invertebrate.

ACKNOWLEDGEMENTS

There are two main factors that contribute to the success of this programme. The project has received significant funding and the authors would like to thank all the grant awarding bodies that have contributed financially to this project: Biffaward, Bristol Water, Cardiff University, Defra's Aggregate Levy Sustainability Fund, Heritage Lottery Fund, ISAC EU Life+, Mohamed Bin Zayed Conservation Fund, Monnow Rivers Association, Natural England, Natural Resources Wales, the Pennon Environmental Trust, Vitacress Conservation Trust, and the Wye and Usk Foundation. The other key element to the continuing success is the communication and support that the programme receives from the South West Crayfish Partnership and all their associated volunteer teams, which includes Bristol Zoological Society, Buglife, The Centre for Environment, Fisheries and Aquaculture Science, The Environment Agency, Nicky Green Associates and the Wildlife Trusts, specifically Hampshire and Isle of Wight Wildlife, Dorset Wildlife Trust and historically the Avon Wildlife Trust. Thank you!

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Manuscript submitted 27 June 2016; revised 16 February 2017; accepted 31 March 2017

Appendix II

Received: 29 January 2018 | Revised: 1 June 2018 | Accepted: 9 June 2018

DOI: 10.1111/are.13766

ORIGINAL ARTICLE

WILEY Aquaculture Research

Determining an effective density regime for rearing juvenile *Austropotamobius pallipes* in a small-scale closed system hatchery

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Funding information
Mohamed Bin Zayed Species Conservation Fund; Bristol Zoological Society

Abstract

With recent advances in aquaculture techniques, captive-breeding of the endangered white-clawed crayfish *Austropotamobius pallipes* for restocking is becoming a widespread conservation method. Establishing optimal stocking densities for aquaculture is essential in maximizing productivity, and increases the likelihood of crayfish survival when released. A 240-day experiment took place using 2-month-old juvenile, captive-born, *A. pallipes*, within a small-scale, closed-circuit hatchery to investigate survival, growth and aggression at three treatment densities, low (100/m^2), medium (200/m^2) and high (300/m^2). Crayfish were counted and measured every 60 days between August 2015 and April 2016. Mean survival rates were high across all three densities (87.7% ± 2.8%). Carapace length was significantly longer at low density than at medium and high densities. While growth rate was not significantly different between treatments, it was significantly higher in the first 2 months, across all three treatments (47.1% ± 6.6%) than in subsequent periods (14.1% ± 5.8%). Size variation within groups increased with density, suggesting that social dominance hierarchies are established with increasing stocking density: dominant individuals are larger and competitively exclude smaller individuals from food resources. Males were significantly larger than females from 6 months of age, (when they could be reliably sexed), in all three treatments. The larger male size suggests that sexual dimorphism begins prior to sexual maturity, with males growing faster and being more dominant and aggressive than females. In conclusion, young-of-the-year *A. pallipes* can be reared at high densities without compromising survival; however, the optimal stocking density that maximizes growth and health is 100/m^2.

KEYWORDS
aggression, conservation, growth, survival

1 | INTRODUCTION

Translocation and reintroduction are widely used as methods for enhancing existing populations and creating new populations of threatened crayfish species in England, Ireland and Wales (Kozák, Füreder, Kouba, Reynolds, & Souty-Grosset, 2011; Souty-Grosset & Reynolds, 2009). The white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858) is endangered throughout its native range in the UK and mainland Europe (Sibley, Holdich, & Richman, 2011). There has been an estimated 50%–80% decline of *A. pallipes*, throughout its global range, over the past 10 years. In south west England, *A. pallipes* has experienced a dramatic 70% decline since the 1970s and a 30% decline in the last 10 years. This decline is thought to be

mainly due to the spread of the invasive American signal crayfish, *Pacifastacus leniusculus* (Dana, 1852) and associated crayfish plague *Aphanomyces astaci*; plus habitat degradation and pollution events (Sibley et al., 2011). In several counties in southern England, *A. pallipes* populations have been completely lost, reduced to very low numbers, or isolated in only a few locations. In response to this decline, captive-breeding hatcheries have been established, including a major facility at Bristol Zoological Gardens, England, which maintains *A. pallipes* brood-stock from highly threatened populations, producing up to 1,000 juveniles per annum for restocking to preserve and enhance in situ populations (Nightingale et al., 2017).

Crayfish aquaculture is an important component of the food industry in some countries, i.e. the American red swamp crayfish (*Procambarus clarkii*, Girard, (1852)) in both Asia and North America (Ackefors, 2000; Romano & Zeng, 2017). Determining optimal rearing densities is one of the key elements in crayfish aquaculture and it is well established that stocking density is directly related to growth and survival of crayfish species (Romano & Zeng, 2017; Savolainen, Ruohonen, & Tulonen, 2004). The effects of stocking density on juveniles have been well studied in some crayfish species with economic value. These include Australian crayfish species (*Cherax* spp.; Jones & Ruscoe, 2000; Naranjo-Páramo, Hernandez-Llamas, & Villarreal, 2004; Rodgers, Saoud, & Rouse, 2006); American red swamp crayfish (*P. clarkii*; Figiel & Miller, 1995; McClain, 1995); plus astacid species such as the signal crayfish (*P. leniusculus*; Ahvenharju, Savolainen, Tulonen, & Ruohonen, 2005; González et al., 2011a; Harlioğlu, 2009; Ulikowski, Krzywosz, & Śmietana, 2006), and noble crayfish (*Astacus astacus*, Linnaeus 1758), (Keller, 1988; Pursiainen, Jarvenpaa, & Westman, 1983).

For juvenile astacid crayfish species, a range of rearing densities from 50/m^2 up to 1,200/m^2, have been investigated, finding that survival is significantly compromised with increasing density, due to intraspecific competition for food and space (González et al., 2010; Harlioğlu, 2009; Savolainen et al., 2004).

Aquaculture of crayfish species for conservation and restocking is a relatively new concept. As with all crayfish aquaculture, it is important to maximize productivity and to produce large, robust crayfish that will have an increased chance of survival when released into the wild. In captivity, *A. pallipes* grows more quickly than in the wild. It is estimated that in situ *A. pallipes* matures in the third or fourth year of life (Reynolds, Celada, Carral, & Mathews, 1992). In contrast, in captivity, the fastest growing males and females can reproduce in the second year of life (Policar, Smyth, Flanigan, Kozák, & Kouba, 2010). Captive-born *A. pallipes* introductions typically occur with young-of-the-year crayfish in order to free up hatchery space for the next hatching attempt (Nightingale et al., 2017). Therefore, if their growth potential in captivity is maximized, this has important recruitment implications, increasing the chances of reproduction in the release year. This in turn should increase the chance of establishment and the success of the wild supplementation. Therefore, a key component for *A. pallipes* aquaculture is to establish an optimal density regime that will produce crayfish of a large enough size that can potentially breed in the second year of life. Chelae autotomy in crayfish can occur when crayfish are housed communally and is an indication that there have been aggressive encounters between individuals (Figiel & Miller, 1995). A lack of chelae can lead to a reduction in survival because the crayfish have injury trauma and may experience an increase of agonistic encounters and reduce their potential to feed optimally. This increased aggression can cause a reduction in fitness, which in turn can lead to increased mortality (Figiel & Miller, 1995; Sáez-Royuela, Carral, Celada, & Pérez, 2001).

There are no known experiments investigating the effect of stocking density on growth and survival of juvenile *A. pallipes*. Previous laboratory experiments, investigating shelter, feeding and temperature regimes when rearing stage-2 juvenile *A. pallipes* employed a wide variation in stocking densities in their research; between 50/m^2 (Sáez-Royuela et al., 2001) and 500/m^2 (Policar et al., 2010).

The aim of this paper was to examine survival, growth and aggression rates of juvenile *A. pallipes*, maintained at differing stocking densities, to establish the optimal stocking density for young-of-the-year crayfish held within a closed-circuit hatchery facility.

2 | MATERIALS AND METHODS

The juvenile *A. pallipes* were hatched from 20 wild-caught, ovigerous females. These females were collected from a local river population in South Gloucestershire, (under Natural England licence), and brought into the hatchery 2 months prior to the experiment commencing and removed once the juveniles were at stage-2; i.e. had undergone two moults, and were free-living. The juveniles were then reared for a further 2 months, which is the most critical survival period, prior to the experiment commencing.

The experiment took place, in an indoor closed-circuit aquaculture facility, in Somerset, England, which consisted of 18 glass tanks (0.12 m^2 bottom area, 45 l^3) with a 2 mm meshed outlet to prevent escape. The tanks were on a closed, recirculating system and water was returned to the tanks via an ultraviolet filter and filtration sump, containing a de-gassing chamber filled with bio-balls and a fluidized sand bed. Turnover rate was four times per hour and total system water volume was 1,200 L. The temperature range was controlled with both coolers and heaters to ensure that there was a maximum temperature variation of no more than 3°C, over a 24-hr period. Water temperature was allowed to fluctuate to reflect natural seasonal variation and therefore varied between 9 and 20°C over the course of the experiment. The photoperiod was natural and therefore fluctuated with season with average values of 12 hr light:12 hr dark.

The study subjects were 432 juvenile, 60 ± 7-day old white-clawed crayfish *A. pallipes*, with a mean carapace length (mm ± *SD*) of 7.24 ± 0.33 mm. The crayfish were randomly assigned into three different treatments: 72 were maintained at low density (100 crayfish/m^2), 144 were maintained at medium density (200 crayfish/m^2) and 216 were maintained at high density (300 crayfish/m^2). Each tank had a 30 mm substrate base layer of coral sand and fine gravel (0.4–1.0 mm diameter). Polycarbonate 10 mm sheeting, held down with substrate, was provided as refuges (two per crayfish). The

crayfish were fed to excess daily at 19:00 hr, on a carefully selected diet, with a proven track record of high survival and growth rates (J. Nightingale, personal observation). The diet included defrosted, plankton mix of bloodworm, *Daphnia* spp., *Cyclops* spp., mysids, krill and rotifers, enriched with New Era frozen food enrichment: a liposome-based product containing vitamins and antioxidants (World Feeds Limited, Thorne, UK). NatuRose, a natural source of Astaxanthin derived from the microalgae *Haematococcus pluvaili* (Dr T&T Health UK Ltd, Northamptonshire, UK), was added to spinach, carrot and chard and offered twice per week. All tanks were gravel-siphoned weekly when 20% of the water was replaced with rainwater, collected from a water reservoir, adjacent to the aquaculture facility.

Water quality was measured weekly using a Colombo Testlab water testing kit (Aquadistri UK Ltd, Cambridgeshire, UK). Chemical levels remained consistent throughout the experiment: ammonia < 0.1 mg, nitrite < 0.1 mg, nitrate < 15 mg, phosphate < 0.2 mg, pH 7.8, calcium ≥ 35 mg/L, general hardness 10 KH, potassium hardness 8 KH and a level of dissolved oxygen > 90%.

2.1 | Data collection and analysis

The experiment took place over 240 days between August 2015 and April 2016. Every 60 days the percentage of surviving individuals in each tank was calculated and each crayfish was examined. Measurements were not taken more frequently to avoid the effects of human handling on the survival, growth and condition of the animals. From day 60, carapace length, was measured from the anterior edge of the rostrum to the posterior edge of the cephalothorax to the nearest 0.01 mm using Vernier 1,500 mm callipers (Moore and Wright, Sheffield). For day 1, carapace length was calculated by taking a photograph of each individual on a known calibrated scale and then using the computer software programme ImageJ (Schneider, Rasband, & Eliceiri, 2012) to determine the length. This reduced stress to the crayfish by minimizing handling time and ensured accurate measurements. Variation in each group size was recorded and percentage growth rate along the time series was calculated by subtracting the new mean carapace length from the previous mean carapace length and dividing this figure by the previous mean carapace length. The percentage of missing chelae, a standard measurement of crayfish aggression (Figiel & Miller, 1995), was also recorded. Five data sets were collected at day 1, 60, 120, 180 and 240. From day 120, the crayfish could be sexed. Average sex ratios in the three density treatments were 42:58 (100/m^2), 43:57 (200/m^2) and 51:49 (300/m^2).

To determine if there were any differences in growth rates, survival or aggression between the three treatment densities, data were examined by using an ANOVA, where variables were tested at the tank level and generalized linear mixed models (GLMMs; function *glmer*, R package *lme4* (Bates, Maechler, Bolker, & Walker, 2015), where variables were tested at an individual level. Goodness-of-fit to normal distributions was checked by running the Shapiro–Wilk test on residuals and prior to using an ANOVA data were log transformed to stabilize the variance. Treatment density (low, medium or high) and sex of crayfish were considered as fixed effects, while tank number was considered a random effect. Interactions between fixed effects were tested within each model; however, all interactions were not significant and therefore dropped from the final models. Only variables that had a significant effect were retained within the models. Statistical analyses were performed using R 3.0.1 (R Development Core Team, 2006).

The experiment was carried out under Natural England Licence and was ethically approved by the University of Bristol Animal Services Unit Ethical Review Committee and the Bristol Zoological Society Welfare and Research Advisory Board.

3 | RESULTS

3.1 | Survival

There was no significant difference in survival (p = 0.88, df = 33, F = 0.13) in the present study, across all three treatments. Average survival rate across all three treatments was 87.7% ± 2.8% (Figure 1).

3.2 | Carapace length and growth rate

Mean carapace length at low density was significantly longer than the mean carapace length at medium and high densities (p = 0.02, df = 370, t = 2.32). Mean carapace length of the medium-density group was also significantly longer than the mean carapace length of the high-density group (p = 0.02, df = 370, t = −2.0; Figure 2a). Males (16.8 ± 0.51 mm) were overall significantly larger (11.3%) than females (14.9 ± 0.58 mm), (p < 0.001, df = 370, t = 6.67; Figure 2b); however, the interaction between sex and treatment on carapace length was not significant. There was no significant difference between overall mean growth rates of the three different treatments (p = 0.9, df = 69, F = 0.1). However, the mean growth rate at the beginning of the time series was significantly higher (47.1% ± 6.6%) between day 1 and day 60 than over each 60-day time period

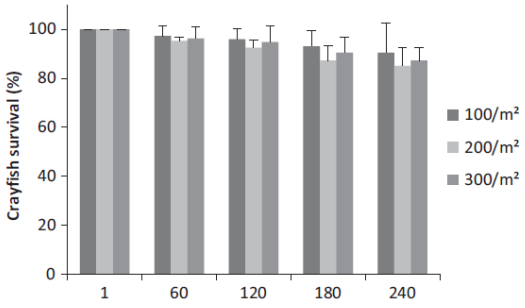

FIGURE 1 Percentage survival of *Austropotamobius pallipes* at different treatment densities: low (100/m^2), medium (200/m^2) and high (300/m^2), over the time series, day 1–day 240. Error bars represent standard deviations

(14.1% ± 5.8%) throughout the rest of the time series ($p < 0.001$, $df = 60$, $F = 76.89$). For the two data sets (day 180 and 240), where comparisons of growth rate of males and females could be made, growth rate was significantly greater (on average 6.8%) in males than females at all densities ($p = 0.004$, $df = 32$, $t = 3.06$; Table 1). There was no significant, interaction between sex and treatment on growth rate.

3.3 | Size variation

Size variation (2.14 ± 0.15 mm), between individuals within treatment groups on day 1, was not significantly different. As the experiment progressed, the size variation, across all groups significantly increased as the experiment progressed ($p < 0.001$, $df = 60$, $F = 34.96$). At day 240, size variation across groups was significantly lower at low density (5.2 ± 0.7 mm) than at high density (7.6 ± 1.2 mm; $p = 0.03$, $df = 34$, $t = -2.2$). Size variation at medium density (6.2 ± 1.0 mm) was not significantly different from high density ($p = 0.4$, $df = 34$, $t = -0.83$; Figure 3a). There was no significant size variation between males and females ($p = 0.22$, $df = 104$, $t = -1.23$; Figure 3b).

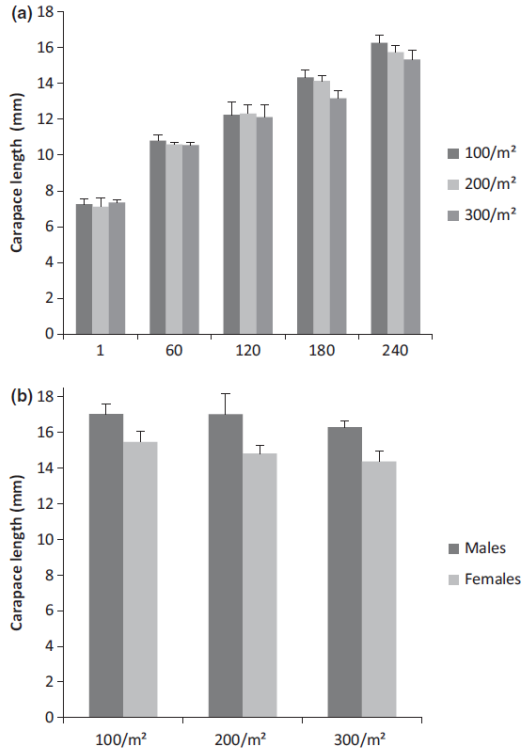

FIGURE 2 Carapace length (mm) of *Austropotamobius pallipes* at different treatment densities: low (100/m^2), medium (200/m^2) and high (300/m^2) over the time series, with (a) males and females combined from day 1 to day 240; and (b) males and females shown at day 240 of the experiment. Error bars represents standard deviations

3.4 | Chelae autotomy

The percentage of individuals showing chelae autotomy increased in the medium- and high-density treatment groups as the experiment progressed; however, due to some of the crayfish starting with missing chelae on day 1 of the experiment, the relative amount of chelae autotomy within the low-density treatment group fell to zero by day 120 and then increased up until the end of the experiment (Figure 4a). The percentage of individuals showing chelae autotomy was greatest in the medium-density group, trending towards being significantly higher than in the low-density group ($p = 0.057$, $df = 370$, $Z = -1.901$). At day 240, relative chelae autotomy (% ±*SD*) was significantly higher in females (29.6% ± 18.3%) than males (16.2% ± 13.7%; $p = 0.004$, $df = 370$, $Z = 2.84$). Males on average experienced 8.9% less chelae autotomy than females; however, the interaction between sex and treatment on relative chelae autotomy was not significant (Figure 4b).

4 | DISCUSSION

There have been several analyses of density-related effects in astacid crayfish that have demonstrated that increasing density results in a reduction in survival and growth rate, greater size variation and more aggressive encounters (González et al., 2010; Harlioğlu, 2009; Naranjo-Páramo et al., 2004; Savolainen et al., 2004). However, there are large disparities between studies as to an acceptable rearing density for juvenile crayfish. What is acknowledged is that there is a critical survival period for juvenile crayfish during the first few weeks of life, (González et al., 2010; Sáez-Royuela et al., 2001). Supplying the correct type of food during this time period may be more important than the density at which the animals are maintained. Historically, many of the experiments investigating juvenile astacid rearing densities used commercially available dry fish diets as a first feed for the crayfish. These diets yield poor survival and growth rates in hatchling *A. pallipes* (Sáez-Royuela et al., 2001) and *P. leniusculus* (Sáez-Royuela, Carral, Celada, Pérez, & González, 2007; Ulikowski et al., 2006). Consequently, some research may well be skewed by mortality and growth deficiencies due to inadequate nutrition rather than due to the treatment densities and the lack of standard methodology in crustacean studies can make results difficult to interpret (Carral et al., 2011; González et al., 2010).

In the current study, the experiment began when the juveniles were 2 months of age, after the critical feeding period had ended, to ensure that the results would be density-related rather than confounded by other husbandry elements. As the objective was to maintain high survival rates, the densities selected for this experiment were based on previous pilot studies and were significantly lower than in some other astacid crayfish experiments where the

TABLE 1 Percentage growth rate at each density treatment shown throughout the time series from day 60 to day 240

			Day 120–180			Day 180–240		
Density/m^2	Day 1–60	Day 60–120	All	Male	Female	All	Male	Female
100	48.8 ± 6.2	13.3 ± 6.8	17.2 ± 4.7	21.2 ± 3.9	14.3 ± 4.9	13.5 ± 3.5	13.0 ± 3.8	12.3 ± 4.1
200	49.0 ± 9.4	16.2 ± 4.9	15.0 ± 6.0	16.0 ± 5.6	12.8 ± 6.2	11.3 ± 1.9	13.6 ± 3.7	9.8 ± 1.0
300	43.5 ± 4.1	14.7 ± 6.7	13.5 ± 6.3	13.5 ± 6.5	12.2 ± 5.8	11.8 ± 1.2	13.3 ± 0.8	10 ± 2.4

Note. Mean ± standard deviations shown.

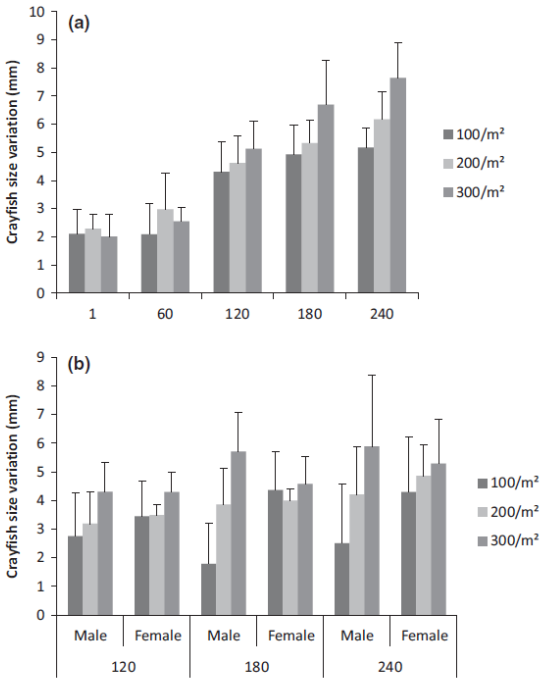

FIGURE 3 Size variation of *Austropotamobius pallipes* at different treatment densities: low (100/m^2), medium (200/m^2) and high (300/m^2) over the time series, with (a) males and females combined from day 1 to day 240; and (b) with male and female *A. pallipes* shown at the last three time series points: day 120, day 180 and day 240. Error bars represent standard deviations

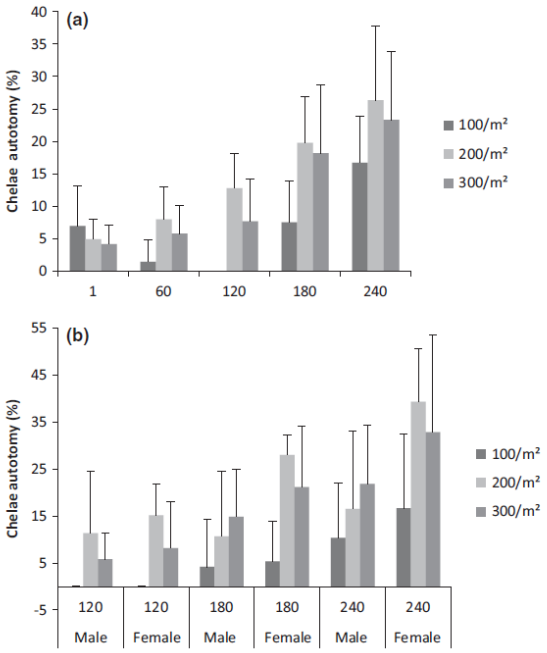

FIGURE 4 Percentage of *Austropotamobius pallipes* experiencing chelae autotomy at different treatment densities: low (100/m^2), medium (200/m^2) and high (300/m^2) over the time series, with (a) males and females combined from day 1 to day 240; and (b) with male and females shown from day 120, day 180 and day 240. Low-density groups are not shown on day 120 as there was no chelae autotomy within these groups at this time point. Error bars represent standard deviations

driver was for commercial rather than conservation purposes, therefore the threshold density for *A. pallipes* may not have been reached.

4.1 | Survival

Density did not significantly impact the survival of *A. pallipes* in this study; high survival rates were achieved at all three treatment densities. It is therefore encouraging to note that up to a relatively high density of 300/m^2, even at 10 months of age, crayfish survival was not compromised. This finding is consistent with research by Policar et al., (2010), where survival rates of up to 80% were achieved when rearing *A. pallipes* hatchlings at an initial density of 500/m^2 and then reducing the density to 115/m^2 after 100 days. In studies with *Pacifastacus lenisuculus* and *A. Astacus*, stage-2 hatchlings were reared at densities of up to 1,200 m^2, with significantly greater survival achieved at densities of up to 400/m^2, suggesting that is the threshold density (González et al., 2010; Savolainen et al., 2004; Ulikowski et al., 2006).

4.2 | Carapace length and growth rate

In the present study, there was a significant difference in carapace length between the different density treatments; there was an inverse relationship between length of carapace and increasing treatment density. This result is consistent with previous studies, which

found that growth diminishes with increased density (Jones & Ruscoe, 2000; Ulikowski et al., 2006). This is likely to be due to increased encounters with other crayfish while foraging, which could reduce the amount of time feeding, even when food is offered in excess. Similar growth rates were recorded in *Cherax quadricarinatus*, a comparatively nonaggressive crayfish species; the number of encounters, even when nonaggressive, increased energy expenditure and reduced foraging opportunities consequently reducing growth rate Growth was most rapid at the beginning of the experiment presented here, with a mean growth rate of 47.6% across all densities, after which the growth rate remained at a steady average increase of approximately 14.1% every 2 months (Jones & Ruscoe, 2000). In general smaller crayfish grow at a higher proportional rate than larger crayfish (Evans & Jussila, 1997; Jones & Ruscoe, 2000).

An unexpected finding was that males were significantly larger than females throughout the time series, even from day 120 of the experiment; i.e. at the age of 6 months when sex could be reliably determined. Growth rate in males was significantly higher than in females; this was particularly marked within the low-density group, possibly because the males were more dominant in the smaller groups, and therefore suppressed the females' growth to a greater extent. Hierarchical dominance is well known in crayfish species; larger animals will rapidly form hierarchies and defend resources from their subordinates, which will suppress the growth rate of the smaller individuals (Alonso & Martinez, 2006; Goessmann, Hemelrijk, & Huber, 2000; Harrison, Hoover, & Richardson, 2006; Herberholz, McCurdy, & Edwards, 2007; Tricarico & Gherardi, 2010; Tricarico, Renai, & Gherardi, 2005). González et al., (2011b) suggested that the dominant crayfish stress the smaller crayfish and prevent them from feeding at an optimal rate, even when food is supplied in excess. Studies with juvenile *P. clarkii* have demonstrated that dominant/subordinate relationships and social hierarchies can form very soon after hatching (Issa, Adamson, & Edwards, 1999; Sato & Nagayama, 2012). It is widely accepted that at sexual maturity, sexual dimorphism occurs and male crayfish grow larger chelipeds and females grow wider tails (Scalici & Gibertini, 2009; Wang, Yang, Zhou, Zhu, & Shan, 2011). A study by Franke, Wessels, and Horstgen-Schwark, (2013), with sub-adult *A. astacus* showed that males grew faster than females; however, there are no known published studies that have found interspecific sex differences in dominance hierarchies or growth rates within juvenile, young-of-the-year crayfish. The observed size difference in the current experiment could be the start of early sexual dimorphism, prior to reaching sexual maturity.

4.3 | Size variation

Size variation was significantly greater in the high-density group than in the medium and low groups. The larger variation in sizes in the higher density treatments may be due to the effects of social dominance being more pronounced when there are an increased number of crayfish present. Size variation was greater in females than males in the low- and medium-density groups, throughout the time series, but was similar to those of males in the high-density group. This could be because the hierarchical dominance of males over the females was more prevalent in the low and medium densities and therefore growth suppression of the females was more noticeable, and so there was a wider range in sizes. Growth rate was faster in the males at all densities, throughout the time series. This increased growth rate occurred even at 6 months of age and therefore the larger animals were predominantly male and consequently suppressed the growth of the smaller animals. The majority of the smaller crayfish within each group were female; however, because crayfish have an intrinsic growth rate (González et al., 2011b), there will be some females that will grow more quickly than individuals of both sexes. This may therefore explain why there was a wider size variation within the females of each group.

4.4 | Chelae autotomy

Chelae autotomy within crustacean species is accepted as an indicator of agonistic aggression (Figiel & Miller, 1995). In captive environments, there is an increasing occurrence of chelae injuries/loss with increasing density as the frequency of attack by conspecifics increases (Harlioğlu, 2009; Savolainen et al., 2004). In our study, rates of chelae autotomy increased as the experiment progressed; as the crayfish grew in size, resource competition increased. Relative chelae autotomy was greater in the medium-density group, across the time series, than in the other two density treatments, which suggests that, at medium densities, there were elevated levels of agonistic aggression. The reason for this might be because at high density the chances of winning an encounter was reduced as resource competition is higher and therefore less dominant animals do not try and defend resources. However at medium density, investing energy in competing for resources was worth the potential cost and therefore less dominant crayfish had more agonistic encounters. Females showed significantly more chelae autotomy than males across the time series. This may be due to males being more aggressive and having greater social dominance over the females than over other males. If the females were more subordinate to the males, there is a higher chance of males winning an aggressive encounter, than one with another male, and therefore more reason to attack a female rather than risk losing an agonistic encounter with another male. Similarly, larger females may be more likely to attack other females rather than the more dominant males.

5 | CONCLUSIONS

This present study illustrates that juvenile, young-of-the-year *A. pallipes* can be maintained for the first 10 months of life at densities up to 300/m^2 without survival being compromised. However, at the higher densities of 200–300/m^2, there will be a wider variation in sizes, an increasing amount of chelae autotomy, and a reduction in mean carapace length. By 6 months of age, there was a significant difference in carapace length and chelae autotomy between the sexes; males were larger than the females and more aggressive, suggesting they were more dominant. Therefore, sexual dimorphism

in *A. pallipes* may start when juvenile and as early as 6 months of age. When breeding *A. pallipes* for wild-release or for ex situ broodstock, maximizing survival and growth of young-of-the-year crayfish is paramount; therefore, the recommendation from this study would be to rear juvenile *A. pallipes* at a maximum density of 100/m^2. Further research is required to establish to explore the theory that *A. pallipes* males are more intrinsically aggressive than females as juveniles and if size-grading and single-sex culturing of this species, from 6 months of age, would improve overall group growth and health.

ACKNOWLEDGMENTS

This work was funded by the Mohamed Bin Zayed Species Conservation Fund and Bristol Zoological Society. Special thanks to Jonathan Williams and Timothy Clements for assisting with husbandry care, during the course of the experiment.

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How to cite this article: Nightingale J, Stebbing P, Taylor N, McCabe G, Jones G. Determining an effective density regime for rearing juvenile *Austropotamobius pallipes* in a small-scale closed system hatchery. *Aquac Res*. 2018;00:1–8. https://doi.org/10.1111/are.13766

Appendix III

Received: 29 January 2018 | Revised: 10 May 2018 | Accepted: 12 June 2018

DOI: 10.1111/are.13774

ORIGINAL ARTICLE

WILEY Aquaculture Research

Assessing the effect of size-grading for rearing young-of-the-year white-clawed crayfish *Austropotamobius pallipes*

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Funding information
Mohamed Bin Zayed Species Conservation Fund; Bristol Zoological Society

Abstract

Crayfish growth rates can vary considerably among individuals from the same brood, and social dominance hierarchies in crustacean species occur frequently. These hierarchies can reduce growth and survival when rearing communal groups. Size-grading and single-sex culturing are the methods used to combat this. A 160-day experiment took place on 288 young-of-the-year captive-born *Austropotamobius pallipes*, within a closed-circuit, indoor aquaculture facility. Crayfish were reared in three treatments (a) equal numbers of large males + small females (LMSF); (b) equal numbers of small males + large females (SMLF); (c) individuals of the same size, equal sex ratio; plus two control groups of single-sex, same sized individuals. Female survival in the LMSF was significantly reduced (52.8%, *SD* = 20.7%), whereas overall survival in all other groups was high (83.1%, *SD* = 15.1%). Male growth (6.3 mm, *SD* = 0.6 mm) was greater than female growth (4.9 mm, *SD* = 0.9 mm) across all groups. Cheliped autotomy was significantly greater (8.8%) in males (26.7%, *SD* = 8.9%) than females (17.9%, *SD* = 2.7%). This study suggests that young-of-the-year juvenile male *A. pallipes* grow faster and are more aggressive than females. Large males will suppress and reduce survival in smaller females whereas small males, when housed with larger females, will still grow faster than the females. We suggest that it is sex and not size that is the main factor that causes dominance hierarchies and growth suppression within juvenile *A. pallipes*. Maintaining juvenile *A. pallipes* in single-sex groups is optimal to ensure high survival and growth rates.

KEYWORDS
aggression, cheliped autotomy, conservation, crayfish, grading, growth

1 | INTRODUCTION

The white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858) is endangered in the IUCN Red List throughout its range in Europe (Füereder et al., 2010). Captive-breeding for reintroduction is becoming a recognized conservation measure to try and halt population declines (Nightingale et al., 2017; Taugbøl & Peay, 2004). High survival rates can be achieved when raising young-of-the-year *A. pallipes*; however, even at low densities, there can be a large variation in size and health of crayfish juveniles within a single brood, even as early as 6-months of age (Ahvenharju, Savolainen, Tulonen, & Ruohonen, 2005; J Nightingale pers. obs.).

Hierarchical dominance structures can develop in fish and crustacean species, and in crayfish intraspecific hierarchies can establish very quickly even without the influence of resource competition (Goessmann, Hemelrijk, & Huber, 2000; Harrison, Hoover, & Richardson, 2006; Issa, Adamson, & Edwards, 1999; Sato & Nagayama, 2011; Tricarico, Renai, & Gherardi, 2005). Dominance hierarchies can result in larger animals suppressing the growth of

smaller individuals, when they competitively exclude them from resources such as food and shelter (Bergman & Moore, 2003; Herberholz, Mccurdy, & Edwards, 2007; Tricarico & Gherardi, 2010). Size-grading, where animals are put into same-sized groups, can help to reduce these effects. The practice of size-grading is well-established within the aquaculture industry where commercial fish farms size-grade the fish to increase survival and growth rates (Gunnes, 1976; Wallat, Tiu, Wang, Rapp, & Leighfield, 2005). Size-grading in the prawn industry is also a standardized procedure and can produce improved growth rates and feeding efficiency when prawns are reared with same-sized individuals (Daniels & D'Abramo, 1994; Tidewell, Coyle, & Dasgupta, 2004).

Most crayfish grading experiments have occurred in large, commercially farmed, species in the family Cambaridae, such as the blue pearl crayfish *Cherax albidus* (Clark, 1936), (Lawrence, Cheng, Morrissey, & Williams, 2000), the redclaw crayfish *Cherax quadricarinatus* (von Martens, 1868) (Curtis & Jones, 1995; Jones & Ruscoe, 2000; Parnes & Sagi, 2002; Rodgers, Saoud, & Rouse, 2006) and the hairy marron crayfish *Cherax tenuimanus* (Smith, 1912) (Qin, Ingerson, Geddes, Kumar, & Clarke, 2001). Historically, the aquaculture of astacid crayfish species, such as *A. pallipes* and the signal crayfish *Pacifastacus leniusculus* (Dana, 1852), had limited success due to high mortality in the critical post-hatching phase, (at stage-2 when exogenous feeding has begun), due to inadequate nutrition (González, Celada, González, et al., 2009; Sáez-Royuela, Carral, Celada, & Perez, 2001). In recent years, feeding regimes for *A. pallipes* have improved, with the use of live *Artemia* spp. being offered in the critical first few weeks post-hatching (J Nightingale pers. obs.) and therefore research into the effects of size-grading stage-2 hatchlings has been possible. There are two known grading experiments that have taken place on juvenile *P. leniusculus* (Ahvenharju et al., 2005; González et al., 2011). However, results from size-grading experiments in fish and crayfish species have been varied, and there is no known published research on the effect of size-grading on growth and survival in *A. pallipes*. Therefore this study had two key objectives: (a) to establish if size-grading and or same-sex culturing would be beneficial during rearing of juvenile *A. pallipes* and (b) to establish what the optimal size and sex ratio for rearing young-of–the year *A. pallipes* is without compromising survival and growth. Both these objectives should assist in the main aim of producing consistently large, young-of-the-year robust crayfish, for wild-release or ex-situ brood-stock for subsequent captive-breeding programmes.

2 | MATERIALS AND METHODS

A 160-day experiment was carried out, within an indoor closed-circuit aquaculture facility in Somerset, England, with 288 5-month old, captive-born *A. pallipes*. The juveniles were hatched from 12 wild-caught, ovigerous females (collected from a local river population in South Gloucestershire, England, under licence from Natural England). Juveniles were measured and assigned, depending on their sex and size, into 24 glass tanks (0.12 m^2 bottom area, 45 L volume) with a 2 mm meshed outlet to prevent animals escaping. Carapace length was measured from the anterior edge of the rostrum to the posterior edge of the cephalothorax to the nearest 0.1 mm using Vernier 1,500 mm callipers (Moore and Wright, Sheffield). The tanks were on a closed recirculating system with a turnover rate of four times per hour (total system water volume 1,200 litres). All water quality parameters were measured weekly using a Colombo Testlab water-testing kit (Aquadistri UK Ltd, Cambridgeshire) to ensure that chemical levels remained consistent throughout the experiment. These included: ammonia <0.1 mg/L, nitrite <0.1 mg/L, nitrate <20 mg/L, phosphate <0.5 mg/L, pH 7.8, calcium 35 mg/L, general hardness 10 KH, potassium hardness 8 KH and a level of dissolved oxygen >90%. All tanks were gravel siphoned and water was changed weekly with 20% of the water replaced with rainwater, collected within a water reservoir, adjacent to the facility. Water temperature varied between 12 and 18°C over the course of the experiment. The temperature range was controlled with coolers to ensure there was a maximum temperature variation of no more than 3°C, over a 24 hr period. The photoperiod was natural and average values were 12 hr light and 12 hr dark. The crayfish tanks, within the system, were randomly assigned to the treatment and control groups. These included three treatments, (a) equal numbers of large males + small females (LMSF); (b) equal numbers of small males + large females (SMLF); (c) individuals of the same size, equal sex ratio, plus two control groups of single-sex, same sized individuals, (Table 1). Twelve crayfish were added to each tank (at an equivalent density of 100 crayfish/m^2).

Each tank had a 30 mm substrate base layer of coral sand and fine gravel (0.4–1.0 mm diameter). Polycarbonate 10 mm sheeting, held down with substrate, provided refuges (two per crayfish), together with 15 mm internal diameter pipe for the larger animals. The crayfish were fed 0.2 g of food, per crayfish, per day, i.e. fed to excess, at approximately 14:00 hours. The diet was a defrosted plankton mix of bloodworm, *Daphnia*, *Cyclops*, mysids, krill and rotifers daily, plus spinach, carrot and pea were offered biweekly. Food items were enriched with New Era frozen food enrichment: a liposome-based product containing vitamins and antioxidants, (World Feeds Limited, South Yorkshire). NatuRose, a natural source of Astaxanthin, derived from the microalgae *Haematococcus pluvaili*, (Dr T&T Health UK Ltd, Northamptonshire), was added to ensure adequate colouration in the crayfish. A lack of caretonoid can result in

TABLE 1 Treatment groups for *Austropotamobius pallipes* with starting mean carapace length (CL) ± standard deviation; 12 crayfish per tank at an equivalent 100/m^2 density

Treatment	Male CL (mm)	Female CL (mm)	Sex ratio	Treatment replicates
Large male, small female (LMSF)	13.2 ± 0.7	9.4 ± 0.7	6:6	6
Small male, large female (SMLF)	10.1 ± 0.3	12.8 ± 0.6	6:6	6
Equal sized male/female	11.5 ± 0.4	11.5 ± 0.5	6:6	5
All-male control	12.1 ± 0.2	–	–	2
All-female control	–	11.2 ± 0.3	–	5

A. pallipes developing blue hues; a similar finding has been seen in black tiger prawn *Penaeus monodon fabricus*, (Measveta, Worawattanamateekul, Latscha, & Clark, 1993). This particular diet combination was chosen as it produces high survival and growth in *A. pallipes* (J Nightingale pers. obs). The experiment ran from November 2016 to April 2017 and was carried out under Natural England Licence and was ethically approved by the University of Bristol Animal Services Unit Ethical Review Committee and the Bristol Zoological Society Welfare and Research Advisory Board.

2.1 | Data collection and analysis

The crayfish were counted, measured, examined and their biometric data recorded at day one and day 160. Missing chelae, a standard measure of aggression (Figiel & Miller, 1995), and stage in moulting cycle were also recorded. Variation in size within each group was recorded and growth rate along the time series was calculated by subtracting the mean end carapace length from the mean start carapace length for each treatment group.

To determine if there were any differences between the survival to the three treatments and controls, data were examined by using nested binomial generalized linear models (function *glm*, R package *lme4*) and log-transformed. To determine if there was any difference in carapace length, growth and cheliped autonomy among the different treatments and the control groups, data were examined using linear mixed models (function *lmer*, R package *lme4*, Bates, Maechler, Bolker, & Walker, 2015). The treatments, plus sex, were considered as fixed effects, and tank was considered a random effect. The alpha level was set at $p < 0.05$. Only variables that had a significant effect were retained in the model. Statistical analyses were performed using R 3.2.5 (R Development Core Team, 2016).

3 | RESULTS

3.1 | Survival

Survival was highest in the all-female control group (90%, *SD* = 7.0%) and lowest in the LMSF group (70.8%, *SD* = 21.1%), (Figure 1). Survival of LMSF group was significantly lower than all other treatments ($p < 0.002$, $df = 251$, $z = -3.1$). Survival (% ±*SD*) between other treatments was not significantly different and was on average 83.1% ± 15.1%. Survival between males and females were exactly the same in the equal-sized group (86.8% ± 13.9%). Survival of the males in the LMSF group was significantly higher (88.9% ± 13.6%) than female survival (52.8% ± 20.7%) i.e. 31.1% greater ($p < 0.001$, $df = 71$, $z = 3.32$). In contrast survival in the SMLF groups was not significantly different between males (80.5% ± 13.6%) and females (88.9% ± 6.8%) ($p = 0.33$, $df = 71$, $z = -0.97$). Survival in the control groups was considerably lower in the all-male (70.8% ± 29.4%) than the all-female groups (90% ± 7.0%), but not significantly so ($p = 0.08$, $df = 41$, $z = -1.75$); however, there were only two male control groups with considerable variation between them. Survival between the two all-male control groups was significantly different ($p = 0.04$, $df = 23$, $z = -2.0$); however, survival between the five all-female control groups was not significantly different ($p = 0.45$, $df = 59$, $z = 0.76$).

3.2 | Carapace length

Carapace lengths (mean ± *SD*) at day-1 were not significantly different between males (11.7 ± 1.1 mm) and females (11.1 ± 1.2 mm). At day-160, males (18.1 ± 1.9 mm) were significantly larger ($p < 0.001$, $df = 201$, $t = 4.4$), than females (16.0 ± 2.1 mm). The LMSF group contained the largest males, with a mean carapace length of 20.1 ± 0.7 mm by day-160, and the SMLF group had the largest females (mean carapace length 18.3 ± 0.9 mm). At the end of the experiment, mean carapace lengths were not significantly different between treatments ($p = 0.14$, $df = 198$, $F = 1.74$; Figure 2).

3.3 | Growth

Growth calculations (i.e., subtracting the starting mean carapace length from the final mean carapace length), showed that the mean growth (mean ± *SD*) across all groups (5.6 ± 1.0 mm) was not significantly different; however, male growth (6.3 ± 0.6 mm) was greater than female growth (4.9 ± 0.9 mm) across all treatment groups and was significantly greater than female growth in the LMSF, equal and single-sex groups ($p = <0.001$, $df = 227$, $F = 22.35$; Figure 3).

3.4 | Cheliped autotomy

Mean cheliped autotomy (% ±*SD*) was 22.3% ± 15.9% over all the treatments (sexes combined) and was not significantly different between treatments. The all-female control group had the least cheliped autotomy (14.6% ± 9.8%) and the SMLF group had the highest

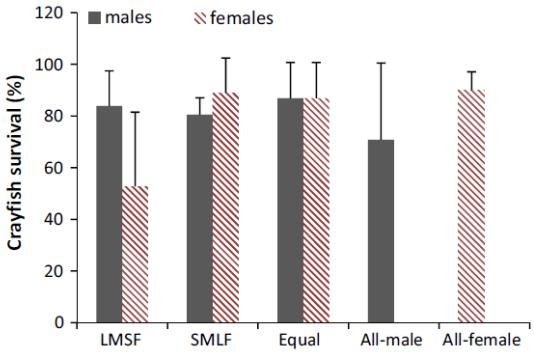

FIGURE 1 Mean percentage survival of male and female *Austropotamobius pallipes* at day-160, within the different treatment and control groups: large male, small female (LMSF); small male, large female (SMLF); equally sized, even sex ratio (Equal); male control group (All-male); female control group (All-female). Error bars represent standard deviations

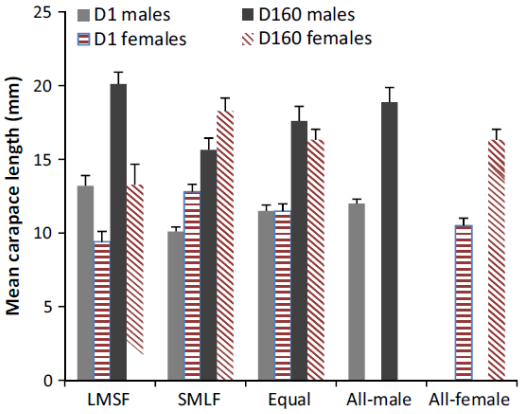

FIGURE 2 Mean carapace lengths of *Austropotamobius pallipes* at day-1 of the experiment (D1), to day-160 (D160), shown for the different treatment and control groups: large male, small female (LMSF); small male, large female (SMLF); equally sized, even sex ratio (Equal); male control group (All-male); female control group (All-female). Error bars represent standard deviations

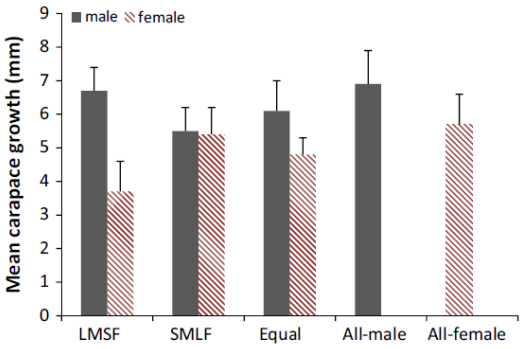

FIGURE 3 Mean carapace growth (mm) of male and female *Austropotamobius pallipes*, throughout the 160-day experiment within the different treatment and control groups: large male, small female (LMSF); small male, large female (SMLF); equally sized, even sex ratio (Equal); male control group (All-male); female control group (All-female). Error bars represent standard deviations

(26.5% ± 23.4%). When the sexes were looked at separately, cheliped autotomy in males (26.7% ± 14.1%) was significantly greater (by 8.7%) than in females (18.0% ± 17.8%) ($p = 0.04$, $df = 203$, $t = -2.1$), throughout all treatments except the equal-sized treatment group, where cheliped autotomy was the same in both sexes (20.7% ± 18.2%), (Figure 4). Crayfish with both chelae present were significantly ($p < 0.001$, $df = 200$, $t = 4.5$) larger than those with missing chelae.

4 | DISCUSSION

4.1 | Survival

There was no significant difference in survival between the equal-sized, SMLF and all-female control group, in which all had high survival; however, there was a significantly higher mortality of females in the LMSF, compared with the other two treatment groups and female control group. This supports previous astacid crayfish studies, which graded juvenile, young-of-the-year *P. leniusculus* into small and large sizes and found that survival was significantly higher in the graded rather than the non-graded groups (Ahvenharju et al., 2005; González et al., 2011). Within the LMSF group, where female survival was significantly reduced, larger males may dominate and kill the females. Figler, Blank, and Peeke, (2005), demonstrated that adult male *Procambarus clarkii* (Girard, 1852) were more likely to attack non-maternal females rather than males or maternal females. In a similar manner, larger male *A. pallipes* may attack the females rather than the other males in the group.

When large females were housed with small males, the survival of the males was not reduced suggesting that aggressive attacks and

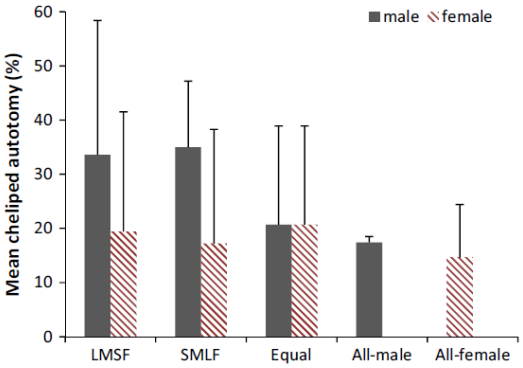

FIGURE 4 Mean percentage of cheliped autotomy for male and female *Austropotamobius pallipes* at day-160 within the different treatment and control groups: large male, small female (LMSF); small male, large female (SMLF); equally sized, even sex ratio (Equal); male control group (All-male); female control group (All-female). Error bars represent standard deviations

hierarchical dominance is predominantly sex-related rather than size-related. Although the survival in the male control group was lower than the average survival, this result needs to be viewed with caution as there were only two replicates for this group in contrast with five or six replicates for each other treatment group (due to the small number of crayfish available). There was also considerable variation in survival (91.6% and 50.0%) between the two different all-male control tanks. The all-female control groups had the highest survival than any of the other females within the other treatments. They also showed the least variability in survival rates between the five different control tanks (survival 90% ± 7.0%). This is supported by single-sex experiments in *C. albidus* (Lawrence et al., 2000) and

C. quadricarinatus (Rodgers et al., 2006); these studies found that there was no significant reduction in male survival when reared in all-male groups.

4.2 | Growth

Males grew faster than females in all treatments, even when males and females were matched for sex and size, suggesting that juvenile *A. pallipes* exhibit sex differences in growth rate. This is in contrast with *C. albidus* where crayfish grow at the same rate until sexual maturity (Woodland, 1967). The all-male *A. pallipes* group had the fastest growth and the lowest male survival suggesting that some males grew large and out-competed the smaller males for resources. The males that were in the LMSF group did not significantly benefit from the fact that they were suppressing the smaller individuals, and the males within the all-male group actually grew faster. This is in contrast with the situation in *P. leniusculus* where larger juveniles benefited from being with smaller individuals, and grew faster than when housed separately (Ahvenharju et al., 2005). The all-female control groups on average grew faster than the females in the other treatment group, suggesting that all-female groups are optimal for growth and that in mixed-sex groups, the females' growth may be supressed by the more dominant males. Both the male and female single-sex groups grew faster than mixed-sex groups as determined by Lawrence et al., (2000) and Rodgers et al., (2006) who found that males and females *C. albidus* and *C. quadricarinatus* grew faster in single-sex rather than mixed-sex groups. However, these studies were with larger species and in the case of Rodgers et al., (2006), the study was with adults rather than juveniles. When juvenile *P. clarkii* were raised in single-sex or mixed-sex groups, there were no differences in growth rate (Figiel, Babb, & Payne, 1991; Wang, Yang, Zhou, Jiang, & Zhu, 2014).

Growth of the females in the LMSF was significantly reduced, suggesting that the larger males dominate the smaller females and supress their growth. Other studies on crayfish have shown that larger individuals dominate smaller animals and will compete for resources (Herberholz et al., 2007; Tricarico et al., 2005). However, in this study, within the LFSM group, male growth was still faster than in females, suggesting that the larger females do not dominate and suppress the smaller males. Some studies on crayfish species such as *C. tenuimanus* (Qin et al., 2001) found that size-grading did not affect growth. This has also been the case for fish species (Sunde, Imsland, Folkvord, & Stefansson, 1998). This indicates that social structure cannot be generalized across species and therefore size-grading has to be assessed at species level. Therefore, it was important to carry out this research rather than assume that size-grading would have similar effects for *A. pallipes* as in the astacid species *P. leniusculus* (Ahvenharju et al., 2005; González et al., 2011).

4.3 | Cheliped autotomy

In all the treatments and control groups, males suffered significantly more cheliped autotomy than females, suggesting that males are more likely to fight each other in competition for resources; this is supported by the female control groups having the lowest amount of cheliped autotomy, suggesting that they are less aggressive when kept in single-sex groups. This study implies that males are more aggressive than females, even before sexual maturity, which is supported by the study of Figiel and Miller, (1995) on juvenile *P. clarkii*, who found that the percentage of chelae loss in males (15.8%) was higher than in females (10.5%). In crayfish species such as *P. clarkii* (Figler et al., 2005), adult crayfish males are more aggressive and will dominate and out-compete females; however, this behaviour has not been reported in juvenile crayfish. A study in juvenile *P. clarkii* reported social dominance behaviour occurring; however, differences between sexes was not investigated (Herberholz et al., 2007). Crayfish with missing chelae were significantly smaller than those with both chelae present, perhaps because they were the subordinate within the social hierarchies and consequently were not efficient at competing for resources such as food. Crayfish with missing chelae will expend energy regenerating a new limb and this may also account for their slower growth rate (Figiel & Miller, 1995; Kouba, Buřič, Policar, & Kozák, 2011).

5 | CONCLUSIONS

This current study found that social hierarchies and dominance in *A. pallipes* start within 6-months of hatching. We suggest that sex, rather than size, plays a more important role in hierarchical dominance, which can lead to a reduction in female survival and growth. In an endangered species, which is being produced for both wild-release and for brood-stock, production of large-sized females, with high survival rates, is paramount. The study indicates that size-grading of juvenile *A. pallipes* is beneficial and can increase survival and growth rates, with all-female groups achieving the best results. The optimal size and sex ratio for rearing young-of-the-year *A. pallipes* would be to split the crayfish into single-sex groups at 6-months of age, when they can be reliably sexed. These groups should be size-graded, with a carapace size differential of no more than 2 mm between individuals, and held at a maximum density equivalent of 300/m^2 up until 10-months of age when the crayfish should then be size-graded again and the density reduced to 50/m^2. Crayfish can then be maintained at this density, within these size-graded groups, up until their release, which ideally should be during their second year, prior to the breeding season (J Nightingale pers. obs.). This size-grading, single-sex grouping strategy would be advantageous, producing larger females that will be ready to breed in their second year. Where brood-stock groups are being maintained for ex-situ breeding-programmes the larger females should be housed with smaller males to ensure that males do not supress female growth or compromise their survival. If equally-sized males and females are housed together the faster male growth rate will result in the males rapidly becoming larger than the females, which will cause them to out-compete the females for tank resources, such as food and shelter, which in turn may increase female mortality.

ACKNOWLEDGMENTS

This research was funded by the Bristol Zoological Society and Mohamed Bin Zayed Conservation Grant. A special thanks goes to Jonathan Williams and Tim Clements for assisting with crayfish care duties during the course of the experiment.

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How to cite this article: Nightingale J, Stebbing P, Taylor N, McCabe G, Jones G. Assessing the effect of size-grading for rearing young-of-the-year white-clawed crayfish *Austropotamobius pallipes*. *Aquac Res*. 2018;00:1–7. https://doi.org/10.1111/are.13774

Appendix IV

Knowl. Manag. Aquat. Ecosyst. 2018, 419, 20
© J. Nightingale *et al.*, Published by EDP Sciences 2018
https://doi.org/10.1051/kmae/2017059

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Knowledge & Management of Aquatic Ecosystems

Journal fully supported by Onema

RESEARCH PAPER OPEN ACCESS

The long-term effects and detection ranges of passive integrated transponders in white-clawed crayfish *Austropotamobius pallipes*

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Abstract – Individual identification of the endangered white-clawed crayfish (*Austropotamobius pallipes*) can provide valuable information when assessing long-term survival of animals released into the wild; currently the most effective method is the use of passive integrated transponders (PIT) tags. A 360 days *ex situ* experiment was undertaken on 20-month, captive-born *A. pallipes* of carapace length (CL): 22–31 mm, to assess growth and survival after PIT-tagging. Thirty crayfish, matched for sex and size, were PIT-tagged, with 30 untagged crayfish as a control. All crayfish survived for the first 60-day post-tagging, indicating that there was no short-term survival effect of the procedure, in controlled conditions. There was no significant difference in survival or growth over the year between tagged and untagged crayfish, indicating that *A. pallipes* (≥22 mm CL) can be PIT-tagged safely. A second *ex situ* experiment investigated the detection range of adult, wild-caught, PIT-tagged *A. pallipes*. Eighteen *A. pallipes* were tagged with either 8 mm or 12 mm tags and added to different treatments (bare tank, tank with substrate, brick refuge, pipe refuge, pipe refuge plus slate), and the distance to detection was measured. Throughout all treatments the *A. pallipes* tagged with 12 mm PIT tags were detected significantly further away (35.6 ± 3.8 mm) than the 8 mm PIT-tagged crayfish.

Keywords: white-clawed crayfish / PIT-tagging / detection / conservation

Résumé – Les effets à long terme et les distances de détection des transpondeurs passifs intégrés dans les écrevisses à pattes blanches *Austropotamobius pallipes*. L'identification individuelle des écrevisses à pattes blanches (*Austropotamobius pallipes*) menacées d'extinction peut fournir des informations précieuses pour évaluer la survie à long terme des animaux relâchés dans la nature; actuellement, la méthode la plus efficace est l'utilisation de transpondeurs intégrés passifs (PIT). Une expérience *ex situ* de 360 jours a été entreprise sur des *A. pallipes* de longueur de carapace (CL) de 20 à 31 mm, âgées de 20 mois nées en captivité, afin d'évaluer la croissance et la survie après marquage PIT. Trente écrevisses, assorties pour le sexe et la taille, étaient marquées PIT, avec 30 écrevisses non marquées comme témoin. Toutes les écrevisses ont survécu pendant les 60 premiers jours après le marquage, ce qui indique qu'il n'y avait aucun effet de l'intervention sur la survie à court terme, dans des conditions contrôlées. Il n'y a pas eu de différence significative dans la survie ou la croissance au cours de l'année entre les écrevisses marquées et les écrevisses non marquées, ce qui indique qu' *A. pallipes* (≥22 mm CL) peut être marquée PIT en toute sécurité. Une deuxième expérience ex situ a examiné la plage de détection des *A. pallipes* adultes capturées à l'état sauvage et marquées PIT. Dix-huit *A. pallipes* ont été marquées avec des marques de 8 mm ou de 12 mm et ajoutées à différents traitements (réservoir nu, réservoir avec substrat, refuge en brique, refuge de tuyaux, refuge de tuyaux et ardoise), et la distance de détection a été mesurée. Au cours de tous les traitements, les *A. pallipes* marquées avec des marques PIT de 12 mm ont été détectées significativement plus loin (35,6 mm SD = 3,8) que les écrevisses marquées PIT de 8 mm.

Mots-clés : écrevisse à pattes blanches / marquage PIT / détection / conservation

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1 Introduction

The white-clawed crayfish *Austropotamobius pallipes*, (Lereboullet, 1858), is globally Endangered throughout its range (Füreder *et al.*, 2010). One of the recognised conservation techniques is to establish ark sites (safe refuges) into which wild populations can be translocated, or captive-born animals introduced (Souty-Grosset and Reynolds, 2009; Nightingale *et al.*, 2017). Evaluation of the long-term success of these ark sites and the levels of recruitment can be greatly assisted by permanent marking and subsequent monitoring of the crayfish being released. If released crayfish individuals can be identified and tracked over an extended time period, within an ark site, this will provide valuable information on the status, long-term viability, and health of these populations.

The standard marking techniques for tracking crayfish have historically included cauterization, hole-punching, marking with correction fluid or oil-based pens/paints and radio-tracking (Abrahamssons, 1965; Guan, 1997; Robinson *et al.*, 2000; Haddaway *et al.*, 2010; Ramalho *et al.*, 2010; Louca *et al.*, 2014). However, none of these methods provides a permanent method of marking that is retained during moulting and some methods, such as cauterization, have been shown to reduce growth rates (Guan, 1997).

There are several options for permanent marking of crayfish. Visible implant elastomer (VIE) is a liquid elastomer that is injected under the skin, allowing identification of a limited number of individuals or groups by colour combinations or implant location. Visible implant alpha tags (VI Alpha) are small, fluorescent tags with an alphanumeric code; both VIE and VI Alpha are designed to remain visible after they have been implanted within the animal (Gotteland, 2013). Both methods have limitations in terms of unique identification, retention rate, long-term readability and, because the tagged animals cannot be detected remotely, they have to be recaptured to be identified (Buřič *et al.*, 2008; Haddaway *et al.*, 2010). Coded micro-wire tags (CWT) are widely used in the fisheries industry. They are very small (1.1 mm × 0.25 mm), and therefore can be implanted without survival being compromised. The tags can be detected using hand-held readers; however, individual identification of live animals is difficult because the tag usually needs to be removed to be read (McMahan *et al.*, 2012). One of the most successful methods of permanent tagging, with easy individual identification, is the use of passive integrated transponders (PIT) tags.

PIT tags are electronic chips, encased within glass, ranging in size from approximately 7–32 mm in length. They remain passive; *i.e.*, dormant, until they are activated by a reader, which emits a close-range electromagnetic field, causing the PIT tag to transmit its unique code. PIT-tagging provides a permanent method to uniquely identify animals (Gibbons and Andrews, 2004). The use of PIT tags for marking animals began in the 1980s, when it was trialled with salmonids in the fisheries industry (Prentice and Park, 1983). With the development of PIT tag readers with extendable antenna, the tags can be read remotely, hence allowing animals to be identified *in situ* without having to be captured or seen. This can potentially provide a very useful tool for monitoring populations of species long-term and validating survival rates when captive-born animals are released into the wild, without the need to physically trap or handle the animals to identify individuals.

The use of PIT tags on fish species is well documented (Prentice and Park, 1983; Roussel et al., 2004). In recent years, this technique has been used on crayfish species and there have been several *in situ* and *ex situ* studies published. Field experiments include studies on *A. pallipes* (Bubb et al., 2008; Louca *et al.*, 2014; Stead *et al.*, 2015), slender crayfish *Orconectes compressus*, (Black *et al.*, 2010), giant Tasmanian crayfish *Astacopsis gouldi* (Shepherd *et al.*, 2011) and signal crayfish *Pascifastacus leniusclus* (Stead *et al.*, 2015). Laboratory experiments include studies on *P. leniusclus* (Wiles and Guan, 1993; Bubb *et al.*, 2002), spiny-cheek crayfish *Orconectes limosus* (Buřič *et al.*, 2008), *O. compressus* (Black *et al.*, 2010), and woodland crayfish *Orconectes hylas* (Westhoff and Sievert, 2013). High survival rates and good growth rates have been reported in most cases, when tagging crayfish >25 mm carapace length (CL). However, previous laboratory studies were relatively short-term, typically less than 60-days in length, with one longer six-month study reported (Bubb *et al.*, 2002).

There have been no known published laboratory trials to assess survival and growth of PIT-tagged *A. pallipes*. There are a few published field studies, which have shown that PIT-tagged *A. pallipes* survived when returned to the wild (Bubb *et al.*, 2010; Louca *et al.*, 2014; Stead *et al.*, 2015). However, in all of these studies, only a proportion of the released tagged crayfish were detected and therefore percentage survival and growth of *A. pallipes*, post-tagging, could not be confirmed.

PIT tags are available in a range of sizes and there have been several studies looking at the detection range of fish species that have been tagged with different sized PIT tags (Morhardt *et al.*, 2000; Burnett *et al.*, 2013). However, there are no known published studies looking at the difference in detection range of crayfish tagged with different sized PIT tags. Bubb *et al.* (2002) and Burnett *et al.* (2013) assessed the specific range at which different sized PIT tags could be detected when placed in a river, but these crayfish were not internally PIT-tagged.

The objectives of the present study were two-fold: (i) to investigate the long-term effects of PIT-tagging on survival and growth of captive-born *A. pallipes*, within the laboratory, and to establish a minimum size at which *A. pallipes* can be safely tagged; and (ii) to investigate the detection range of PIT-tagged captive-born *A. pallipes* with 8 mm versus 12 mm PIT tags, within a variety of laboratory conditions. Both experiments should help to inform the efficacy of using PIT tags for long-term monitoring of *A. pallipes in situ* and the safe minimum size at which *A. pallipes* can be PIT-tagged.

2 Materials and methods

2.1 Investigating the effect of pit-tagging on growth and survival *of A. pallipes ex situ*

A year-long experiment was conducted in an outdoor, flow-through, aquaculture facility in Somerset, United Kingdom. Sixty, captive-born, 20-month *A. pallipes* were used in the experiment: 30 males and 30 females. The juveniles were hatched from 20 wild-caught, ovigerous females (collected

Table 1. Mean carapace lengths (CL mm) of *A. pallipes* within the treatment and control groups for each of the replicate tanks (Tank 1–3).

Treatment/control	Tank 1 CL mm	Tank 2 CL mm	Tank 3 CL mm
Male tag	27.4 ± 4.0	27.5 ± 4.5	25.6 ± 3.3
Male untagged	28.1 ± 7.1	27.3 ± 1.2	26.1 ± 3.6
Female tag	25.6 ± 1.1	26.1 ± 2.6	24.8 ± 2.7
Female untagged	24.4 ± 1.7	25.0 ± 3.5	24.6 ± 3.0

from a local river population within South Gloucestershire, under licence) that were reared for the first year in a nearby indoor, closed-circuit aquaculture facility and then moved to the outdoor aquaculture facility ten-months prior to the experiment commencing.

The crayfish were split into three groups, 20 crayfish in each, with an equal sex ratio and, within each sex, an equal mean CL and weight. Each tank group was then divided into tagged (treatment) and control sub-groups, with equal sex ratio (Tab. 1).

Crayfish in the treatment sub-groups were tagged with Trovan ID100A/1.4, (RFID Systems LTD, East Yorkshire), 8 mm × 1.4 mm PIT tags and animals in the control group were left untagged. For the tagging procedure, the crayfish were held around the cephalothorax and an incision made, with a sterile 2 mm-gauge, hypodermic needle, through the cuticle and abdominal muscle of the third ventral abdominal segment (*i.e.*, between the second and third set of pleopods). The tag was then injected into the abdominal muscle and the crayfish was scanned with a Trovan LID-575 midrange reader (RFID Systems LTD, East Yorkshire) and the tag code recorded.

Each group of 20 crayfish was then randomly assigned to one of three experiment tanks (0.9 m^2 bottom area), on continuous flow-through from a local water reservoir and with an excess of refuges, (engineered bricks and PVC pipes, no substrate). Water temperature was allowed to fluctuate naturally with the incoming water and was recorded hourly with an aquatic TinyTag data logger (Gemini Data Loggers Ltd, West Sussex). Temperature varied seasonally between 5.8 and 22.5 °C, with no more than 2.8 °C variation over a 24 h period. Water quality was monitored monthly using a Colombo Testlab water-testing kit (Aquadistri UK Ltd products, Cambridgeshire). Chemical levels remained constant throughout the experiment: ammonia <0.1 mg/l, nitrite <0.1 mg/l, nitrate 15 mg/l, phosphate <0.1 mg/l, pH 8.0, calcium 35 mg/l, general hardness 12 KH, potassium hardness 10 KH and a level of dissolved oxygen >80%. The photoperiod was natural and therefore fluctuated with season; average values were 12 h light and 12 h dark. No supplementary feeding occurred during the experiment; the crayfish foraged on live invertebrates and plant matter, existing within the tanks. This naturally occurring food supply was in constant supply from the incoming water. The experiment ran for 12-months, from the end of January 2016 until beginning of January 2017. At the end of the experiment, all surviving tagged crayfish were X-rayed with a Roentgen 703 machine (C and G Medical) and processed with a Direct Digitizer Regius model 110 (Konica Minolta), to assess the internal position of the PIT tags.

Table 2. Mean carapace lengths (CL mm) of *A. pallipes* within the two PIT-tagged treatment groups: 8 mm and 12 mm tags.

Treatment	Male CL mm	Female CL mm	Male: female ratio
8 mm × 1.4 mm PIT-tag	34.5 ± 1.5	35.1 ± 3.2	5:4
12 mm × 2.1 mm PIT-tag	40.9 ± 3.2	38.9 ± 1.7	4:5

2.1.1 Data collection and analysis

The crayfish were counted every 60 days; *i.e.*, on day 1, 60, 120, 180, 240, 300 and 360. On each counting day, crayfish were scanned and biometric data recorded. CL was measured from the anterior edge of the rostrum to the posterior edge of the cephalothorax to the nearest 0.1 mm using Vernier callipers (Moore and Wright, Sheffield). The crayfish were dried with paper towel and weighed to the nearest 0.1 g using a digital weighing scale (Smart Weigh SWS600). Missing chelae, a standard measure of aggression, (Figiel and Miller, 1995) and stage in moulting cycle, (*i.e.* whether inter-moult, pre or post moult), were also recorded. During the breeding season reproductive status was recorded; *i.e.*, if females were in reproductive glair (development of glair glands on the ventral abdomen of the female), egg production or had spermatophores (deposited by males) present.

To determine if there was any difference in the survival of the tagged treatment and the untagged control, data were log transformed and examined by using generalized linear models (function *glm*, R package lme4, Bates *et al.*, 2015). To determine if there was any difference in growth of the tagged and untagged group, data were examined using linear models (function *lm*, R package, Bates *et al.*, 2015). The treatment, control groups and sex were considered as fixed effects, and tank was considered as a random effect. The alpha level was set at $p < 0.05$. Only variables that had a significant effect were retained in the model, and the most appropriate model was identified by using the Akaike's Information Criterion corrected for small sample sizes (AICc). Where models were considered equivalent; *i.e.*, AICc < 2, the model with the fewest parameters was chosen (Burnham *et al.*, 2011). Statistical analyses were performed using R 3.2.5.

2.2 Investigating the detection rates of tagged *A. pallipes ex situ*

Eighteen, wild-caught adult *A. pallipes* crayfish (collected from the River Itchen, Hampshire), which had been maintained in an outdoor aquaculture facility for 9–13 months, were PIT-tagged one week prior to the experiment commencing, using the technique described in method 2.1 (Tab. 2).

An experimental tank (2000 mm × 500 mm × 750 mm) was used and water from the crayfish aquaculture system was added to a depth of 650 mm. The tank was set up in one of five different treatments: (i) bare tank; (ii) tank with gravel substrate (to a depth of 20 mm); (iii) gravel substrate plus pipe refuge (50 mm diameter); (iv) gravel substrate plus brick refuge (50 mm diameter); (v) gravel substrate plus pipe refuge with slate (15 mm thickness) over pipe. The PIT-tagged

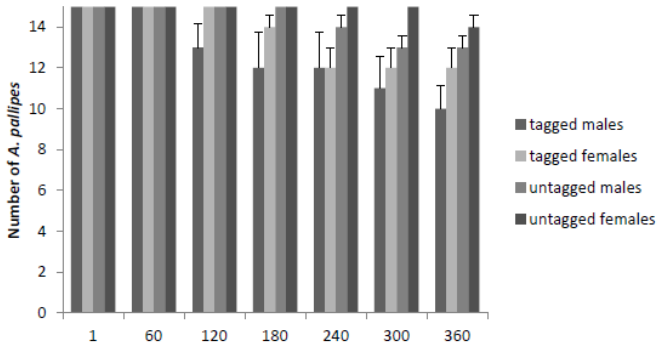

Fig. 1. Survival of PIT-tagged male and female *A. pallipes* compared with untagged males and females, shown throughout time series from day 1 to 360 of experiment, $n = 30$. Error bars represent standard deviations.

crayfish were added, one-by-one, to the experimental tank and the range of detection of each crayfish in each of the different treatments was measured using a single-coil waterproof (IP54 rated) Trovan ANT-610F square antenna attached to a Trovan LID-65 decoder box (RFID Systems LTD, East Yorkshire). The same crayfish was subjected to all five treatments sequentially and then removed and the next crayfish tested. For treatments iii, iv and v, refuges were added and the crayfish was put inside the refuge before the detection range was measured. Both experiments were carried out under Natural England Licence and were ethically approved by the University of Bristol ethics committee and the Bristol Zoological Society Welfare and Research Advisory Board.

2.2.1 Data collection and analysis

The antenna was placed in the tank under the surface of the water and moved towards the crayfish until the decoder box produced an audible noise, signifying that the PIT tag had been detected. The distance between the crayfish and the antenna was then measured to an accuracy of 1 mm using a 300 mm rule. The process was repeated three times for each crayfish and the mean value calculated.

To determine if there were any differences between the PIT tags of variable size and the distance to detection in the five different treatments, data were examined by using linear models (function *lm*, R package lme4, Bates *et al.*, 2015) with normal distribution. The five different treatments, sex and tag size were considered as fixed effects. Model selection was based on the criteria (as outlined in 2.1.1).

3 Results

3.1 Investigating the effect of pit-tagging on growth and survival of *A. pallipes ex situ*

3.1.1 Survival

All crayfish survived up until day 60. From day 60 to 360, several crayfish went missing and were therefore presumed dead, although their bodies/gastroliths were not recovered; the crayfish could not have escaped as the outflows were meshed and the tanks lidded. Eight tagged crayfish died in total, with CLs at the start of the experiment ranging from 22 to 32 mm: males 22–32 mm and females 25–27 mm. Two tagged crayfish died prior to each of the May (day 120), July (day 180) and September (day 240) counts and one tagged crayfish died prior to each of the November (day 300) and final January (day 360) count. Five of the eight crayfish (CL: 25–32 mm) that died had moulted successfully at least once. The other three crayfish (CL: 22–27 mm) died before moulting could be confirmed. From day 180, three untagged crayfish died in total, with starting CL: 22–29 mm. All untagged crayfish had undergone two successful moults prior to death. The two males (CL: 26 mm and 29 mm) died between August (day 180) and November (day 300) and the only untagged female to die (initial CL = 22 mm), was found dead at the end of the experiment, CL = 33.4 mm. Although only one untagged female died in comparison with 5 tagged males, there were no significant effects of sex ($P = 0.31$, d.f. = 56, $z = -1.0$) or tagging (pooled sex: $P = 0.1$, d.f.= 56, $z = 1.63$) on survival rates (Fig. 1).

3.1.2 Growth

CL at the start of the experiment were not significantly different between tanks ($P = 0.16$, d.f. = 56, $F = 1.97$) or treatments ($P = 0.63$, d.f. = 56, $F = 0.24$) but males were significantly larger (mean ± SD) (27.0 ± 2.6 mm) than females (25.0 ± 2.1 mm); ($P < 0.001$ d.f. = 44, $F = 44.88$). After adjusting for the differences in starting length for males and females, the model showed that there was no significant effect of treatment ($P = 0.61$, d.f. = 48, $t = -0.27$) or tank on final CL ($P = 0.48$, d.f. = 46, $t = -0.71$). There was a significant effect of sex; over the course of the experiment, on average the males' CL increased by 8.6 ± 1.1 mm) and the females increased by 7.5 ± 1.5 mm), showing that the males grow faster than the females ($P < 0.001$, d.f. = 46, $t = 3.41$), (Fig. 2).

3.1.3 Moulting events

All moulting events occurred between the end of March (day 60) and the end of September (day 240). First moults had all occurred between day 60 and day 120. Of the tagged crayfish, 90% moulted successfully and it was unconfirmed if the three tagged crayfish that died between counts had already moulted because their bodies were not recovered. Males with

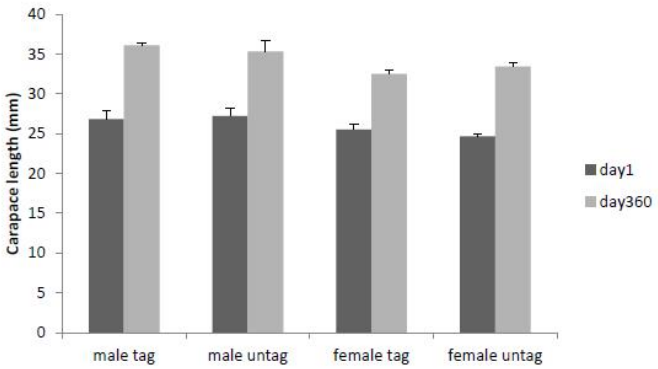

Fig. 2. Carapace length (mm) of PIT-tagged male and female *A. pallipes* compared with untagged males and females, at the beginning (day 1) and end of the experiment (day 360) $n = 30$. Error bars represent standard deviations.

CL < 29 mm moulted three times, and males with CL > 29 mm moulted twice. Females with CL < 26 mm moulted three times, and females with CL > 26 mm moulted twice. There was an inverse relationship between moult increments; *i.e.*, the increase in size of crayfish between moults, and CL and the percentage moult increment decreased as the crayfish grew. Moult increments decreased with an increase in size of crayfish from a maximum of 5.2 mm CL increase (18.8% moult increment) down to a 2 mm CL increase (5.9% moult increment). Females on average grew slower than males at all sizes.

The crayfish were X-rayed at the end of the experiment. In 25% of the crayfish, the PIT-tag had remained within the abdomen, and in 75% of the crayfish, the PIT-tag had moved into the cephalothorax and was positioned close to the dorsal cuticle, adjacent to the hepatopancreas (Fig. 3).

3.1.4 Fecundity

All tagged female *A. pallipes* came into reproductive glair and 80% of the untagged females came into glair, which was visible at the September count (day 240). At the November count (day 300), three tagged and one untagged female had spermatophores present and one tagged and one untagged female had a single fertile egg attached to their abdomens. Both eggs were still present and viable at the end of the experiment (day 360).

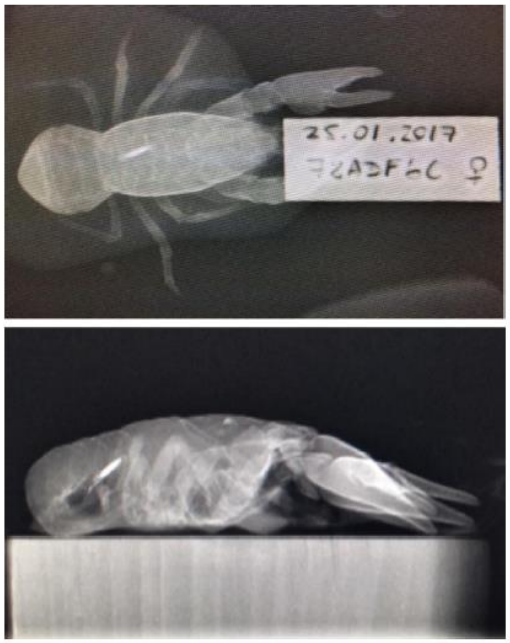

Fig. 3. X-rays of PIT-tagged *A. pallipes* taken one-year post-tagging.

3.2 Investigating the detection rates of tagged *A. pallipes ex situ*

There was a significant difference in detection range between crayfish tagged with 8 mm tags to crayfish tagged with 12 mm tags in all five treatments ($P < 0.001$, d.f. = 79, $t = 9.4$). Crayfish tagged with 12 mm tags were detected by the PIT tag antenna on average 35.6 mm (SD = 3.8 mm) further away than the crayfish tagged with 8 mm PIT tags. There was no significant difference in distance detection rates with bare tanks and tanks with a gravel substrate ($P = 0.55$, d.f. = 79, $t = 0.59$). There was a significant difference between detection range of crayfish in a bare/substrate tank versus within bricks ($P < 0.001$, d.f. = 79, $t = -11.90$), pipe ($P < 0.001$, d.f. = 79, $t = -10.47$) or pipe plus slate ($P < 0.001$, d.f. = 79, $t = -10.37$). There was no significant difference between detection rate of tagged crayfish within bricks versus pipes ($P = 0.16$, d.f. = 79, $t = 1.45$) or bricks versus pipe plus slate ($P = 0.13$, d.f. = 79, $t = 1.53$). There was also no significant difference between pipes versus pipes plus slate ($P = 0.92$, d.f. = 79, $t = 0.10$) (Fig. 4). There was no significant difference in detection rate between males and females ($P = 0.59$, d.f. = 83, $t = -0.53$), or crayfish of different sizes ($P = 0.12$, d.f. = 77, $t = 1.57$) (Fig. 4).

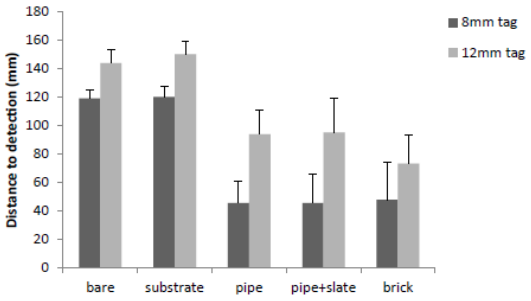

Fig. 4. Comparison of detection ranges of *A. pallipes* tagged with 8 mm or 12 mm passive integrated transponders, within five different tank treatments, $n = 8$. Error bars represent standard deviations.

4 Discussion

The current study found that there was no significant difference in survival, growth or fecundity between *A. pallipes* injected with 8 mm PIT and the untagged control animals. All of the crayfish survived for at least the first 60 days of the experiment, and there was a 100% retention rate of PIT tags. This experiment was conducted with a fairly small number of animals due to the endangered status of the species. It would not have been ethical to tag a large number in case survival was significantly compromised by the procedure.

These experimental findings are supported by previous laboratory trials tagging other crayfish species. In a 50-day experiment undertaken by Wiles and Guan (1993), survival and growth of *P. leniusculus* tagged with 12 mm tags was not significantly different to untagged crayfish if CL > 26 mm. Bubb *et al.*, (2008), undertook a 182-day experiment and found that survival and growth of *P. leniusculus*, CL: 33.7–61.4 mm, tagged with 12 mm tags, was not significantly different to untagged crayfish, (93.3 and 96.7% survival rate respectively). In a laboratory experiment with *O. hylas*, Westhoff and Sievert (2013), randomly tagged 96 crayfish CL: 15.3–33.3 mm, (44 were untagged as a control). They found that mortality dropped below 20% if crayfish CL > 23 mm and CL > 26 mm were tagged with 8.5 mm and 12.5 mm tags, respectively. However, they also experienced a high mortality of untagged crayfish (20%), which may suggest there were underlying husbandry issues that might have masked the results of the experiment.

In a laboratory-based experiment with *P. leniusculus*, Wiles and Guan (1993), used 13 mm tags and injected the tags either at the base of the fourth pereopod or at the first pleopod ventrally into the cephalothorax, close to the internal organs. They observed unusual behaviour after tag insertion with crayfish stretching out legs and or chelipeds after the procedure and in crayfish CL < 25 mm, 47.6% died within ten days. They concluded that the PIT tags caused internal organ damage in the smaller crayfish and temporary reduction in leg movement in the some of the larger specimens. In this current experiment, the PIT tags were injected lower down the crayfish, into the muscle of the third segment of the abdomen, to reduce the risk of damaging internal organs whilst the tag was being inserted (Buřič *et al.*, 2008). The difference in tagging procedure may explain why smaller crayfish could be tagged without survival being compromised, and no temporary reduction in crayfish mobility was observed. The X-rays of the tagged *A. pallipes* taken on day-360 showed that most of the tags had only moved slightly from where they were injected into the abdomen and were positioned either just into the cephalothorax (next to the hepatopancreas) or still just slightly further up the abdomen (Fig. 3).All of the *A. pallipes* in this study survived the first 60 days indicating that tagging does not cause mortality in the short-term, under controlled conditions, and, up to day 180, there were only three deaths (10%) of PIT-tagged individuals. After day 180, similar numbers of tagged (4) and untagged (3) crayfish died, suggesting that these mortality events were not connected to the tagging event. This is supported by Wiles and Guan (1993), who concluded that crayfish mortalities occurring a few weeks after the tagging procedure could not be attributed to the physical tagging procedure because control animals also died.

Mortality events in the current experiment could be linked to moulting. The crayfish were held at a density equivalent of 5.8 /m^2 and therefore, during moulting, they could be susceptible to antagonistic encounters from tank-mates. There was no supplementary feeding during the experiment, which might also have had an effect. Bubb *et al.*, (2008) and Wiles and Guan (1993), housed the experimental crayfish individually and therefore removed any potentially negative effects of tank-mates or competition for resources such as food and shelter.

This study is the first known laboratory trial of PIT-tagging *A. pallipes*. There are several published field studies on PIT-tagged *A. pallipes* released into rivers, which indicate that a large proportion of tagged crayfish survive at least short-term. These field studies did not investigate survival or growth rates of individual crayfish over time or minimal size of crayfish that could be safely tagged, (Bubb *et al.*, 2008; Louca *et al.*, 2014; Stead *et al.*, 2015). Therefore, the current study is the first to illustrate that growth and survival of both sub-adults and adult *A. pallipes* is not compromised by the PIT-tagging procedure.

During this experiment, both tagged and untagged crayfish successfully moulted up to three times from spring through to autumn, and growth was not significantly different between tagged and untagged crayfish. Laboratory studies on other crayfish species also found that growth was not affected by PIT-tagging (Wiles and Guan, 1993; Westhoff and Sievert, 2013). Bubb *et al.*, (2006) found that although growth was not significantly different, the tagged crayfish were 10% smaller than untagged individuals by the end of the experiment; tagged male crayfish were 4.7% larger than untagged males, whereas untagged females were 3.9% larger than tagged females.

In this current experiment, the captive-born crayfish were only 2.5 years old at the beginning of the breeding season, and therefore, were potentially too immature to successfully produce large clutches of eggs. This has also been seen in other groups of 2+year captive-born crayfish (pers. obs.); however, it could be that keeping the crayfish with an equal ratio of males at a density of 5.8 /m^2 caused other males to interrupt mating causing egg loss during egg-laying. The effect of PIT tagging on the reproductive success *A. pallipes* could not be comprehensively established. As all tagged females came into glair, two females produced viable eggs and signs of successful mating were observed it does not appear that breeding activity is impaired. This is supported by Wiles and Guan (1993), who

tagged ovigerous *P. leniusculus*, which then carried their eggs full-term.

This study shows that *A. pallipes* with a CL of 22 mm could be PIT-tagged without survival or growth being compromised. When releasing captive-born crayfish, the individuals will rarely be larger than this size and therefore tagging with larger-sized PIT tags is not a suitable option as this may well compromise survival. When the detection range of the 8 mm tagged crayfish was compared with 12 mm tagged crayfish the average difference in range was marginal (35.6 mm), which indicates that tagging with 8 mm tags is a good compromise. Using a single-coil antenna, the 8 mm tagged crayfish could be detected to a maximum distance of 120 mm when not within refuges, which was reduced to a maximum distance of 60 mm when the crayfish was inside a refuge. Despite detection range limitations, it was encouraging to note that even when the crayfish was within a brick refuge, the PIT-tag antenna could still detect the animal. Larger, multiple-coil antennae are available, which can increase the detection range; however, they are heavier and more expensive. In practice, it can be very difficult to reliably detect crayfish remotely *in situ*, and recapture through trapping is the preferred method. Burnett *et al.*, (2013) compared detection rates and range of detection for tags of sizes 12, 23, and 32 mm. The tags were attached to rocks underwater and the range of detection was an average of 120, 202, and 290 mm, respectively. When tagged fish were tested, detection efficiency significantly increased with size of tag from 55% for 12 mm, 91% for 23 mm and 97% for 32 mm tags. However, 23 mm tags are too big for even the largest of *A. pallipes*, which have a maximum CL of up to 55 mm (Matthews and Reynolds, 1995). The smallest crayfish that were tagged with an 8 mm tag had a minimum CL of 22 mm; *i.e.*, the tag was 36% of the size of the carapace. Working on this principle, if a 23 mm tag to be used, the crayfish would need to have a CL of 63 mm, minimum.

When captive-born crayfish are released into the wild, this is typically done with yearling animals. The ideal minimum size for release is at a CL of 22 mm or above to make them less prone to predation and more likely to breed in the year of release. At this size, it also allows them to be safely PIT-tagged prior to release. However, PIT are still relatively expensive (€1.1/tag); in comparison to other internal markers, such as VIE and CWT, which are considerably cheaper options (a few cents per tag). These other tagging options are suitable for crayfish <22 mm CL, (due to their smaller size); however, they have their limitations. Haddaway *et al.* (2010) tested VIE with *A. pallipes* (CL: 9.5–31.1 mm), and found there was an 87.9% retention rate and a 36.0% tag migration rate over a 103-day period. They did not find a significant decrease in survival; however, survival in both the tagged (64.4%) and untagged (60.0%) groups was fairly low. Gotteland (2013) found no significant difference in survival when using VI Alpha, but experienced 33.0% mortality during a 60-day trial using VIEs with *A. pallipes*. In an experiment with the American lobster, *Homarus americanus* (H. Milne Edwards, 1837), McMahan *et al.*, (2012) fitted CWTs and had an average 96% retention rate, CL: 12–30 mm. Where large groups of crayfish require a group identification system at low cost, VIE could potentially be a solution. However, with this technique, identification of individual live animals would be difficult, tag retention is not 100%, there is an issue with tag migration and therefore, long-term tag visibility.

In conclusion, *A. pallipes* can be tagged with 8 mm PIT at a minimum CL of 22 mm, without survival or growth being compromised., Care must be taken to ensure that the tag is injected into the muscle of the second or third segment of the ventral abdomen so that there is no risk of damaging internal organs during the insertion process. Although *A. pallipes* tagged with 8 mm PIT tags are detected at reduced distances to those with 12 mm PIT tags (mean difference 35.6 mm) this drawback is outweighed by other benefits conveyed by using the smaller tag. An 8 mm tag allows captive born, sub-adult crayfish to be tagged, prior to release, and reduces the risk of internal organ damage to crayfish irrespective of size. Although PIT-tagging is more expensive than other internal marking methods, it currently offers the only 100% reliable method of permanently identifying individual animals and detecting them without having to recapture the individuals.

Acknowledgements. This work was funded by the Centre for Environment, Fisheries and Aquaculture Science, Mohamed bin Zayed Species Conservation Fund and Bristol Zoological Society. Thank you to Bristol Water for providing an aquaculture facility for the research. Thanks to Nathan Edmonds from the Centre of Environment, Fisheries and Aquaculture Science, Lowestoft, UK for assistance with this experiment.

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Cite this article as: Nightingale J, Stebbing P, Taylor N, McCabe G, Jones G. 2018. The long-term effects and detection ranges of passive integrated transponders in white-clawed crayfish *Austropotamobius pallipes*. *Knowl. Manag. Aquat. Ecosyst.*, 419, 20.