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EFFECT OF ENVIRONMENTAL TEMPERATURE ON ASPECTS OF THE DEVELOPMENTAL ECOPHYSIOLOGY OF THE SEMI-TERRESTRIAL AMPHIPOD ORCHESTIA GAMMARELLUS

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Ellen Tully, 2020

EFFECT OF ENVIRONMENTAL TEMPERATURE ON ASPECTS OF THE DEVELOPMENTAL ECOPHYSIOLOGY OF THE SEMI-TERRESTRIAL AMPHIPOD *ORCHESTIA GAMMARELLUS*

By

ELLEN TULLY

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Research Masters has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

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ABSTRACT

THE EFFECT OF ENVIRONMENTAL TEMPERATURE ON ASPECTS OF THE DEVELOPMENTAL ECOPHYSIOLOGY OF THE SEMI-TERRESTRIAL AMPHIPOD *ORCHESTIA GAMMARELLUS*

BY ELLEN TULLY

Ecophysiological responses vary during development in ways that may determine the ecological and evolutionary consequences of climate change. How temperature alters processes vital to organismal homeostasis (e.g. cardiac and gastro-intestinal (GI) behaviour) and survival (e.g. thermal tolerance) at different developmental stages is underdeveloped. Consequently, my thesis investigated key aspects of the thermal biology (specifically cardiac output, the ontogeny of GI behaviour and upper thermal limits) of an ecologically-important, brooded species, the semi-terrestrial amphipod *Orchestia gammarellus*. Little is known of the earliest embryonic responses, particularly with respect to direct developing brooded species. This is in-part due to a lack of high-throughput technologies capable of visualising stages of early development. This was achieved by firstly developing a platform for autonomous image acquisition and analysis, Embryophenomics, subsequently used to investigate effects of temperature on the ecophysiology of *O. gammarellus*. The thermal history of embryos *in situ* was recorded during the reproductive period and used to inform incubation temperatures. A relatively small increase in temperature (5 °C) greatly increased the rate of GI movement and yolk usage throughout development, supporting the hypothesis that the gut is an engine for yolk

dispersal. However, there was little support for the hypothesis that GI movement was stimulated by hypoxia and therefore the gut does not appear to have the precardiac function found in other crustacean embryos. This is the first qualitative and quantitative investigation of changes in GI behaviour with ontogeny in any crustacean, adding to previous descriptions of development generally. Despite being in a thermally stable environment *in situ* there are profound changes in upper thermal limits of embryos throughout development. This does not fit the current paradigm that thermal tolerance is necessarily governed by selection pressure. Overall the ecophysiological responses to temperature of *O. gammarellus* would not have been predictable based on the life history and thermal environment of the embryos.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

"No panegyrist of the Amphipoda has yet been able to invoke anything like popular enthusiasm in their favour"

-Rev Thomas R. R. Stebbing, 1899

1.1 Developmental ecophysiology

Ecophysiology is the study of how the environment, both physically and biologically, interacts with physiological processes at the organismal level (Nature, 2020). Over the last few decades, the field of ecophysiology has aimed to bring mechanistic understanding to ecological change by applying an ecological and evolutionary context to physiological study. It has done so either under the name ecophysiology or its *alias*' – conservation physiology (Wikleski and Cooke, 2006; Homyack, 2010; Cooke and O'Connor, 2010; Coristine *et al*., 2014), comparative animal physiology (CAP) (Withers, 1992; Prosser, 1991) and more recently comparative developmental physiology (CDP) (Burggren, 2005; Warburton *et al*., 2006).

Ecophysiological studies of early life-stages lag significantly behind that of adults despite the importance of juvenile forms to adult traits and survival. The interaction between environment and physiology during ontogeny may produce traits that are advantageous to juvenile forms but disadvantageous to adults, highlighting the definitive need of life-history studies for the integration of physiology and evolution. Developmental ecophysiology attempts to unite aspects of development, physiology and evolution and is simply described by Burggren (2005) as "how developing animals work" in an ecological and

evolutionary context. It is widely recognised that physiological processes at the "organismal level" are not static and vary considerably through-out ontogeny (Adolph, 1968; Spicer and Gaston, 1999; Warburton *et al*., 2006) for a variety of vertebrate and invertebrate species of oviparous and viviparous reproductive nature. External environmental factors, such as temperature, oxygen and salinity, can act on critical periods of development to alter gene expression, resulting in phenotypic variation (Burggren and Warburton, 2005; Warburton *et al*., 2006; Rundle and Spicer, 2006; Spicer and Rundle, 2007). Phenotypic expression of embryonic and larval stages could have consequences for growth, survival and fecundity of later life-stages (e.g. Harrison *et al*., 2011) and influence population dynamics through altered time taken to reach sexual maturity, reproductive success and survival.

Arguably the impact of changing environmental conditions on early developmental stages is as, if not more important, than the adult stage. Yet, embryonic and larval forms remain severely underrepresented in ecophysiological studies (Pandori and Sorte, 2018). This is, in part, due to the lack of automated high-throughput technologies capable of visualising and measuring dynamic stages of early development. The few technologies capable of high content screening of early life-stages are costly, tend to be restricted to specific aspects of the phenotype and are non-transferable between species (Houle, 2010; Rozenblatt-Rosen *et al.* 2017) *Embryo Phenomics* is a high-throughput open source platform that allows automated phenotypic data collection and processing for aquatic embryos (Tills *et al.*, 2018), comprised of OpenVIM, an open source video microscope, and EmbryoCV, an analytical software (Chapter 2). The Embryophenomics platform has so far been used to

assess the response of a variety of species as disparate as jellyfish (Tills *et al*., 2016), amphipods (Tills *et al.*, 2018) and gastropods (Tills *et al.*, 2013, 2018; Truebano *et al.*, 2018) to multiple abiotic environmental drivers on several temporal scales. Thus, it has provided an ideal technological platform for the exploration of developmental ecophysiology.

As we progress into the Anthropocene (IPCC, 2018), ecological consequences of global warming will likely be governed by varying ontogenetic responses to elevated temperature (Kingsolver *et al*., 2011; Radchuk *et al.,* 2012; Carlo *et al*., 2017). Temperature has a profound effect on biological function for all levels of biological organisation i.e. from biological molecules to entire ecosystems (Hochachka and Somero 2002). At a biochemical level, an increase in environmental temperature will result in increased rates of reaction, and at more acute temperatures, protein denaturation (Hochachka and Somero, 2002) and the production of heat shock proteins (HSP), all at an energetic cost to the individual (e.g. Krebs and Feder, 1997). Embryonic stages have tighter energy constraints than adults as approximately 80 % of their energy budget is used for growth (Warburton *et al*., 2006). Oviparous species are energetically closed systems, provisioned with a finite amount of energy (yolk) at conception. Therefore, the costs incurred by both chronic and acute thermal conditions may prove more damaging to egg laying species than species with other reproductive modes. At an organismal level, elevated temperatures are known to increase the overall rate of development (Spicer and Burggren, 2003), which has been documented to have deleterious effects in some species (e.g. Temple *et al*., 2001; Pankhurst and King, 2010). Altered developmental rates extend to

timing of sexual maturation with implications for fecundity, recruitment and mortality ultimately affecting population dynamics with ecological repercussions.

So far, much attention has been focused on “model organisms” such as the fruit fly *Drosophila melanogaster* and zebrafish *Danio danio*. Major advances in modern biology have stemmed from these models and the intensive research for which they have been a focus. However, lessons can be learned from “exotic animals”, those not traditionally utilised when addressing questions of intra- and inter-specific physiological diversity. The vast majority of what we know about interactions between temperature and physiological variation at the organismal level, particularly of those with complex life-histories, is derived from non-brooding species (i.e. species that develop, either directly or indirectly, outside the maternal/paternal environment). Ontogenetic variation in physiology of non-brooding species has largely been related to changes in selection pressures (e.g. Bowler and Treblanche, 2008 and references therein), particularly in traits such as thermal tolerance - as individuals experience different thermal regimes (e.g. Miller *et al*. 2013) and develop varying capabilities to behaviourally avoid thermal extremes (Bowler and Treblanche, 2008; Radckuk et al., 2012) throughout ontogeny. When drawing potential patterns and generalities of aspects of developmental physiology, limiting studies to just non-brooding modes of reproduction restricts our ability to make informed prediction about species with other reproductive modes. Since ecophysiological studies ideally require an ecologically important model species (Warburton *et al*., 2006), one of the groups that may meet all of the model species criteria are the globally-distributed, ecologically-important talitrid amphipods.

1.2 Why talitrid amphipods?

The Order Amphipoda is comprised of approximately 10,000 described species divided into six suborders: Pseudingolfiellidea, Hyperiidea, Colomastigidea, Hyperiopsidea, Senticaudata (described in Lowry and Myers, 2013) and Amphilochidea (Lowry and Myers, 2017). Around 81 % of all amphipod species occur in marine and estuarine habitats. Only 3 %, all within the parvorder Talitridira, have successfully colonised the supralittoral and terrestrial realms (Lowry and Myers, 2013, 2017; also see Spicer *et al*.,1987; Lincoln 1979; Morritt and Spicer, 1998)

Talitrids brood their young within a ventral pouch known as the marsupium – formed of ventral thoracic segments and oostegites, interlacing plate-like projections that stem from the medial surfaces of the thoracic coxae (Morritt and Spicer 1996a,b; Browne *et al.* 2005). The marsupium in semi-terrestrial species such as NW European *Orchestia gammarellus* provides a semi-closed, maternally-controlled aquatic environment (Spicer and Taylor, 1994; Morritt and Spicer, 1996a,b, 1998, 1999; Morritt and Richardson 1998) in which embryos directly develop into hatchlings and are maintained until full physiological competency is achieved (Morritt and Spicer, 1999). The female manipulates this aquatic environment by redirecting urine, secreted from antennal glands, along facial and ventral groves (Morritt and Spicer, 1996b; shown diagrammatically for males in Spicer and Taylor, 1994) into the marsupium. Maintaining the brood in a solution 1/3rd the strength of seawater (S = 11) (Morritt and Spicer, 1996b). This passive brood care enables females to raise young in dilute salinities (10

% sea water) (Morritt and Stevenson, 1993) which they would otherwise be unable to tolerate (Morritt and Spicer, 1996a,b, 1999). Similar marsupial fluid control was found in the euterrestrial amphipod, *Mysticotalitrus cryptus* (Morritt and Richardson, 1998). There are other behaviours within the Amphipoda more generally; both marine (Dick *et al*., 2002) and freshwater (Dick and Elwood, 2006; Dick *et al.*, 1998) species have been shown to exhibit active brood care. The freshwater amphipod *Crangonyx pseudogracilis*, uses abdominal flexing, beating of the pleopods and physical rotation of the eggs within the brood to maximise ventilation of the young (Dick *et al*., 1998). No such behaviour has been documented for semi-terrestrial or terrestrial talitrids, but that is not to say that it does not occur.

This maternally provisioned brooded system does not only serve to benefit our understanding of amphipod biology generally but also to lay a foundation for comparative developmental ecophysiological study of other brooded species currently underrepresented in the literature. Our lack of knowledge of the response of talitrids to elevated temperatures restricts our ability to accurately predict the response of this ecologically important group (sect. 1.4) to local temperature change and to global climatic change. Despite detailed notes of development of few amphipod species (Browne *et al.*, 2005), the impact of temperature on the timing of key developmental events, the form and function of organs and physiological processes remains largely unknown for members of the Talitridae. The study of developmental ecophysiology under current day and future climate projections (IPCC, 2018) should inform our understanding of the potential biological impacts of climate change on this ecologically important species.

Thermal tolerance, commonly used to assess species vulnerability to climate change, is known to vary throughout ontogeny (e.g. Truebano *et al*., 2018). Thermal tolerance estimates have been established for some adult talitrid species (e.g. Moore and Francis, 1986a) but, to my knowledge, do not exists for earlier developmental stages. Intertidal and supralittoral species are thought to be living at their upper thermal tolerance limits and are therefore expected to be particularly vulnerable to climatic warming (Stillman, 2003; Somero, 2010; Nguyen *et al*., 2011) due to an inability to extend these limits *via* acclimation (see Appendice 1). We urgently need to understand the key effects of temperature on aspects of developmental ecophysiology of an abundant, ecologically important supralittoral semi-terrestrial member of the Talitridae.

1.3 Aims and objectives

The aim of my thesis is to investigate aspects of the thermal biology (specifically the ontogeny of gastro-intestinal (GI) movements and upper thermal limits) of an ecologically important widespread and abundant brooded talitrid amphipod during early development. *Orchestia gammarellus* was identified as an ideal model for the reasons outlined below (sect. 1.4).

To help achieve this aim I engaged in a collaboration to develop a bioimaging technique, *Embryo Phenomics*, which formed an integral part of my data acquisition and analysis. Therefore, **Chapter two** aims to introduce *Embryo Phenomics*, an open-source high throughput platform for phenomics in aquatic embryos. This consists of an Open-source Video Microscope (*OpenVIM*), with experimental control over the embryonic environment, and a Python package *Embryo Computer Vision* (*EmbryoCV*) for high-dimensional measurement of phenomic traits from video datasets.

My objectives are to demonstrate how *Embryo Phenomics* (1) enables the quantification and integration of inter- and intra-individual temporal change in morphological, physiological and behavioural traits with high resolution and to an unprecedented scale, in a largely automated workflow; (2) can be used to quantify integrated organismal responses to environmental change, using experiments assessing: i) chronic; ii) acute; and iii) interactive effects, of environmental stressors.

Chapter three aims to document the ontogeny and development of the different GI movements in embryonic talitroidean amphipods at different, ecologically relevant environmental temperatures. I test the hypothesis that embryonic gut movements in talitroidean amphipods function as an extracardiac pump, dispersing yolk to growing and developing embryonic tissue, and aiding in the delivery of oxygen to developing tissues.

If gut movements are related to yolk dispersal then (1) it should be possible to visualise yolk release and relate it to particular gut movements; (2) tracing the subsequent movement of the released yolk in the embryo should be possible; (3) an increase in environmental temperature should change the frequency and/or character of gut movements to release more yolk to meet heightened metabolic demand. If gut movements are related to oxygen demand, then (1) acute exposure to environmental hypoxia should result in a corresponding increase in the frequency of gut movements to enhance circulatory function during this period of heightened demand and (2) circulation should be more pronounced at higher temperatures.

These predictions will be tested by (1) first describing the ontogeny and nature of the gut movements from their first appearance through to hatching, (2)

quantifying rates of gut movements in eggs cultured at two different ecologically realistic environmental temperatures at different times during development and (3) determining visually whether, and how frequently, yolk is extruded from the gut and once released where it goes in the embryo; (4) testing whether the rate of gut movements increase in response to environmental hypoxia. The rationale for, and temperatures chosen in, this experiment were informed by some preliminary work determining the microhabitat temperatures experienced *in situ* by talitroidean amphipods during their breeding season presented in section 1.4.

Chapter four aims to provide the first investigation of ontogenetic variation in upper critical thermal limits (CT_{max}) and thermal sensitivities (z) of any talitroidean species in the context of improving understanding of what drives ontogenetic shifts in thermal tolerance in a brooded system.
This shall be achieved using a static assay technique and model framework pioneered by Rezende et al. (2014) whereby the time taken for different developmental stages to reach lethal end points at different static temperatures is recorded and used to plot thermal death time (TDT) curves. TDT curves will be used to predict the critical thermal maximum (CT_{max}) and thermal sensitivity (z) of each developmental stage. Thermal tolerance landscapes will detail survival probability of each stage to extreme thermal challenge. Natural history observations made by myself and colleagues shall be discussed as a backdrop to the interpretation of the laboratory results.

Appendice 1 is broadly related to the central theme of temperature effects on semi-terrestrial talitroidean amphipods but deviates from the focus on the early

developmental ecophysiology of the chosen model species, *O. gammarellus* (sect. 1.4) and is therefore not included as a formal thesis chapter. It does, however, provide valuable insight into other aspects of the effect of temperature, specifically low temperature acclimation, on the ecophysiology of talitrids and has therefore been included as an appendice.

The study determines whether the talitrid sandhopper *Deshayesorchestia* (formerly *Talorchestia*) *deshayesii* has the ability to acclimate to temperature change. John Spicer (University of Plymouth) and I, together with our international collaborators Dr. Ursula Janas and Ms Marta Tykarska (University of Gdańsk) tested two hypotheses. First that torpor (inactivity) is a consequence of not being able to acclimate, and that in a warming world the temperature at which torpor is induced will be unchanged and so the incidence of torpor will decline. Second, that the ecosystem service of strandline decomposition, to which talitrids are a major contributor, will change as a consequence of an inability to acclimate, and that in a warming world not only will there be more time for feeding but that feeding activity will increase, i.e. an inability to acclimate will mean that feeding rate will increase proportionally with an increase in temperature.

1.4 Why *Orchestia gammarellus?*

"The context of ecophysiology is ecology, so the model species chosen must be ecologically important" (Warburton *et al*., 2006). *Orchestia gammarellus*, in common with many other supralittoral talitrids, is an ecologically important member of the wrack fauna and can be found under loose stones and cast up

wrack of shores and estuaries throughout the Atlantic coast of North America and Europe; from the coasts of Norway (Lincoln, 1979), the hot springs (Morritt and Ingólfson, 2000) and southern coast of Iceland (Ingólfsson, 1977; Henzler and Ingólfson, 2008) to the Mediterranean (Lincoln, 1979). In some instances, *O. gammarellus* has been observed to live a completely terrestrial existence, residing 120 m above sea-level on St Kilda, Scotland (Bagenal, 1957). Omnivorous detritivores (Bowers, 1964; Moore and Francis, 1985), they are largely responsible for the breakdown of organic material and subsequent flux of nutrients into the intertidal food web and inshore waters (Stenton-Dozey and Griffiths, 1983; Lastra *et al*., 2008). Details of *O. gammarellus* feeding preferences are available (Moore and Francis, 1985) but rates and quantities of wrack processing by this species are few (e.g. Marsh, 2007) Such figures do exist for talitrids more broadly, for example of fifteen sandy shore sites in California, talitrid amphipods, *Megalorchestia* spp., were the most abundant wrack-associated species at all but three sites with a mean abundance of 85 to 10,200 individuals. m^2 (Dugan *et al*., 2003). On South African shores, talitrids contributed as much as 90 % to the total biomass of wrack-associated fauna (Stenton-Dozey and Griffiths, 1983), responsible for up to 50 % of Kelp consumption at some sites (Griffiths *et al*., 1983). *Orchestia gammarellus* is one of the most abundant faunal species associated with the supralittoral strandline (Jones, 1948) and undoubtedly plays a key role in nutrient cycling in both semi-terrestrial and in some instances terrestrial environments.

The breeding period of *Orchestia gammarellus* is primarily governed by temperature, with the exception of thermal sites in Iceland where photoperiod is thought to have a more critical role (Ingolfson *et al*., 2007). In temperate

regions, the species breed over spring and summer months. In Millport, Scotland, breeding initiates towards the end of April (air temperature ~ 7 °C) and ceases in October (air temperature ~ 10 °C) (Moore and Francis, 1986b). Similar observations were made by Morritt and Stevenson (1993) and Persson (1999) whom observed ovigerous females between the months of May - September on the shores of the Isle of Cumbrae, Scotland and Kalmar Sound, Southern Baltic Sea respectively. Whereas those at the more southern end of their range reproduce year-round (e.g. Dias and Sprung, 2003). Breeding in this species has been shown to be induced by temperature alone, illustrated by Morritt and Stevenson (1993) whom induced breeding by incubating winter populations at T = 15 °C. Elevated temperature is also known to shorten both the time taken to reach sexual maturity (Ingólfson *et al*., 2007) and the gestation period of this species (Tully, unpubl. obs.). The study of developmental ecophysiology under current day and future climate projections (IPCC, 2018) will help us understand the potential biological impacts of climate change on this ecologically important species. Information on the species natural thermal environment is essential to provide ecological context to laboratory results (Warburton *et al*., 2006, p.163). Unfortunately such information is absent from many similar studies.

In addition to its ecological importance and availability of thermal breeding history, *O. gammarellus*, was chosen as our model because (1) it is similar, and closely-related to, the widely used talitroidean model species, *Parhyale hawaiensis;* (2) the eggs are larger and easier to manipulate than *P. hawaiensis* and successful culture techniques have been developed for *O. gammarellus* eggs *in vitro* (Morritt and Spicer, 1996b) (3) the eggs develop and show good survival

in the bioimaging apparatus used to track embryonic development in invertebrates (see Chapter 2).

1.5 Thermal biology of strandline environment

The strandline environment refers to the line of seaweed and other marine debris discarded by the tide above the highwater mark. This dynamic environment varies in physical and chemical properties (Bucklund, 1945; Marsh, 2007), despite its complex nature, there are general traits characteristic of all strandline habitats. The wrack habitat buffers temperature oscillations to create an environment that increases in thermal stability with depth (Moore and Francis, 1985). Even shallow wrack habitats have been shown to significantly buffer thermal extremes (Marsh, 2007), creating a more stable thermal environment than the surrounding beach. Thermal measurements of the wrack environment have been conducted down to depths of 30 cm in some cases (e.g. Moore and Francis,1985; Marsh, 2007). Few studies link actual thermal habitat measurements with organismal biology. Therefore, to provide an ecological backdrop to the laboratory data presented in Chapters 2 - 4, I recorded the temperature of the strandline environment adjacent to Coxside Marine Station (Plymouth, UK) from the surface to a depth of 10 cm. This was the mean depth where ovigerous females were found quiescent during the breeding season (late Feb - early Oct 2019).

Temperature sensors (HOBOware, U23-004 Pro V2 Temp Logger with 1 x External Waterproof Sensor, accuracy ± 0.21 °C), positioned throughout the females occupied habitat (Fig. 1.1), collected environmental temperature data

(°C) at 15 min intervals for two weeks during spring (27th Feb - 13th Mar 2019 (Fig. 1.2 A)) and summer (31st May - 13th June 2019 (Fig. 1.2 B)) to establish the thermal conditions experienced by adults, eggs and juveniles during the breeding season.

Figure 1.1 HOBOware temperature sensor set up: four sensors secured to a steel rod in air 5 cm above the wrack, on the wrack surface (0 cm) and at 5 cm and 10 cm depth into the wrack. (Photograph by E. Tully).

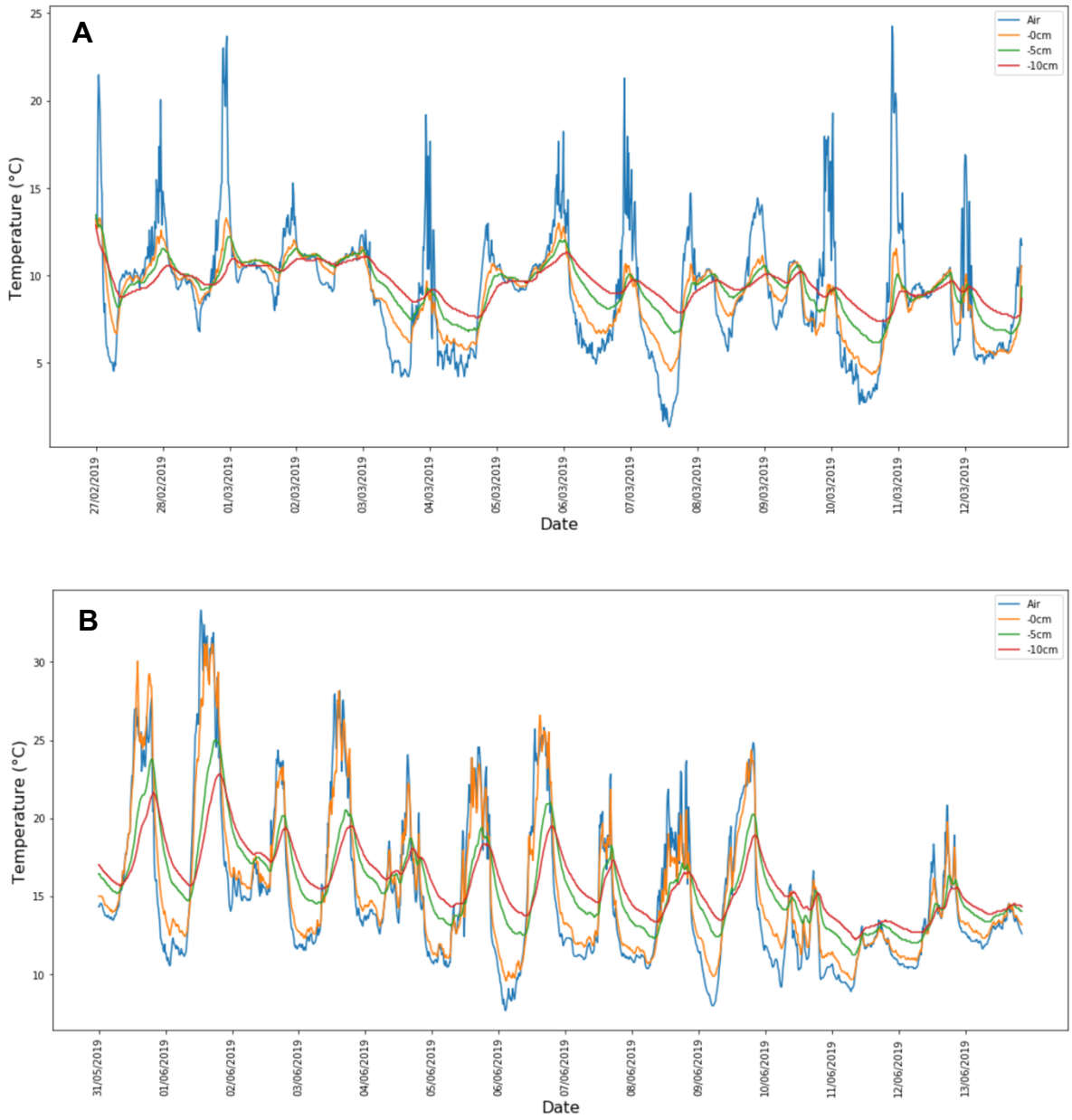

Figure 1.2 Temperature of strandline environment during A) Spring (27th Feb - 12th Mar 2019) and B) Summer (31st May - 13th June 2019).

Thermal depth profile curated using temperature data collected by HOBOware temperature sensors in air 5 cm above the surface (blue), at 0 cm on the surface (orange), 5 cm (green) and 10 cm (red) depth into the wrack habitat for two weeks during A) Spring (27th Feb - 12th Mar 2019) and B) Summer (31st May - 13th June 2019).

The strandline habitat buffers *O. gammarellus* from the widely fluctuating air temperatures, producing an environment that increases in thermal stability with depth. Diurnal temperature fluctuations decreased from T = 20 °C and 25.6 °C at the surface to T = 5 °C and 14.3 °C at 10 cm depth, during spring and summer respectively. During intermittent observations made *in situ* over the course of the breeding season (11:00 – 15:00, Feb - Oct 2019), no females were observed on the surface. During spring, torpid (inactive) and active ovigerous females were found between 1 – 10 cm depth with the greatest abundance between 5 and 10 cm (~ 2 per 5 cm^3). Ovigerous females were more abundant (~ 5 per 5 cm^3), more active and more evenly distributed throughout the sediment during summer months. Naturally the number of juveniles increased throughout the summer season, their abundance is difficult to estimate since females often eject premature hatchlings from the marsupium when disturbed (Tully and Spicer, unpubl. obs.).

CHAPTER 2: A HIGH-THROUGHPUT AND OPEN-SOURCE PLATFORM FOR *EMBRYO PHENOMICS*: TALITRID AMPHIPODS AND GASTROPOD MOLLUSCS AS AQUATIC MODEL ORGANISMS

Much of this chapter was published as Tills, O., Spicer, J.I., Grimmer, A., Marini, S., Jie, V.W., **Tully, E.** and Rundle, S.D., 2018. A high-throughput and open-source platform for embryo phenomics. *PLoS biology*, *16*(12), p.e3000074.

E. Tully contributed to the testing of the concept, ground truthing the method, carried out the experiments using *Orchestia gammarellus* and contributed to the writing of the manuscript.

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2.1 ABSTRACT

The paucity of high-throughput technologies for the generation of phenomic data is a serious bottleneck in Biology, especially for the investigation of dynamic processes such as embryonic development. Presented here is a high-throughput platform for phenomics in aquatic embryos comprising *OpenVIM*, an open-source video microscope, and *EmbryoCV*, a Python package for automated phenotyping of embryos. This is *Embryo Phenomics,* a modular platform that enables the quantification of inter- and intra-individual temporal change in the phenome of developing aquatic embryos with unprecedented resolution, incorporating morphological, physiological, behavioural and proxy traits. The broad-scale applicability of *Embryo Phenomics* is demonstrated in a range of chronic and acute experiments, where phenomic data are produced for > 600 embryos generated from video comprising > 30 M images. While the challenges of phenomics are significant, the rewards are substantial. *Embryo Phenomics* can acquire and process data capturing functional, temporal and spatial responses in the earliest, most dynamic life stages and is game-changing for those studying development, and phenomics more broadly.

2.2 INTRODUCTION

2.2.1 Phenomics

Phenomics is the acquisition of high-dimensional phenotypic data on an organism-wide scale (Houle, 2010). It is both analogous and complementary to genomics and has a similar capacity to facilitate advances in Biology. However, in many ways the challenges of phenomics dwarf those faced at the advent of modern genomics (Bilder, 2008; Rozenblatt-Rosen *et al.,* 2017). The information content of phenomes is far greater than genomes (Houle *et al*., 2010; Kültz *et al*., 2013) and, whereas genomics can benefit from standardised tools and technologies that are readily applicable to different species and scales of study, phenomics requires the development and application of less generic approaches. Consequently, the dearth of transferrable technologies for high-dimensional acquisition of phenomic data remains the greatest bottleneck to the advancement of phenomics and is recognised as a key challenge in Biology (Houle *et al*., 2010; Rozenblatt-Rosen *et al*., 2017)

Technologies for acquiring high-resolution phenomic data enable studies that use combinatorial analyses unimaginable using manual approaches (Mezey and Houle, 2005). Such analyses permit the relationships and interactions among multiple traits to be explored and integrated and, for phenomic data, have greater discriminatory and explanatory power than univariate approaches, thereby providing novel biological insight (Alexandrov *et al.*, 2016; Pound *et al.*, 2017). High dimensional phenotyping also enables the identification and measurement of ‘proxy’ traits – phenome-level measures that are not detectable

using manual observation (Kültz *et al.*, 2013). In plant root phenomics, such proxy traits have been shown to possess strong discriminatory power and include automated traits with natural manual trait equivalents, and novel measurements for which there was no manual trait equivalent (Pound *et al.*, 2017). Another key asset of datasets acquired using high-throughput phenomic technologies is the ability to incorporate biological complexity in the interrogation of a particular experimental response. For example, analysis of phenomic data for mice identified a combinatorial signal from > 200 phenotypic traits that was predictive of a Huntingdon disease genotype (Alexandrov *et al.*, 2016). Such studies point to the power of phenomics to transform our understanding of phenome-level response, *via* the development and application of appropriate technologies.

2.2.2 Techniques for acquiring phenomics data

The rewards of phenomics will arguably be greatest when applied to quantify aspects of the phenome for which current methods are most limiting. Embryonic development is a dynamic process with high levels of intra-individual temporal, spatial and functional change meaning that traditional approaches to its quantification at the whole-organism level can never be thorough. Furthermore, in addition to high levels of intra-individual change, embryonic development also exhibits high levels of inter-individual variation (Tills *et al.*, 2013) and therefore one approach to its study is to adopt longitudinal observation of large numbers of embryos (Spicer and Gaston, 1999). This approach necessitates compromises in: i) the number of embryos studied; ii) the number of phenomic measures quantified; iii) the precision with which measurements are made; and

iv) the temporal resolution of measurements. Such compromises occur not only because of the highly dynamic nature of biological development, but due to the complexity and interconnectedness of biological responses through time. Therefore, automated technologies with the capability to make measurements through development offer huge potential to alleviate a major bottleneck in the application of phenomics.

There are examples of where automated longitudinal observation of developing embryos has been used effectively for commercial applications. Automated non-invasive imaging and analysis of cytokinetic patterns in early stage cell-cleavages in human embryos can predict survival to the blastocyst stage with > 93 % sensitivity (Wong *et al.*, 2010). Consequently, time-lapse imaging is now routinely offered as an option in IVF treatments to select embryos for implantation on the basis of early cytokinetic parameters significantly increasing success rates (Fishel *et al.*, 2017).Technologies used for non-human, high-throughput screening include the ImageXpress system for widefield cellular imaging (Leet *et al.*, 2014; Lantz-McPeak *et al.*, 2016) and the Ethovision platform (Noldus *et al*., 2001), for use with zebrafish embryos and larvae, with a focus on tracking (Horzmann *et al.*, 2017). A limitation of commercial platforms remains their transferability and applicability to different species, research questions and study designs. Consequently some laboratories are turning to self-build, open-source solutions and open-source hardware (Pitrone *et al.*, 2013; Baden *et al.*, 2015) and software (Gentleman *et al.*, 2004; Schindelin *et al*., 2012) are becoming increasingly central to Biology. They have advantages including greater opportunities for educational engagement (Jahr *et al.*, 2016), accelerated innovation, reduced cost, reduced

redundant problem solving in different laboratories and more rapid advancement of scientific discovery *via* a greater return on investment (Pearce, 2015). Such accessible technologies for high-throughput phenomics during the dynamic and sensitive process of embryonic development are urgently required, particularly within the context of assessing the impacts of global change (Kültz *et al.*, 2013).

2.2.3 Aim and Objectives

The aim of this chapter is to introduce *Embryo Phenomics*, an open-source high throughput platform for phenomics in aquatic embryos that consists of an Open-source Video Microscope (*OpenVIM*), with experimental control over the embryonic environment, and a Python package *Embryo Computer Vision* (*EmbryoCV*) for high-dimensional measurement of phenomic traits from video datasets.

The objectives are to demonstrate how *Embryo Phenomics* (1) enables the quantification and integration of inter- and intra-individual temporal change in morphological, physiological and behavioural traits with high resolution and to an unprecedented scale, in a largely automated workflow; (2) can be used to quantify integrated organismal responses to environmental change, using experiments assessing: i) chronic; ii) acute; and iii) interactive effects, of environmental stressors.

These two objectives will be achieved by using two ecologically-important species with radically different patterns of development: *Orchestia gammarellus*

and *Radix balthica*. Details of *O. gammarellus* ecological significance is detailed in section 1.5. *R. balthica* is a freshwater gastropod mollusc and has been used extensively in developmental ecophysiology studies (Salinger *et al*., 2009; Tills *et al*., 2011; Spicer *et al*., 2011; Rundle *et al*., 2011).

The effect of temperature on *O. gammarellus* embryos detailed in this chapter and in chapters 3 and 4 relied on the use of the Bioimaging system and *Embryophenomics* approach described here. In this chapter, the technology is applied to look at changes in heart rate during early development when *O. gammarellus* is reared at two ecologically realistic temperatures. Chapters 3 and 4 utilise this technology to investigate the effects of temperature on gastro-intestinal movements and yolk usage (Chapter 3) and changes in upper critical thermal maximum temperature (Chapter 4) both through embryonic development.

2.3 MATERIALS AND METHOD

2.3.1 *OpenVIM*: Open-source video microscope

Image acquisition in the *Embryo phenomics* platform is achieved using *OpenVIM*, a modular, custom assembled, video microscope that enables long-term automated image acquisition of large numbers of aquatic embryos. An *OpenVIM* is modular and can incorporate a range of different components, enabling a wide range of operating parameters (see original publication - Tills et al., 2018). The *OpenVIM* configuration applied in the current studies is a Charge Coupled Device camera (monochrome, resolution: 2048 x 2048; Pike

421B, Allied Vision Technology, Stradtroda, Germany) connected to high-depth of field optics (magnification: 20 – 200 x) VHZ20R, Keyence, Milton Keynes, UK) inverted and mounted in an aluminium frame atop which an XY motorised stage (Scan, Marzhauser, Wetzlar, Germany) is fixed (Fig. 2.1). The motorised stage houses an incubation chamber (T range: min = 10 – 15 °C below ambient, max = 60 °C; Bold Line Cryo, OkoLab, Naples, Italy) for multiwell plates, providing temperature control and high levels of humidification to minimise evaporation. Dark field lighting is provided by an LED ring light (LDR2-42-SW2, CCS, London, UK) mounted above the incubation chamber. The camera and motorised stage are synchronised using the ImageJ (Schindelin *et al*., 2012) plugin MicroManager (Edelstein *et al*., 2014).

Image acquisition is performed using the Multidimensional Acquisition function in MicroManager. A sequence of images is acquired of each individual embryo in succession and this process is repeated for the duration of the experiment using a Beanshell script. Images are stored as individual sequences of TIFF format 16 bit images with accompanying metadata and these are written to 6 TB hard drives (ST6000DM004, Seagate, Dublin, Ireland) using a hard drive enclosure (TeSU, DATOptic, California, USA) for offline processing using *EmbryoCV*.

2.3.2 *EmbryoCV*: A machine vision Python class for *Embryo Phenomics*

The software component of the *Embryophenomics* platform is *EmbryoCV* (www.embryocv.org), a Python class written in Python 2.7. *EmbryoCV* has dependencies including the open-source libraries OpenCV (Bradski, 2000), Sci-Kit (Jones, Oliphant and Peterson, 2001), Numpy (Oliphant, 2007), Pandas

(McKinney, 2010), XArray (Hoyer and Hamman, 2017), Matplotlib (Hunter, 2007) and Pyqtgraph. Python is an increasingly popular language with biologists (Dudley and Butte, 2009) and was used to develop *EmbryoCV* to maximise the utility and extendibility of this platform by potential users.

2.3.2.1 Workflow

The code of *EmbryoCV* is structured as a series of functional modules – EmbryoCV.py, dataHandling.py, imageAnalysis.py, dataIntegration.py, dataAnalysis.py, eggUI.py, but the user experience is intentionally simple, consisting of the following user callable functions.

(i) Initiating an *EmbryoCV* analysis: Users begin by creating an instance of *EmbryoCV* and providing it with some information about the experiment. *EmbryoCV* will generate a results file for each embryo and extract information from MicroManager metadata for each image sequence, including the time of acquisition for each image. Furthermore, at this stage *EmbryoCV* attempts to locate the egg in each image sequence.

Command: experiment = embryocv.embryocv('pathToFiles','new',scale = micrometers_per_pixel , species = 'speciesname').

(ii) Validating egg identification: An optional stage during which a user interface is used for modifying the egg ROI size, shape and position prior to beginning the more computationally demanding aspects of the analysis.

Command: experiment.validateEggs()

(iii) Quantify embryo traits: Measurement of size, shape, position and multiresolution blockwise signals are made from every image within each image sequence and stored to the embryo results files. Multiresolution blockwise signals of mean pixel intensity are produced at different spatial resolutions 1 x 1 (whole embryo ROI), 2 x 2, 4 x 4, 8 x 8, 16 x 16. Power spectral density within different temporal frequencies are then calculated using Welch's method, from the signal module of Scipy (Jones *et al*., 2001; Welch, 1967) to produce a spectrum of power within different frequency bands within different resolutions of each image sequence.

Command: experiment.quantifyAllEmbryos()

(iv) Integrate embryo traits: Raw measurements from preceding step are integrated to form biologically relevant measures including time-specific measurements such as minimum, maximum and mean size and movement at each time point during the experiment, but also global measurements such as growth rate. Frequency analysis is also applied to the blockwise signals generated in the preceding step to quantify energy within different frequency bands. At this stage data are transformed into a dynamically accessible XArray Dataset

Command: experiment.savePhenomeMeasuresForAllEmbryos()

(v) Focussed data analysis optional steps:

a. Generate summary reports: produces PDF reports for individual embryos including developmental time series of time, movement patterns and energy within different frequency bands.

   Command: experiment.generateSummaryReports('pathToSave')

b. Quantify and model cardiac rates: identify cardiac rates from within the frequency data output and fit either a segmented (*R. balthica),* or linear (*O. gammarellus)* model. Time series of cardiac rates, including parameters from the model are output.
   Command: experiment.measureHeartRateFoAllEmbryos('pathToSave')
c. Quantify lethal endpoints: Use data from previous processes to identify lethal endpoints in different stage embryos, optimised to work with E3, E7 and E11 stage *R. balthica* embryos, using either; reductions in energy within particular frequency bands or sudden increases in size indicative of a failure of osmotic control, or a combination of the two.
   Command: analysis.identifyLethalEndPoints('pathToSave')

The experiments described in this paper were analysed on Apple Mac computers (MacPro, 12 core, 64 GB RAM). The most computationally intensive stage of *EmbryoCV* is quantifyAllEmbryos (Fig. 2.1) during which the embryo is segmented (approx. 20 frames s^{-1}) and data are stored to disk as a Pandas Dataframe *via* pickle. A high proportion (95 %) of the 30.03 M images acquired by *OpenVIM* across the four experiments analysed were successfully analysed by *EmbryoCV*.

2.3.3 Manual Validation

2.3.3.1 Spatial accuracy

Assessment of the accuracy of the embryo size measurements produced by *EmbryoCV* was performed by comparison of manual and automated

measurements of embryo area (Fig. 2.3). Images (n = 656) were randomly selected from Experiment 3 and were presented to users (n = 7) for manual analysis in Fiji (Schindelin *et al*., 2012) with a simple ActionBar user interface. Users applied the polygon tool to draw manually around the perimeter of the embryo and the results were recorded to file, together with the image ID, using an ActionBar macro. The area of these manually recorded outlines was determined using the same OpenCV procedure as for the outlines generated by *EmbryoCV*.

1.3.3.2 Experimental designs

Experiments 1 - 3 used *Radix balthica* embryos produced in the laboratory from a source population at Chilton Moor on the Somerset Levels, UK (51.19°N 2.88°W) and maintained in the laboratory for a minimum of seven days prior to experiments. Snails were maintained at 15 °C in 20 L aquaria containing Artificial Pond Water (APW, ASTM 1980) with 90 mg. L^{-1} [Ca^{2+}] (Rundle et al 2004). Water was changed twice weekly and snails were fed lettuce and spinach *ad libitum*. Eggs were dissected from egg masses under low power microscopy (Leica MZ12, x 40) and were placed in individual wells of a 96 well microtitre plate (Greiner Bio-One) before being inserted into the incubation chamber of the *OpenVIM* within the relevant treatment solution (Experiments 1 - 2: APW, Experiment 3: APW and APW combined with Instant Ocean to form a salinity of 7 parts per thousand) and at the relevant treatment temperature Experiment 1: T = 20, 25 and 30 °C, Experiment 2: T = 36 °C, Experiment 3: T = 20, 25 and 30 °C. Water was changed every 2 days.

Orchestia gammarellus were collected by hand from beneath wrack cast up at HW on the upper shore, Mount Batten beach (50.35°N 4.13°W) and were maintained in aquaria (vol. = 1 L) on damp filter paper at T = 15 °C and were fed carrot *ad libitium*. Embryos were harvested from mothers within 48 h of collection, *via* extraction, using a paint brush, from the brood pouch. They were then staged and inserted into a pre-warmed microtitre plate at the relevant treatment temperature (T = 15 and 20 °C). Water was changed every two days. Automated image acquisition using *OpenVIM* and subsequent image analysis using *EmbryoCV* was performed for each experiment as outlined in Supplementary 1.

1.3.3.3 Temporal accuracy

Heart rate measurements performed by *EmbryoCV* were validated by comparison with heart rate measurements made by performing manual video analysis. The heart rate of *R. balthica* is significantly slower than in *O. gammarellus* and had a more regular heart rhythm. Consequently, manual counts of the number of heartbeats visible within the video at a particular time point were considered a reliable benchmark against which to compare the heart frequency identified for *R. balthica* embryos by *EmbryoCV*.

Orchestia gammarellus has an irregular heart rhythm including extended periods of asystole and therefore it was necessary to record the timing of individual heartbeats to enable a representative heart frequency to be calculated*, via* analysis of beat to beat timings. A Fiji macro was used to record the active frames in an image sequences at which each heartbeat occurred *via*

manual pressing of a space bar and this was subsequently used to generate a time-series of beat-to-beat timings. Owing to the influence of asystole in producing a non-normal distribution of beat-to-beat timings, the median beat-to-beat timing was calculated, and this was used in comparison with the heart rate quantification performed by *EmbryoCV*.

In Experiment 2 the accuracy of lethal endpoints for *R. balthica*, recorded as time to death, was assessed using a comparison with manually determined lethal endpoints for all embryos. Mortality was defined as the time at which all visible signs of life ceased, including body movements and heart function. Manual video analysis was used to determine the time of death and this was compared with the lethal endpoints determined using the identifyLethalEndPoints function in *EmbryoCV*. 93 % of the 157 embryos analysed were identified correctly as exhibiting a lethal endpoint during the experiment. Of the 7 % that were misidentified 25 % were identified as having a lethal endpoint, contrary to the lack of manually identified lethal endpoint and 75 % had no lethal endpoint identified. Concordance between manual and automated lethal endpoints was high in all three developmental stages (Fig. 2.3).

2.4 RESULTS

2.4.1 Overview of *Embryophenomics* platform

The *Embryophenomics* platform (www.embryophenomics.org) consists of open-source hardware (Figs. 2.1 and 2.2A, *OpenVIM*, www.openvim.org) and

software (Fig. 2.2 C, *EmbryoCV*, www.embryocv.org). *OpenVIM* performs long-term imaging of large numbers of aquatic embryos with coupled environmental control (Fig. 2.2 B). *EmbryoCV* is a Python package for automated and robust analysis of the resultant video to extract and integrate large quantities of data to form biologically relevant phenome-level measurements (Fig. 2.2 D - 2.2 E).

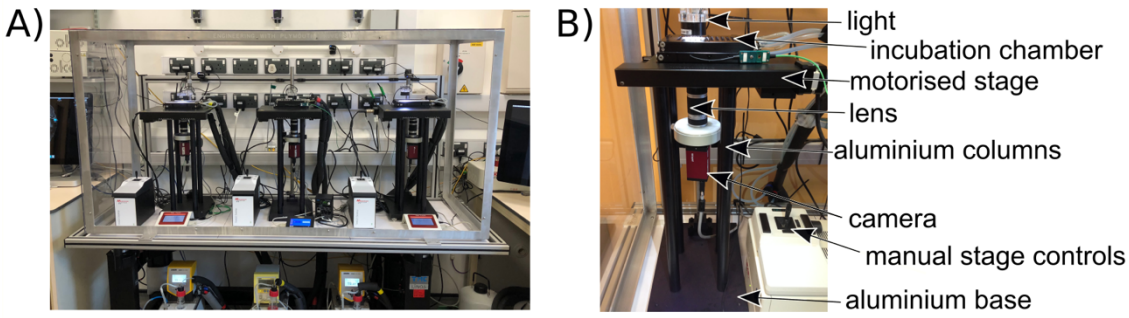

Figure 2.1 A) Photograph of three OpenVIM systems at University of Plymouth on a vibration insulating imaging table. B) Annotated photograph of a single OpenVIM system.

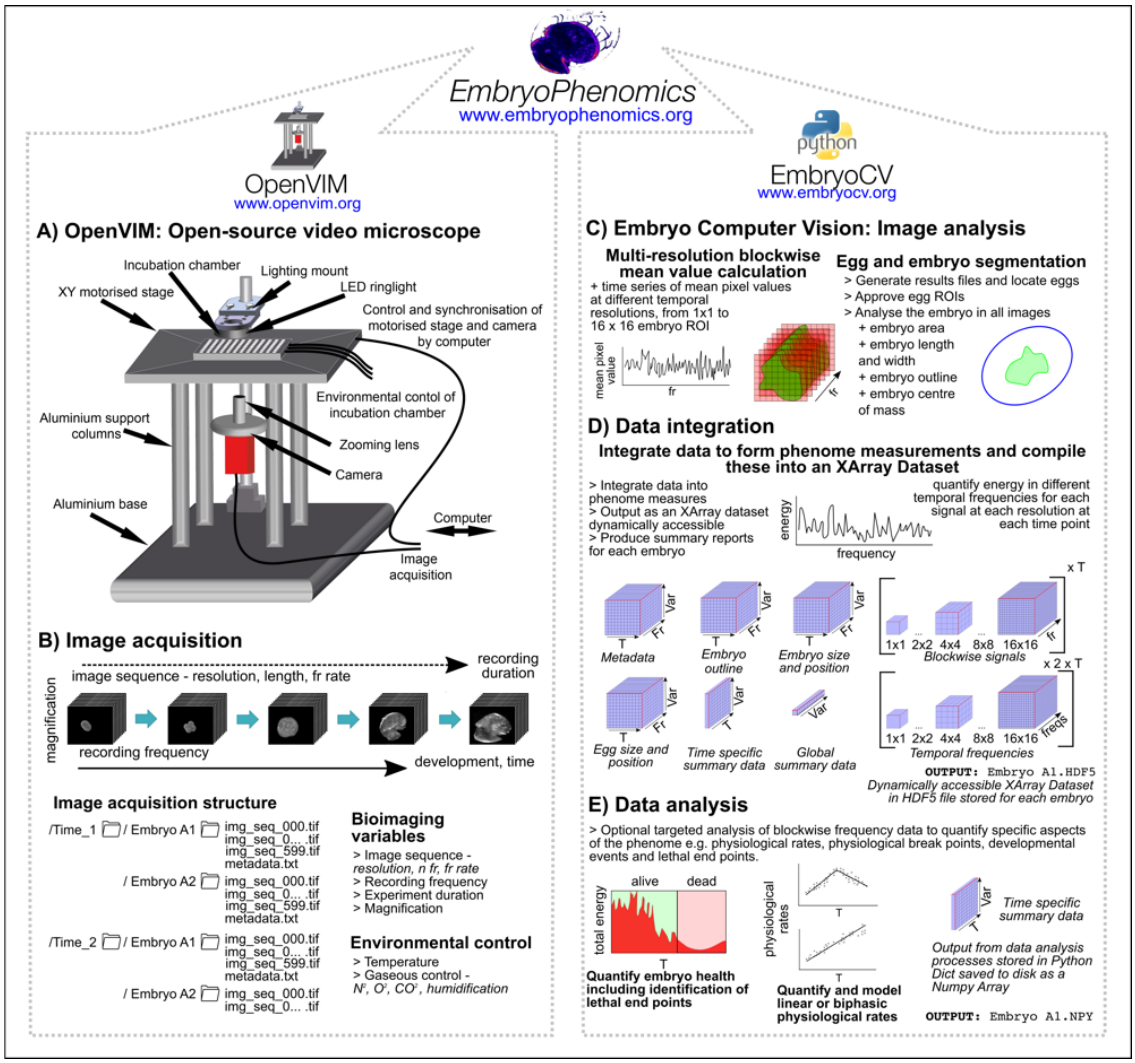

Figure 2.2 Schematic outline of the *Embryophenomics* platform. A) *OpenVIM* – technical drawing of the *OpenVIM* bioimaging hardware with labelling of its different components. B) Image acquisition using *OpenVIM* – bioimaging and environmental variables controlled using *OpenVIM* in the process of performing an experiment and acquiring images. The structure of image acquisition and storage is outlined. C) *EmbryoCV* – outline of the processes within the three functional modules of *EmbryoCV*; Image Analysis, Data Integration and Data Analysis. T = time, Var = phenomic variable, fr = frame, freqs = frequency. Data are output for each embryo as; Pandas Data Panels (McKinney, 2010) from the Image analysis stage (Fig. 2.2 C), HDF5 Xarray Dataset files (Hoyer and Hamman, 2017) from the Data Integration stage (Fig. 2.2 D) and Python

Dictionaries in the Data analysis stage (Fig. 2.2 E). The structure of data within each stage is outlined including their different dimensions.

OpenVIM can acquire images of large numbers of developing embryos at different temporal scales. The resultant image-sequence time series can be used to visualise short-term changes in the physiology (e.g. heart rate) and behaviour (e.g. spinning and crawling rates) of embryos in real-time throughout the course of an experiment, and longer-term changes in form and function (e.g. morphometrics, physiology) through ontogeny. The use of high depth-of-field optics (see Materials and methods) enables long-term and fully automated simultaneous recording of large numbers of embryos for the duration of development, including species with embryonic life history stages lasting many weeks, or even months.

Analysis of the image time-series produced by *OpenVIM* is performed offline, following image acquisition, by *EmbryoCV*, a purpose-built Python based package (www.embryocv.org). The *EmbryoCV* software performs analysis and extraction of data from every image sequence acquired (Fig. 2.2 C - Image Analysis) and these are integrated to form datasets that include morphological, behavioural, physiological and proxy measurements. Proxy measurements include the measurement of mean pixel intensities (i.e. grey level intensities) at multiple-resolutions within each frame of an image sequence to form time series signals that are subsequently analysed to quantify energy within different temporal frequency bins within each (Fig. 2.2 D, see Materials and method for more information). These proxy traits can be used for quantification of both

holistic (lethal and sublethal classification) and specific measures (quantification and modelling of cardiac rates) of embryo health and physiology (Fig. 2.2 E).

The accuracies of measurements made by *EmbryoCV* were assessed *via* comparison with manual quantification of spatial and temporal traits equivalent to the measurements performed by *EmbryoCV* in a randomised subset of images from each experiment. Concordance between measurements made manually and those made by *EmbryoCV* was high for both spatial and temporal measurements (Fig. 2.3, see Materials and methods for more information).

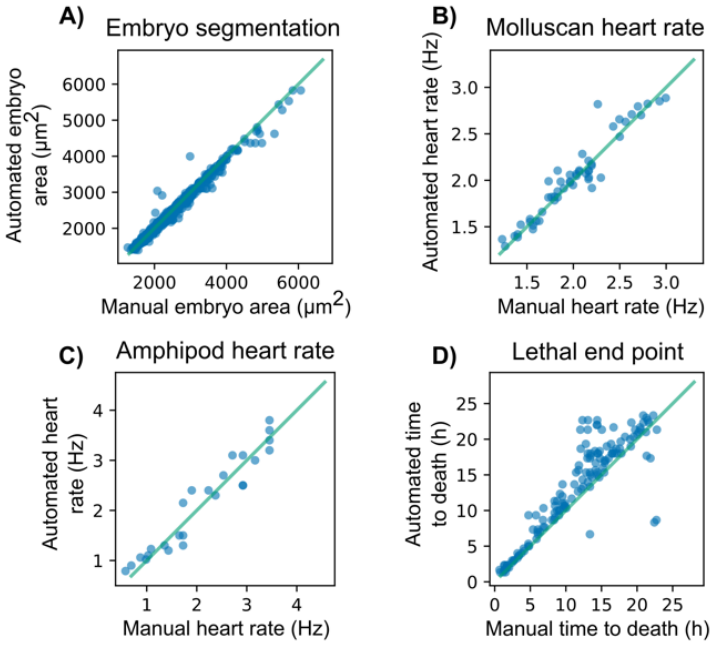

Figure 2.3 Comparison of data produced by *EmbryoCV* with manually quantified measures. A) Area of *Radix balthica* embryos determined by both manual drawing of ROIs and automated embryo segmentation (r_{614} = 0.989, p < 0.001); B) Heart rate of *R. balthica* embryos determined using both manual video analysis and the automated *EmbryoCV* identifyHeartRateForAllEmbryos process (r_{52} = 0.956, p < 0.001); C) Heart rate of *Orchestia gammarellus* determined using manual video analysis and the automated *EmbryoCV* identifyHeartRateForAllEmbryos process (r_{25} = 0.961, p < 0.001); D) Time to death for *R. balthica* embryos at E3, E7 and E9 developmental stages was determined using manual video analysis and automated measurements were generated using the lethalEndPoint *EmbryoCV* process (r_{140} = 0.892, p < 0.001). 1 Hz = 1 cycle.s^{-1}.

The ability of the *Embryophenomics* platform to quantify integrated organismal responses to environmental change was assessed using four different experimental designs - i) chronic, ii) acute, and iii) interactive effects. Furthermore, we demonstrate the transferability of the platform using two

ecologically important species with different patterns of development – the freshwater gastropod mollusc *Radix balthica*, and the marine supralittoral amphipod *Orchestia gammarellus*. These experiments required the acquisition of > 30 M images of 623 embryos by *OpenVIM,* of which 95 % were successfully analysed using *EmbryoCV.* Four percent of embryos were removed manually from the analysis due to abnormal development and of the remaining embryos, 98 % were successfully characterised using the *EmbryoCV* workflow (see Tills et al.., 2018 publication for supporting information).

2.4.2 Experiment One. Developmental responses to chronic elevated temperatures

We examined phenome-level differences of embryos maintained in contrasting environments from the first cell division until hatching (Fig. 2.4). One cell stage snail *Radix balthica* embryos were placed in three temperatures (n = 48 for 20, T = 25 and 30 °C) and recorded using *OpenVIM* for 30 sec, at 20 frames.s^{-1}, every hour for the duration of their development. This generated a total of 17.73 M images for all 144 embryos. These were subsequently analysed by *EmbryoCV*. The embryo was successfully identified and measured by the *Image Analysis* stage of *EmbryoCV* in 16.32 M of the acquired images (92 %) and in 143 of the 144 embryos recorded using the *OpenVIM*. Further to the core *EmbryoCV* steps of Image Analysis and Data Integration an additional Data Analysis process (Fig. 2.2) was used to quantify the ontogeny of cardiac function for each embryo and to fit an appropriate model to these data. The ontogeny of cardiac function in *Radix balthica* exhibits a segmented pattern and consequently a segmented regression model was applied to the heart rate

measurements extracted for each embryo within the *EmbryoCV* function measureHeartRatesForAllEmbryos.

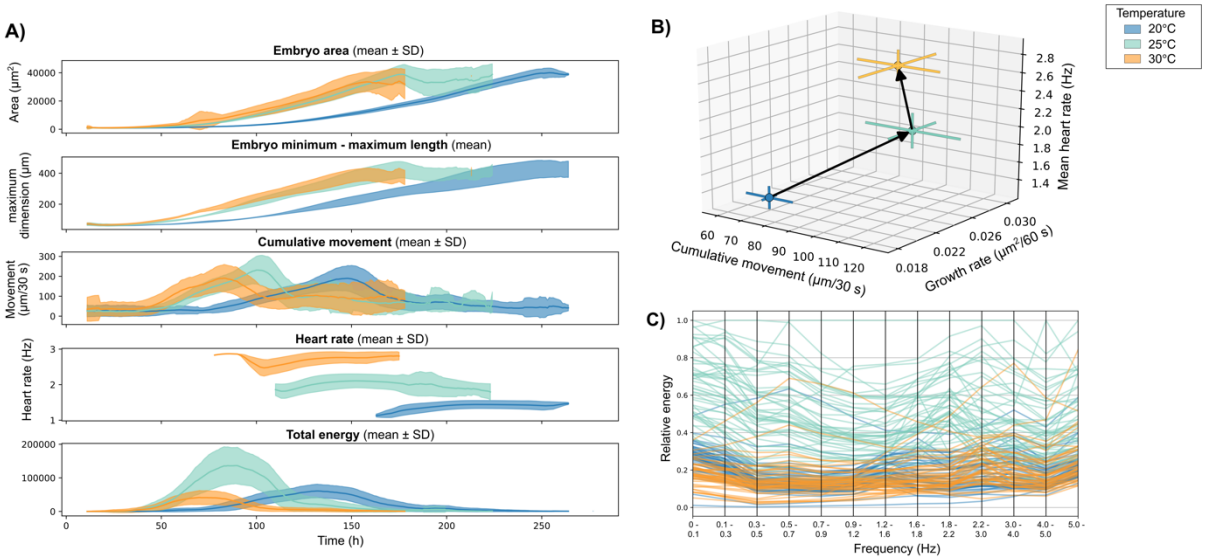

Figure 2.4 A) Developmental time series of a range of phenome-components in *Radix balthica* cultured under contrasting temperatures; B) The response of morphological growth rate, physiological (heart rate) and behavioural (cumulative movement) parameters of *R. balthica* embryos cultured under contrasting temperatures (mean ± 1.SD); C) Parallel coordinate plot of the relative energy within different frequency bin ranges during the development of individual *R. balthica* embryos cultured under contrasting temperatures.

The measurements made by *EmbryoCV* detected significant effects of temperature on growth rates ($F_{(2,51.6)} = 212.28$, $p < 0.001$) rates of movement ($F_{(2,64.7)} = 666.17$, $p < 0.001$) and heart rates ($F_{(2,59.6)} = 661.7$, $p < 0.001$), but also a marked difference in the direction and magnitude of responses between temperature increments (Fig. 2.4 B). An increase in movement, growth rates and heart rate was evident in embryos grown in T = 25 and 30 °C, growth rate and rates of movement decreased marginally yet heart rate was significantly

accelerated, well beyond the net increase observed between T = 20 and 25. Restriction in the extent to which growth rates and rates of movement can be accelerated in response to temperature are of biological interest and suggest that chronic exposure to higher temperatures may prove problematic for *R. balthica*. This is further supported by the reduction in 'Total Energy' proxy trait in the time series at T = 30 °C, compared to both T = 20 and 25 °C. Total energy is a measure of the power within pixel intensity (i.e. grey level) fluctuations at all temporal frequencies in the embryo. Consequently, a reduction in the Total Energy proxy trait (Fig. 2.4 A) is indicative of a reduction in the overall activity of embryos including both net and gross embryo movements. In addition to a reduction in Total Energy there was also a corresponding reduction in the energy across frequency bands in T = 30 °C embryos (Fig.24 C)

2.4.3 Experiment two. Lethal endpoints for different developmental stages

To assess the capability of the *Embryophenomics* platform for measuring extreme (i.e. lethal) biological responses we used an acute 24 h exposure of three developmental stages (n = 63, 56 and 64 for E3, E7 and E9, respectively) of *Radix balthica* embryos to an elevated temperature of 36 °C. *OpenVIM* recorded individual embryos for 30 sec, at 20 frames per second (fps), every 20 min for 24 h. This generated a total of 7.7 M images for all 183 embryos and these were subsequently analysed by *EmbryoCV*. We assessed the ability of *EmbryoCV* to quantify the occurrence of lethal endpoints (time to death) within developmental stages that exhibit different forms of biological response. In addition to the core *EmbryoCV* processes of Image Analysis and Data Integration an additional Data Analysis process – identifyLethalEndPoint was

used to identify time to death for each embryo, from the data that had been collected in the preceding stages, including size, movement and energy within different frequency bands.

Ninety three percent of the 183 embryos studied were correctly identified as exhibiting a lethal endpoint. Concordance with manually determined lethal endpoints was high for all three developmental stages (E3 = 98.8 %, E7 = 99.3 %, E9 = 99.3 %, Fig. 2.5). The sensitivity of the three developmental stages E3, E7 and E9 to elevated temperature, as measured by the median lethal time for a proportion of individuals (LT), was significantly different (LT_{25} – $F_{(2,20)}$ = 4.98, p = 0.018, LT_{50} – $F_{(2,20)}$ = 11.09, p < 0.001, LT_{75} – $F_{(2,20)}$ = 14.04, p < 0.001). The earliest developmental stage (E3) had a lower LT_{50} (8.6 ± 1.82) and LT_{75} (4.11 ± 0.98) than the two later stages (E7: LT_{50} = 22.94 ± 3.21, LT_{75} = 14.59 ± 2.31. E9: LT_{50} = 16.21 ± 1.81, LT_{75} = 13.28 ± 1.21), indicating a greater sensitivity to thermal stress. These early stage embryos exhibited a ciliary-driven spinning behaviour and their lethal endpoint coincided with a loss of osmotic control made visual by a rapid increase and subsequent decrease in embryo area. Automated peak identification was used in the *EmbryoCV* function identifyLethalEndPoint to identify this lethal endpoint. E7 stage embryos possess a weak heartbeat and a transitionary form of locomotion consisting of part ciliary driven gliding and part muscular crawling on the egg capsule wall, whereas E9 stage embryos possess a strong heartbeat, radula movements and muscular contractions. Lethal endpoints in E7 and E9 stage embryos were indicated by a loss of cardiac function and a cessation of gross embryo movements. The *EmbryoCV* identifyLethalEndPoints function identified this response in these later developmental stages and detected lethal endpoints, *via*

a decrease in the relative energies within specific summed frequency bins within the energy proxy traits.

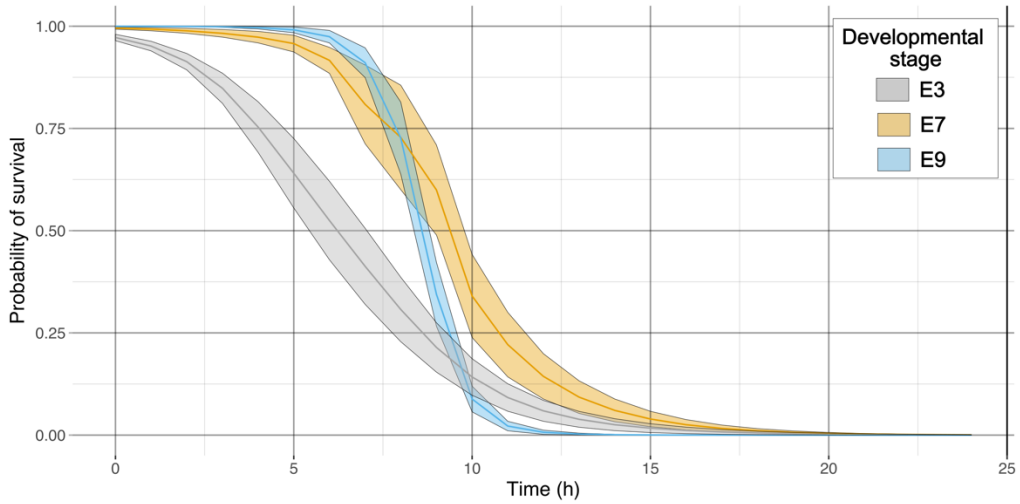

Figure 2.5 Duration of exposure dependant probability of survival curves at 36 °C for different developmental stages of *Radix balthica*.

2.4.4 Experiment three. Short-term responses to multiple stressors

The ability of the *Embryophenomics* platform to detect complex multifaceted responses within multistressor experiments was assessed using a 24h exposure of E3 stage *Radix balthica* embryos (n = 288) to contrasting temperatures (T = 20, 25 and 30°C) and salinities (S = 0 or 7). *OpenVIM* recorded embryos for 30 sec, at 20 frames. s^{-1} for 24 h. This generated a total of 17.73 M images for all 288 embryos and these were subsequently analysed by *EmbryoCV* (Fig. 2.5). There were significant effects of temperature and salinity on both growth rate and movement (Growth rate: temperature - $F_{(2, 261)}$ = 107.02, $p < 0.001$, salinity - $F_{(1, 261)}$ = 157.06, $p < 0.001$, Movement: temperature - $F_{(2, 273)}$ = 17.06, $p < 0.001$, salinity - $F_{(1, 273)}$ = 43.08, $P < 0.001$).

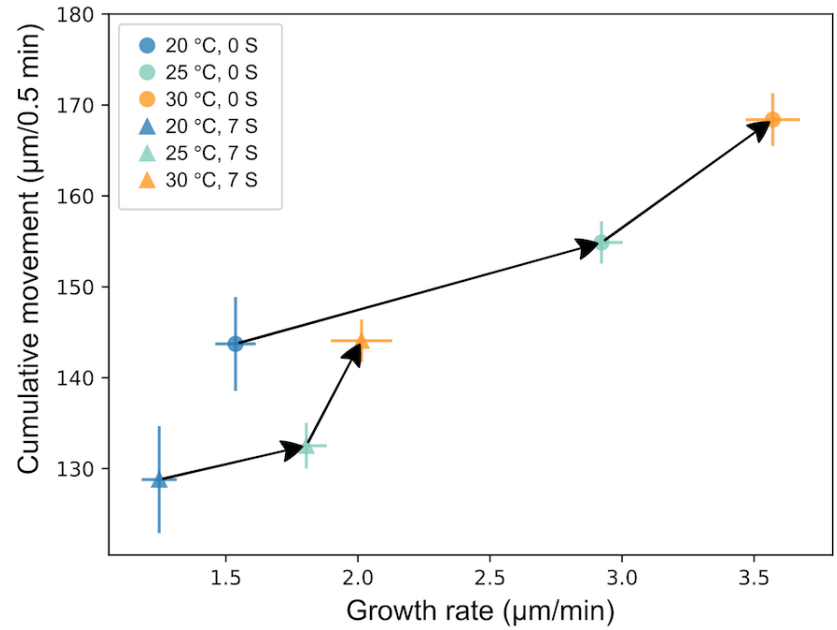

Figure 2.6 Phenomic responses of E3 developmental stage *Radix balthica* (n = 277) exposed to combinations of temperature and salinity for a period of 24 h (means ± 1 S.D.).

In S = 0 *EmbryoCV* identified an increase in both cumulative movement and growth rate that were associated with an increase in temperature from 20 to 25 °C; however, from 25 to 30 °C the increase in growth rate was markedly reduced, despite a similar increase in movement (Fig. 2.6). In S = 7 both growth rate and movement were reduced at each temperature, relative to the S = 0 salinity treatment. Furthermore, the proportionate magnitude of response comparing T = 20 to 25 °C and T = 25 to 30 °C was more pronounced in S = 7, indicating that the ability to tolerate elevated temperatures was compromised by the addition of a salinity stress.

2.4.5 Experiment four. Effects of temperature on the ontogeny of cardiac function

The *EmbryoCV* software and *OpenVIM* hardware have been developed to be versatile and extendible to a wide range of species and scientific applications. A core process within *EmbryoCV* is the use of a multi-resolution blockwise signal quantification that is capable of quantifying embryonic traits with different functional forms and of capturing embryo responses as holistic proxy measures.

During the development of *Radix balthica* the embryo exhibits both ciliary driven rotation and a muscular crawling behaviour within the egg capsule. Furthermore *R. balthica* possesses a two-chambered globular heart and undergoes the process of torsion in which its mantle cavity (where the heart is located) rotates by 180 °. The automated quantification of cardiac function in *R. balthica* is demonstrated in Experiment 1 and here we apply the same process to quantifying the response of the supralittoral talitrid *Orchestia gammarellus* during a 24 h period in contrasting temperatures (Fig. 2.7). The development and morphology of *O. gammarellus* is markedly different to *R. balthica.* *Orchestia gammarellus* fills its egg capsule and possesses a tubular heart positioned dorsoventrally and with a cardiac rate approximately double that of *R. balthica.* Despite these differences, *EmbryoCV* achieved high levels of concordance for measurements of cardiac rate compared to manual measurements for *O. gammarellus* (see Fig. 2.3 C).

OpenVIM recorded *O. gammarellus* embryos maintained at either 15 or 20 °C for 54 sec, at 38 fps, every hour from 24 – 48 h after the initial onset of cardiac

function. Rates of increase in heart rate from 24 to 48 h after first heart function were significantly different (H_1 = 5.33, p = 0.021) and approximately four times faster in 20 °C than 15 °C. Such high rates of acceleration in the physiological ontogeny of cardiac function in response to a 5 °C increase in temperature are interesting and suggest a high thermal sensitivity of *O. gammarellus.*

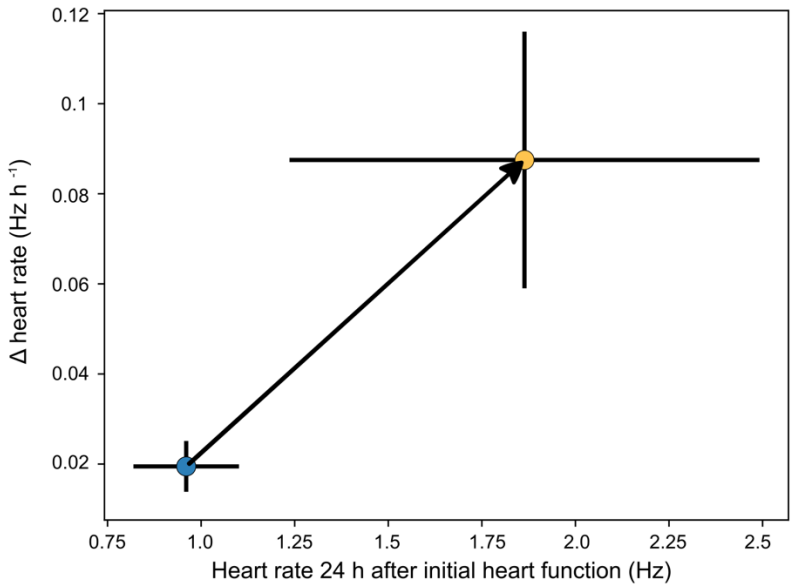

Figure 2.7 Early cardiac ontogenic response to elevated temperature in *Orchestia gammarellus* (n = 8). Blue = 15 °C, Orange = 20 °C; (means ±1 S.D.)

2.5 DISCUSSION

2.5.1 *Embryo Phenomics* – a versatile platform

Here we demonstrate the ability of the open-source *Embryophenomics* platform to quantify complex phenome-level responses of large numbers of developing aquatic embryos. *Embryo Phenomics* is broad in its scope and we demonstrate its application to a broad range of experimental designs, ranging from two-week, to 24 h experiments, and demonstrate its application to two species. These experiments include assessment of responses to acute and chronic thermal stress, arguably one of the greatest current threats to Biodiversity and one to which early life stages can have heightened sensitivity (Spicer and Gaston, 1999; Truebano *et al*., 2018). *Embryo Phenomics* was also used to quantify the response of 288 early developmental stage *Radix balthica,* embryos to combined thermal and saline stress, and revealed an inhibitory effect of salinity on growth and movement that contrasted with the effects of elevated temperature. The generation of high-dimensional data is a pre-requisite to phenomics and, owing particularly to the dynamic nature of embryonic development, this capability will facilitate advances in our understanding of this pivotal life stage. *EmbryoCV* and *OpenVIM* are versatile, extendible and open-source technologies applicable to a range of species and study designs. Our aim is that these resources will develop a community of users, in a similar model to that seen with the OpenSPIM project (Pitrone *et al*., 2013). In contrast to more restrictive and focussed technologies for embryo phenotyping (Steenbergen *et al*., 2011; Vogt, 2009), both *OpenVIM* and *EmbryoCV* are intentionally modular, versatile, high-throughput and

transferrable to species with contrasting developmental patterns. *Embryo Phenomics* can therefore underpin the generation of data describing whole-organism responses with a scale, diversity and quality that are required to study embryonic development in a manner befitting phenomics, and which compares with that produced using molecular -omic technologies.

2.5.2 Lessons from experiments

Concordance between manual and *EmbryoCV* determined measures of embryo spatial and temporal characteristics was high (Fig. 2.3). The blockwise signal and frequency workflow was effective at locating and quantifying both the two-chambered globular heart of *R. balthica*, but also the elongated, tubular heart of *Orchestia gammarellus*. The typical maximum cardiac rate of *R. balthica* at T = 20 °C is 1.5 Hz, whereas *O. gammarellus* has a higher cardiac rate of 3.5 Hz, and with sustained periods of diapause, presenting challenges in the effective manual quantification of rate. Here, for *O. gammarellus,* to account for the skew introduced by diapause, beat-to-beat timings were recorded manually and the rate calculated from the median timing was closely correlated with the frequency measurements produced by *EmbryoCV*. For *R. balthica* direct comparisons of manually determined rate, i.e. counts of heart beats, were closely correlated with *EmbryoCV* determined rates. Movements of the embryo meant that the heart and other features of interest were not always visible in the image. Consequently, *EmbryoCV* applies spectral frequency analysis across different areas of the embryo and attempts to identify biologically relevant frequencies for modelling heart rate. These data then inform the fitting of a model to the development of heart rate and, the resultant cardiac rates

predicted from this model were closely correlated to manual measures for both species in Experiments 1 and 4. A similar workflow also underpins the automated identification of lethal end points across three developmental stages of *R. balthica*, in Experiment 2. Here, developmental-stage specific algorithms identifying rapid increases in size indicative of a loss of osmotic control, rapid reductions in energy within particular frequency bands indicative of a lack of physiological activity, or a combination of the two, was highly effective at producing classifications of lethal endpoints closely correlated with those ascertained manually. Further optimisation and mining of the data produced by *Embryo Phenomics* will allow further tailored endpoints to be identified, including responses that while present, are not immediately apparent to human observers in the multi-temporal video produced by *OpenVIM.*

2.5.3 Perspectives

Phenomics offers the potential to facilitate rapid advances in our understanding of biology *via* the generation of a quantity and quality of phenotype-level data that is more appropriate to addressing questions focused on understanding the complexities of organismal biology. The *Embryophenomics* platform provides the technological capability for the study of organismal development in a way that captures temporal, spatial and functional diversity at both inter- and intra-individual levels – a task identified as a major challenge in biology (Mueller *et al.*, 2015, Spicer and Gaston, 1999). Here we demonstrate the capability of *OpenVIM* to document the development of large numbers of aquatic embryos (n > 600) in experimental treatments ranging from long- (> 240 h) to short-term (24 h) exposures. Of the > 30 M images acquired across the four experiments

described here *EmbryoCV* successfully extracted phenome-level data from 95 %, producing high dimensional data describing morphological, physiological and behavioural embryo responses.

CHAPTER 3: ONTOGENY OF GASTROINTESTINAL TRACT MOVEMENTS IN AMPHIPOD EMBRYOS CULTURED AT DIFFERENT ENVIRONMENTAL TEMPERATURES

E. Tully conducted material collection and maintenance, planned and carried out the experiments, analysing the data and wrote the chapter.

3.1 ABSTRACT

The gastrointestinal (GI) tract plays a vital role in digestive processes and contributes to the regulation of homeostasis throughout the life cycle of many invertebrate species, even functioning as an extra cardiac pump during early embryonic development of some crustaceans. Although our knowledge of the development of GI tract morphology is good, our knowledge surrounding the development of GI behaviour and function has not kept pace. Temperature is arguably one of the most important environmental variables in early development (Chapter 1) as it generally alters the rate and even pattern of developmental and physiological processes. Changing environmental temperature may alter the rate and pattern of GI behaviour and function with consequences for yolk usage (a finite resource in oviparous species) and dispersion to developing tissues. Consequently here I investigate the effect of two environmentally realistic temperatures (15 and 20 °C) on the ontogeny of GI behaviour and function throughout embryonic development of oviparous talitrid amphipod *Orchestia gammarellus*. The prevalence of a precardiac function was determined by measuring changes in GI behaviour under hypoxia. Individuals cultured at 20 °C expressed 2.4 x faster development than those reared at 15 °C but followed the same sequence of developmental milestones. The rate of peristaltic wave motion was significantly affected by temperature ($p < 0.001$) and time ($p < 0.001$) with a clear interactive effect between the two ($p < 0.001$). Yolk usage, a proxy for energy expenditure, also showed a significant response to temperature ($p = 0.014$) and time ($p < 0.001$) with combined effect ($p < 0.001$). Thermal and temporal effects on GI behaviour and yolk remained significant when time was standardised to make direct comparisons between key developmental stages. There was no effect of hypoxia on GI behaviour,

suggesting the GI tract does not function as a pre-cardiac pump in this species. It is evident that temperature has a profound effect on the rate of development and GI movement and on the rate at which yolk is used. High rates of mortality (98 %) at 20 °C may warn of downstream consequences for growth and survival of later life stages.

3.2 INTRODUCTION

The gastrointestinal (GI) tracts of invertebrates are often as complex and variable in structure as those of vertebrates (Ahearn, 1988; Wright and Ahearn, 2010; Welcome, 2018). While considered principally as engines of digestion, the GI tract has long been recognised as exhibiting a wide range of physiological roles which contribute to organism homeostasis (Pavlov, 1902; Ahearn, 1988; Ahearn *et al*., 1992; Vannier *et al*., 2014; Navarrete del Toro and García-Carreño, 2019). For both adults and embryos in taxa as disparate as echinoderms, pycnogonids and crustaceans, GI tract movements are suggested to have a role in the internal circulation of body fluids (Maynard, 1961; Spicer, 2006; Woods *et al.*, 2017). Rhythmic or peristaltic movements of the GI tract are some of the first behaviours observed during the embryonic development of aquatic crustaceans (Berrill, 1969, 1973; Corner, 1977, 1978; Browne *et al*., 2005). Movement of the GI tract, in some groups, appears to create circulation in the extracellular spaces, even before the ontogeny of a functional cardiovascular system (Spicer, 2006). The embryonic stage of invertebrates is particularly sensitive to environmental temperature (Zippay and Hofmann, 2010; Truebano *et al*., 2018), and therefore in a warming world (IPCC 2018), changes in GI movement as a result of changes in thermal environment may negatively impact organismal homeostasis, with consequences for survival. For example, an increase in rate (a known effect of elevated temperature) of GI movement as a result of increased environmental temperature may change the frequency or amount of yolk released from GI structures. Too much yolk released too soon, or too little, and the embryo may run out of energy. While our understanding of the morphological development of the gut in crustaceans is relatively good (e.g.

Brunet *et al*., 1999; Browne *et al*., 2005) there are few descriptions and experimental investigations of the appearance and development of GI movements in embryonic stages, with no studies accounting for the potential effects of temperature. Temperature is known to influence biological rates; from biochemical rates of reaction at the cellular level (Hock and Somero, 2002) to the timing and order of developmental events at the organismal level (Tang, 2000; Temple, 2001; Fuhrman *et al*., 2018). Thermal influence on the behaviour and function of GI tract is likely, but still to my knowledge undescribed for crustaceans.

Talitroidean amphipods are becoming key models in the study of development (Tosetto *et al*., 2016; Kao *et al*., 2018; Sun and Patel, 2019) and particularly the development of function (e.g. Morritt and Spicer, 1999; Tills *et al*., 2018). During organogenesis, the endoderm and visceral mesoderm form the GI tract around a central yolk in the talitroidean amphipod *Parhyale hawaiensis* (Gerberding *et al*., 2002; Browne *et al*., 2005). The pattern of yolk volume change during development has been described qualitatively for *P. hawaiensis* (Browne *et al*., 2005) but the detail of the development of GI movements and the extent of these movements, how they develop and how they are related to dispersing yolk through-out the embryo have not been investigated. Furthermore, while the effects of environmental temperature on embryonic development has been characterised for many a number of taxa, including crustaceans (Styf *et al*., 2013), and amphipods in particular (Steele and Steele, 1973; Dick *et al.,* 1998; Xue *et al*., 2018), how temperature alters embryonic physiology and behaviour, (including GI development and movement), is largely unstudied. Some work has been carried out on the effects of culture temperature on heart function (e.g.

Storch *et al*., 2009), and enzyme function (e.g. Lemos *et al*., 2003) but compared with temperature effects on the timing of appearance of key developmental stages (particularly hatching), our knowledge of GI function is underdeveloped.

Consequently, the aim of this study is to document the ontogeny and development of the different GI movements in embryonic talitroidean amphipods at different, ecologically relevant, environmental temperatures. I test the hypothesis that embryonic gut movements in talitroidean amphipods function as an extracardiac pump, aiding in the delivery of oxygen to developing tissues and dispersing yolk to growing and developing embryonic tissue. If gut movements are related to yolk dispersal then (1) it should be possible to detect yolk release and relate it to particular gut movements; (2) tracing the subsequent movement of the released yolk in the embryo should be possible; (3) an increase in environmental temperature should change the frequency and/or character of gut movements to release more yolk to meet heightened metabolic demand; if gut movements are related to oxygen demand then (1) acute exposure to environmental hypoxia should result in a corresponding increase in the frequency of gut movements to enhance circulatory function during this period of heightened demand and (2) circulation should be more pronounced at higher temperatures.

These predictions will be tested by (1) first describing the ontogeny and nature of the gut movements from their first appearance through to hatching, (2) quantifying rates of gut movements in embryos cultured at two different ecologically realistic environmental temperatures at different times through development, (3) determining visually whether, and how frequently, yolk is extruded from the gut and once released where it goes in the embryo and (4) testing whether the rate of gut movements increase in response to environmental hypoxia.

3.3 MATERIALS AND METHODS

3.3.1 Collection and maintenance

Adult beachfleas *Orchestia gammarellus* (body length, from base of antennae to tip of telson = approx. 2 cm) were collected by hand from beneath cast up wrack on the high shore at Mount Batten Bay, Plymouth, Devon, UK (50°21′25.23″N, 4°07′37.21″W) during August 2016. Individuals were transported in large plastic bags to the laboratory at the University of Plymouth within 4 h of collection. Here, ovigerous females were isolated, removed and maintained in a number of aquaria (vol. = 5 L, T = 15 ± 1 °C, 100 % humidity, 12 h : 12 h light:dark cycle), each lined with moist tissue paper (S = 11) for 48 h before use as described below. Individuals were not fed during this time, but they did consume the tissue within the aquaria.

3.3.2 Embryo harvesting and incubation

Ovigerous females carrying early stage embryos were identified by eye and removed from the holding aquaria. The development stage of individuals in each of the broods was established by removing a single ‘representative’ embryo from the marsupium with a fine, moist paintbrush. The embryo was transferred to a Petri dish (diam. = 5 cm) containing dilute sea water (S = 11) and examined under low power magnification (x 40). The developmental stage of the embryo was ascertained according to the detailed scheme of Browne et

al (2005) for the related talitroidean amphipod *Parhyale hawaiaensis*. Broods at development stage 18 (Browne *et al*., 2005) were selected for incubation at one of two nominal temperatures (T = 15 or 20 °C). In total 50 individuals were incubated per treatment temperature (at either T = 15 or 20 °C). Each embryo was placed in one of 96 wells within the incubation chamber of the bioimaging system (described in Chapter 2) containing pre-heated (T = 15 or 20 °C) diluted sea water (vol. = 200 µL, S = 11). The humidity within each incubation chamber was maintained at 90 % to minimise evaporation and to stabilise the salinity of the water that eggs were incubated in. Salinity of water in each of the wells was monitored daily using a digital refractometer (Hanna HI 96822, Befordshire, UK. Resolution, S = 1) and showed no change during the course of the incubation. Thereafter each embryo (n = 50 and 50 per T = 15 and 20 °C respectively) was imaged as follows in section 3.3.3.

3.3.3 Image acquisition

Images of individual embryos were captured using a 4 MP monochrome camera (Allied Vision Technology Pike 421B, Stemmer® Imaging, Surrey, UK) operating with 700 x 700 pixels. The camera was connected to a zooming optic lens system (Keyence VHZ20R), which was inverted beneath a motorized XY stage (Marzhauser Scan) and controlled using Micromanager software package (Edelstein *et al*., 2010). See Chapter 2 for full details of bioimaging system set up. Each embryo was recorded for 60 sec (38 frames per second, 16 bit pixel depth, 40 ms exposure), producing a stack of 2280 images every hour for each embryo up until the time of hatching or death.

3.3.4 Image analysis

All image stacks were loaded into the image analysis software Fiji (Schinelin *et al*., 2012) for analysis. Original stacks were converted from 16 bit to 8 bit grey scale and manipulated using Fiji's "threshold tool" to improve the distinction between the light and dark components of the embryo, thereby enhancing GI structural outlines and movement via binary thresholding. To account for faster rates of development under warmer treatment conditions (T = 20 °C), stacks were analysed every 5 h for embryos developing at 20 °C and every 15 h for those developing at 15 °C. To standardise for differences in developmental time between treatments, the time taken for each individual to develop fully (n = 10 per treatment), from the stage where GI movement begins (stage 22) to the final stage of development (stage 30), was used to create embryo-specific measures of standardised percentage developmental time (where S22 = 0 % and S30 = 100 %) and these were rounded to the nearest 5 %. Relative developmental time was used to divide data into developmental stages (in accordance with stage list, sect. 3.4.1.1) for stage by stage comparison. Consequently, the effect of culture temperature, chronological time (h), relative developmental time (%) and stage of development on GI movement and yolk utilisation was assessed independently to decouple time effects.

3.3.4.1 Quantifying frequency of peristaltic movement

Peristaltic wave frequency was quantified as the number of complete peristaltic waves (refer to Fig. 3.4 for diagram) occurring in 60 sec. This frequency was recorded every 15 h and 5 h for individuals reared at 15 (n = 35) and 20 °C (n = 25), respectively, from the time of first occurrence of a full peristaltic wave (stage 23) until death/ hatching. Individuals that died prior to the onset of peristalsis were not analysed.

To analyse temporal and thermal effects on the rate of GI movement; peristaltic wave frequency data were compared at 15 h intervals (n = 35 and 25, T = 15 and 20 °C, respectively), at 5 % relative developmental time increments (n = 10 per T = 15 and 20 °C) and at relative developmental stages (n = 10 per T = 15 and 20 °C). To assess temperature dependant changes in the frequency of GI movements with developmental stage, Q_{10} temperature coefficients were calculated from peristaltic wave frequency means for each stage of development (n = 10 for both T = 15 and 20 °C) using equation 3.1:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\left(\frac{10}{T_2 - T_1}\right)} \quad \text{(Eqn. 3.1)}$$

Where T_1 = 15, T_2 = 20 and R_1 and R_2 correspond to the mean rate of movement at T = 15 and 20 respectively. T_1 and T_2 were controlled as the independent variables and R_1 and R_2 were measured as the dependent variables.

3.3.4.2 Quantify volume of yolk

To estimate changes in yolk volume; the total pixel area covered by the egg capsule, midgut and caeca structures (Fig. 3.1) were estimated at 15 h and 5 h intervals (for T = 15 and 20 °C respectively) using the “Polygon” tool and “Measure” function in Fiji (Schindelin, 2012).

Total yolk area (%), i.e. the percentage area of the embryo covered by yolk when viewed laterally, was quantified by adding midgut and caeca (singular) pixel areas together and dividing by the pixel area of the egg capsule. All individuals analysed (n = 6 and 7 per T = 15 and 20) were in lateral orientation (Fig. 3.1), with good lighting and with the entire egg capsule in shot. To analyse temporal and thermal effects on yolk usage rates; change in percentage total yolk area (as a proxy for yolk usage rate) was measured at two culture temperatures over different temporal scales (chronological time, developmental time and developmental stage).

To describe the effect of culture temperature on transfer of yolk between developing midgut and caecum structures, the area of the caecum (plural) was estimated by multiplying the area of one visible caeca by two.

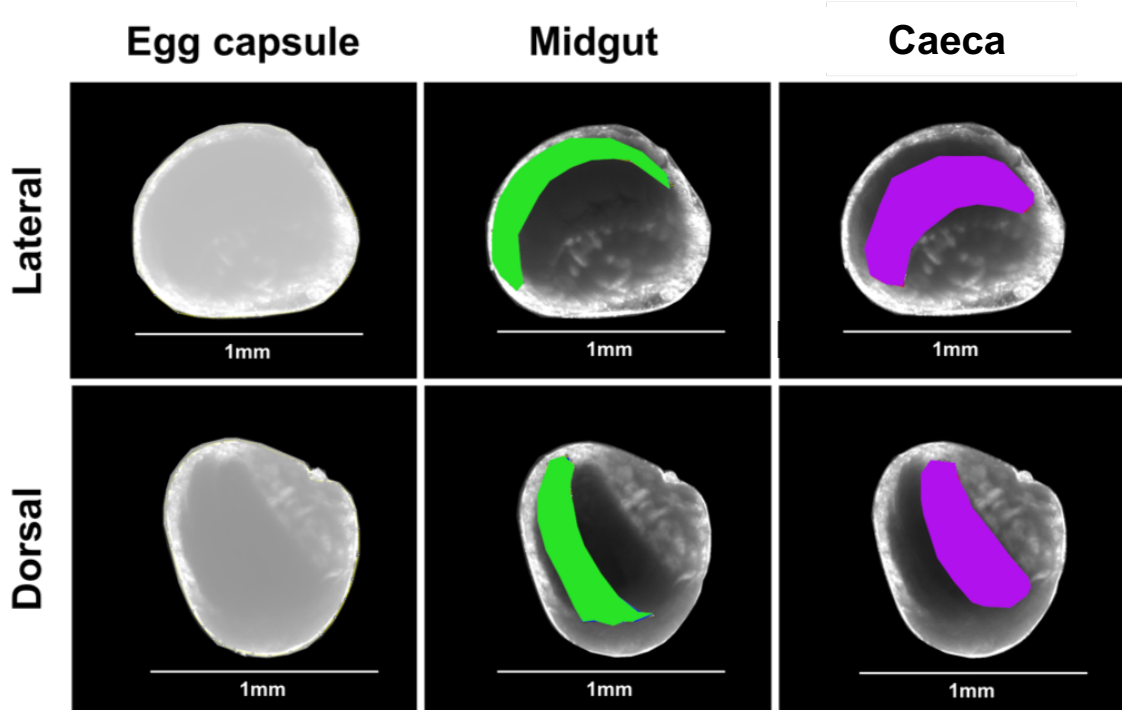

Figure 3.1 Stage 25 (Fig. 3.2) of embryonic *Orchestia gammarellus*, lateral and dorsal view of whole egg capsule (white), midgut (green) and caeca (purple).

3.3.5 Effect of hypoxia on GI movement

To investigate whether environmental hypoxia altered the rate of GI movements, indicative of its pre-cardiac function, the following experiment was carried out. Embryos were placed in a watchglass (diam. = 2 cm) containing sea water (vol. = 1 – 2 mL, S = 32) at room temperature (T = 23.6 °C) and the watchglass was located on the stage of a binocular microscope (x 40 mag.). The water in the watchglass was aspirated by gas mixtures produced by a precision gas mixing apparatus (Wöstoff, Buchum, Germany). The initial gas mixture (20 % O_2/ 80 % N_2) was aspirated for about 10 min before it was

replaced by a hypoxic mixture (2 % O_2/ 98 % N_2) for about a further 10 min. Preliminary tests showed no significant difference between nominal and measured oxygen concentration (PO_2). PO_2 was not measured at the time of the experiment to avoid disturbance effects that may stimulate embryonic movement. The number of peristaltic waves observed across the GI was viewed under normoxic and hypoxic conditions, under low power magnification (x 10 - 14) and recorded manually for a 60 sec period. Any other GI behaviour due to the hypoxic treatment was also noted.

3.3.6 Statistical analysis

All data analysis was performed in R studio (Rstudio Team, 2016). All data were log transformed to meet normality criteria. Shapiro wilks test for normality and Levene's test for homogeneity of variance were carried out on all transformed data and the assumptions were met. A two-way analysis of variance (ANOVA) was used to test for effects of culture temperature and different measures of time (chronological time, relative time and developmental stage) on the rate of GI movements. Linear regression analysis gave slopes of lines for all yolk data. Analysis of covariance (ANCOVA) was used to establish if there was an effect of temperature on the total yolk area of the caeca when the total yolk area of the midgut was controlled for as a covariate. In the absence of such relationships the effect of hypoxia on gut movements was tested using a paired 't' test.

3.4 RESULTS

3.4.1 Description of ontogeny of GI movements

The embryonic development of *Orchestia gammarellus* generally (stages 1 - 30), and GI development specifically, are very similar to those described in detail by Browne *et al*., (2005) for another talitroidean, *Parhyale hawaiensis.* Although the chronology of development differed significantly between the two species, the sequence of developmental stage marks were identical for *O. gammarellus* developing at 15 and 20 °C. Therefore, the following stages described below for *O. gammarellus* supplement the existing stage list for *P. hawaiensis* and provide detail of GI behaviour not previously recorded for stages 22 - 30. Stages 1 - 21 exhibited no observable GI movement and will therefore not be repeated here (Refer to Browne *et al*. 2005 for details).

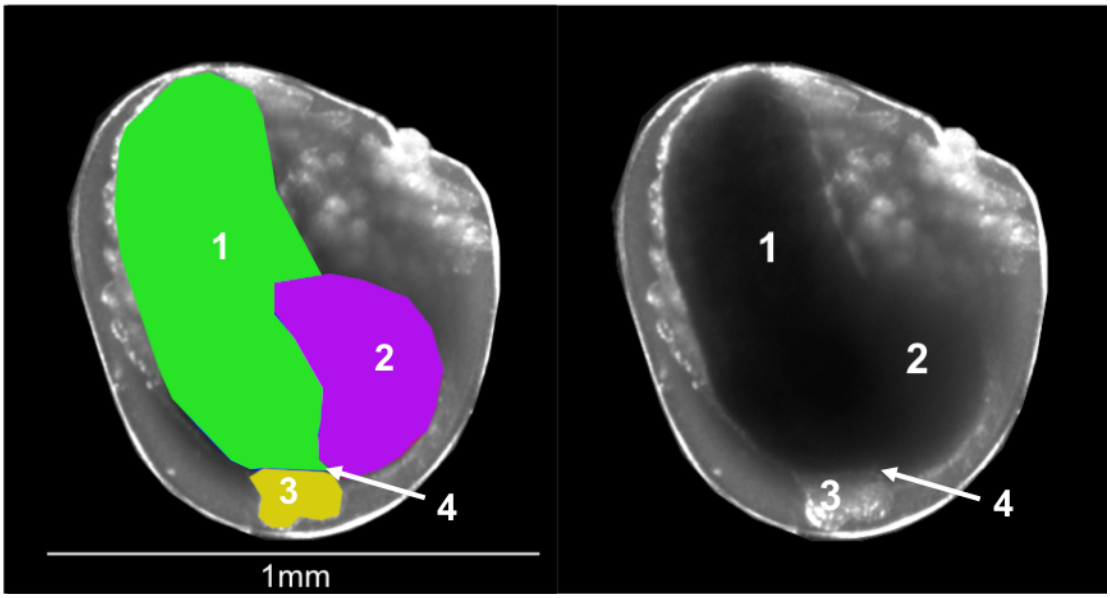

Figure 3.2 Stage 22, illustration of features described in stage list: (1) midgut (green), (2) single digestive caeca (purple), (3) dorsal organ and (yellow) and (4) foregut midgut junction.

3.4.1.1 Stage list

Stage 22 (S22 = 0 %): The midgut (Fig. 3.2), a straight tube with two pairs of digestive caeca and completely envelops all yolk contained within the embryo. The two dorsal anterior caeca appear as small almost transparent sacks (Fig. 3.2) attached dorsolaterally at the foregut midgut junction (Fig. 3.2). At this stage, the first GI movements appear as a single sporadic pulse; a full contraction of the caecum sack, marking the start of caecum extension in the posterior direction. Note that this movement is not the same as a peristaltic wave (Fig. 3.4) as it occurs as a single contraction, not as serious of contractions (Fig. 3.4).

Stage 23 (S23 = 5 – 10 %): As the two dorsal anterior caeca - secretory organs consisting of a pair of blind tubes that open into the anterior of the midgut (Browne *et al*., 2005) - begin to extend in unison, the first regular peristaltic wave motions (Fig. 3.4) can be seen (~ 1 wave.min^{-1} at T = 15 °C and ~ 2 wave.min^{-1} at T = 20 °C) traveling the short length of the caecum structure. Waves typically, but not exclusively, initiate as a contraction in the centre of the caeca (Fig. 3.4), dissimilar to the exclusively anterior to posterior waves briefly outlined by Browne *et al*. (2005). Each caeca operates with its own rhythm and direction of peristaltic wave travel. No muscular twitching can be seen at this time.

Stage 24 (S24 = 15 %): This stage marks the midpoint of caecum extension in the posterior direction towards the abdomen. The midgut has thinned, and the

caecum have approximately doubled in area when compared to the start of S23.

Stage 25 (S25 = 20 %): The extension of the caeca to the rear of the abdomen is now complete, some yolk has been transferred from the developing medial midgut to the dorsal anterior caeca but much yolk remains in the midgut (more so than is seen in *P. hawaiensis* at this stage). The midgut appears as a thick strip between the two caecum that's movements are characterised by increasingly frequent peristaltic waves. These waves, while typically emanating from the centre of the caecum, normally travel the length of the caeca tube in both the posterior and anterior directions (Fig. 3.4). A wave may also originate at the anterior or posterior end of the caecum tube and radiate away from the site of contraction in either direction along its length. Each caeca continues to operate its own rhythm, site of contraction (anterior, posterior or mid caeca) and direction of peristaltic wave travel, which seems unique to the individual and can change unpredictably with ontogeny. This is the case in embryos cultured at both 15 and 20 °C.

Stage 26 (S26 = 30 %): The caeca continue to increase in girth as yolk continues to transfer from the midgut to the caeca. The tubular heart, a bilaterally symmetric single chamber structure positioned dorsally above the midgut, has formed and begins to beat. Faint eyespots become visible and slight muscular twitching begins. Here key developmental milestones (i.e. the start of heartbeat and eyespots) parallel that of *P. hawaiensis*, yet the caeca of

O. gammarellus appear to continue to fatten at this stage whereas yolk stores of *P. hawaiensis* begin to deplete.

Stage 27 (S27 = 40 %): The midgut is largely transparent and not visible when the GI tract is viewed laterally, and the caeca's yolk stores begin to diminish. Waves continue to increase in frequency (~ 3 wave.min^{-1} and ~ 5 wave.min^{-1} at T = 15 and 20 °C) and muscular twitching motions become more regular.

Stage 28 (S28 = 45 – 70 %): Yolk at the anterior and posterior ends of the caeca have diminished, giving the appearance of tapered ends as the yolk recedes towards the center of the caeca. Peristaltic wave frequency often reaches its peak for individuals reared at T = 15 °C (~ 5 wave.min^{-1}) at this stage, however for individuals reared at T = 20 °C at S28 a wave frequency of ~ 10 wave.min^{-1} is observed and this reaches a maximum at S29. Muscular twitching continues to increase in intensity and frequency.

Stage 29 (S29 = 75 – 95 %): The heart is beating strongly, and muscular twitching can be intense and regular (30 movements.min^{-1}, at T = 20 °C). Wave frequency peaks in this stage for individuals reared at T = 20 °C (~ 12 wave.min-1). The caeca are well defined, the dense yolk within having considerably diminished, giving the caeca a thinner appearance compared to previous stages. At this stage the gut often shows weaker peristaltic wave movement; occasionally exhibiting a quivering, vibrating motion during this stage opposed to strong defined waves. The peristaltic waves pausing can last for > 1 min.

Stage 30 (S30 = 100 %): Flexing the length of its body, the juvenile uses its telson to pierce the embryonic membrane and ‘kick free’ from the embryonic capsule, immediately entering a state of coordinated motion. The GI tract is visible through the cuticle with the juvenile caeca appearing dense with residual yolk.

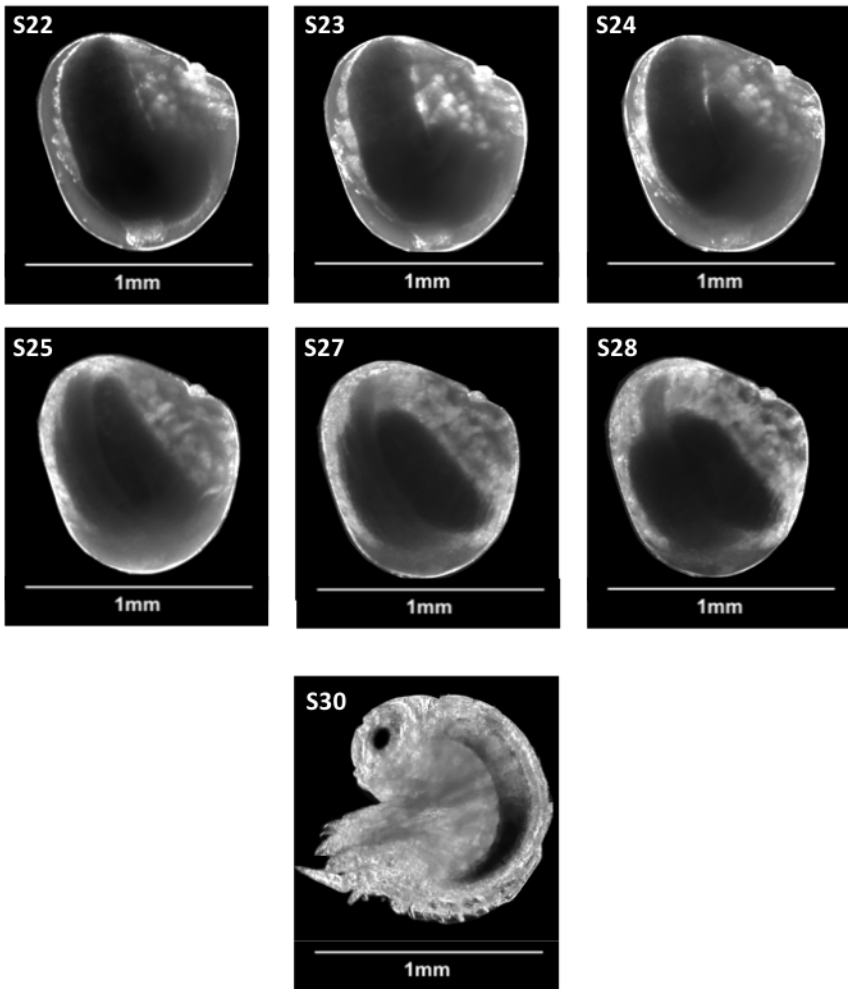

Figure 3.3 Stages 22, 23, 24, 25, 27 and 28 of GI development of *Orchestia gammarellus* (dorsal view) and of hatching stage 30 (lateral view) captured using video microscopy techniques described in Chapter 2 and section 3.2.

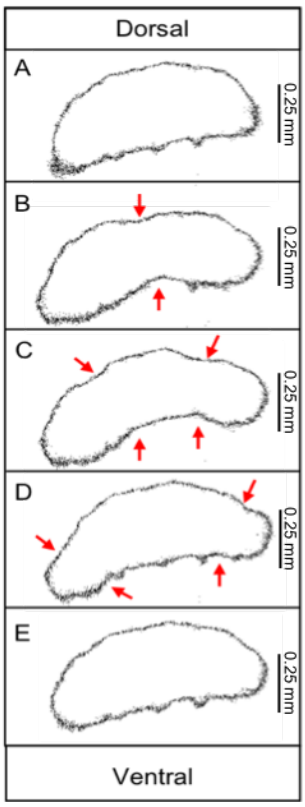

Figure 3.4 Outline of caecum (S26) isolated using threshold tool, image analysis software Fiji (Schindelin, 2012) to show change in form of GI structure during peristaltic motion. Arrows indicate the direction of contracting movement. A) Caeca relaxed; B) contraction mid-caeca, indicative of the origin of a peristaltic wave; C) sequential contractions in the anterior and posterior direction move the wave towards both ends of the tube; D) contractions terminate at the end of the tube; E) caeca returns to a relaxed position similar to A.

3.4.2 Visualising yolk release and relating it to GI movements

From S25 – S30, once the caecum had fully extended (S25), yolk was seen on several occasions to be released from GI structures into the intracellular space (Fig. 3.5) in “plumes” – concentrated clouds of yolk (area > 2 % egg capsule). Yolk plumes did not correlate to GI movements recorded here but seemingly co-

occurred with distinct haphazard global twitching movements within the embryo. Although twitching behaviour was more frequent in embryos exposed to T = 20 °C, the incidences of yolk extrusion remained few and did not appear to differ between the two treatments. Yolk plumes were not quantifiable, given their sporadic and apparent infrequent occurrence although this observation may be an artefact of the data collection method (i.e. hourly sampling as opposed to continuous observation).

Yolk movement was not traceable, as plumes of yolk dissipated quickly (< 1 sec) and required greater image resolution than that achieved here.

Yolk was only observed to be released close to the head and dorsal organ (Fig. 3.5 A), in both treatments. It is possible that yolk is extruded in other regions not captured during the imaging process.

As the embryo developed from S22 to S30 a continuous translocation of yolk from the midgut to the attached caecum was evident (Fig. 3.8), this is likely powered by peristaltic wave action (Browne *et al*., 2005).

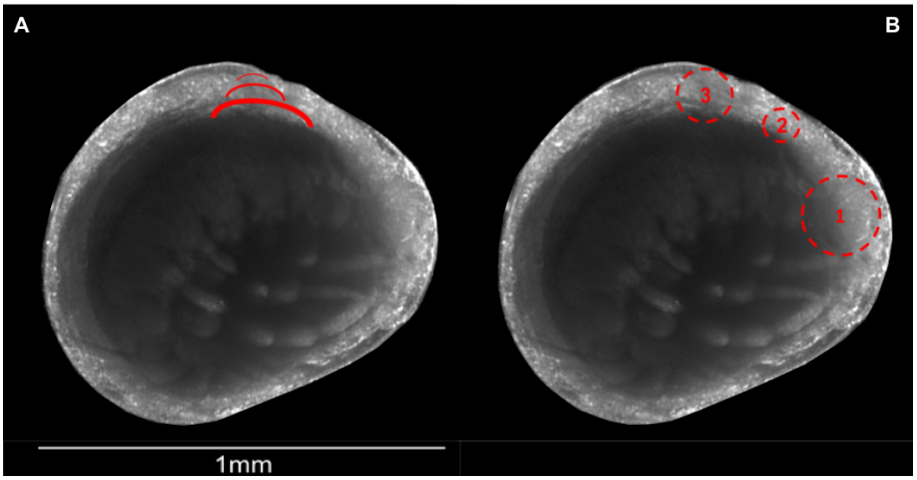

Figure 3.5 *Orchestia gammarellus* embryo captured using video microscopy techniques described in Chapter 2. A) Red lines represent a typical plume of yolk ejected from the gut into extracellular space. B) Circles 1 - 3 represent regions of the embryo where plumes of yolk have been observed (1) in the head region, (2) anterior to the dorsal organ, (3) posterior to the dorsal organ.

3.4.3 Effect of temperature on developmental rate and mortality

The rate of morphological development generally, and of the GI tract specifically, was 2.4 x greater when individuals were incubated at T = 20 °C compared to T = 15 °C. The ontogeny of the GI tract lasted on average 230 h at T = 15 °C and 95 h at T = 20 °C from the start of gut movement (S22) to hatching (or S30). Despite temporal differences in the ontogeny of GI structures, stages of gut development were sequentially identical for individuals reared in both temperatures. Mortality was high across treatments, with 94 and 98 % mortality at T = 15 and 20 °C respectively. Unlike natural thermal cycles experienced in situ, incubation at constant static temperature (despite 15 and

20 °C being within *O. gammarellus*' natural thermal range) may exert a degree of thermal stress resulting in the high mortality observed.

3.4.4 Effect of development and temperature on GI movements

Presented in Figure 3.6 is the effect of incubation temperature on peristaltic wave frequency during the embryonic development of *O. gammarellus*. Two-way ANOVA (only including comparable data i.e. 0 – 105 h) detected significant effects of time ($F_{(1,339)} = 56.12$, $p < 0.001$) and of treatment ($F_{(1,339)} = 7.19$, $p < 0.01$) on the rate of GI movements, with a clear interaction between them ($F_{(1, 339)} = 53.10$, $p < 0.001$). There was an unmistakeable visible difference in gut movements between the two temperatures. At T = 15 °C mean gut movements of individuals 15 h in culture were approx. 1 movement.min^{-1}. This rose steadily with time to a maximum after 165 h of approx. 4 movements.min^{-1} before diminishing back to ~ 1 movement.min^{-1} immediately pre hatching (225 h). At T = 20 °C the number of movements after 15 h was approximately double that observed at T = 15 °C and increased to a maximum of approx. 8 movements.min^{-1} at 75 h. This was followed by a decline to approx. 6 movements.min^{-1} at 120 h with no individuals surviving 135 h.

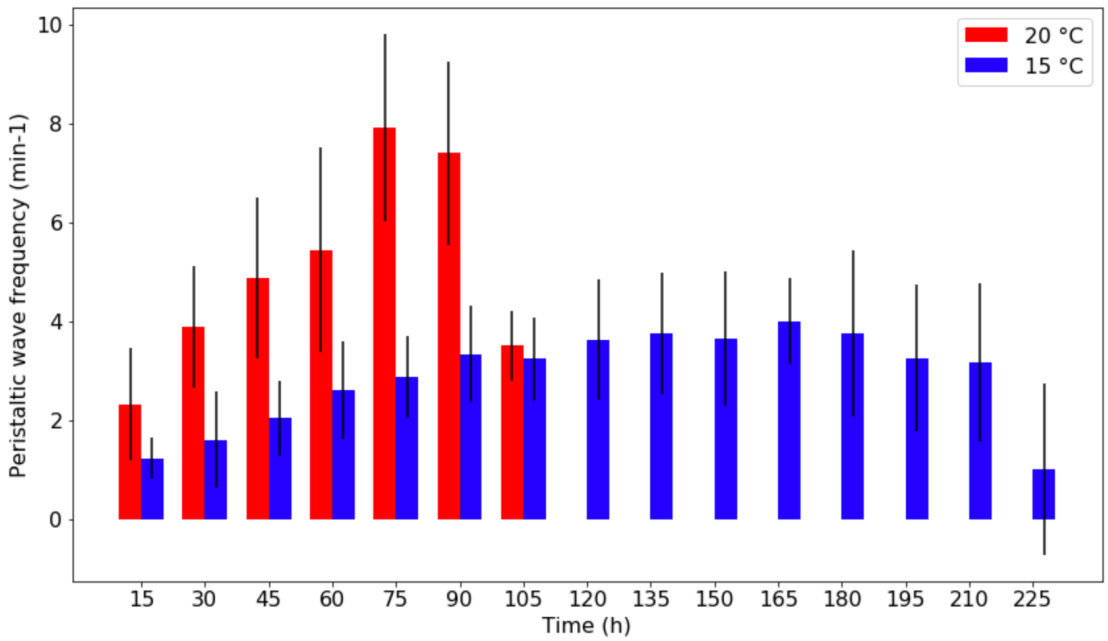

Figure 3.6 Frequency of peristaltic waves (mean ± S.D., n = 35 and 25 for 15 and 20 °C respectively) at different times during embryonic development for *Orchestia gammarellus* reared at T = 15 and 20 °C.

After standardising GI movement data to correspond to developmental time (see Materials and Methods) a two-way ANOVA on data split in to quartiles (≤ 25; > 25 – 50; > 50 – 75; > 75 %) was performed and this revealed significant effects of developmental period ($F_{(1,76)}$ = 82.19, $p < 0.001$) and of culture temperature ($F_{(1,76)}$ = 98.30, $p < 0.001$) on the rate of GI movements, with a significant interaction between them ($F_{(1,76)}$ = 14.43, $p < 0.001$).

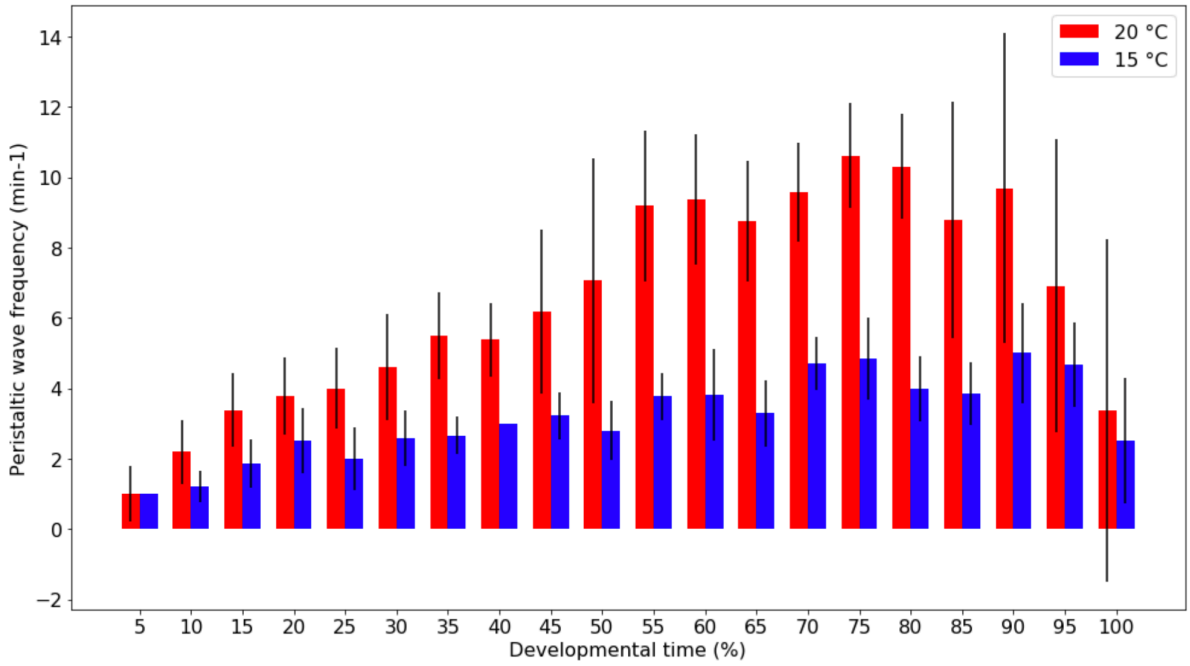

Figure 3.7 Frequency of peristaltic waves (mean ± S.D., n = 10 per 15 and 20 °C respectively) at relative developmental percentage time points during embryonic development of *Orchestia gammarellus* reared at T = 15 and 20 °C

GI movement data was further compartmentalised into comparable developmental stages (Fig. 3.8). A two-way ANOVA of this data revealed significant effects of developmental stage ($F_{(1,145)} = 32.06$, $p < 0.001$) and of treatment ($F_{(1,145)} = 37.11$, $p < 0.01$) on the rate of GI movements, however, similar to developmental time, there was no significant interaction ($F_{(1, 145)} = 2.25$, $p = 0.135$).

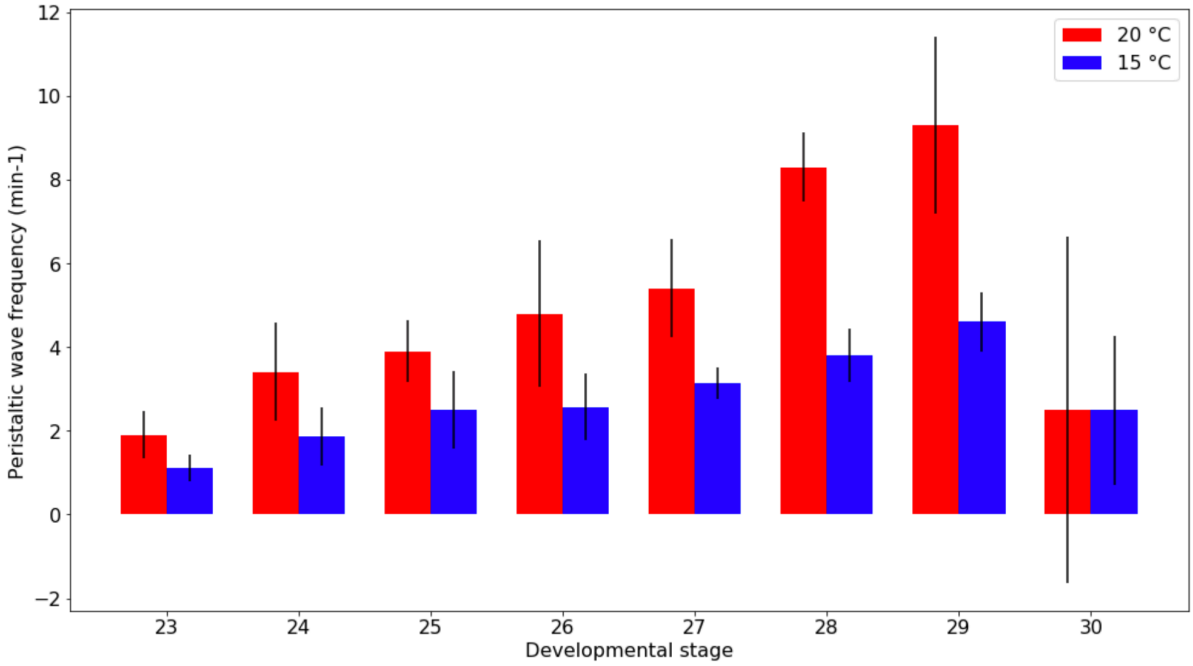

Figure 3.8 Frequency of peristaltic waves (mean ± S.D., n = 10 per 15 and 20 °C) at different stages during embryonic development for Orchestia gammarellus reared at T = 15 and 20 °C.

Q_{10} values presented in Figure 3.9 show more than a doubling of rate of GI movements (< Q_{10} of 2) at all stages with a 5 °C increase in culture temperature with the exception of stage 30. Disparities in rate peaked at stage 28, where rate of GI movement was > 4.5 x greater for individuals reared at 20 °C compared to 15 °C, declining to an equal rate at stage 30.

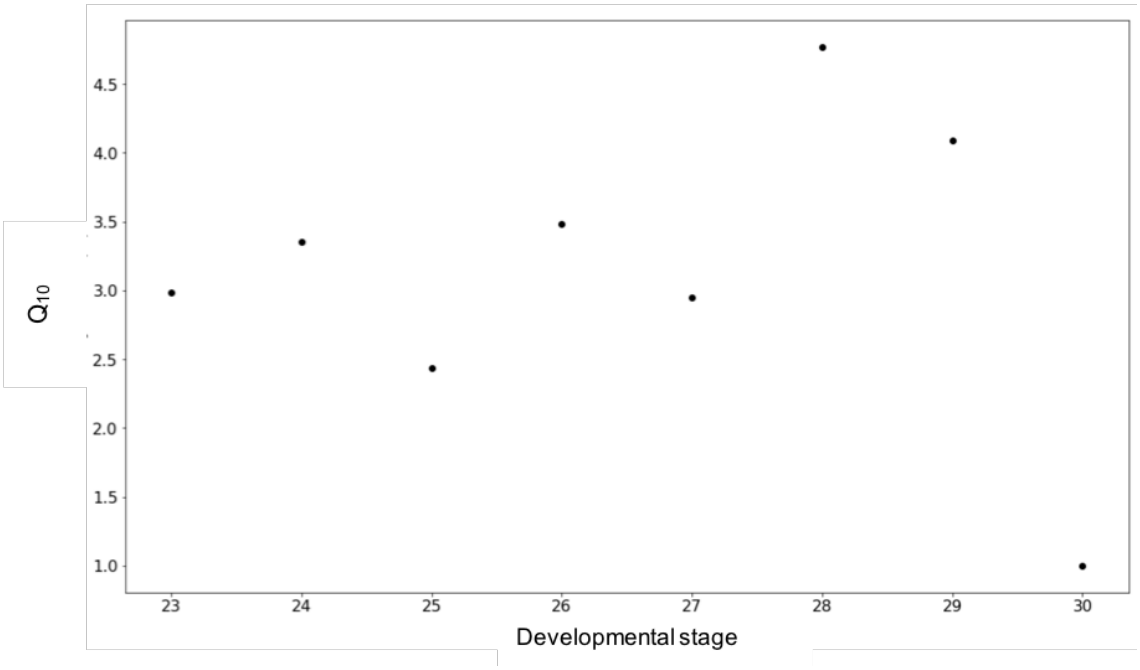

Figure 3.9 Temperature coefficient Q_{10} values calculated from peristaltic wave frequency means for each developmental stage of embryonic *Orchestia gammarellus*.

3.4.5 Effect of development and temperature on yolk usage

Figure 3.10 presents the effect of incubation temperature on total percentage yolk area per unit time (as an indices of yolk usage) during embryonic development. ANCOVA detected a significant effect of culture temperature ($F_{(1,230)} = 6.49$, $p = 0.011$) and time ($F_{(1,230)} = 243.83$, $p < 0.001$) on the total yolk area, with a significant interaction between the two ($F_{(1,230)} = 42.9$, $p < 0.001$) (Fig. 3.9) i.e. rate of yolk utilization was greater at T = 20 °C than 15 °C.

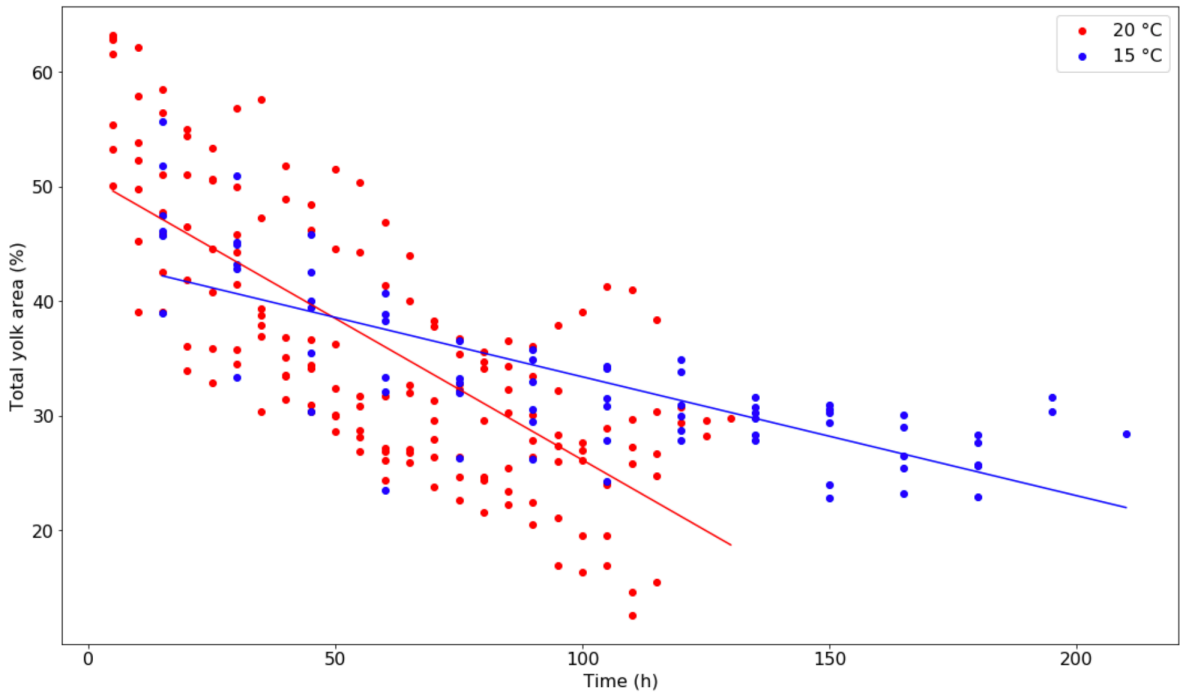

Figure 3.10 Percentage of total yolk area at different times throughout embryonic development of *Orchestia gammarellus* for T = 15 °C (line of best fit Yolk area = 45.59 – 0.1179 chronological time) and T = 20 °C (line of best fit Yolk area = 52.32 – 0.2656 chronological time)

Figure 3.11 presents the effect of temperature on yolk usage when time is standardised (to account for an average 2.4 x faster development at 20 °C) and expressed as % total development time. Two-way ANOVA shows a significant effect of culture temperature ($F_{(1,232)} = 6.08$, $p = 0.014$) and developmental time ($F_{(1,232)} = 345.75$, $p < 0.001$) with a significant interaction between these variables ($F_{(1,232)} = 10.10$, $p < 0.001$), i.e. again yolk utilisation is greater at T = 20 °C compared to 15 °C even when developmental time is standardised.

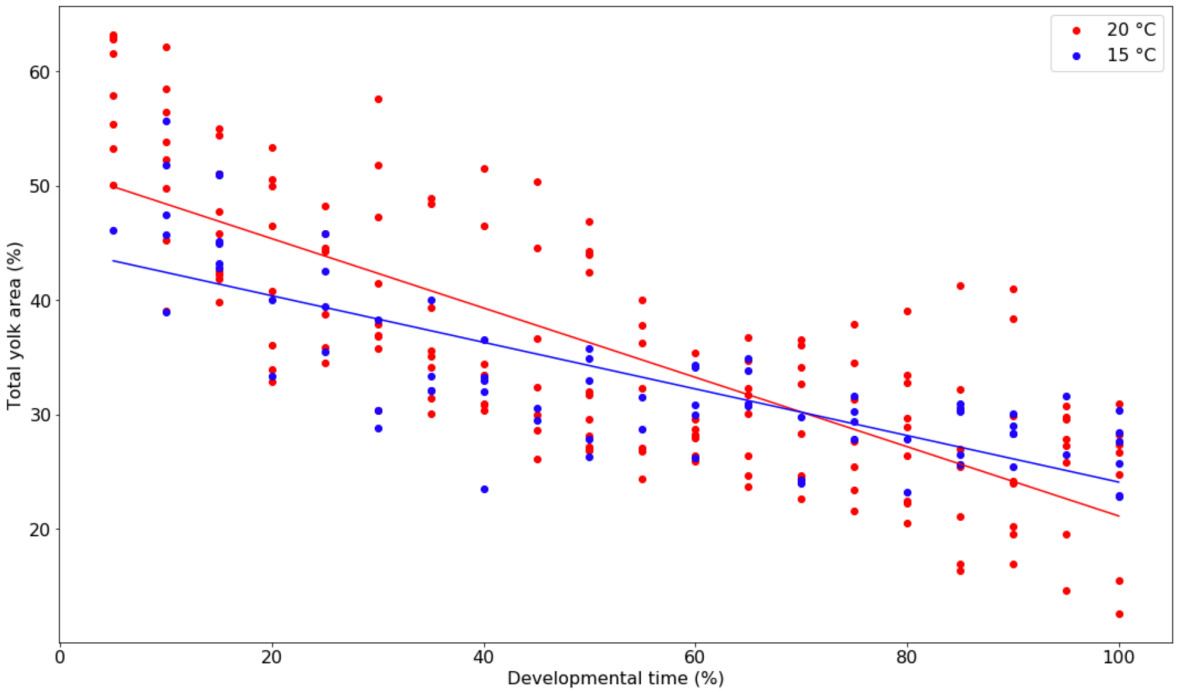

Figure 3.11 Percentage of total yolk area at different relative developmental percentage time points throughout embryonic development for *Orchestia gammarellus* T = 15 °C (line of best fit Yolk area = 44.46 – 0.2036 developmental time) and T = 20 °C (line of best fit Yolk area = 51.43 – 0.3031 developmental time)

When developmental time is segmented into comparable developmental stages (Fig. 3.12), two-way ANOVA confirmed a significant effect of temperature ($F_{(1,91)}$ = 10.16 , $p < 0.001$) and developmental stage ($F_{(1,91)}$ = 219.44 , $p < 0.001$) on yolk usage rate, with a significant interaction between them ($F_{(1,91)}$ = 6.217, p = 0.014). This suggests that, across a multitude of temporal scales, including stages of development, yolk utilisation is consistently higher for individuals reared at 20 °C.

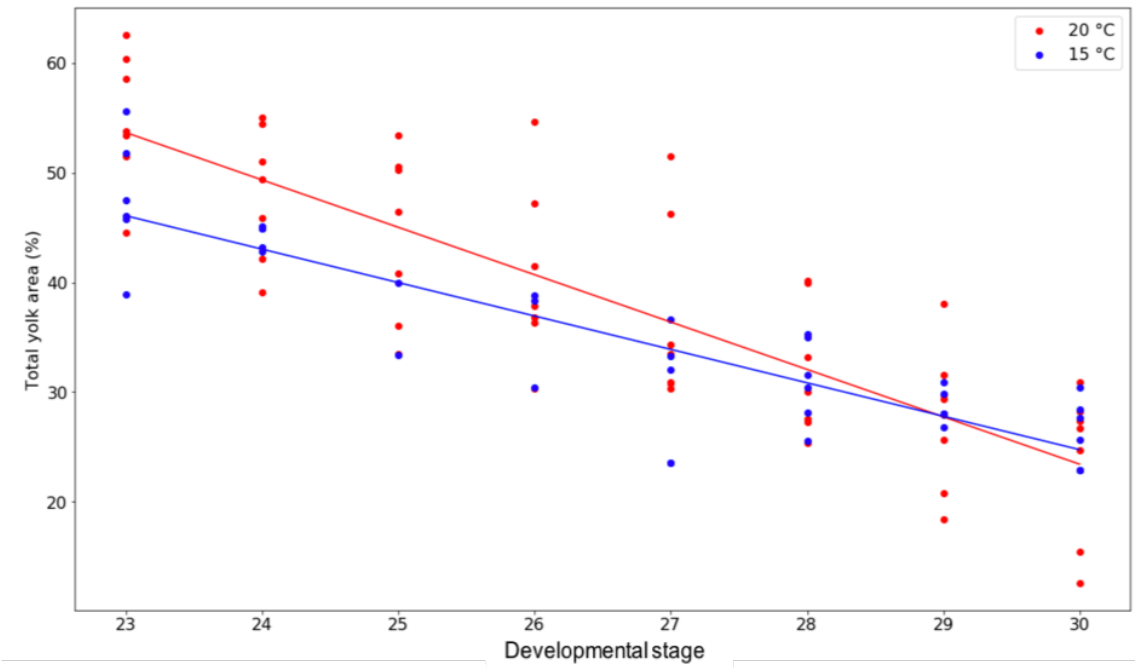

Figure 3.12 Percentage of total yolk area at different stages throughout embryonic development for *Orchestia gammarellus* T = 15 °C (n = 6; line of best fit Yolk area = 116 – 3.045 developmental stage) and T = 20 °C (n = 7; line of best fit Yolk area = 152.9 – 4.317 developmental stage).

3.4.6 Effect of temperature on yolk transfer between GI structures

During GI ontogeny yolk is transferred from the medial midgut cavity to the expanding caeca. Regression analysis confirmed a linear relationship between midgut and caeca yolk area for both treatments (R^2 = 0.68 and 0.62 for T = 15 and 20 °C, respectively – see Fig 3.12 for slopes). Temperature does not appear to effect this relationship, as ANCOVA (performed to examine the effects of culture temperature on total yolk area of the caecum, whilst controlling for midgut yolk area) showed no significant effect of treatment on caeca yolk area ($F_{(1,128)}$ = 274.56, p = 0.191).

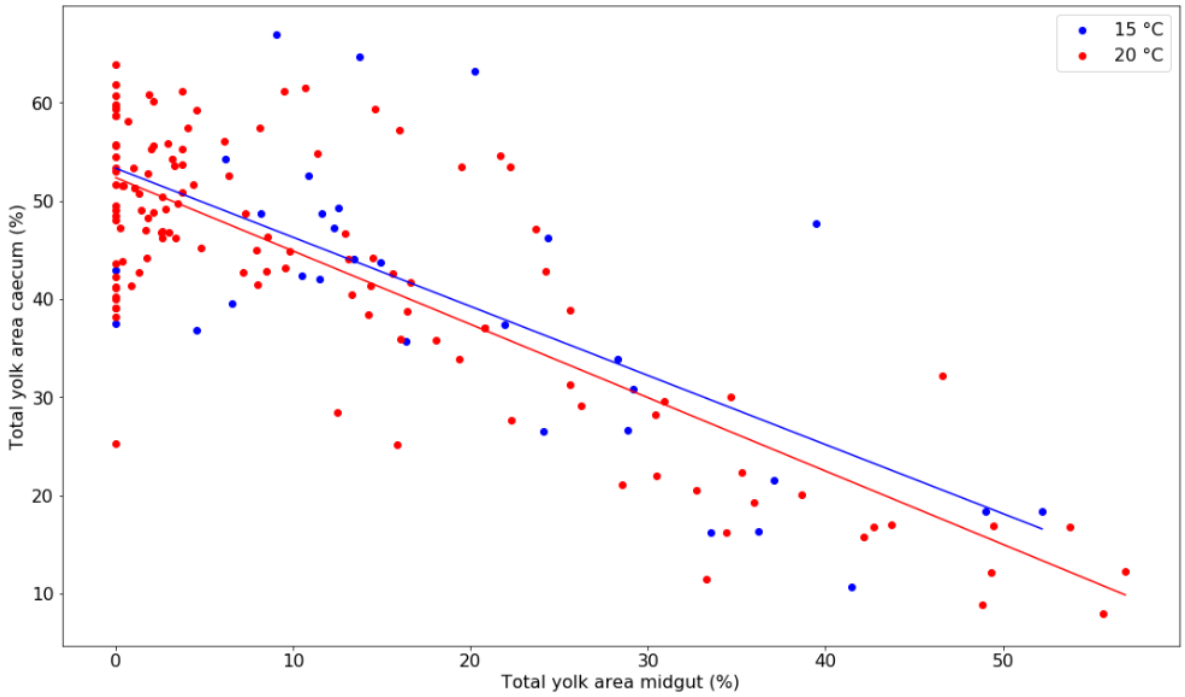

Figure 3.13 Relationship between the total amount of yolk (presented as % area) within caecum structures relative to midgut structure for *Orchestia gammarellus* reared at 15 (n = 6, line of best fit Yolk area = 53.3 – 0.7029 x) and 20 °C (n = 6, line of best fit Yolk area = 52.33 – 0.7031 x).

3.4.7 Effect of hypoxia on GI movements

There was no effect of acute hypoxia on the frequency of GI movements in early or late stage individuals (paired 't' test, t = 0.54, n = 6, p = 0.611) with a mean frequency of 2.8 - 3.0 movements.min^{-1}. There was, however, an apparent increase in sporadic muscle twitching behaviour in the embryo during exposure to low oxygen.

3.5 DISCUSSION

I set out to test the hypothesis that embryonic gut movements in talitroidean amphipods function as an extracardiac pump, dispersing yolk to growing and developing embryonic tissue, and aiding in the delivery of oxygen to developing tissues. While doing so I have produced the first detailed description and quantification of GI movements from their appearance in early development (S22), through to hatching (S30) of any amphipod, and to my knowledge, any crustacean species. All stages of morphological development (S1 - 30) of *O. gammarellus* and particularly with respect to gut formation, exhibited temporal, but no sequential intraspecific differences in developmental milestones compared with that previously described for the only other talitroidean amphipod for which we have comparable information, *P. hawaiensis* (Browne *et al*., 2005). To test my proposed hypothesis, a number of predictions were made.

3.5.1 GI movement and yolk dispersal

First, if gut movements were related to yolk dispersal, it should be possible to measure yolk release, trace the subsequent movement of the released yolk and second relate it to particular GI movements in the embryo (Predictions 1 and 2). It was possible, to some extent, to visualise yolk release from the gut and visually follow subsequent movement of that yolk within the embryo. On occasion, dense yolk plumes were released from the GI tract and dispersed to surrounding tissues, however, these releases were few, sporadic, short lived (> 1 sec) and unquantifiable. Plumes were not obviously linked with GI

movements. Instead, these releases seemed to co-occur with distinct global twitching movements within the embryo. Similar twitching movement have been observed previously in crustacean embryos, e.g. mysids (Berrill, 1971, 1973) and lobsters (Styf *et al*., 2013), Berrill (1973) suggested that these movements are associated with trialling developing muscular systems (Berrill, 1973). Given that the incidence of such twitching moments could be induced by particular stresses (e.g. the effect of hypoxia described below) it is possible that these behaviours could result in release of yolk to meet heightened metabolic demand.

As embryos developed, significant reduction in the total yolk area of GI structures was observed, indicative of GI yolk release. This supports the hypothesis that the gut is releasing nutrients to growing and developing tissues. Given the infrequency of yolk plumes observed, it is likely that nutrient release from the gut is occurring on a finer, less visible, scale. It was possible to quantify changes in yolk area, making it possible to indirectly measure rates of yolk release, satisfying prediction 1 to some extent. However, as it was not possible to trace the release of yolk nor relate it to specific GI movement, prediction 2 is not met.

3.5.2 Temperature

As for most ectotherms, metabolic rate of crustacean eggs increases with temperature (e.g. Styf *et al*., 2013; García-Guerro *et al*., 2003; García-Guerro, 2010; Naylor *et al*., 1999). On this basis, the third prediction was that an

increase in environmental temperature should change the frequency and/or character of gut movements to release more yolk to meet heightened metabolic demand. While the yolk pluming was not related to temperature, gross GI movements were. A 5 °C change in culture temperature had a pronounced effect on GI tract peristalsis, with warm incubated embryos (T = 20 °C) expressing a significantly higher frequency of movement throughout ontogeny (with the exception of stage 30). The calculated Q_{10} varied from a minimum of 1 (stage 30) to a maximum of greater than 5. This figure is greatly in excess of the value of 2 which typifies the effect of temperature on biochemical reactions *in vitro.*

In *O. gammarellus*, using the visual measurement method employed here, yolk area (as a proxy for yolk usage) did appear to decline with a significant effect of culture temperature on the rate of usage. This compliments findings of the effects of temperature on other crustaceans, e.g. the embryonic stages of the Norway lobster *Nephrops norvegicus* (Styf *et al*., 2013), the crayfish *Cherax quadricarinatus* (García-Guerro *et al*., 2003) and the prawn *Macrobrachium americanum* (García-Guerro, 2010) where there was a clear increase in yolk consumption with increased temperature.

Although I was not able to observe directly, or trace where the yolk went (Predictions 1 and 2), a significant increase in GI movement and in yolk utilization, as evidenced by the disappearance of the yolk, overall supports the hypothesis that embryonic gut movements in talitroidean amphipods disperse yolk to growing and developing embryonic tissue.

3.5.3 The effect of oxygen

Finally there is evidence from some crustaceans (Spicer, 2006; Woods *et al*., 2017) and gastropod mollusc (Bitterli *et al.,* 2012;) species that if, for whatever reasons, oxygen supply does not meet demand in developing embryos, this stimulates movement of extracardiac structures such as the gut, and internal movement generally, to provide accessory cardiovascular function. Embryos of *O. gammarellus* did not exhibit any increase in peristaltic wave frequency in response to environmental hypoxia, suggesting that the GI movements we observed do not have any significant role in the circulation of extracellular fluid in *O. gammarellus* embryos. The GI movements certainly contribute to mixing of embryonic extracellular fluids, although this appears to be a passive consequence rather than an active mechanism. Thus my study does not support the hypothesis that GI movements are a circulatory mechanism to pump more oxygen round the body, although any temperature related increase in GI movement should facilitate enhanced oxygen diffusion indirectly.

3.6 CONCLUSION

There is reasonable support from this study for the hypothesis that embryonic gut movements in talitroidean amphipods function as an extracardiac pump, dispersing yolk to growing and developing embryonic tissue. There is a clear effect of environmental temperature on this function, as elevated incubation temperature resulted in a significant increase in GI movement and yolk usage.

Although yolk utilisation does appear to increase with temperature, it is unknown if the energetic costs of (amongst other things) increased GI movements out weights this supply. High pre-hatching mortality could be linked to a mismatch between energetic demand and supply leading to the demise of the embryos before hatching at moderate temperatures. Consequently, in the next chapter the thermal tolerance of embryos of *O. gammarellus* at different stages of development will be determined while also trialling a novel method and technique for determining the upper thermal tolerance of animals, particularly in their embryonic stages.

CHAPTER 4: ONTOGENETIC SHIFTS IN UPPER THERMAL TOLERANCE LIMITS DURING EARLY DEVELOPMENT OF THE BROODED AMPHIPOD, *ORCHESTIA GAMMARELLUS*

E. Tully conducted material collection and maintenance, planned and carried out the experiments, analysing the data and wrote the chapter.

4.1 ABSTRACT

The assessment of species sensitivity to elevated temperature is essential to our understanding of current and future organismal responses to climate change. Currently upper thermal tolerance limits, used to infer species resistance to thermal change, are based almost entirely on adult data, ignoring potentially more sensitive early life-stages. How and why thermal limits vary with ontogeny is poorly understood, with current knowledge primarily derived from few assessments of species which develop externally to the maternal/ paternal environment (non-brooding species). Our knowledge of the effect of ontogeny on thermal tolerance traits in species developing within the parental environment (brooded) is severally lacking. Consequently, I aim to assess how and why thermal tolerance changes with ontogeny for an ecologically important brooded species, *Orchestia gammarellus,* in the context of improving understanding of what drives ontogenetic shifts in thermal tolerance in a brooded system. This was achieved using thermal death time curves, which incorporate the intensity and duration of heat stress, to calculate upper thermal limits (CT_{max}) and sensitivity (z) estimates for multiple developmental stages. CT_{max} and *z* estimates were used to generate thermal tolerance landscapes, enabling estimation of survival probability of each life stage to acute thermal stress. Pre-cardiac embryos had the highest CT_{max} and lowest *z* value relative to post-cardiac and juvenile individuals, suggesting a greater ability to tolerate short exposure to thermal extremes and poorer ability to withstand prolonged exposure to moderate temperatures relative to later stages. Post-cardiac stages displayed a distinct pattern in knockdown time that is absent in pre-cardiac individuals, suggesting a relationship between cardiac output, temperature and

upper thermal limits. This study is the first to illustrate that thermal tolerance varies across multiple early life-stages of a brooded species and provides the first assessment of its kind for any member of the Amphipoda. I conclude that underlying patterns and potential mechanisms in shifts in thermal tolerance at the individual level are essential to improve the accuracy of predicting organismal responses to local and global environmental change.

4.2 INTRODUCTION

Upper thermal tolerance limits are frequently used to infer species vulnerability to changes in environmental temperature (Deutsch *et al*., 2008; Somero, 2009; Chown *et al*., 2010; Sunday *et al*., 2010, 2012) and form an integral part of modelling the effects of climate change (Madiera *et al*., 2012; Molinos *et al*., 2016) and formulating mitigation efforts. Predictive models, such as species distribution models, have recently begun to incorporation biological mechanisms to predict species responses to climatic change (Riddell *et al*., 2016; Carlo *et al*., 2017) but almost exclusively formulate predictions based on adult data (e.g Buckley, 2008; Deutsch *et al*., 2008; Randin *et al*., 2009; Kearney, 2013). Quantifying thermal tolerance at such coarse resolutions maybe misleading since thermal tolerance varies considerably with ontogeny; a phenomenon that has been documented for a variety of vertebrate (e.g. Winne and Keck, 2004; Komoroske *et al*., 2014; Turriago *et al*., 2015) and invertebrate (Bowler and Treblanche, 2008; Diederich and Pechenik, 2013; Miller *et al*., 2013; Radchuk *et al*., 2012; Truebano *et al*., 2018) species, with early life-stages often expressing heightened sensitivity to environmental change (Zippay and Hofmann, 2010; Schiffer *et al*., 2014; Truebano *et al*., 2018). To effectively utilise thermal tolerance estimates in any context there is a recognised need to incorporate the effects of ontogeny (Bowler and Treblanche, 2008; Kingsglover *et al*., 2011; Radchuk *et al*., 2012; Levy *et al*., 2015). However, our current understanding of patterns and underlying mechanisms that drive ontogenetic shifts in thermal tolerance, particularly during early development, is severely underdeveloped.

The study of non-brooding species – those whose young develop outside the maternal/ paternal environment – has yielded several testable hypotheses, both adaptive and non-adaptive that aim to address mechanisms underlying ontogenetic shifts in thermal tolerance. Physiological re-organisation associated with moulting events and transitions between life-stages (e.g. as a result of metamorphosis in amphibians and arthropods – reviewed in Spicer and Gaston, 1999) has been proposed as a potential driving force in some groups, for example the insects (Bowler and Treblanche, 2008; Kingsglover *et a*l., 2011), and may explain abrupt transitions in heat tolerance between life-stages. Such as those expressed in the marine gastropod, *Littorina obtusata*, where both early and late stage embryos expressed a critical thermal maxima (CT_{max}) 7.2°C lower than adult individuals (Truebano *et al*., 2018). Disparities within the same life-stage can be just as, if not more, extreme than between life-stages. For the toad, *Bufo woodhousii fowleri*, the difference in CT_{max} between early and late stage tadpoles was far greater than between pre-metamorphic tadpoles and post-metamorphic juveniles (CT_{max} of ~ 5 °C and ~ 0.5 °C, respectively) (Sherman, 1980), this suggests thermal tolerance is shifting in response to more than physiological re-organisation alone.

Natural selection is considered a primary driving force in ontogenetic shifts in temperature tolerance (Huey *et al*., 2012). The energetic costs associated with the maintenance of high thermal tolerance limits, e.g. energy required for the upregulation of heat shock proteins (HSP) and changes in enzyme abundance (Hochachka and Somero, 2002), is thought to generate selection for lower thermal tolerance where possible so that the individual might capitalise on potential energy savings. This has led to predictions that life-stages inhabiting

thermally stable environments and those capable of behaviourally avoiding thermal extremes (Bogert effect – Huey *et al*., 2003) will present depressed upper thermal limits. Many studies are conducive with this theory. For example, neonate Diamondback water snakes (*Nerodia rhombifer*) that hunt in thermally variable shallow waters expressed a higher temperature tolerance than adults that reside in more stable deep water (Winne and Keck, 2005). Similarly, Miller *et al*. (2013) found embryonic and juvenile stages of porcelain crab (Genus *Petrolithes*) that inhabit the thermally variable intertidal expressed a higher thermal tolerance than larval zoea found in the relatively more thermally benign pelagic. For some species, more mobile stages capable of behaviour thermoregulation, exhibit a general decrease in heat tolerance with mobility (e.g. Marais *et al*., 2009; Huey *et al.,* 2003) but for others the reverse has been shown. Sessile eggs of the blowfly, *Calliphora erythrocephala*, expressed the lowest temperature tolerance of all larval, pupate and adult stages (Davidson, 1969) despite having no means of avoiding thermal extremes. Similarly, a review of abiotic stressors on marine embryos and larvae showed that, generally, mobile larvae are more heat sensitive than less mobile embryonic stages (Prezeslawski *et al*., 2014). The idea of selection being a primary driver becomes more complex when considering brooded species, as they, in theory, experience a similar thermal regime throughout their life-history. Embryos of subtidal and intertidal population of sessile marine gastropod, *Crepidula fornicata*, expressed significantly greater thermal tolerance than the adult females that brooded them (Diederich and Pechenik, 2013) despite being maintained within the mantle cavity of the mother and therefore exposed to exactly the same thermal environment. To expand our current knowledge of

how and why thermal tolerance changes with development, we must account for a variety of developmental strategies and modes.

Brooded species offer an excellent model system, where two key influences, variation in thermal regime and mobility both currently thought to be responsible for life-history stage variation in thermal tolerance, are either absent, or greatly reduced. Brooders that possess a direct mode of development further remove aspects of physiological reorganisation, such as metamorphosis, attributed to thermal tolerance shifts in some groups. Based on current perceptions of what drives age related shifts in tolerance traits, it may be predicted that thermal tolerance limits of brooded species will vary little with ontogeny in comparison to those of non-brooded species. In the case of *C. fornicata* an indirect developing brooded species, we know this not to be true. With little comparative literature, it is not known if current knowledge of patterns and mechanistic thought to underly thermal tolerance can be employed to predict the fate of brooded species.

This study aims to (1) investigate how upper thermal tolerance shifts during the early development of a direct developing brooded species and (2) explore why such changes occur in the context of improving understanding of what drives ontogenetic shifts in thermal tolerance in a brooded system.

This will be achieved using a method and mathematical model framework proposed by Rezende *et al*. (2014) to assess the upper critical thermal limits (CT_{max}) and sensitives (z) of embryonic and juvenile forms of a novel model organism.

Upper thermal tolerance limits (or CT_{max}) are typically quantified using either the “dynamic method”, in which rate of warming is increased at a constant or variable rate until a critical point is reached (i.e. physiological collapse), or the “static method” whereby temperature that results in 50 % or 100 % mortality is determined from a plot of percentage mortality at multiple constant (static) temperatures. Irrespective of the methodology, CT_{max} is typically presented as a single value; an oversimplification since thermal tolerance is a function of both intensity and duration of thermal stress. Here I shall employ a static method and mathematical framework proposed by Rezende *et al*. (2014) to quantify the interactive effects of intensity and duration of thermal stress. Thermal Death Time curves (TDT) are kinetic models from which CT_{max} (the average time taken for an individual to die within 1 min at a set temperature) and *z* (a constant that characterises the sensitivity to thermal change) estimates can be obtained for each life-stage. The association between temperature, time and mortality can be described using a thermal tolerance landscape (Rezende *et al*., 2014) also known as survival probability plots. Thus, I shall present upper thermal tolerance, not as a single value but as a survival probability estimated embedded in both temperature and time to investigate how upper critical thermal limits (CT_{max}) and sensitivities (*z*) change with ontogeny.

The common beachflea of north-western European shores, *Orchestia gammarellus*, is an ideal model organism with which to address this aim. The marsupium – brood pouch – of this semi-terrestrial amphipod species provides a semi-closed, maternally-controlled aquatic environment (Spicer and Taylor, 1994; Morritt and Spicer, 1996a, 1998, 1999; Morritt and Richardson, 1998) in which embryos directly develop in to hatchlings and are maintained until full

physiological competency is achieved (Morritt and Spicer, 1999). Thermoregulatory behaviour, in which mothers burrow down into the wrack habitat (sect. 1.4), keeps the developing brood at a relatively stable temperature compared to that of the surface intertidal (sect. 1.5). The mother and young appear to experience the same buffered thermal environment throughout development, providing a developmental environment with suppressed fluctuations in environmental temperature.

4.1 MATERIALS AND METHODS

4.3.1 Collection and maintenance

Adult *Orchestia gammarellus* (body length 2 – 3 cm) were collected by hand from beneath cast up wrack on the high shore at Mount Batten Bay, Plymouth, Devon, UK (50°21′25.23″N, 4°07′37.21″W) during the spring-summer breeding season (Morritt and Spicer, 1996) June – September 2018 and May – July 2019. Individuals were transported in large plastic bags to the laboratory at the University of Plymouth within 4 h of collection. Here, ovigerous females were identified and maintained in a number of aquaria (vol. = 5 L, T = 15 ± 1°C, 100 % humidity, 12 h: 12 h light:dark cycle), each lined with moist tissue paper (sal. = 11) for < 48 h before experimental use to prevent acclimation to laboratory conditions. Individuals were not fed during this time, although they did consume the tissue within the aquaria.

A single individual was removed from each brood to be examined under low power magnification (x 10 – 40) and assigned to a recognisable (Fig. 4.1), and constrained developmental stage (i.e. “pre-heart”, “post-heart” and “hatchling” individuals) following the criteria presented in Tab. 4.1.

Table 4.1 Classification criteria of early developmental stages

Stage name	Criteria
Pre-heart	Embryonic stage. Presence of gut movement, absence of heartbeat
Post-heart	Embryonic stage. Presence of heartbeat
Hatchling	Juvenile, hatched from egg capsule and residing within the marsupium

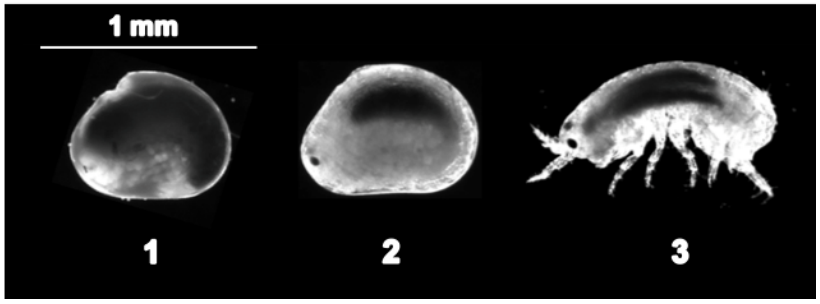

Figure 4.1 Developmental stages of *Orchestia gammarellus* used in this study: (1) pre-heart, (2) post-heart and (3) hatchling.

4.3.2 Experimental protocol

Pre-heart (n = 41), post-heart (n = 45) and hatchling (n = 47) stages were harvested from mothers within 48 h of collection, *via* extraction, using a moist paint brush, from the brood pouch and inserted into a pre-warmed 96 well microtitre plate (vol. = 200 µL, S = 11) at one of four static nominal treatment

temperature (T = 33.5, 35, 36.5 and 38 °C). Precautions were taken to ensure consistent conditions within the incubation chamber (a heated chamber mounted on a motorized XY stage, mobilised above a high powered lens – see Chapter 2, Fig. 2.1 B); including the maintenance of 100 % humidity to minimise evaporative loss and potential changes in salinity (no change in salinity throughout incubation confirmed using a refractometer Hanna HI 96822). A maximum capacity of n = 8 per chamber per test was chosen to minimise thermal fluctuations within the chamber (< 0.5 °C) and the time taken to start image acquisition.

4.3.3 Image acquisition

Images of individual embryos were captured using a 4 MP monochrome camera (Allied Vision Technology Pike 421B) operating with 750 x 750 pixels. The camera was connected to a zooming optic lens system (Keyence VHZ20R), inverted beneath a motorized XY stage (Marzhauser Scan) and controlled using Micromanager software package (Edelstein et al., 2010). Each individual was recorded for a period of 20 secs (imaging parameters = 20 frames per second, 8 bit pixel depth, 40 ms exposure) every 15 min for 24 h. See Chapter 2 for full details of bioimaging system set up.

4.3.4 Image analysis

Image stacks (400 images per stack acquired hourly) were compiled into time-lapse video and loaded into biological imaging analysis software Fiji (Schindelin

et al., 2012). Time-lapse enabled efficient approximation of death time of individual, followed by inspection of individual image stacks at 15 min intervals to determine point of death to the nearest 15 min. The time taken to reach a lethal end point (defined by the criteria in Tab. 4.2) was recorded for every individual (Tab. 4.3) at each of the four nominal temperatures (T = 33.5, 35, 36.5 and 38 °C).

Table 4.2 Lethal end point per developmental stage

Stage name	Lethal end point criteria
Pre-heart	Termination of gut movement
Post-heart	Termination of heart function
Hatchling	Termination of heart function

Table 4.3 Number of individuals (n) per stage per temperature treatment

Stage name	Temperature (°C)			
	33.5	35	36.5	38
Pre-heart	n = 8	n = 10	n = 14	n = 19
Post-heart	n = 14	n = 11	n = 10	n = 10
Hatchling	n = 12	n = 17	n = 10	n = 8

4.3.5 Data analysis

Time to reach lethal end point was recorded per individual per treatment (Tab. 4.3). Survival probability plots (thermal tolerance landscapes) for each stage were generated using lethal endpoint times of each individual within each

treatment. Thermal death time (TDT) curves were constructed from lethal end points at four static assay temperatures. Upper thermal limits (CT_{max}) and thermal sensitivities (z) were estimated using the equation of Rezende et al. (2014) in the form:

$$\log_{10} t = \frac{(CT_{max} - T)}{z} \quad \text{(Eqn. 4.1)}$$

where t is time to death (min), CT_{max} is the upper critical thermal limit (°C) at 1 min ($\log_{10}$ t = 0), T corresponds to the assay temperature and z is the temperature change (°C) required for a 10-fold change in survival times, and thus quantifies the sensitivity to temperature change. T was controlled as the independent variable and t was measured as the dependent variable. CT_{max} and z respectively were calculated using the following rearrangements of Egn. 4.1:

$$CT_{max} = \frac{\text{- intercept}}{\text{slope}} \quad \text{(Eqn. 4.2)}$$

$$z = \frac{1}{\text{slope}} \quad \text{(Eqn. 4.3)}$$

Normality checks and Levene's test for homogeneity of variance were carried out on all data and the assumptions were met prior to creation of linear model to determine goodness of fit. Data analysis was performed in R studio (Rstudio Team, 2016).

4.4 RESULTS

Variability in survival time with developmental stage was captured by a semi-logarithmic model with a high goodness of fit (pre-heart, R^2adj = 0.12, $F_{(1,39)}$ = 6.669, p = 0.0137; post-heart, R^2adj = 0.41, $F_{(1,34)}$ = 31.69, p < 0.001; hatchling,

R^2adj = 0.51, $F_{(1,45)}$ = 47.97, $p < 0.001$) (Fig. 4.2 A). The interpolation at the abscissa represents CT_{max}, or the tolerated temperature following 1 min of exposure (Eqn. 4.1), whilst z corresponds to the reciprocal of the slope. The pre-heart embryonic stage exhibited the highest CT_{max} (65.5 °C) and the highest z (18.9) values relative to post-heart (CT_{max} = 49.1°C, z = 7.2) and hatchling (CT_{max} = 45.3 °C, z = 5.1) stages (Fig. 4.2 B). A trade-off between CT_{max} and z is seen (Fig. 4.3 B) for all stages, i.e. high CT_{max} comes at the expense of high z.

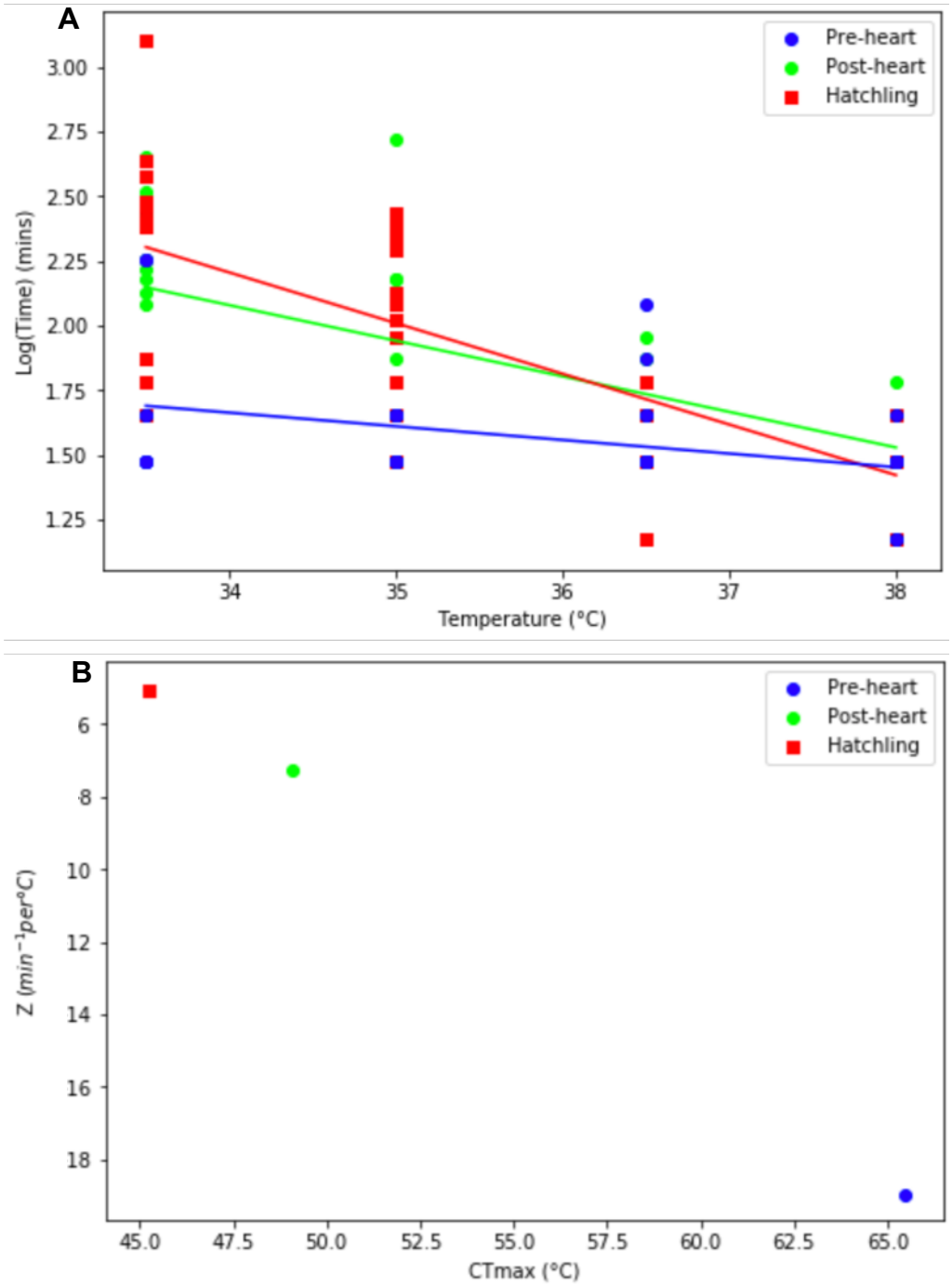

Figure 4.2 A) Thermal death time curves (TDT): survival times of three developmental stages of *O. gammarellus* at different acute thermal exposures (T = 33.5, 35, 36.5 and 38 °C) plotted on a logarithmic scale with line of best fit. B) Relationship between upper critical thermal limits (CT_{max}) and sensitivity to temperature change (z) for three developmental stages of *O. gammarellus*.

Average survival times (± SD) at T = 33.5 °C were shortest for pre-heart embryos (71 ± 67 min), relative to post-heart (167 ± 118 min) and hatchling (290 ± 333 min) stages. Survival time was lowest for all stages at 38 °C, reduced to 28 min ± 9, 37 min ± 12.7 and 30 min ± 8 for pre-heart, post-heart and hatchling stages respectively.

Post-heart and hatchling thermal tolerance landscapes (Fig. 4.4) show two distinct patterns of survival, grouped between the two upper (T = 36.5 and 38 °C) and two lower (T = 33.5 and 35 °C) temperatures. Data for pre-heart embryos show no such pattern.

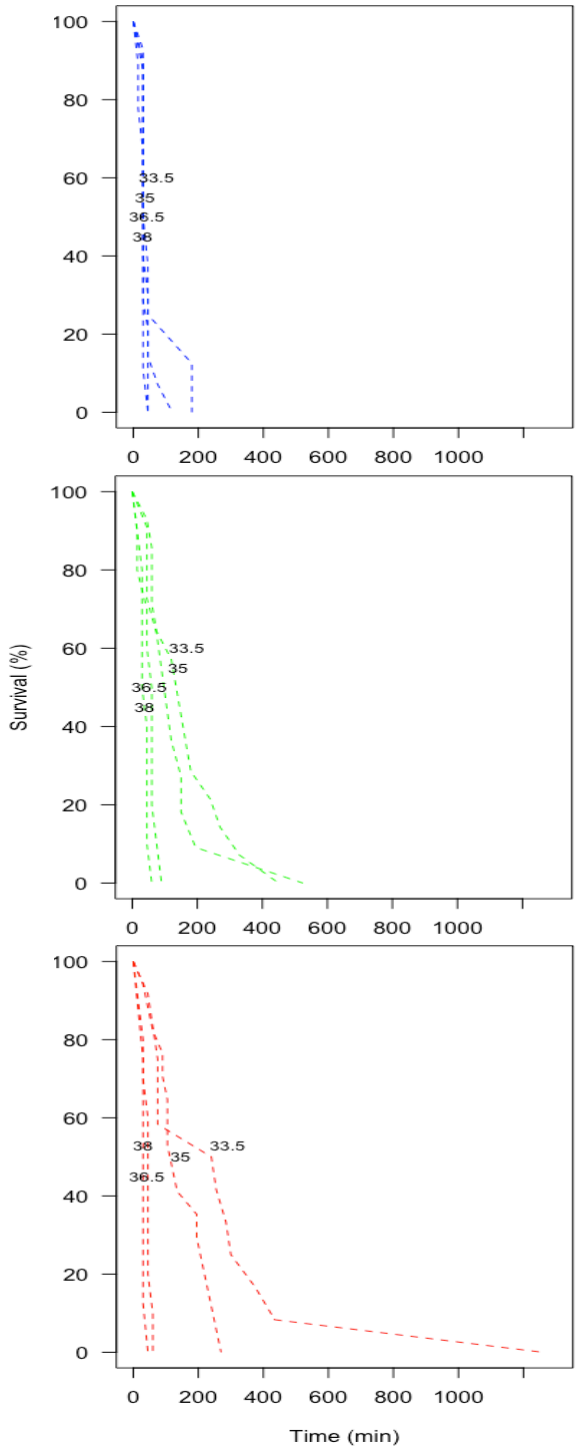

Figure 4.3 Thermal tolerance landscapes for *O. gammarellus* at three different developmental stages; pre-heart (blue), post-heart (green) and hatchling (red) at 33.5, 35, 36.5 and 38 °C respectively. Refer to Tab 4.3 for number of individuals per stage per treatment.

4.5 DISCUSSION

4.5.1 How does CT_{max} and z change with ontogeny

Thermal tolerance of three developmental stages of brooding species *O. gammarellus* were assessed using a mathematical model framework (Rezende *et al*., 2014) and were presented as TDT curves and thermal tolerance landscapes. Upper critical thermal limits (CT_{max}) and sensitivities (z) varied significantly between development-stages with an overall sequential decline in acute tolerance (CT_{max}) and increase in ability to withstand moderate thermal challenge (z) with increasing developmental age (Fig 4.3 B). The relationship between thermal tolerance, temperature and time for each stage shows a typical dose-response relationship (i.e. the higher the temperature the less time the individual is able to tolerate it). Inter and intra -specific variability in this relationship has been documented for many species (e.g. Tang *et a*l., 2000; Armstrong *et al.*, 2009; Truebano *et al*., 2018; Santos *et al*., 2011; Rezende *et al*., 2014) and is typically attributed to the adaptive response of species to different thermal regimes throughout their life-histories (Miller *et al*., 2013; Truebano *et al*., 2018). For example, Truebano *et al*. (2018) found embryonic littorinid snails that develop in relatively constant thermal environments below algal fronds to possess a lower acute tolerance (CT_{max}), but better ability to survive longer under less extreme temperatures (low z), than adults that graze in the thermally variable intertidal.

Interestingly, *O. gammarellus* exhibit distinct variation in heat tolerance with developmental stage but in the absence of obvious changes in microhabitat selection or mobility. Pre-heart embryos expressed the highest CT_{max} and

highest z values of the three developmental stages (Fig. 4.2) which suggests they are less sensitive to temperature change (i.e. large fluctuations in environmental temperature will have a minor impact on survival) relative to later stages at the cost of tolerating high temperatures for a short amount of time (Santos *et al*., 2011; Rezende *et al*., 2014). The adaptive value of this is ambiguous as field observations indicate a relatively consistent thermal regime across all stages of development (sect.1.5). The occurrence of ontogenetic variation in tolerance traits of brooded species in the absence of obvious selective forces challenges adaptive theories. This begs investigation of mechanistic determinants of upper thermal tolerance limits.

4.5.2 Why does CT_{max} and z change with ontogeny

The mechanisms underpinning upper thermal tolerance limits in ectotherms are under continuous study and rigorous debate (Luttershmidt and Hutchingson, 1997; Klok *et al*., 2004; Pörtner, 2010; Verbeck *et al*., 2016; Portner *et al*., 2017; Jetfelt *et al*., 2018; MacMillian, 2019). Since standard metabolic rate increases with temperature, related to elevated energetic costs associated with increased rates of reaction and protein maintenance (Hochachka and Somero, 2002), much attention has been directed towards organismal capacity for oxygen delivery in relation to oxygen demand in the bid to predict lethal thermal limits (e.g. Ern *et al*., 2015, 2016; Verbeck *et al*., 2016; Payne *et al*., 2015). The Oxygen- and Capacity-Limited Thermal Tolerance (OCLTT) hypothesis predicts that lethal end points occur when oxygen demand outweighs supply (Frederich and Pörtner, 2000; Pörtner 2001, 2002, 2010; Portner *et al*., 2017). When applied to development, the OCLTT hypothesis predicts that ontogenetic shifts

in thermal tolerance should directly correlate to the individuals changing ability to deliver oxygen (e.g. Youngblood *et al*., 2019). If oxygen delivery limits heat tolerance the mismatch between supply and demand should increase with growth (increased metabolic demand), suggesting later life-stages should express a lower thermal tolerance than earlier stages of development. This is conducive with the results of this study, as CT_{max} declined with developmental age. The greatest disparities in both CT_{max} and z were observed between pre-cardiac and post-cardiac (i.e post-heart and hatchling) stages (Fig. 4.2). Crustaceans, including talitrid amphipods, rely on convective oxygen transport by the cardiovascular system. Therefore, cardiac organogenesis should improve oxygen circulation, increasing oxygen delivery (Frederich and Portner, 2000) to tissues, resulting in increased thermal tolerance in post-cardiac individuals. Although a decline in CT_{max} values with cardiogenesis does not support this, it may explain why both post- cardiac stages exhibited higher percentage survival than pre-cardiac individuals at the lower two assay temperatures (Fig. 4.3 B&C). There was a sharp decline in survival for all stages at 36.5 °C, creating a threshold in survival probability between the upper two and lower two assay temperatures for post-cardiac stages. Previous studies have described similar thresholds as representing the onset of anaerobic metabolism (terming the temperature at which this occurs as T_{crit}). Since anaerobic metabolism is ~15 fold less efficient than aerobic, survival time is limited once the T_{crit} threshold has been reached (e.g. Santos *et al*., 2011; Payne *et al*., 2015).

When oxygen demand is greater than supply then physiological collapse is inevitable. However, whether oxygen lack is always the limiting the factor

causing collapse is hotly debated. Although multiple studies of thermal limits in crustaceans do appear to point oxygen limitation (e.g. Frederich and Portner, 2000; Storch *et al*., 2009, 2011) there is considerable controversy as to the generality of this finding (e.g. Ern *et al*., 2014; Verberk *et al*., 2016; Youngblood *et al*. (2018) found a decrease in thermal tolerance during the progression of early development in grasshopper, *Schistocerca americana,* as predicted by the OCLTT. However, this decline was not oxygen-limited as hypoxia had no effect on heat tolerance limits at any stage of development. Since the onset of anaerobic metabolism can be determined by the presence of secondary metabolites, such as L-lactate in the haemolymph of crustaceans (Frederich and Pörtner, 2000), oxygen limitation in various development stages of *O. gammarellus* could be assessed by testing for the presence of such compounds at T_{crit} (around 36.5 °C in this instance). However, such markers should be used cautiously as the presence of metabolites associated with anaerobic metabolism, such as alanine, may provide a false narrative as they can result from changes in other metabolic pathways (see Bayley *et al*., 2010). It is likely that insufficient oxygen delivery is one of many limiting physiological processes, such as the failure of protein function, membrane function and ion regulation, under acute thermal challenge prior to or in conjunction with the onset of anaerobic metabolism (Clark *et al*., 2013; Ern *et al*., 2015; Schulte, 2015; Verberk *et al*., 2016).

When contesting the OCLTT Jutfelt *et al*. (2018) question “why animals would allow tissue hypoxia to become severe enough to inflict performance declines… when possessing the functional capacity to significantly increase oxygen delivery to tissues” such as increasing ventilation and respiratory processes.

This statement may be applicable when considering autonomous adult stages of many species, but developing young may simply not yet possess the mechanisms to do so. Life-stages likely differ in their mechanistic thermoregulatory capabilities, be it oxygen regulation or otherwise, giving rise to variation in upper thermal tolerance with ontogeny.

4.6 CONCLUSION

Despite the brooded nature of this species and its comparatively stable thermal environment throughout development *Orchestia gammarellus* is one of many invertebrate and vertebrate species that show variation in upper thermal tolerance traits with ontogeny. Selection of thermal tolerance traits based on the thermal regimes encountered at various life-history stages maybe a good predictor of ontogenetic shifts in non-brooded species but cannot be employed to determine ontogenetic shifts here. The mechanistic basis underlying heat tolerance variation with development is likely set by physiological limits, such as oxygen limitation, that change throughout ontogeny and play a pivotal role in dictating survival throughout the life-histories of this species and many others. An exploration of physiological limits and how they change as a result of ontogeny is therefore critical to determine how brooded species, and indeed many others, will respond to climatic change. Ontogenetic variation in thermal limits may have downstream consequences for ecological balance. The results of this study support the importance of assessing thermal tolerance on a stage by stage basis, to form part of the wider understanding of responses to environmental change scenarios.

CHAPTER 5: CONCLUSION

5.1 Thesis aim

The overall aim of my thesis was to investigate the effects of environmental temperature on aspects of the developmental ecophysiology of a brooding species. I investigated this specifically by considering the effects of temperature on the ontogeny of GI movements and upper thermal limits in embryos of an ecologically important, widespread and abundant talitrid amphipod species, *Orchestia gammarellus* (see rationale outlined in section 1.4 for choice of this model species)

5.2 Development of bioimaging system

To achieve this aim I first was involved in developing a bio-imaging technique, *Embryo Phenomics*, which was to form an integral part of my data acquisition and analysis. Therefore, my first experimental chapter (Chapter Two) introduces *Embryo Phenomics*, an open-source high throughput platform for phenomics in aquatic embryos. This consists of an Open-source Video Microscope (*OpenVIM*), with experimental control over the embryonic environment, and a Python package *Embryo Computer Vision* (*EmbryoCV*) for high-dimensional measurement of phenomic traits from video datasets. My objectives were to demonstrate how *Embryo Phenomics* (1) enables the quantification and integration of inter- and intra-individual temporal change in morphological,

physiological and behavioural traits with high resolution and to an unprecedented scale, in a largely automated workflow and (2) can be used to quantify integrated organismal responses to environmental change, using experiments assessing: i) chronic; ii) acute; and iii) interactive effects, of environmental stressors.

To fulfil those objectives, I helped develop a high-throughput platform for phenomics in aquatic embryos comprising *OpenVIM*, an open-source video microscope, and *EmbryoCV*, a Python package for automated phenotyping of embryos. This is *Embryo Phenomics,* a modular platform that enables the quantification of inter- and intra-individual temporal change in the phenome of developing aquatic embryos with unprecedented resolution, incorporating morphological, physiological, behavioural and proxy traits. The broad-scale applicability of *Embryo Phenomics* is demonstrated in a range of i) chronic, ii) acute and iii) interactive experiments, where phenomic data are produced for > 600 embryos generated from video comprising > 30 M images. While the challenges of phenomics are significant, the rewards are substantial. *Embryo Phenomics* can acquire and process data capturing functional, temporal and spatial responses in the earliest, most dynamic life stages and is game-changing for those studying development, and phenomics more broadly.

5.3 GI movements – engines of yolk supply and oxygen transport?

The next experimental chapter (Chapter 3) documented the thermal sensitivity of the ontogeny and development of a key embryonic behaviour/physiological function in *O. gammarellus*, namely gastro-intestinal (GI) movements. I tested

the hypothesis that embryonic gut movements in talitroidean amphipods function as an extracardiac pump, dispersing yolk to growing and developing embryonic tissue, and aiding in the delivery of oxygen to developing tissues. If gut movements were related to yolk dispersal then (1) it should have been possible to detect yolk release and relate it to particular gut movements; (2) trace the subsequent movement of the released yolk in the embryo and (3) an increase in environmental temperature (T = Δ5 °C) should change the frequency and/or character of gut movements to release more yolk to meet heightened metabolic demand. If gut movements are related to oxygen demand, then (1) acute exposure to environmental hypoxia should result in a corresponding increase in the frequency of gut movements to enhance circulatory function during this period of heightened demand and (2) circulation should be more pronounced at higher temperatures. These predictions were tested by

(1) *first describing development generally, focussing specifically on the ontogeny and nature of the gut movements from their first appearance through to hatching*. General development was very similar to that already described by another talitroidean amphipod, *Parhyale hawaiensis*.

(2) *quantifying rates of gut movements in eggs cultured at two different ecologically realistic environmental temperatures at different times through development*. Individuals reared at T = 20 °C expressed faster development and greater mortality than those reared at T = 15 °C (2 % and 4 % hatching success respectively). The rate of peristaltic wave motion was significantly greater ($P < 0.001$) for embryos reared at 20 °C compared to 15 °C. Greater rates of mortality at 20 °C may warn of downstream consequences for growth and survival of later life stages.

(3) determining visually whether, and how frequently, yolk was extruded from the gut and once released where it went in the embryo;

The rate of yolk depletion, a proxy for energy expenditure, increased at the higher temperature as did the rate of gut movements, thus supporting the hypothesis that gut movements acted as an engine of yolk dispersal.

(4) *testing whether the rate of gut movements increase in response to environmental hypoxia*.

There was no apparent effect of hypoxia on GI behaviour, therefore the hypothesis that the GI tract functions as a pre-cardiac pump, responding to oxygen reductions in this species was not supported.

5.4 Thermal limits change with development even in a direct developing brooded species

The third experimental chapter (Chapter 4) investigated how upper thermal tolerance shifts during the early development of *O. gammarellus* and (2) explored why such changes occur. This was done using a method and mathematical model framework proposed by Rezende *et al*. (2014) to assess the upper critical thermal limits (CT_{max}) and sensitives (z) of embryonic and juvenile forms of *O. gammarellus*. I used thermal death time curves, which incorporate the intensity and duration of heat stress, to generate upper thermal limits (CT_{max}) and sensitivity (z) estimates for multiple developmental stages of this amphipod. CT_{max} and *z* estimates were used to calculate thermal tolerance landscapes, enabling estimation of survival probability of each life stage to acute thermal stress. Based on current perceptions of what drives ontogenetic shifts in thermal tolerance, it may be predicted that thermal limits would vary

very little between stages of this direct developing brooded species. This was not the case. Pre-cardiac embryos had the highest CT_{max} and lowest *z* value relative to post-cardiac and juvenile individuals, suggesting a greater ability to tolerate short exposure to thermal extremes and poorer ability to withstand prolonged exposure to moderate temperatures relative to later stages. Post-cardiac stages displayed a distinct pattern in knockdown time that is absent in pre-cardiac individuals, suggesting a relationship between cardiac output, temperature and upper thermal limits.

This study is the first to illustrate that thermal tolerance varies across multiple early life-stages of a direct developing brooded species and provides the first assessment of its kind for any member of the Amphipoda. I conclude that underlying patterns and potential mechanisms in shifts in thermal tolerance at the individual level are essential to improve the accuracy of predicting organismal responses to local and global environmental change.

5.5 Perspectives

My thesis investigated the effects of environmental temperature on key aspects of the thermal biology of talitrid amphipod, *O. gammarellus*. In doing so, I have contributed to knowledge of ontogenetic variability in the response of this species, and of brooded species more broadly, to changes in mean and acute environmental temperature.

Environmental temperature experienced during early development *in situ* for *O. gammarellus* significantly affected processes vital to organismal homeostasis;

altering rates of GI movement, yolk utilisation and heart rate in ways that exceeded expectation based on typical effects of temperature on biochemical reactions *in vivtro*. Embryonic and juvenile stages expressed marked variation in their ability to tolerate thermal extremes, with distinct patterns in pre and post cardiac individuals, suggesting that mechanisms underlying heat tolerance are likely set by physiological limits, such as oxygen limitation, that change throughout ontogeny. It was not possible to predict the outcomes of the studies presented in chapters 2 and 3 due to the lack of literature surround the effects of temperature on early development, particularly with regard to brooded systems. This knowledge gap is, in part, related to the lack of technologies capable of high resolution, autonomous image acquisition needed in the study of early development. The development of *Embryo Phenomics* was central to all experimental chapters and its application here should serve to inspire further study of developmental ecophysiology.

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SUPPLEMENTARY 1. Overview of experimental treatments, designs, and image-acquisition parameters

Treatment	N embryos imaged	Acquisition details (RS, FR, ROI, data aquired, magnification)	Duration (min-max; h)	Total images acquired	Successfully analysed (embryos; images)	Embryos manually removed (insufficient sata; abnormal development)
Experiment 1		RS = 600 frames,				
20 °C	48	repeared hourly;	292-367	7.53 M	42; 7.14 M	0; 6
		FR = 20 fps; ROI				
25 °C	48	= 750 x 750 px;	292-364	5.37 M	44; 4.66 M	0; 4
		bit depth =16 bits;				
30 °C	48	image data = 21	196-269	4.83 M	32; 4.51 M	1; 14
		TB; phenome				
		data = 510 GB;				

		magnification = 200x				
Total	144			17.73	119; 16.32 M	1; 24
Experiment 2		RS = 600 frames, repeared 20 min; FR = 20 fps; ROI = 750 x 750 px; bit depth =16 bits; image data = 9.2 TB; phenome data = 121 GB; magnification 200x				
36 °C, St = E3	63		24 h	2.52 M	58; 2.44 M	0; 0
36 °C, St = E7	56		24 h	2.42 M	56; 2.41 M	0; 0
36 °C, St = E9	64		24 h	2.76 M	64; 2.75 M	0; 0

Total	183			7.7 M	178; 7.6 M	0; 0
Experiment 3		RS = 600 frames, repeared hourly; FR = 20 fps; ROI = 750 x 750 px; bit depth =16 bits; image data = 5 TB; phenome data = 44.17 GB; magnification = 200x				
20 °C, S = 0	48		24 h	720,000	46; 679,650	2; 0
20 °C, S = 5	48		24 h	720,000	46; 681,720	2; 0
25 °C, S = 0	48		24 h	720,000	48; 704,160	0; 0
25 °C, S = 5	48		24 h	720,000	46;681,030	2; 0
30 °C, S = 0	48		24 h	720,000	47; 698,655	1; 0

30 °C, S = 5	48		24 h	720,000	44; 636,240	4; 0
Total	288			4.3 M	277; 4.24 M	11; 0
Experiment 4		RS = 600 frames, repeared hourly; FR = 20 fps; ROI = 750 x 750 px; bit depth =16 bits; image data = 5 TB; phenome data = 44.17 GB; magnification = 200x				
15 °C	4		24 h	201,600	4; 201 600	0; 0
20 °C	4		24 h	201,600	4; 201 600	0; 0
Total	8			403,200	8; 402,200	0; 0

Sample video and data set

TOTAL	**623**		**30.13 M**	**582; 28.52 M**	**12; 24**

Abbreviations: fps, frames per second; FR, frame rate; GB, gigabyte; M, million; px, pixels; ROI, regin of interest; RS, recording schedule; TB, terabyte.

APPENDICES 1: PATTERN OF TEMPERATURE ACCLIMATION IN THE SANDHOPPER *DESHAYESORCHESTIA DESHAYESII* (CRUSTACEA: AMPHIPODA) CHALLENGED EXISTING UNDERSTANDING OF WINTER 'HIBERNATION' IN SANDHOPPERS, WITH CONSEQUENCES FOR PREDICTING CLIMATE CHANGE EFFECTS

Much of this appendices was submitted for publication as Tykarska, M., **Tully, E.**, Urszula, J. and Spicer, J.I. 2019. Pattern of temperature acclimation in the sandhopper *Deshayesorchestia deshayesii* (Crustacea: Amphipoda) challenges existing understanding of winter 'hibernation' in sandhoppers, with consequences for predicting climate change effects.

E. Tully contributed to the study conception and design, material collection, preparation, data collection and contributed to writing the manuscript.

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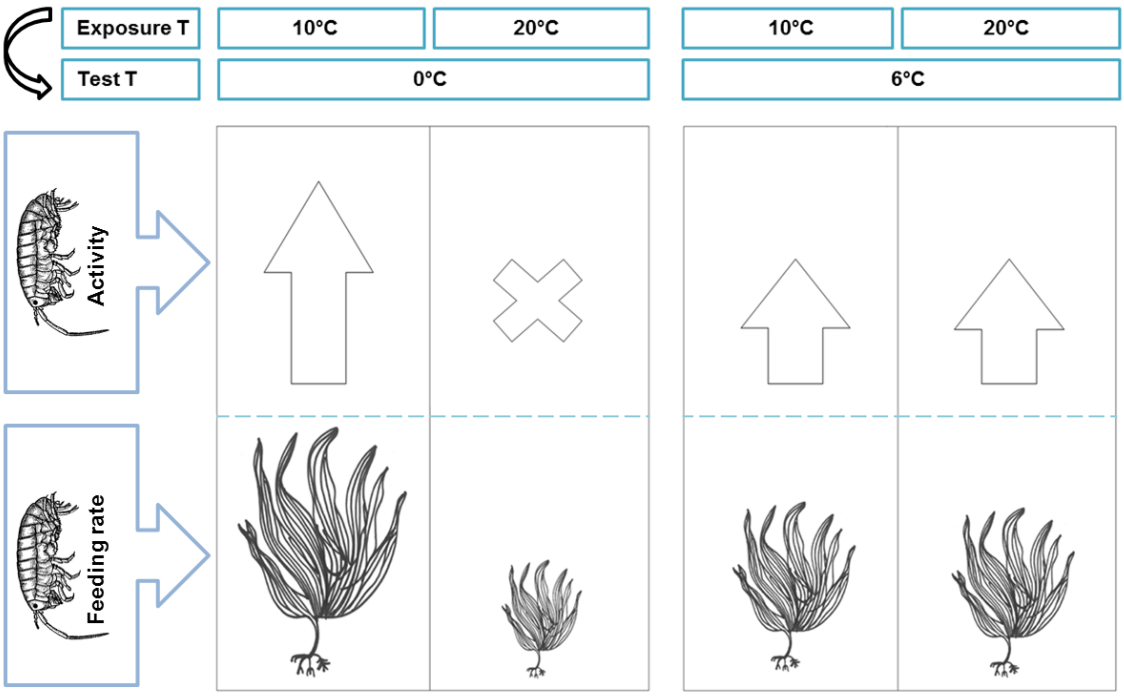
A1.1 ABSTRACT

Talitrid amphipods inhabiting the coastal zone serve as a link between marine and terrestrial ecosystems by feeding on stranded macroalgae. The aim of this study was to determine whether the temperature to which talitrids were previously exposed alter their activity, feeding rate and mortality when tested at low environmental temperatures. Amphipods collected at Wembury Point, Devon, UK (50°18'57.8"N 4°05'40.3"W) during October 2018 were exposed to two different environmental temperatures (T = 10 and 20 °C) and tested at 0 and 6 °C. When the exposure temperature was low sandhoppers were able to maintain activity and feeding in freezing temperatures. When exposure temperature was high *D. deshayesii* became torpid and reduced their activity and feeding at freezing temperature. We predict that in a warming world, the incidence of torpor and feeding will be influenced not just by a direct effect of temperature but also in combination with interactions with acclamatory ability.



A1.2 INTRODUCTION

Sandhoppers are semi-terrestrial, fossorial amphipods belonging to the family Talitridae (Bousefield, 1984). They range from sub-polar to tropical regions and so encounter a variety of thermal regimes (Spicer *et al*., 1987; Morritt and Spicer, 1998). Like all ectotherms, sandhoppers regulate their body temperature by behavioural means (Williams, 1980; Morritt and Spicer, 1998). Low latitude populations remain active year-round (e.g. Prato *et al*., 2009; Ayari and Nasri-Ammar, 2011; Ayari-Akkari *et al*., 2014), whereas it has long been thought that higher latitude populations, of at least some species, overwinter in deep burrows. In their burrows they are in a state of, what has been referred to as, “hibernation” (e.g. Bate, 1862; Edwards and Irving, 1943; Den Hartog, 1963; Spicer *et al*., 1994; Węsławski *et al*., 2000). The term ‘hibernation’ usually refers to endotherms that reduce their activity, but still control, their body temperature and metabolism (Davenport, 1992). As the sandhoppers, in common with other ectotherms, are unable to exert physiological control over their body temperature, it is perhaps better to refer to the reduced activity and metabolism in overwintering sandhoppers as torpor. Torpor has been observed in some sandhopper species, for example *Talitrus saltator*, overwintering in France (Palluault, 1954), Scotland (Spicer *et al.,* 1990) and Poland (Tykarska unpubl. obs.), but is notably absent in southern populations, e.g. those found in the Mediterranean (Geppetti and Tongiorgi, 1967; Scapini *et al*., 1992). The prevalence of torpor in high latitude populations suggests that the behaviour is vital to surviving low temperature conditions experienced during the winter months at the northern end of the species range. It also suggests that

sandhoppers are unable to acclimate - adjust their physiology to maintain physiological rate in the face of environmental change - to low temperatures, meaning that low temperature torpor is obligatory, a suggestion first made by Edwards and Irving (1943).

With mean and extreme global temperatures set to increase on average by 4.6 °C by 2100, the frequency of warm episodes experienced by high latitude populations is predicted to rise (IPCC, 2014). A recent study by Glandon et al. (2018) predicted that under climate change scenarios overwintering would be shorter for the blue crab *Callinectes sapidus* and survival would improve. The potential impact of any temperature increase on the incidence of torpor will depend on an individual's ability to acclimatize to, amongst other things, elevated environmental temperatures. Acclimatization is the ability of an individual to modify key physiological processes in order to maintain normal physiological function in the face of environmental change *in situ* (Precht, 1958; (Spicer and Gaston, 1999)). The scope for such phenotypic plasticity can be estimated in the laboratory by quantifying the capacity for change (or lack of it) in physiological response to a single environmental factor under laboratory conditions, termed acclimation (Precht, 1958; Spicer and Gaston, 1999). Although acclimation and acclimatization are not analogous (Spicer and Gaston, 1999), there is good evidence to suggest that one may be inferred from the other (Somero, 2010).

We do have some information on the acclimatory ability of sandhoppers and talitrids in general. Marsden (1989) found that seasonal 'acclimatized' winter and summer individuals of the sandhopper *Bellorchesita* (as *Talorchestia*)

quoyana showed near identical respiratory rates to individuals kept at mean winter (5 °C) and summer (20 °C) temperatures. However, such physiological modification in other talitrid species has proved ambiguous. Edwards and Irving (1943) noted no modification of metabolic rate in *Americorchestia* (as *Talorchestia*) *megalopthalma*, as they claimed there was no difference in the respiratory rates between individuals from winter and summer populations. However, this finding was later disputed by Rao and Bullock (1954), who found that individuals of winter and summer population of *A. megalopthalma* displayed different Q_{10} values, thus indicating some ability to acclimatize.

The sandhopper *Deshayesorchestia* (formerly *Talorchestia) deshayesii* is a common inhabitant of the high shore on sandy beaches of European (Bulnheim and Scholl, 1986) and Mediterranean coasts (Prato *et al*., 2009; Ayari and Nasri-Ammar, 2011). Its distribution extends as far north as the North Sea (Kalbrink, 1969) and the Baltic Sea (Tykarska *et al*., 2019) although its *locus typicus* is believed to be Egypt on the Mediterranean Sea (Zettler and Zettler, 2017). In the southern part of its range *D. deshayesii* can encounter temperatures of 25.9 ± 1.8 °C (Prato *et al.,* 2009) or even 32.8 °C (Ayari-Akkari *et al*., 2014). Even in more northern locations it can encounter high temperatures, e.g. during the particularly hot summer of 2018 on Portwrinkle beach, SW England, *D. deshayesii* was jumping and walking, on having been disturbed by the incoming tide, when the temperature of the surface reached 42.3 °C (Spicer and Ellis unpubl. obs.) a temperature close to, or even exceeding that lethal to other talitrid species (Edwards and Irwing, 1943; Backlund, 1945; Moore and Francis, 1986). Whilst neither its low or high thermal tolerance of *D. deshayesii* has been determined, it evidently has a wide

tolerance range. Certainly, it becomes torpid during winter in the southern Baltic Sea and overwinters in burrow in the sand (Tykarska unpubl. obs.). Bate and Westwood (1863) expressed some doubt as to whether it became torpid during the winter months on the coast of Britain, but there is some evidence that in SW England they move higher up the shore in winter where they can, during the day, be found torpid in their burrows (Tully and Spicer unpubl. obs.). Thus, with a predicted increase in the prevalence of warmer winters, knowing the extent to which *D. deshayesii* is able to acclimate to higher environmental temperatures is important in understanding what underpins low-temperature torpor. Talitrid amphipods, including *D. deshayesii* play a pivotal role in the breakdown of organic detritus and subsequent regulation of nutrient supply to intertidal sediments and inshore waters (Griffiths and Stenton-Dozey, 1981; Lastra et al., 2018) and the impact of that role may increase if the incidence of winter torpor declines and feeding rate increases proportionally with an increase in temperature.

Consequently, the aim of this study is to determine whether *D. deshayesii* has the ability to acclimate to temperature change. We test two hypotheses. First that torpor is a consequence of not being able to acclimate, and that in a warming world the temperature at which torpor is induced will be unchanged and so the incidence of torpor will decline. Second, that the ecosystem service of strandline decomposition, to which talitrids are a major contributor, will change as a consequence of an inability to acclimate, and that in a warming world not only will there be more time for feeding but that feeding activity will increase, i.e. an inability to acclimate will mean that feeding rate will increase proportionally with an increase in temperature. We tested these hypotheses by

exposing *D. deshayesii* for a week to two different environmental temperatures (T = 10 and 20 °C) before quantifying their activity and their rate of feeding at two low test temperatures 0 and 6°C. We made four predictions. (1) If there is no acclimation there will be a doubling of feeding rate (i.e. Q_{10} = 2) when comparing two exposure temperatures with a ΔT = 10°C and (2) no difference in feeding rate when tested at equivalent temperatures but (3) no change in the incidence of torpor in sandhoppers tested at 0 and 6°C, even after exposure to 10 °C compared to 20 °C, with (4) activity decreasing with decreasing test temperature. Mortality was also recorded as was the water content of sandhoppers, the latter measure taken as a measure of physiological condition giving an indication if desiccation played a role in either mortality or the physiological responses observed when sandhoppers were tested at low temperatures. Exposure temperatures (ET) of 10 and 20 °C were chosen to produce a 10 °C difference but still using temperatures well within the environmental tolerances of this species. The test temperatures encompassed the extremes of what this species experiences when buried in the sand during the winter months (Tully and Spicer unpublished).

A1.3 MATERIALS AND METHOD

A1.3.1 Animal collection and maintenance

Amphipods were collected by hand from the strandline at high tide from the sandy-shore at Wembury Point, Devon, UK (50°18'57.8"N 4°05'40.3"W) during October 2018. Individuals were stored in plastic bags and transferred to the laboratory facilities at the University of Plymouth within 2 h of collection. Here, individuals were divided equally between 6 plastic aquaria (vol. = 1L)

provisioned with cast up wrack, *Laminaria digitata* (which acted as a food source and as shelter) and white roll moistened with sea water (S = 33). Three aquaria were kept at T = 10 °C and a further 3 at T = 20 °C (12 L: 12 D photoperiod) using computerised temperature-controlled environments. The contents of each aquarium were sprayed with sea water (S = 33) every 2 days to maintain 100 % humidity within.

A1.3.2 Experimental design

After 7 days of exposure to one of two different temperatures (ET = 10 or 20 °C) *D. deshayesii* of a similar body length (~ 1.5 cm) were removed and placed individually into (diam. = 2.3 cm, h = 14.7 cm) test tubes and run at test temperatures of either 0 or 6 °C for 48 h in complete darkness. Each test tube also contained a disc of *L. digitata* (diam. = 2.0 cm) of known mass. Discs were cut with a cork borer (diam. = 2.0 cm), washed with deionised water, blotted dry and weighed using an analytical balance (Sartorius, MC1 Analytic AC2105, accurate to 0.1 mg). Each test tube was plugged with cotton wool moistened with sea water (S = 33) and further sealed with paraffin film (Parafilm M, Bemis). Control test tubes, containing a disc of *L. digitata* but without an amphipod, were used to estimate changes in the disk that could not be attributed to amphipod feeding. High accuracy thermometers (HH806AU by Omega) were placed inside the test tubes at random to verify the thermal accuracy and stability (accuracy ± 0.05 % rdg + 0.3 °C, temporal fluctuations ± 0.1 °C) of the temperature treatments.

After 48 h exposure, activity was assessed by assigning survivors to one of two activity states. Individuals that displaying any activity at all (locomotor, twitching, flexing) in the first 60 sec after being stimulated were designated as 'active'. Individuals that remained inactive for > 60 sec after being continually manually stimulated were designated as torpid. Mortality was expressed as the % of those individuals in a treatment that showed signs of decay and/or failed to respond to manual stimulation with fine forceps. Survivors were quickly removed from test tubes, flash frozen (T = - 80 °C) and also weighed using the analytical balance mentioned above. Individuals were then re-weighed after drying in an oven (T = 60 °C) for 2 days. A physiological condition index was estimated as the water content of an individual (dry: wet mass ratio) expressed as a percentage (Lucas and Beninger 1985).

At the same time *L. digitata* discs were removed from each test tube using a pair of fine forceps, blotted with white roll to remove faecal matter and weighed using the same analytical balance as described above.

The amount of food consumed by an individual was calculated by subtracting initial from final mass of their *L. digitata* disc. Feeding rate was expressed as *L. digitata* wet mass loss [mg] per total wet animal mass [g] per h.

5.3.3 Statistical analysis

The effects of exposure and test temperatures (and their interactions) on activity and mortality were carried out using two-way permutational multivariate analysis of variance (PERMANOVA), based on Euclidean distance matrices, with 999 unrestricted permutation of raw data, sums of squares type III and Monte Carlo

asymptotic *p*-value. *Post hoc* pairwise permutation t-test were used to identify differences between levels of significant factors.

The effects of exposure and test temperatures (and their interactions) on feeding rate was carried out using a two-way ANOVA with NIR Fisher *post-hoc* test, after a square root transformation. Differences in feeding rate between 'control' groups at exposure temperatures of T = 10 and 20 °C were tested using a Mann-Whitney *U*-test. The level of significance chosen in each case was $p \leq 0.05$. Prior to the statistical analyses, normality of the data was tested with Kolmogorov-Smirnov test. All statistical analysis were conducted using R 3.4.4., Dell Statistica 13.1 or PRIMER v6.

A1.4 RESULTS

A1.4.1. Activity

There was a significant difference in activity of *D. deshayesii* exposed to T = 10 and 20 °C (Fig A1.1). At T = 10°C 30 % of individuals were active while at T = 20 °C 90 % of individuals were active.

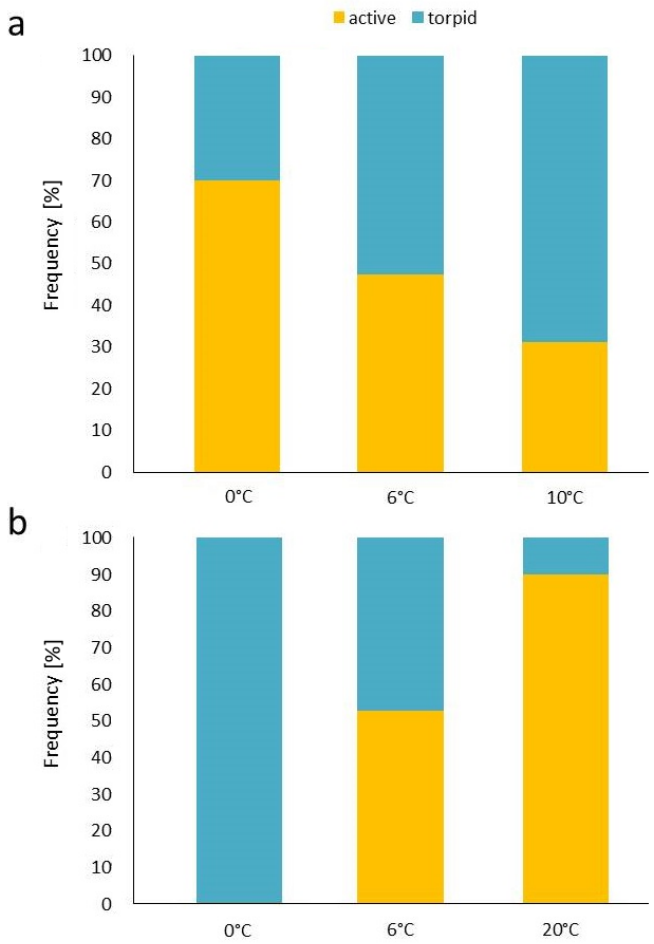


Figure A.1 Activity of *D. deshayesii* exposed to T = 10 °C (a) and 20 °C (b) and tested at two low environmental temperatures. (T = 0 and 6 °C).

There was a significant effect of exposure temperature on activity but there was also a significant interaction between exposure temperature and test temperature (Tab. A1.1). There was, however, no significant effect of test temperature on activity of individuals exposed to T = 10 °C. Activity did differ significantly between T = 0 °C and 6 °C in the individuals exposed to T = 20 °C (Tab. A1.1). Activity decreased with a decrease in temperature. The difference in the activity between individuals at the exposure temperatures was particularly evident at the lowest temperature, T = 0 °C. Here all of the individuals exposed to T = 20 °C were torpid while 70 % of the individuals acclimated to T = 10 °C were active (Fig. A1.1).

Table A1.1. Two-way PERMANOVA and pair-wise tests to evaluate the effects of exposure and test temperature and their interaction on the activity of D. deshayesii.

Factor	*df*	*MS*	*Pseudo-F*	*p(MC)*
Exposure temperature	*1*	*1.82*	*3.88*	***0.04***
(ET)	*1*	*0.22*	*0.46*	*0.51*
Test temperature (TT)	*1*	*2.55*	*5.45*	***0.02***
ET x TT	*56*	*0.47*		
Res	*59*			
Total				
Pair-wise tests		*Groups*	*t-values*	*p(MC)*
Term				
ET		*10 - 20*	*1.97*	*0.07*
TT		*0 - 6*	*0.68*	*0.52*
ET x TT (ET = 10)		*0 - 6*	*1.16*	*0.27*
ET x TT (ET = 20)		*0 - 6*	*2.20*	***0.03***

A1.4.2 Feeding rate

The feeding rates of *D. deshayesii* exposed at T = 10 °C and 20 °C were significantly different (Mann-Whitney *U*-test, $p = 0.04$). Average feeding rate at 10 °C was 12 mg g^{-1} h^{-1} and in 20 °C was 18 mg g^{-1} h^{-1} (Fig. A1.2).

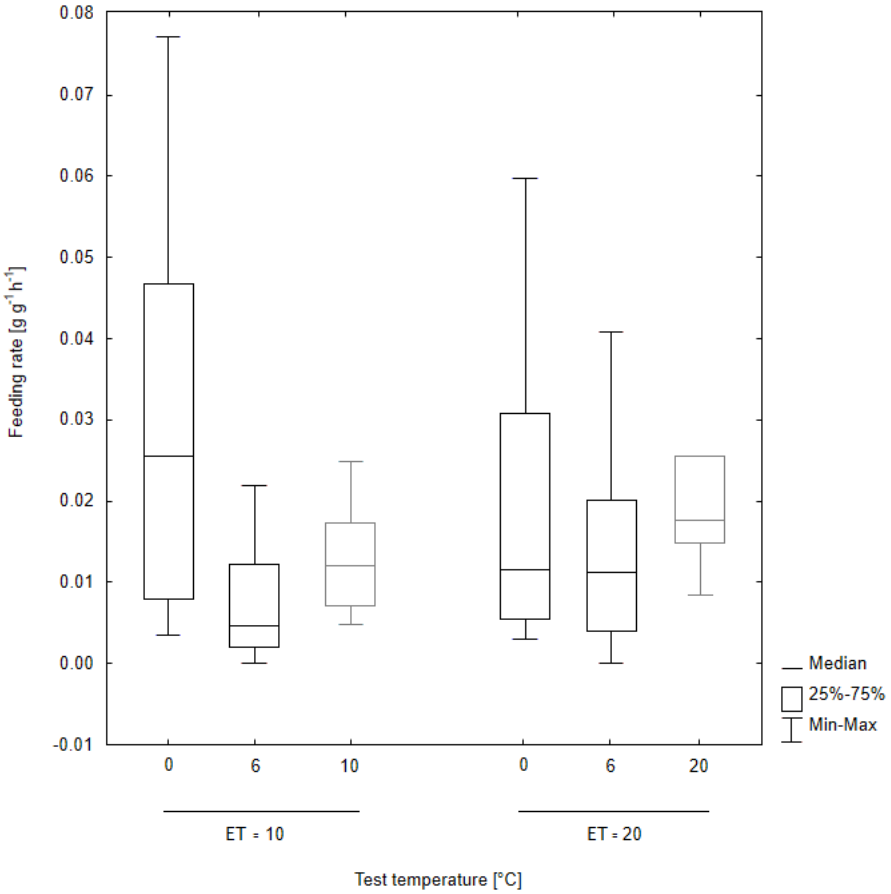


Figure A1.1. Feeding rate of D. deshayesii after 7 days exposed to one of two different temperatures (ET = 10 or 20 °C) and tested at 0 and 6 °C).

There was no significant effect of exposure temperature on the feeding rates tested at T = 0 and 6 °C (Tab. A1.2). There was, however, a significant effect of test temperature on feeding rate and significant interaction between exposure and test temperature (Tab. A1.2).

In individuals exposed to T = 10 °C feeding rate was significantly different between the tested temperatures of 6 and 0 °C (Tab. A1.2). At T = 6 °C average feeding rate was 5 mg g^{-1} h^{-1} and at 0 °C it was 26 mg g^{-1} h^{-1}. However for individuals exposed to T = 20 °C the feeding rate remained at a low level in

each of the different temperatures tested (Fig. A1.2). At 6 °C average feeding rate amounted to 11 mg g^{-1} h^{-1} and at 0 °C it was 12 mg g^{-1} h^{-1}. Perhaps counterintuitively, it was at a test temperature of 0 °C in individuals exposed to T = 10 °C that the greatest feeding rate was recorded (Fig. A1.2).

Table A1.2. Two-way ANOVA and post-hoc test to evaluate the effects of exposure (10, 20 °C) and test temperature (0, 6 °C) and interactions between them on feeding rate of *D. deshayesii*

Factor	df	MS	F	*p*
Exposure temperature (ET)	1	0.00	0.04	0.85
Test temperature (TT)	1	0.03	8.75	**0.00**
ET x TT	1	0.01	3.99	**0.05**
Res	51	0.00		
Total	54			

		P			
Post-hoc test		ET=10, TT=0	ET=20, TT=0	ET=10, TT=6	ET=20, TT=6
	ET=10, TT=0				
	ET=20, TT=0	0.20			
	ET=10, TT=6	**0.00**	0.07		
	ET=20, TT=6	**0.02**	0.52	0.11	

A1.4.3 Water content

There were no significant differences (and no interactions) detected in the physiological condition index of *D. deshayesii* as a result of any of the experimental treatments employed (ANOVA, $p > 0.1$). Water content in *D. deshayesii* varied between 56 to 83 % (mean 67 % ± 5.12 S.D.).

A1.4.4 Mortality

The mortality of *D. deshayesii* exposed to T = 20 °C was high, compared with the individuals exposed to T = 10 °C where no mortality was observed (Tab. A1.3). At a test temperature of 6 °C the mortality was the same, irrespective of exposure temperature. At a test temperature of 0 °C mortality was high (33 %) among the individuals exposed to 20 °C, whereas no mortality was observed among individuals exposed to T = 10 °C (Tab. A1.3).

Table A1.3. Mortality of D. deshayesii from 2 exposure temperatures, tested at three different environmental temperatures.

Exposure temperature [°C]	Test temperature [°C]	Mortality [%]
10	0	0
10	6	5
10	10	0
20	0	30
20	6	5
20	20	33

There was a significant effect of exposure, but not test temperature on mortality (Tab. A1.4). There was some indication (though not significantly different) of an interaction between exposure and test temperature in the group exposed to T = 20 °C (Tab. AI.4).

Table A1.4. Two-way PERMANOVA and pair-wise tests to evaluate the effects of exposure and test temperature and their interaction on the mortality of *D. deshayesii*

Factor	df	MS	Pseudo-F	*p*(MC)
Exposure temperature (ET)	1	0.3	4.20	**0.05**
Test temperature (TT)	1	0.13	1.87	0.18
ET x TT	1	0.30	4.20	0.06
Res	56	4.00		
Total	59	4.58		

Pair-wise tests

Term	Groups	*t*-values	*p*(MC)
ET	10 – 20	2.05	**0.05**
TT	0 - 6	1.37	0.17
ET x TT (ET = 10)	0 – 6	0.70	0.48
ET x TT (ET = 20)	0 – 6	1.96	0.06

A1.5 DISCUSSION

This study is a first step in predicting the impact of warmer winters on the ecology and physiology of temperate sandhopper populations. Talitrid amphipods, such as *D. deshayesii* are an integral component of the macrofaunal community responsible for breakdown of wrack in the supralittoral zone (Stenton-Dozey and Griffiths, 1983; Robertson and Lucas, 1983) assimilating nutrients and providing a direct food item for birds, fish and larger invertebrate predators (Backlund, 1945) excreting ~ 30 % of nitrogen consumed, *via* faecal waste (McLachlan and MacGwynne 1986), in addition to bioturbating sediment and fragmenting debris for use by other consumers (Griffiths and Stenton-Dozey, 1981,1983). They may contribute as much as 70 % to the wrack decomposition on sandy shores (Lastra *et al*., 2018). Therefore,

if there was no temperature acclimation of torpor frequency and feeding rate, then increased climate-related warming of even just a few degrees may increase the annual rate of breakdown of organic detritus, increasing the export of nutrients to inshore waters (McLachlan and MacGwynne, 1986). In addition, warming would result in the negation of low-temperature induced torpor. This would decrease residence time in deep overwintering burrows and so change the number of amphipod prey available to both invertebrate and vertebrate predators.

Consequently, the aim of this present study was to see if *D. deshayesii* is able to acclimate. We found some evidence of acclimation but interpreting our findings is more challenging than we initially predicted because of an unexpected discovery. That is sandhoppers, previously exposed to T = 10 °C, showed no significant difference in torpid behaviour, and the greatest feeding rate recorded, at the lowest test temperature used (T = 0 °C).

While there was no significant effect of exposure temperature on feeding (supporting prediction 1) there was some effect on torpor (not fully supporting prediction 3), i.e. there was some evidence of acclimation. However, the nature of the significant effects of test temperature on torpor and feeding were not as predicted and, as such, complicate the interpretation of predictions 1 and 3. Sandhoppers previously exposed to T = 20 °C showed a reduction in activity with a reduction in test temperature with all individuals becoming torpid in freezing temperatures (supporting prediction 2). However, previous exposure to T = 10 °C appeared to reduce the incidence of torpor in individuals experiencing freezing temperatures (not supporting prediction 2) and survival was better at this exposure temperature. Therefore, while there is clearly evidence of

acclimation, we must, at present, reject the hypothesis that torpor is primarily a consequence of an inability to acclimate.

In line with prediction 4 there was an increase in the frequency of torpor with all individuals entering torpor within 48 h in the freezing test temperature, but only for sandhoppers exposed to T = 20 °C, not 10 °C. Furthermore, there was greater mortality observed at each test temperature in sandhoppers exposed to T = 20 °C compared with 10 °C. There is anectodal evidence from the UK (Tully and Spicer unpubl. obs) for a similar response, whereby individuals that experience winter warm periods *in situ*, may incur increased mortality during cold spells. This may have further ecological implications regarding decomposition and nutrient cycling (Marsh, 2007).

While there was an effect of test temperature on feeding, this was only significant for sandhoppers previously exposed to T = 10 and not 20 °C. Furthermore in sandhoppers previously exposed to T = 10 °C, counterintuitively feeding rate was greatest at the lowest temperature tested.

With no comparable literature available, generalising these results must be done with caution as the limits of acclimatization are genetically determined and may therefore differ both intra- and inter- specifically (Spicer and Gaston, 1999). Moreover, it should be noted, the long-term response might be different, taking into account the short time-scale of our study. Also, in our experiment we did not allow amphipods to bury in sand as they would *in situ*, which may interfere with their response to low temperature.

A1.6 CONCLUSION

How overwintering of various taxa will be affected by climate change is a currently a focus of intense interest and concern (Bale and Hayward, 2010; Abarca *et al*., 2019; Glandon *et al*., 2019). Given the results of our study we suggest that exposure to more frequent and intense warm periods in winter associated with climate change has the potential to alter torpor and feeding in overwintering *D. deshayesii* populations at their northern range but perhaps not in such a simple and straight-forward way as first envisaged. The temperature which *D. deshayesii* are exposed to previously clearly modifies torpor, feeding and mortality. When the exposure temperature was low sandhoppers were able to maintain activity and feeding in freezing temperatures. When exposure temperature was high *D. deshayesii* became torpid and reduced their activity and feeding at freezing temperature. If these results hold *in situ* across *D. deshayesii* populations, then we predict that, in a warming world, the incidence of torpor and feeding will be influenced not just by a direct effect of temperature but also in combination with interactions with acclamatory ability in some, but not all, physiological processes.

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