

2013

# Developmental timing in aquatic embryos: linking intraspecific heterochrony and evolution

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<http://hdl.handle.net/10026.1/1402>

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<http://dx.doi.org/10.24382/3377>

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**DEVELOPMENTAL TIMING IN AQUATIC EMBRYOS:  
LINKING INTRASPECIFIC HETEROCHRONY AND  
EVOLUTION**

By

**OLIVER A. TILLS**

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

**DOCTOR OF PHILOSOPHY**

**School of Marine Science and Engineering**

**Faculty of Science**

2013

## **Developmental timing in aquatic embryos: linking intraspecific heterochrony and evolution**

The main aim of this thesis is to understand the extent to which intraspecific variation in developmental event timing might provide the raw material from which heterochronies may originate. To this end I studied the timing of a suite of both morphological and physiological events in the embryonic development of *Radix balthica*, a species of aquatic snail known to exhibit event timing variation during the embryonic period (Tills et al. 2010; Rundle et al. 2011) and that sits within an evolutionary clade, in which extensive heterochrony has been documented (Smirthwaite et al. 2007). I found that variation in embryonic developmental event timing within *R. balthica* is pervasive (Chapters 2 - 5) and distributed primarily at low (inter-individual and egg mass), rather than high (population) biological levels (Chapter 3). This variation also appears to have a genetic basis (Chapter 2) and to be heritable (Chapters 4 and 5). Examination of the development of function in the cardiovascular (CV) system in Chapter 5 also revealed extensive variation, including differences between egg masses in the timing of aspects of this development, and differences between populations in the rates of change in heart rate during different phases of ontogeny. Variation in CV development also had effects on life history, which suggest that altered embryonic development might have implications for Darwinian fitness (Chapter 5). This thesis demonstrates that intraspecific variation in developmental event timing represents a fundamental link between ontogeny and phylogeny and that study of altered timing at the inter-individual level provides the opportunity to address questions concerning its evolvability and implications.

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**Appendix 2** – Tills, O., Spicer, J.I., and Rundle, S.D. 2010. Salinity-induced heterokairy in an upper-estuarine population of the snail *Radix balthica* (Mollusca: Pulmonata). *Aquatic Biology*, 9: 95-105.

**Appendix 3** – Tills, O., Rundle, S.D., Salinger, M., Haun, T., Pfenninger, M., and Spicer, J.I. 2011. A genetic basis for intraspecific differences in developmental timing? *Evolution and Development*, 13: 542-548.

**Appendix 4** – Tills, O., Bitterli, T., Culverhouse, P., Spicer, J.I., and Rundle, S.D. A novel application of motion analysis for detecting stress responses in embryos at different stages of development. *Manuscript in prep.*



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## ACKNOWLEDGEMENTS

Altered timing of developmental events was a topic first introduced to me by Simon Rundle and John Spicer during the third year of my degree. They discussed this subject with a contagious enthusiasm and this sparked my desire to study in this area. Without their advice, encouragement and patience discussing ideas and strategies (and regularly re-discussing and reaching different conclusions), this work would not have happened. Simon Rundle as my primary supervisor has given me an incredible level of support and guidance for which I am extremely grateful. I have also really appreciated John Spicer's inspirational words, which he has a talent for delivering just prior to my breaking point.

In the lab Ann Torr has been key in facilitating this research through her culture of snails and providing the solution for any technical issues I came to her with. Marie Hawkins has also been a great help in the lab, perhaps most notably for her (better than mine!) carpentry skills and an annoying (but useful) ability for knowing the answer to any technical or aquaculture question. Manuela Truebano Garcia has provided valuable advice to me on the genetic aspects of this thesis and I appreciate her guidance. Thanks to Tabitha Bitterli for her help in developing the bio-imaging technology and also, when I was unavailable, for coming into the lab at weekends to check everything was still running smoothly. When presented with an analytical problem, on which I would usually stew and become increasingly frustrated, Andy Foggo was a source of immense help, providing great advice and support for different statistical approaches. Piero

Calosi also provided great advice on statistical analysis and I thank him for this. Thanks to Phil Culverhouse, my third supervisor for this PhD, for advice and guidance on the development of bio-imaging technology.

Owing to the large volume of video collected during the course of this PhD, it quickly became evident that help would be needed to efficiently analyse this. For this, I have relied on the dedication of a team of undergraduate students; James Hinchcliffe, Rachael Coppock, Patrick Jones, Lucy Hawkins and Matthew Crossland and I am extremely grateful to them for their help with data collection.

Rick Preston and Neil Fewings were pivotal in designing and machining (this process was repeated several times) novel bio-imaging systems for imaging aquatic embryos. Without their expertise, patience and the confidence they instilled in me that nothing was impossible, development of the bio-imaging systems, which were key to much of this research, would not have been possible.

Ian McFazden has been central in generating business cases to facilitate the development of technology and ideas in this PhD. He was able to efficiently see the big picture to recognise opportunities for its development, without losing sight of the science and I don't feel this would have been possible working with anyone else and therefore I am extremely grateful to him. Funding for the development of technology during this PhD has been provided from the Higher Education and Innovation Fund (HEIF) by both the Marine Institute and the Department for Research and Innovation, and I am grateful to them for this.

Thanks to Markus Pfenninger, Moritz Salinger and Timm Haun from Frankfurt University for carrying out the genetic analysis used in Chapter 2.

Finally thanks to my friends and family for offering support and encouragement, during both emotional ups and downs. Most notably this thanks goes to my wife Laura for her incomprehensible patience with me for the past three (and a bit) years, she has been an amazing and never-ending support on which I could always rely.

## **AUTHOR'S DECLARATION**

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

This study was financed by Plymouth University.

Microsatellite analysis in Chapter 2 was performed by Markus Pfenninger, Moritz Salinger and Timm Haun.

### **Publications**

**Tills, O.**, Rundle, S.D., Salinger, M., Haun, T., Pfenninger, M., and Spicer, J.I. 2011. A genetic basis for intraspecific differences in developmental timing? *Evolution and Development*, 13: 542-548.

Spicer, J. I., Rundle, S. D., and **Tills, O.** 2011. Studying the altered timing of physiological events during development: It's about time... or is it? *Respiratory Physiology and Neurobiology*, 178: 3-12.

### **Conferences**

2011 - Marine Institute Conference, Plymouth: Seminar Presentation.

Word count of the main body of thesis: 34,153

Signed

Date

## CHAPTER 1

### INTRODUCTION

#### 1.1 Studying developmental event timing

Biologists have long been fascinated by the development of individual organisms (ontogeny) and the timing and sequence of the assembly of their constituent parts during ontogeny. Darwin described development and embryology as, “one of the most important subjects in the whole round of natural history” (Darwin 1878, p 386) and commented that, “the adaptation of the larva to its conditions of life is just as perfect and as beautiful as in the adult animal” (Darwin 1859 p 440). Therefore study of the time during ontogeny at which developmental events occur has had a long, rich, and at times controversial, history (discussed by Gould 1977).

Arguably the greatest controversy within this subject has focussed on the work of Haeckel (1834 - 1919). He proposed in his Biogenic Law that ontogeny ordinarily proceeded *via* a uniform acceleration of the entire development of ancestral adult stages (Haeckel 1866) with evolutionary change normally occurring by the addition of new developmental stages to the end of this ancestral ontogeny. This, ‘terminal addition’, Haeckel proposed, was the main method of evolution, thereby preserving the ancestral ontogeny present during earlier stages of development. Under the Biogenic Law, novelty, or evolutionary change, should occur only towards the end of ontogeny. However, Haeckel was aware that this was not always the case, with the timing of some developmental events moving seemingly independently and not recapitulating an ancestral ontogeny. Haeckel coined the term ‘heterochrony’ for these ‘prominent

exceptions to the rule of evolution by change in developmental timing' (Haeckel 1866).

Haeckel was a strong supporter of Darwin's theory of evolution by natural selection and in 1866 he proposed the term "ecology" to embody Darwin's ideas. However, Haeckel's interest in the relationship between ontogeny and phylogeny stemmed not just from a desire to understand the link between development and evolution, but to trace phylogenetic trees by identifying ancestral developmental events in descendants at an earlier stage (critically reviewed in Gould 1977 and Richardson and Keuck 2002). To provide support for his Biogenetic law Haeckel produced drawings of various species of vertebrate at different stages of embryonic development to illustrate that these species recapitulated the same ancestral stages (Haeckel 1866). Haeckel's drawings certainly did show marked similarities between stages in different species, but there were concerns about the accuracy of these drawings (e.g. Sedgwick 1894). There were also theoretical objections to the Biogenetic law during the late nineteenth and early twentieth centuries (e.g. Garstang 1922; Gould 1977; Richardson and Keuck 2002).

Walter Garstang, a British zoologist, summarised his objection to Haeckel's law with the phrase, 'ontogeny does not recapitulate phylogeny, it creates it' (Garstang 1922). He considered variation in early development commonplace, coming to this conclusion from his work with marine invertebrate larvae, which show elaborate morphological specialisation during their time as plankton. Garstang (1922) concluded that such specialisation during early life stages was ubiquitous and not the result of a recapitulation of ancestral ontogenies. Instead

he proposed that evolutionary divergence could occur at any time during ontogeny, a view shared by many others during this period (Müller 1869; Balfour 1875; Lillie 1899; Sedgwick 1909; McMurrich 1912).

The Biogenic Law fell out of favour during the twentieth century, partly due to frustration from embryologists and zoologists in using it as a tool for investigating causation during ontogeny (discussed in Holland 2011). However, despite the criticisms of Haeckel's law (e.g. Gould 1977), it still held some support for a good part of the 20<sup>th</sup> century. In 1997, Richardson and colleagues re-assessed the drawings Haeckel produced to illustrate his theory, using large numbers of tail bud stage vertebrate embryos, and they found Haeckel's drawings to be both idealised and inaccurate (Richardson et al. 1997). This demonstration of inaccuracies in the evidence provided by Haeckel to support his ideas offered a conclusion to most of the support for his pervasive law, which was further undermined by subsequent studies (e.g. Bininda-Emonds et al. 2003; Richardson and Keuck 2002). Although Haeckel's 'theory of recapitulation' was ultimately disproven, it did raise awareness of both the role that altered timing of developmental events might play in evolution and the study of variation within and between species during different periods of development, both of which are now exciting areas of research within the field of evolutionary developmental biology (evo-devo) (Richardson 1995; Bininda-Emonds et al. 2003).

The perceived importance of heterochrony in evolution has grown from the mid-20th century, fuelled by the writings of de Beer, (1958), who aimed to distance heterochrony from Haeckel's theory of recapitulation. The result was that

heterochrony soon came to be considered as one of the key potential processes linking development and evolution (Gould 1977; McKinney and McNamara 1991; Raff 1996; Hall 1999).

The word heterochrony has been defined in many ways (e.g. de Beer 1958; Haeckel 1866; Raff and Wray 1989; Richardson 1995; Reilly et al. 1997; Gould 2001; McNamara 2012), but in this thesis I will adopt the one used by many physiologists (Spicer 2006): 'changes in the relative timing of developmental events between ancestors and their descendants' (Spicer and Rundle 2006). Heterochrony has proven to be the norm, in terms of its frequency of occurrence (reviews e.g. Mckinney and McNamara 1991; McNamara and McKinney 2005; Spicer et al. 2011 – Appendix 1), contrary to Haeckel's belief that it was the exception.

## **1.2 Contemporary approaches to investigating developmental sequences**

In 1977 Gould published 'Ontogeny and Phylogeny' and arguably this book marked the time at which heterochrony became a common word within the vocabulary of evolutionary biology (discussed in McNamara 2012). However, Gould had a much more restricted use of the term heterochrony, than earlier workers such as de Beer, moving from changes in relative timing to focussing on changes in size and shape. Alberch et al. (1979) took Gould's work further by extending heterochrony to encompass changes to growth rates. The terminology and analytical approaches put forward, however, were largely unsuitable for those not studying morphological characters, thereby impeding the study of these traits within the context of heterochrony (e.g. Smith 2001; Spicer and Gaston 1999). The two main difficulties with the approaches



proposed by Gould and Alberch for application to non-morphological traits, are: (i) size was often used as a surrogate for time, assuming a linear relationship between size and age; and (ii) the analytical approaches were restricted to events measured in terms of either size or shape, which for many traits (e.g. differentiation of organ systems, gene expression, segmentation) are unsuitable measures (discussed in Smith 2001; 2002).

Although most heterochrony research was, and still is, performed using traits presented within this size/shape framework, there are alternative approaches to the analysis of altered timing and these can be categorised according to the way they treat time: (i) the use of developmental sequences (i.e. ranking/numbering developmental events by the order in which they occur) (e.g. Smirthwaite et al. 2007; Rundle 2011; Tills et al. 2011 – Appendix 2); (ii) relative timing (i.e. timing of one event relative to the duration of a period of development) (e.g. Germain and Laurin 2009; Laurin and Germain 2011); or (iii) absolute time (i.e. timing of an event in real time) (e.g. Mourabit et al. 2010; Tills et al. 2010 – Appendix 2; Tills et al. 2011 – Appendix 3). Within ontogeny, absolute time is highly species-specific and therefore the use of developmental sequences and relative timing are preferred for interspecific comparisons as they help to control for differences in the overall rate of development.

The most sophisticated tools for analysing altered timing are probably those designed for application to developmental sequences. These include approaches for investigating altered timing within a phylogenetic context (event pair cracking- Bininda-Emonds 2004) and for analysis of complex patterns of variation in ontogenic itineraries (Ontogenic Sequence Analysis (OSA) - Colbert

and Rowe 2008). However, in sequence analyses, events are only considered to alter in their timing if they switch positions with another event and, therefore, the temporal resolution with which altered timing can be detected is relatively low. The use of relative timing is a step up from sequence analysis, in terms of the ability to detect altered developmental event timing, and recently the application, and suitability, of event pairing to relative timing data, to allow analyses within a phylogenetic context has been promoted by Germain and Laurin (2011). Finally, the use of absolute timing data is mostly restricted to intraspecific investigations of altered timing as there is no standardisation to control for the overall rate of development.

### **1.3 Altered event timing within and between species**

The contemporary study of heterochrony consists mainly of interspecific comparisons (e.g. Jeffery et al. 2002; Smirithwaite et al. 2007; Mitgutsch 2009; Fabrezi 2011). This has been fruitful and has led to the recording of heterochronies in a wide range of groups, and across a diverse range of traits (reviewed in McKinney and McNamara 1991; Smith 2003; McNamara and McKinney 2005; Spicer et al. 2011 – Appendix 1). It is becoming increasingly apparent, however, that altered developmental event timing is also present, and measureable, within species (discussed in Spicer et al. 2011 – Appendix 1). Therefore, what is the relationship between intraspecific altered developmental event timing and interspecific heterochrony?

The definition of heterochrony presented in Sect 1.1 (altered timing of developmental events between ancestors and descendants) implies an interspecific phenomenon, so the question is does this exclude ancestor-

descendant altered timing within species? This question is complicated by the need to first consider whether altered timing is the result of developmental plasticity or has a genetic underpinning. Spicer and Burggren (2003) aimed to make this distinction clear by coining the term *heterokairy* which they defined as, “plasticity in the timing of the onset of physiological regulatory systems or their components”. Distinguishing environmentally-induced altered timing (plasticity) from altered timing, caused, not by an environmental stimulus, but genetic differentiation, is paramount to understanding the implications of intraspecific altered timing (discussed in Spicer et al. 2011 – Appendix 1).

Heterokairy has been demonstrated in many groups, including crustaceans (Spicer and El-Gamal 1999; Terwilliger and Ryan 2001; Spicer and Eriksson 2003), molluscs (Tills et al. 2010 – Appendix 2; Rundle et al. 2011), fish (Sakamoto 1993; McCormick 1994) and amphibians (Warkentin, 1995, 2000, 2002, 2005; Warkentin et al. 2005). Increasingly, however, reports of intraspecific differences in event timing not induced by an environmental stimulus, are also being made (Gomez-Mestre et al. 2008, 2010; Rogge and Warburton 2008; Warkentin 2007, 2011, a,b; Pan and Burggren 2010; Mourabit et al. 2010).

Work by Warkentin and colleagues investigating altered timing of hatching in tree frogs provides some evidence of how differences at the inter- and intra-specific levels might be linked (e.g. Warkentin 2007; 2011a). A study using the toad, *Bufo americanus* investigated hatching plasticity in response to a pathogenic water mould and this suggested that: (i) induced early hatching had evolved independently of early hatching without this stressor; and (ii) heritability

for hatching age was higher in controls than in the stressed individuals (Gomez-Mestre et al. 2008). In addition, a recent review by Warkentin (2011b) showed that environmentally-induced hatching in the Amphibia is common and has evolved independently in distant lineages to similar environmental cues, and within some clades multiple cues. Looking to the invertebrates, altered timing of developmental events have also been revealed in embryos of a freshwater snail, *Radix balthica*, both in response to, and in the absence of, biotic (Rundle et al. 2011) and abiotic (Tills et al. 2010 – Appendix 2) environmental stressors.

It appears that altered timing at the intra-specific level, a lower biological level than has typically been the focus of heterochrony research, is prevalent. Variation in developmental event timing with a genetic underpinning is perhaps not too surprising, after all, variation at this level is most likely the raw material form which heterochronies were formed. However, it does demand a re-evaluation of what is meant by the term heterochrony and what the relationship might be between altered timing at the within and between species levels.

#### **1.4 Gene regulatory mechanisms and genetic basis for altered timing**

Altered timing is pervasive as a pattern at different hierarchical levels (reviewed by McKinney and McNamara 1991; Raff 1996; Spicer and Rundle 2006; Spicer et al. 2011 – Appendix 1), but what do we know of the gene regulatory mechanisms underpinning altered timing and secondly of its genetic basis? In what follows I discuss possible gene regulatory mechanisms before going on to look at their genetic basis.

#### 1.4.1 Gene regulatory mechanisms

In a recent review paper, Spicer et al. (2011) (Appendix 1) suggested that haemoglobins in humans are probably the physiological system for which we know the most about the underlying genetic mechanisms for altered timing, due to their clinical relevance in combatting the symptoms of Sickle Cell Disease and  $\beta$ -cell thalassemia (Thein et al. 2009; Bower and Orkin 2011; Sankaran and Nathan 2011). Humans usually express the  $\gamma$ -globin gene during fetal development and this produces fetal haemoglobin, which has a higher oxygen affinity than adult haemoglobin, thereby enhancing oxygen delivery from the mother to the fetus. Ordinarily the production of fetal haemoglobin occurs over a period of several months, however significant increases can also occur over just a few days, in humans (Sankaran et al. 2009) and also baboons (Desimone et al. 1978). Close to the time of birth fetal haemoglobin is suppressed, and replaced, *via* expression of the adult  $\beta$ -globulin gene, by adult haemoglobin. The mechanism underpinning the switch from fetal to adult haemoglobin has received considerable research focus, but is still far from clear. What is clear, however, is that there are at least four chromosomes that play a role in regulating production of fetal haemoglobin (reviewed in Spicer et al. 2011 – Appendix 1). There is also considerable natural variation in the timing of this transition both within and between human populations (Leonova et al. 1996; Rottgardt et al. 2010) and also between species (Opazo et al. 2008). Yet despite this being one, if not the, best-studied example of altered timing of a physiological system, we still do not yet understand the regulatory pathways responsible.

The soil nematode *Caenorhabditis elegans* is perhaps the species for which we know most about the mechanisms underlying altered morphological developmental event timing. Work with *C. elegans* has documented the complete cell lineage of its 'wild type' development, which has proven largely invariant (Sulston and Horvitz 1977; Kimble and Hirsh 1978; Sulston et al. 1983). This cell lineage information allows comparison of the relative timing of developmental events at the level of individual cell divisions and differentiations, at both inter- and intra-specific levels. This information has been used to understand the mechanisms responsible for heterochrony, which occurs mainly in the cuticle morphology and moulting behaviour, induced via chemicals or radiation (Ambros and Horvitz 1984). This work has identified six genes that can be mutated to cause heterochrony (Chalfie et al. 1981; Ambros and Horvitz 1984) and analysis of these mutations suggests that heterochrony can be caused by just small base-pair deletions in these genes, which appear to control the relative timing of developmental events (Rukuvín et al. 1989).

Together these examples demonstrate that we have made considerable progress towards understanding the potential genetic mechanisms leading to altered developmental event timing in some well-studied systems. It is also evident, however, that for most researchers this degree of study of the mechanistic basis of altered timing is unobtainable and that in the case of the fetal Hb example, hundreds of studies have still not fully resolved the mechanism underlying the altered timing of the production of this relatively simple molecule.

#### 1.4.2 Genetic basis of altered timing

Understanding the nature of the genetic basis for intra-specific variation in altered timing should be a pivotal step in disentangling the link between inter- and intra-specific variation, but is an area of investigation that has been largely overlooked in heterochrony research. Noticeable exceptions include the work of Gomez-Mestre et al. (2008) suggesting a heritable component to hatching age in *Bufo americanus* and a study of polychromatism in a species of cichlid, *Nechromis omnicaeruleus*, which hints at a heritable component to the timing of transition in colour (Maan et al. 2005).

Perhaps the best method for investigating the genetic basis of altered timing will prove to be the use of experimental evolution. Experimental evolution can be broadly divided into “Laboratory natural selection” (LNS) and “Artificial selection” (Rose and Garland 2009). Laboratory natural selection studies allow ‘natural’ selection to proceed in the lab, with no selective culling, typically in individuals or populations exposed to different treatments. Artificial selection, involves the experimenter selecting breeders to use for the next generation, based on either single, or multiple, trait values (Garland 2003).

The application of selection studies within the field of evolutionary physiology has become commonplace over the past decade (Bennett 2003; Garland 2003; Swallow and Garland 2005), but has so far not been utilised to address questions relating to altered timing or, more specifically, the extent to which intraspecific altered timing might be the raw material from which heterochronies arise. The reason for this is uncertain, but Darwin did comment, “that natural selection will always act very slowly” (Darwin 1859, p108) and Rose and

Garland (2009) discussed that this belief has led to very old patterns of research in which the focus all too often jumps to higher biological levels (e.g. population or species comparisons). In Chapters 4 and 5 I use perhaps the simplest form of breeding experiment, a direct parent-offspring comparison with no environmental stressor. This experimental design allowed me to compare the timing of developmental events in a (selfing) parent, with the timing of the same events in their offspring and thereby determine the degree of inter-generational similarity. Parent-offspring comparisons are relatively common in biology and have revealed hundreds of heritable traits, including, amongst others: fecundity in *Drosophila* (Sgro and Hoffmann 1997); tarsus length in the pied flycatcher (Alatalo and Lundberg 1986); susceptibility to ectoparasites in kittiwakes (Boulinier et al. 1997); and growth rates in cattle (Carter 1959).

### **1.5 Thesis aims and objectives**

The main aim of this thesis was to investigate to what extent intraspecific variation in developmental event timing could contribute to the origin/genesis of heterochrony.

This was achieved by addressing three objectives:

(1) I described, in detail, the pattern with which variance in intraspecific developmental event timing occurs at different biological levels (Chapters 2 and 3);

(2) I then investigated the extent to which this variation might have a genetic underpinning (Chapters 2, 4 and 5);



(3) Finally, I explore the implications of altered timing for an individual's life history (Chapter 5).

The chapters in this thesis are structured as follows:

In Chapter 2 I investigate whether, in embryos taken from a wild population, levels of difference in inter-individual developmental event timing are linked to levels of inter-individual genetic dissimilarity.

In Chapter 3 I address how variation in developmental event timing is partitioned between the levels of population, egg mass and inter-individual, with the aim of better understanding the ecological and evolutionary implications of this variation.

In Chapter 4 I use a parent-offspring comparison to investigate the degree to which variation in developmental event timing has a parental origin. Determining cross-generational similarity in event timing will help elucidate what role intraspecific variation might play in the generation of heterochronies.

In Chapter 5 I explore within and between generational variation in the development of aspects of cardiovascular (CV) function, including the timing of CV developmental events. This physiological system was chosen as its functional development can be visualised with ease during the embryonic period.

In my final chapter (Chapter 6), I provide a general discussion of my thesis including its implications for our understanding of the origins of heterochrony and potential future directions in the study of altered timing.

## 1.6 Model organism used in this study

### 1.6.1 Gastropods as a model for studying altered event timing

In gastropods the fundamentals of morphological (Raven 1966) and physiological (Wilbur and Yonge 1966) development were described in the late nineteenth to mid-twentieth century. This early work included descriptions of cell lineages (Blochman 1882; Delsman 1912) and these facilitated the investigation of phylogenetic signals in cell lineage across 30 species of gastropod (Hyman 1951; Lindberg and Guralnick 2003). Gastropods have also provided a good model for neurobiology, in particular for the study of learning and memory (e.g. Dickinson and Croll 2003).

A group of air breathing gastropods, the Lymnaeidae, were the first invertebrate group in which heterochrony was investigated within a quantitative framework (Smirthwaite et al. 2007) using (as a basis) the work of Morrill (1982) who detailed the development and culture of *Lymnaea* and also Cumin (1972), who described developmental stages in *Lymnaea stagnalis*. Smirthwaite et al. (2007) described fourteen developmental events, both physiological and morphological, spread throughout the embryonic development of 12 species in this group and discovered relatively large numbers of heterochronies in events that included first heart beat, eye spot formation and body flexing. Heterochronies were found to have occurred at familial, generic and species levels in the phylogeny and several occurred repeatedly, and independently. Smirthwaite et al. (2007) showed that the occurrence of heterochronies in these functional events, within this invertebrate group, had occurred with a similar frequency to that seen in the vertebrates suggesting that heterochrony may have had a prominent role in the

evolution of this group. Here, I use many of the same developmental events described by Smirthwaite et al. (2007), but also some others (e.g. velum and shell formation – Chapter 4) and these are described within the chapters in which they feature.

Further work with snails from the Lymnaeidae has revealed environmentally induced altered timing in many of the developmental events used in Smirthwaite et al. (2007). Using an estuarine population of *Radix balthica* (Linnaeus, 1758), Tills et al. (2010) demonstrated salinity induced heterokairy in developmental events including veliger, eye spot formation, first heartbeat and hatching. This study also discovered significant differences in event timing between embryos from different egg masses in control treatments and in salinity treatments the magnitude of plastic response was also different between egg masses. Further work by Rundle et al. (2011) also revealed heterokairy in this group, but in this instance in response to kairomones from a fish predator. They exposed embryonic *Radix balthica* and *R. auricularia* to predator cue and discovered species-specific responses. *Radix auricularia* exhibited earlier eye spot formation, first heart beat and body flexing whereas *R. balthica* showed earlier foot attachment and crawling, but later mantle muscle flexing. Interestingly the direction of altered developmental timing in these two species resulted in more similar development in a stressed environment than control, and not all embryos responded to the predator cue. *Radix balthica* is known to use crawling behaviour to escape predators and therefore it is interesting that crawling is an event bought forward in embryos exposed to predator cue and to consider the potential post-hatch advantage such early development of this behaviour might entail.

### 1.6.2 *Radix balthica* as a model study species

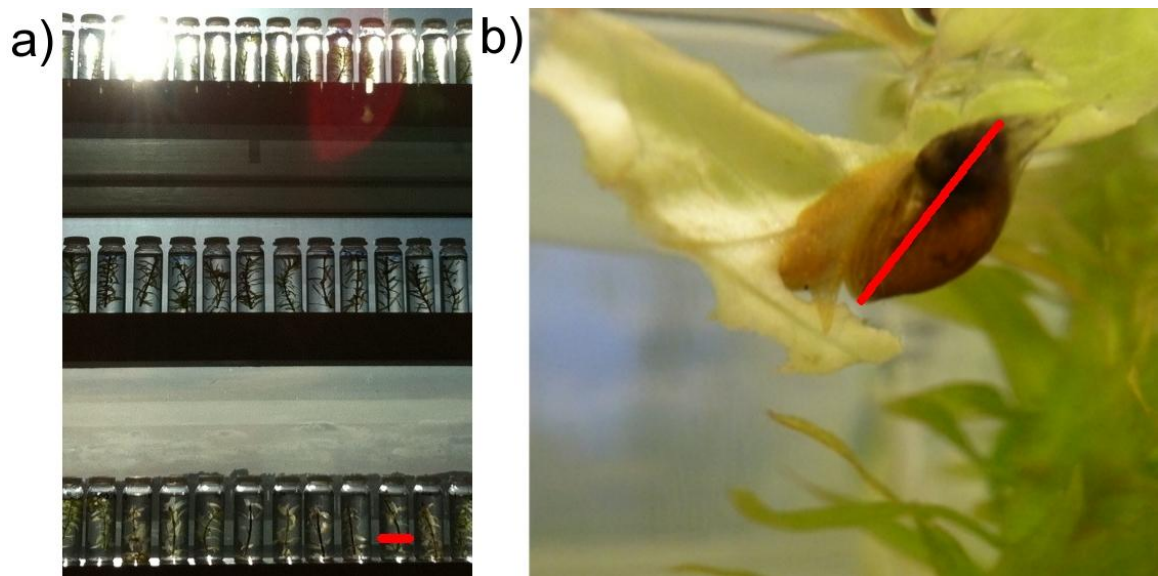
The common pond snail, *Radix balthica* (formerly *Lymnaea peregra*) is part of the Lymnaeidae family (Bassomatophora) and is widely distributed throughout European streams, lakes and ponds (Pfenninger 2006). *Radix balthica* is a highly plastic species, in terms of its morphology (Lakowitz et al. 2008; Pfenninger 2006; Brönmark et al. 2011; Schneibs et al. 2011), behaviour (Åhlgren and Brönmark 2012), development (Tills et al. 2010 – Appendix 2; Rundle et al. 2011) and reproduction (Evanno et al. 2006; Pfenninger et al. 2011; Haun et al. *in press*). Also, despite being a freshwater species, it is found in the brackish waters of the Baltic sea (Råberg and Kautsky 2007) and in UK estuaries (Tills et al. 2010 – Appendix 2).

In 1920 a large scale breeding experiment using *R. balthica* was begun on the rooves of various London hospitals and universities, which lasted for over ten years. *Radix balthica* were maintained both in pairs and individually, in which case offspring were produced via selfing, in glass jars containing Canadian pondweed, *Elodea densa*. This breeding study was performed to investigate the heritability of sinistrality (left-handed coiling) in shell and body coiling, making use of rare, but naturally occurring sinistral snails, in this largely dextral (left-handed coiling) species (Boycott and Diver 1923; Boycott and Diver 1925; Boycott et al. 1931). This study involved the breeding of over a million snails and demonstrated the potential of *R. balthica* in breeding experiments.

*Radix balthica* lays transparent eggs (approx. 1 mm length) within a gelatinous egg mass and these can subsequently be dissected from the egg mass with relative ease, cultured individually, and their development observed (e.g.

Smirthwaite et al. 2007; Tills et al. 2010, 2011 [Appendices 2 and 3]; Rundle et al. 2011).

Here, for the studies detailed in Chapters 4 and 5, I adopted a similar approach to Boycott and Diver in culturing *R. balthica*. I maintained individual snails in glass jars, with pieces of *Elodea densa*, on shelving positioned in a west facing window, in a 15°C controlled-temperature (CT) facility (Figure 1). Time to maturity and egg laying was approximately eight months and eggs were most commonly deposited on the *E. densa*. Hatchling and juvenile snails primarily grazed on the *Elodea*. However, from three weeks after hatching, *Lactuca sativa* (lettuce) was used to supplement the diet and to promote growth. Mortality during the three weeks after hatching was approximately 50%, but low thereafter (~ 5%). This method of long-term culture proved to be effective and required fairly low levels of maintenance.



**Figure 1.1** a) Jars used to culture hatchling snails, containing *Elodea densa*, positioned in the window. Red line indicates the 20 mm width of a single jar.; b) Mature *Radix balthica* feeding on lettuce. Red line indicates the 10 mm length of the snail's shell.

## 1.7 Measuring developmental event timing

### 1.7.1 A purpose-built bio-imaging system

Embryonic development of *R. balthica* takes place over approximately 10 days at 20°C and previous work with this species has identified a suite of functional physiological and morphological developmental events occurring throughout this period, which can all be observed under low power magnification (x 10) (Smirthwaite et al. 2007). Given the central importance of being able to measure the timing of functional events, with high temporal resolution, in large numbers of embryos, I decided to invest several months into the research and development of a bio-imaging system that would facilitate the observation of a greater number of embryos, and with higher frequency, than is feasible using manual methods. The requirements of this system were: i) image quality (high resolution and frame rate); ii) depth of field (sufficiently high to encompass the depth of an egg, thereby ensuring embryos are in focus regardless of their position in the egg); and, iii) automation (system must be capable of running for months at a time). It soon became apparent that none of the microscopes on the market could meet these requirements and therefore I trialled individual components with a view to designing and constructing a system in house.

The resulting system comprises a four megapixel, monochrome, shutterless machine vision camera (Pike 421, Allied Vision Technologies, Germany) connected to a zooming optic lens (VHZ20R, Keyence, Japan) (Fig. 2). The optics are then inverted and mounted in an aluminium frame, which was machined 'in-house'. Above the optics a robotic XY stage (Optiscan, Prior, England) is mounted and this is fitted with the appropriate insert for multiwell

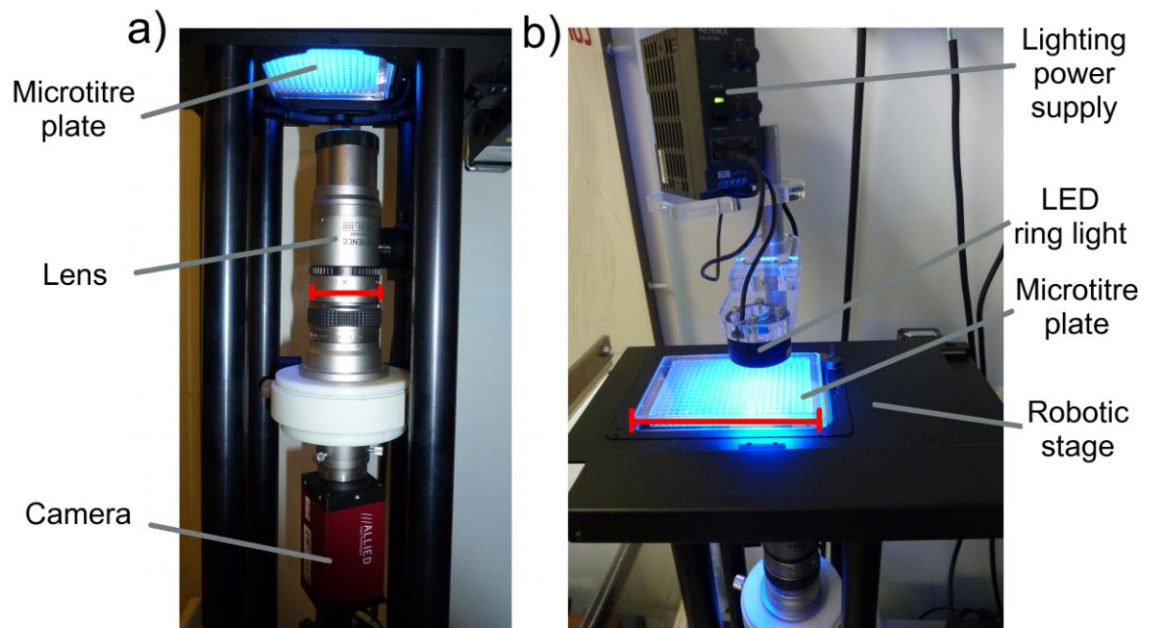
plates. Darkfield lighting is provided by a light emitting diode (LED) ring light (Keyence, Japan), which is mounted above the robotic stage using a plastic mounting bracket designed and also machined in house.

An Apple Mac Pro was used to control the system and was connected to the camera via a Firewire800 cable. The robotic stage was connected to the Mac Pro via a Universal Serial Bus (USB) to serial adapter (USA-19HS, Keyspan, USA). Camera and stage were controlled using the open source software Micromanager (Edelstein et al. 2010), which runs as a plugin to Image J, a popular image analysis software, and provides drivers for both this camera and stage. To facilitate safe and efficient data capture an external Serial ATA (eSATA) Peripheral Component Interconnect (PCI) card was installed in the Mac Pro, and, to this, an external hard drive array was connected (6 drive). This arrangement allowed data to be written simultaneously to mirrored hard drives and meant that, in the event that one hard drive failed, the remaining intact drive would still contain a complete copy of the data, and image capture would continue uninterrupted. Use of this array also allowed removal and addition of hard drives whilst the system was still running. For data storage I used 2 terabyte (TB) Seagate Barracuda 3.5 inch with a spin-rate of 7200 revolutions per minute (RPM) hard drives owing to their speed and the reputation of Seagate.

The imaging system was housed in a 20°C controlled-temperature (CT) facility and embryos were maintained, when being imaged, in individual wells of a clear 384 well microtitre plate (Elkay, UK) with a lid. Embryos were cultured in artificial pond water (APW; ASTM 1980) containing 90 mg/litre of calcium

(Rundle et al. 2004) and this was changed in each of the wells daily via manual pipetting.

Appendix 4 describes the use of this bio-imaging system for the application of motion analysis for developmental staging and assessment of embryonic stress responses.



**Figure 1.2** The purpose built bio-imaging system with labelled components. a) Inverted camera and lens mounted beneath the motorised microscope stage (red line = 50 mm length); b) LED lighting unit providing dark-field lighting to a 384 well multi-well plate positioned on the motorised microscope stage (red line = 130 mm length).

### 1.7.2 Measures of developmental event timing

In this thesis I chose, for the most part, to analyse developmental events in terms of their absolute timing (measured as time from the first cell division). However, I also analyse events in terms of their relative timing in Chapter 3 and position within the developmental sequence for some analyses in Chapter 2. The decision to focus mainly on absolute timing was made as this thesis



concerns intra-specific comparisons and therefore I wanted to conserve as much temporal information about event timing as possible.

## CHAPTER 2

### A GENETIC BASIS FOR INTRA-SPECIFIC DIFFERENCES IN DEVELOPMENTAL TIMING

Published as – Tills, O., Rundle, S.D., Salinger, M., Haun, T., Pfenninger, M., and Spicer, J.I. 2011. A genetic basis for intraspecific differences in developmental timing? *Evolution and Development*, 13(6): 542-548.

#### 2.1 Summary

Heterochrony, altered developmental timing between ancestors and their descendants, has been proposed as a pervasive evolutionary feature and recent analytical approaches have confirmed its existence as an evolutionary *pattern*. Yet the mechanistic basis for heterochrony remains unclear and, in particular, whether intraspecific variation in the timing of developmental events generates, or has the potential to generate, future between-species differences. Here I make a key step in linking heterochrony at the inter- and intra-specific level by reporting an association between inter-individual variation in both the absolute and relative timing (position within the sequence of developmental events) of key embryonic developmental events and genetic distance for the pond snail, *Radix balthica*. I report significant differences in the genetic distance of individuals exhibiting different levels of dissimilarity in their absolute and relative timing of developmental events such as spinning activity, eye spot

formation, heart ontogeny and hatching. This relationship between genetic and developmental dissimilarity is consistent with there being a genetic basis for variation in developmental timing and so suggests that intraspecific heterochrony could provide the raw material for natural selection to produce speciation.

## **2.2 Introduction**

In the *Origin of Species* Darwin (1859) asked “at what period of life (do) the causes of variability, whatever they may be, generally act” (p. 8), highlighting that variation early in development could be important for natural selection. Haeckel (1866) subsequently championed the notion of a link between early development and evolution, proposing in his Biogenetic law that an organism’s ontogeny was a brief and rapid recapitulation of its phylogeny (Gould 1977). This ‘law’ was subsequently refuted, most notably by De Beer (Olsson et al. 2009; Richardson et al. 2009), who, like Darwin, suggested that evolutionary innovation could occur throughout development. De Beer’s theories on embryology played a crucial role in the Modern Synthesis, yet it is his views on heterochrony, altered developmental timing, that have had greatest longevity.

Despite his discredited theory of recapitulation, Haeckel did identify the occurrence of heterochrony, the altered timing of developmental events between ancestors and descendants, which has had greater longevity and has been proposed to be a pervasive evolutionary feature (Gould 1979). Recent analytical approaches have demonstrated the occurrence of heterochrony within vertebrate phylogenies, confirming the occurrence of heterochrony as an evolutionary *pattern* (Spicer and Rundle 2006; Richardson et al. 2009; Fiser et

al. 2008; Richardson et al. 2002; Weisbecker et al. 2008; Maxwell et al. 2010). Despite this body of evidence, however, the key question of what generates heterochrony still persists (Spicer et al. 2011 – Appendix 1).

Intra-specific variation in the timing of developmental events is an aspect of development that has been rarely examined due to the assumption that variation at the intra-specific level is low relative to inter-specific heterochrony (Spicer and Gaston 1999). However, the number of studies reporting altered timing of developmental events at the intra-specific level is increasing, perhaps due to the use of larger sample sizes (Cubbage and Mabee 1996; de Jong et al. 2009; Mabee and Trendler 1996; Schmidt and Starck 2004; Spicer and Rundle 2007; McKinney and McNamara 1991). Given the existence of variation in developmental event timing at both the inter- and intra-specific level, a key challenge is to elucidate whether there is a relationship between these two levels of variation, in particular whether intra-specific variation in developmental timing generates, or has the potential to generate, between-species heterochrony (Spicer et al. 2011 – Appendix 1).

Here I investigate whether altered developmental timing might provide the raw material for natural selection to fix heterochronies by asking if variation in the timing of developmental events within a species has a genetic basis. Observations of freshwater snail embryos from the Lymnaeidae provided the first evidence for extensive heterochrony among species, on both internal and terminal branches, in an invertebrate phylogeny (Smirthwaite et al. 2007). Snail embryos have advantages over those of vertebrates as they allow the generation of continuous developmental data whereas studies of vertebrate

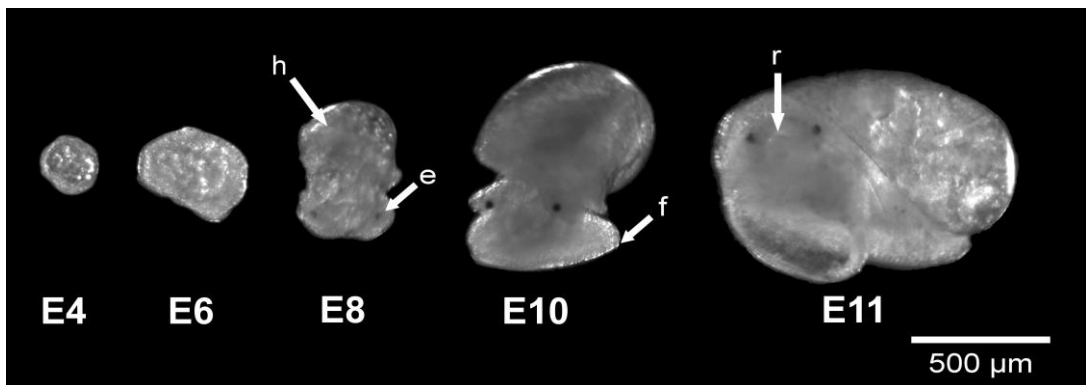
development typically rely on one-off measurements of individuals at discrete developmental stages (Bininda-Emonds et al. 2003). At the same time, intra-specific changes to the developmental sequence of *Radix balthica* embryos, the snail I use here, have been reported in response to both biotic (Rundle et al. 2010) and abiotic (Tills et al. 2010 – Appendix 2) stressors making this a relevant species for investigating intra-specific variation in developmental timing. I used a standard suite of developmental events (Smirthwaite et al. 2007) to identify developmental event sequences within *Radix balthica* (Fig. 2.1, Table 2.1) and compared differences in developmental timing to strength of genetic relation, measured using eight polymorphic microsatellites (Salinger and Pfenninger 2009; Nei et al. 1983).

## **2.3 Materials and Methods**

### **2.3.1 Embryonic development**

Egg masses of *R. balthica* as identified by DNA-barcoding (Pfenninger et al. 2006) were collected at low tide on the 9<sup>th</sup> of June, 2009 from a single nine metre stretch of shore in the upper estuary of the River Dart, Totnes, Devon, UK (50°26'19" N, 3°41'24" W). They were removed to the laboratory at Plymouth and examined under low power (x 10) (Leica MZ12) with those egg masses containing eggs that had not developed beyond the 2-cell division stage being used in the study. Six eggs from each of 11 egg masses were dissected under low power and placed in multi-well trays (vol. per well = 24 ml, 24 wells per trays) containing Artificial Pond Water (APW) (ASTM 1980) with 90 mg l<sup>-1</sup> [Ca<sup>2+</sup>] (Rundle et al. 2004) with a single egg in each well. Eggs within multi-well trays were cultured in a controlled-temperature facility (T = 20°C) under a 12: 12 light:

dark cycle. Treatment solution in each of the wells was changed daily under low power magnification at which time the developmental events undergone by each embryo were recorded (see Fig. 2.1 and Table 2.1 for description of the developmental events used). Both morphological and physiological developmental events were used and these events were chosen as they have undergone heterochrony between species within the Lymnaea group, of which *Radix balthica* is a member (Smirthwaite et al. 2007). When examining embryos for 'non-morphological' developmental events, such as the appearance of the first heart beat, embryos were monitored for several minutes to identify the onset of that developmental event. Embryonic development lasted an average of 13 days (Fig. 2.2).



**Figure 2.1.** Developmental stages (E4, E6, E8, E10, E11) of *Radix balthica* embryos (Cumin 1972). The location of the developmental events; eye spot formation (e), foot attachment (f), heart beat (h) and radula function are indicated.

<b>Developmental event</b>	<b>Description of event</b>
Spinning activity	Rotating using cilia.
Trochophore	Spherical liver cells form a helmet like velum within the egg.
Veliger	Foot formation and shell secretion. Symmetrical development of two ends of the embryo.
Hippo	Asymmetrical development with further development of the foot and shell. This is the major stage of organogenesis, the head becomes more conspicuous from the rest of the body, as the middle part pinched in.
Ontogeny of eyes	Formed on the head, further pigmentation of eye spots occurs as they develop.
First heart beat	Chamber type heart forms and begins beating on the far back of the shell, which moves closer towards the head as the embryo grows.
Initial foot attachment	Foot attaches to the capsule wall enabling the embryo to crawl.
Radula activity	The radula situated in the head starts actively sucking the capsular fluid in.
Hatching	Rupture of the egg capsule, enabling the embryo to emerge out of it.

**Table 2.1** Descriptions of the developmental events recorded (Smirthwaite et al. 2007). Each developmental event was recorded when it was first observed, observations were made every 24 h.

### 2.3.2 Genetic analysis

Once snails hatched they were preserved separately in microcentrifuge tubes (Eppendorf, vol. = 0.6 ml) containing 70% ethanol. DNA extraction was accomplished using a glass fibre DNA extraction method after a protocol developed by the Canadian Centre of DNA Barcoding (Ivanova et al. 2006).

All snails were genotyped for 8 highly polymorphic microsatellite loci (Salinger et al. 2009) by collaborators at Frankfurt University. Multiplex Microsatellite amplification was carried out using Quiagen Type-it™ microsatellite PCR Kit with fluorescent dye labeled forward primers. PCR products were separated using ABI Prism 3730 sequencer (Applied Biosystems) with GeneScan™ 500-LIZ™ size standard (Applied Biosystems). Microsatellite allele lengths were analysed using GENEMAPPER 4.0 software (Applied Biosystems).

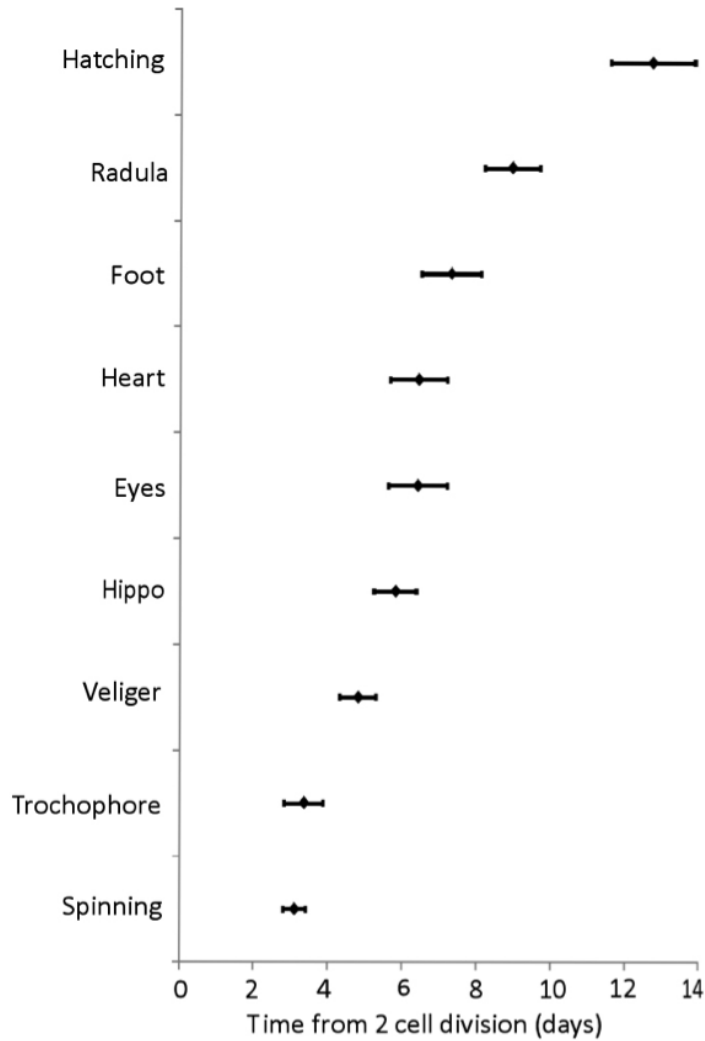
Genetic analysis was successful for forty-eight embryos and these data together with the embryonic development data were used in the analysis. Pair-wise genetic distance among individuals was estimated as Nei's standard genetic distance (Nei et al. 1983) (Table 2.2). This genetic distance measure is a rather conservative estimate of genetic relatedness, as it does not partition between recent co-ancestry and the background relatedness of individuals belonging to the same population. There was no significant relationship between the genetic distance of eggs from different egg masses and their topographical distance at the collection site. AMOVA revealed that 96% of molecular variance is at the individual level and only 3% is distributed between egg masses (Among egg masses -  $R_{10} = 0.034$ ,  $p = 0.01$ ; Among individuals -  $R_{104} = 0.994$ ,  $P = 0.01$ ) and therefore I did not include egg mass in the analysis.

### 2.3.3 Analytical approach

Developmental data were analysed both as the absolute time of onset of developmental events and as the position of events within the developmental sequence (relative timing) for each snail on which microsatellite analysis was

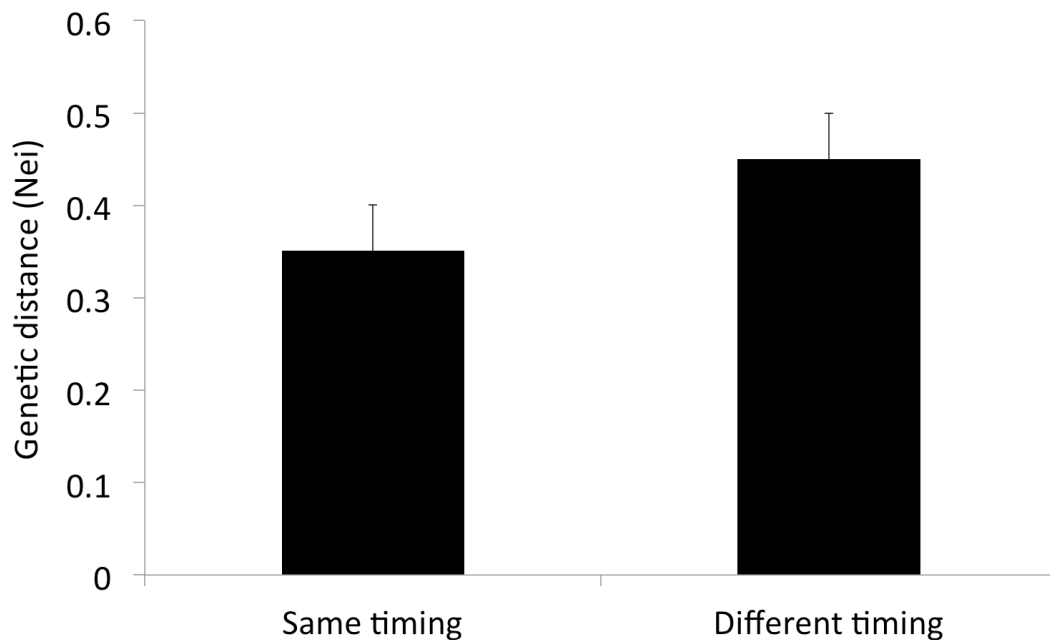


successful (N = 48). The relative timing of developmental events provides a potentially better method of comparing an embryo's timing of decision making and therefore could be more useful in understanding the mechanism underlying heterochronic change (Smith 2001). To allow comparisons of developmental difference between individuals the developmental data for each individual was subtracted from that for every other individual, and the direction of all differences expressed as positive values. This provided a dataset of developmental differences between all possible pair-wise individual comparisons.



**Figure 2.2** Time of onset (mean  $\pm$  1 S.D.), from the 2-cell division stage, of nine embryonic developmental events.

A Generalised Linear Model with log linked interval level response was used to test for differences in the genetic distance of individuals with different levels of developmental dissimilarity and *post-hoc* Tukey's tests were used for pair-wise comparisons.



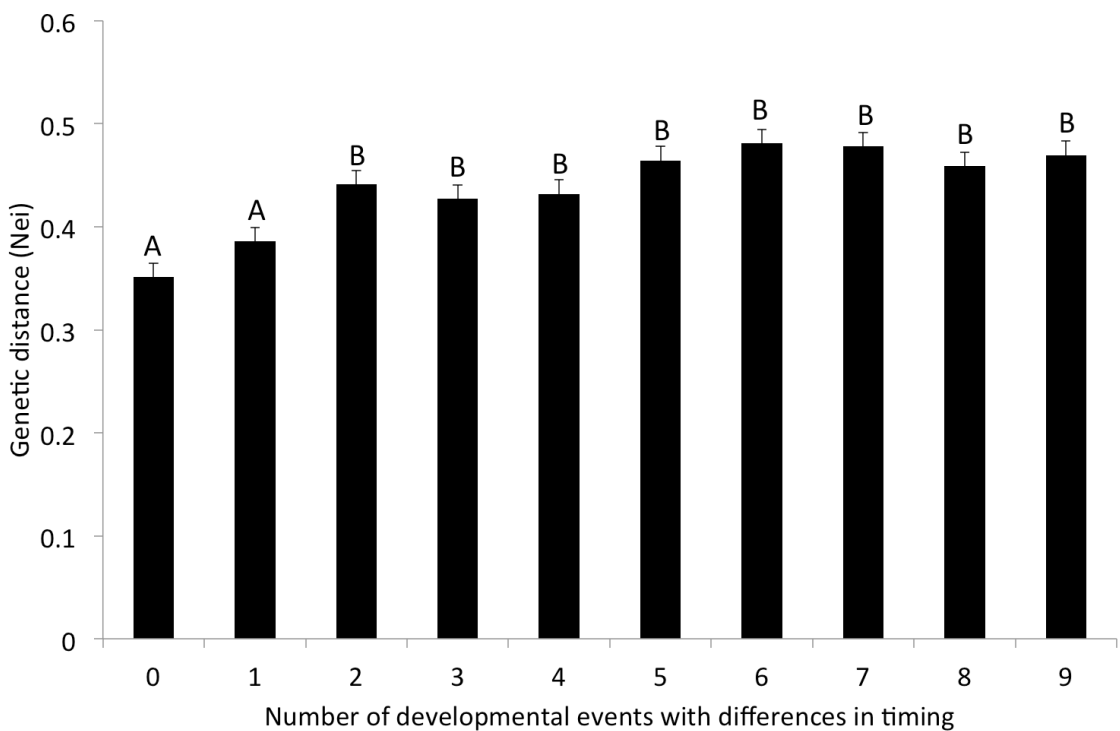
**Figure 2.3.** Genetic distance of individuals with no significant differences in the time of onset of nine embryonic developmental events compared with individuals exhibiting a difference. Values are means  $\pm$  1 SD.

## 2.4 Results

### 2.4.1 Absolute time of onset of developmental events

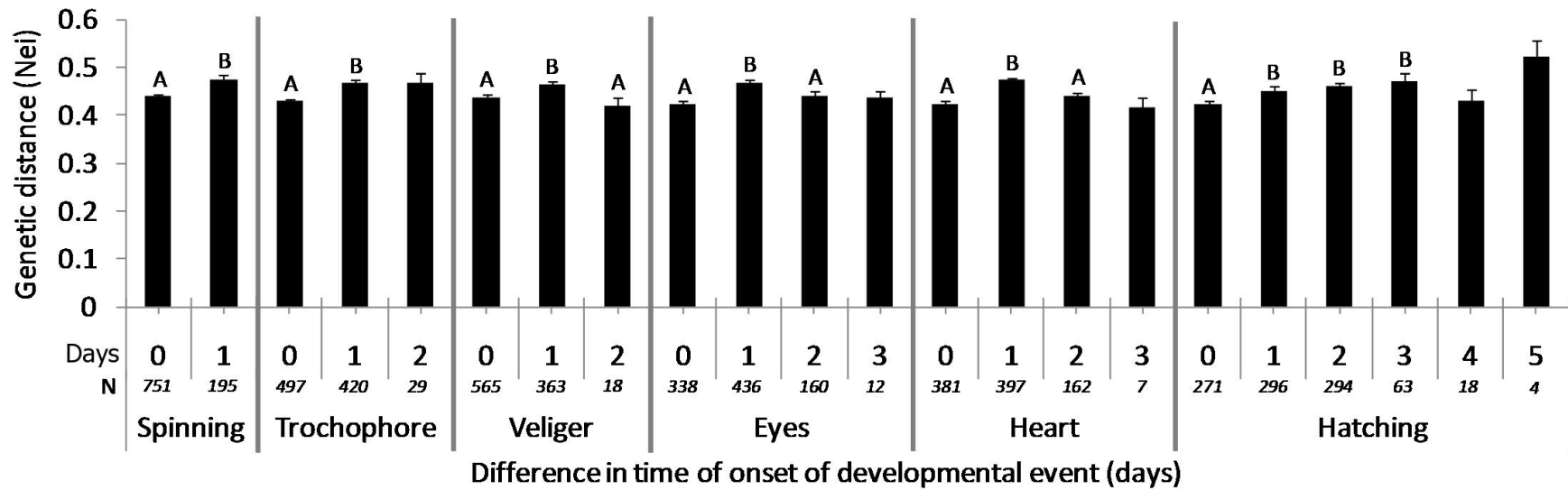
Variation in the time of onset of developmental events increased as development progressed (Fig. 2.2). I found clear evidence for between-embryo differences in the absolute time of onset of several developmental events that was associated with their genetic relation: individuals with the same time of onset of all nine developmental events used here had higher genetic similarity than individuals with any differences ( $\chi^2_{946, 1} = 13.437$ ,  $P < 0.001$ ) (Fig. 2.3). Individuals ranged from having zero to nine developmental events that differed in their time of onset and there were significant differences in the genetic

distance of individuals with these different levels of developmental dissimilarity ( $\chi^2_{946, 9} = 47.486, P < 0.001$ ) (Fig. 2.4). Individuals with either no events, or a single event, with differences in their time of onset were more genetically similar than individuals with two or more events with differences in their developmental timing (**0 Events** vs **1** –  $P = 0.002$ ; **0** vs **2** –  $P = 0.008$ ; **0** vs **3** –  $P = 0.006$ ; **0** vs **4** –  $P = 0.006$ ; **0** vs **5** –  $P < 0.001$ ; **0** vs **6** –  $P < 0.001$ ; **0** vs **7** –  $P < 0.001$ ; **0** vs **8** –  $P < 0.001$ ; **0** vs **9** –  $P < 0.001$ . **1 Event** vs **2** –  $P = 0.005$ ; **1** vs **3** –  $P = 0.028$ ; **1** vs **4** –  $P = 0.02$ ; **1** vs **5** –  $P < 0.001$ ; **1** vs **6** –  $P < 0.001$ ; **1** vs **7** –  $P < 0.001$ ; **1** vs **8** –  $P < 0.001$ ; **1** vs **9** –  $P = 0.001$ ).



**Figure 2.4.** Genetic distance of individuals with different numbers of developmental events with differences in their time of onset. Different letters above error bars, within each developmental event, indicate significant differences in genetic distance as indicated by *post-hoc* Tukey tests.

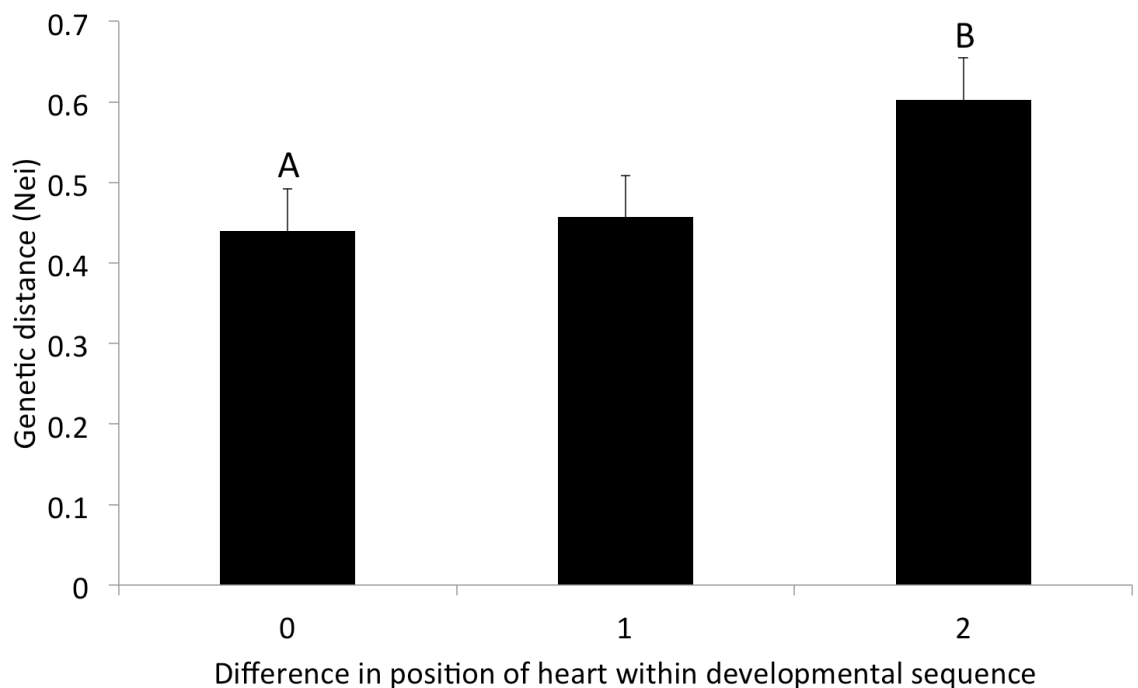
There were also event-specific genetic relationships with significant differences in the genetic similarity of individuals with altered time of onset of several developmental events (spinning,  $\chi^2_{946, 1} = 9.65$ ,  $P = 0.006$ ; trochophore,  $\chi^2_{946, 2} = 16.46$ ,  $P < 0.001$ ; veliger,  $\chi^2_{946, 1} = 8.216$ ,  $P = 0.016$ ; eyes,  $\chi^2_{946, 3} = 20.749$ ,  $P < 0.001$ ; heart,  $\chi^2_{946, 3} = 22.817$ ,  $P < 0.001$  and hatching,  $\chi^2_{946, 5} = 13.874$ ,  $P = 0.016$ ) (Fig. 2.5). The majority of pairwise comparisons between individuals for the developmental events spinning, trochophore and veliger revealed no developmental differences (Fig. 2.5). However, several of the developmental events occurring later in development (eyes, heart and hatching) have more pairwise comparisons revealing differences in their time of onset. Each developmental event had fewer pairwise comparisons revealing the greatest level of difference in developmental timing than comparisons where there was no difference.



**Figure 2.5.** Genetic distance of individuals with various differences in the time of onset of several developmental events. N = the number of pairwise individual comparisons revealing that level of difference in the timing of that developmental event. Different letters above error bars, within each developmental event, indicate significant differences in genetic distance as indicated by *post-hoc* Tukey tests.

#### 2.4.2 Relative timing of developmental events

Variation was also observed between embryos in the relative timing (position within the developmental sequence) of several developmental events (eye spot formation, first heart beat and foot attachment), but only embryos with dissimilarity in the position of first heart beat within their developmental itinerary had differences in their genetic dissimilarity: embryos with the greatest difference in the relative position of first heart beat (two positions) had lower genetic similarity than those with no difference ( $\chi^2_{946, 2} = 9.159, P = 0.01$ ) (Fig. 2.6).



**Figure 2.6.** Genetic distance of individuals with various levels of difference in the position of heart ontogeny within the developmental sequence ( $\chi^2_{946, 2} = 9.159, P = 0.01$ ). Values are means  $\pm$  1 S.D. Different letters above error bars, within each developmental event indicate significant differences in genetic distance as indicated by *post-hoc* Tukey tests.

1	a	0.000																												
	b	0.422	0.000																											
	c	0.268	0.340	0.000																										
	d	0.198	0.349	0.340	0.000																									
2	a	0.429	0.310	0.613	0.369	0.000																								
	b	0.198	0.412	0.340	0.250	0.369	0.000																							
	c	0.323	0.500	0.453	0.412	0.369	0.162	0.000																						
	d	0.470	0.613	0.715	0.571	0.417	0.399	0.256	0.000																					
	e	0.429	0.411	0.613	0.470	0.143	0.470	0.227	0.250	0.000																				
	f	0.323	0.511	0.512	0.448	0.357	0.297	0.198	0.042	0.214	0.000																			
3	a	0.271	0.547	0.328	0.235	0.369	0.209	0.334	0.470	0.512	0.360	0.000																		
	b	0.636	0.584	0.756	0.547	0.441	0.573	0.485	0.411	0.441	0.485	0.485	0.000																	
	c	0.328	0.411	0.470	0.268	0.143	0.268	0.268	0.500	0.286	0.429	0.227	0.512	0.000																
	d	0.599	0.547	0.798	0.511	0.399	0.537	0.448	0.369	0.399	0.448	0.521	0.037	0.470	0.000															
	e	0.583	0.549	0.517	0.583	0.400	0.500	0.382	0.583	0.400	0.583	0.598	0.348	0.259	0.299	0.000														
4	a	0.386	0.563	0.584	0.375	0.470	0.349	0.323	0.126	0.369	0.183	0.448	0.396	0.470	0.360	0.549	0.000													
	b	0.297	0.500	0.483	0.287	0.470	0.261	0.261	0.167	0.369	0.146	0.360	0.396	0.470	0.360	0.549	0.037	0.000												
	c	0.537	0.448	0.554	0.386	0.369	0.412	0.297	0.340	0.369	0.422	0.345	0.297	0.268	0.261	0.333	0.297	0.297	0.000											
	d	0.511	0.474	0.584	0.386	0.512	0.474	0.599	0.369	0.613	0.485	0.433	0.485	0.512	0.448	0.667	0.261	0.297	0.313	0.000										
	e	0.287	0.599	0.441	0.287	0.571	0.250	0.261	0.298	0.470	0.297	0.271	0.448	0.470	0.412	0.500	0.261	0.172	0.287	0.412	0.000									
5	a	0.399	0.429	0.598	0.214	0.264	0.429	0.399	0.215	0.264	0.268	0.369	0.483	0.264	0.441	0.600	0.143	0.185	0.298	0.227	0.399	0.000								
	b	0.512	0.584	0.681	0.584	0.549	0.613	0.584	0.598	0.549	0.655	0.596	0.298	0.549	0.256	0.431	0.512	0.512	0.399	0.470	0.470	0.598	0.000							
	c	0.688	0.625	0.756	0.625	0.613	0.688	0.625	0.613	0.662	0.636	0.396	0.613	0.360	0.382	0.563	0.563	0.448	0.537	0.599	0.571	0.382	0.000							
6	a	0.625	0.547	0.756	0.636	0.429	0.573	0.573	0.685	0.571	0.599	0.610	0.599	0.429	0.563	0.583	0.636	0.636	0.537	0.636	0.750	0.584	0.571	0.724	0.000					
<b>Egg mass</b>		1	2				3				4				5				6											
<b>Individual</b>		a	b	c	d	a	b	c	d	e	f	a	b	c	d	e	a	b	c	d	e	a	b	c	a					
6	b	0.000																												
	c	0.298	0.000																											
	d	0.431	0.098	0.000																										
	e	0.433	0.512	0.500	0.000																									
	f	0.485	0.268	0.264	0.625	0.000																								
7	a	0.431	0.465	0.459	0.382	0.382	0.000																							
	b	0.573	0.483	0.465	0.698	0.422	0.396	0.000																						
	c	0.459	0.411	0.431	0.750	0.224	0.382	0.183	0.000																					
	d	0.495	0.453	0.480	0.756	0.227	0.431	0.126	0.084	0.000																				
	e	0.511	0.554	0.583	0.750	0.412	0.431	0.323	0.323	0.227	0.000																			
8	a	0.422	0.268	0.250	0.537	0.448	0.431	0.636	0.537	0.512	0.375	0.000																		
	b	0.511	0.453	0.465	0.625	0.349	0.264	0.422	0.349	0.268	0.162	0.198	0.000																	
	c	0.584	0.453	0.465	0.613	0.256	0.313	0.340	0.256	0.268	0.185	0.185	0.042	0.000																
	d	0.599	0.685	0.833	0.448	0.537	0.549	0.724	0.662	0.655	0.599	0.636	0.474	0.441	0.000															
	e	0.483	0.431	0.459	0.429	0.357	0.259	0.453	0.500	0.549	0.512	0.613	0.613	0.549	0.714	0.000														
9	a	0.610	0.441	0.465	0.500	0.349	0.299	0.371	0.474	0.411	0.422	0.662	0.511	0.441	0.662	0.185	0.000													
	b	0.636	0.483	0.348	0.412	0.474	0.465	0.334	0.573	0.512	0.474	0.662	0.573	0.512	0.662	0.328	0.224	0.000												
	c	0.547	0.423	0.348	0.360	0.422	0.396	0.386	0.610	0.512	0.573	0.636	0.636	0.584	0.573	0.268	0.261	0.198	0.000											
	d	0.386	0.512	0.500	0.573	0.235	0.431	0.474	0.297	0.310	0.386	0.511	0.386	0.399	0.537	0.441	0.485	0.573	0.485	0.000										
10	a	0.636	0.483	0.382	0.500	0.375	0.431	0.360	0.474	0.411	0.537	0.662	0.500	0.429	0.625	0.429	0.349	0.235	0.386	0.412	0.000									
	b	0.485	0.596	0.549	0.448	0.386	0.362	0.412	0.511	0.470	0.599	0.724	0.599	0.542	0.599	0.298	0.360	0.360	0.224	0.323	0.287	0.000								
	c	0.554	0.549	0.459	0.328	0.429	0.259	0.655	0.571	0.549	0.613	0.542	0.512	0.465	0.441	0.429	0.429	0.571	0.411	0.411	0.571	0.411	0.000							
11	a	0.448	0.441	0.417	0.662	0.573	0.348	0.599	0.662	0.655	0.500	0.438	0.474	0.542	0.875	0.411	0.511	0.599	0.573	0.537	0.511	0.573	0.613	0.000						
	b	0.369	0.514	0.459	0.613	0.470	0.317	0.685	0.542	0.598	0.470	0.298	0.298	0.382	0.685	0.583	0.714	0.714	0.756	0.470	0.643	0.685	0.583	0.256	0.000					
<b>Egg mass</b>		6	7				8				9				10				11											
<b>Individual</b>		b	c	d	e	a	b	c	d	e	a	b	c	d	e	a	b	c	d	a	b	c	a	b	c					

**Table 2.2.** Pairwise matrix containing the genetic distance (Nei et al. 1983) for 48 individuals distributed between 11 egg masses.



## 2.5 Discussion

At present, the precise mechanistic basis for the observed heterochronic differences in developmental timing between closely-related species is unclear. Here, I have provided clear evidence for inter individual variation in developmental event timing in the gastropod *Radix balthica* which has a genetic basis. This variation could provide the raw material on which selection may act, leading to the formation of heterochrony. These developmental events have undergone heterochrony within *R. balthica*'s recent evolutionary past, which adds strong support for the hypothesis that intraspecific variation, such as seen here, has led to the formation of heterochrony within this phylogeny. At present, however, I am unable to go further and identify the mechanism underlying this link between developmental and genetic differences.

The developmental events that showed timing (both absolute and relative) shifts in this study (i.e. spinning activity, trochophore stage, veliger stage, eye spot formation, first heart beat, foot attachment and hatching) have also been shown to be important in distinguishing species within the evolutionary clade of which *R. balthica* is part (Smirthwaite et al. 2007). Although I am not inferring a direct link in this instance, the fact that the timing of these events has been demonstrated to be labile both within and between species does suggest a possible link between these two levels of variation (Rundle et al. 2010, Tills et al. 2010 – Appendix 2). It is important to remember that the timing of developmental events is inherently non-independent; for example, the insertion of time early in development can have a cascading effect leading to the later occurrence of subsequent events. Whilst this non-independence limits the

ability to infer the mechanisms underlying changes in developmental timing it can help us to understand the relationship between different developmental events.

*Radix balthica* is a mixed mating simultaneous hermaphrodite and therefore within a population can have varying levels of independence between family lineages, with potentially poor blending (Jarne and Delay 1990; Haun et al. submitted). This population structure has perhaps allowed for differences in developmental sequence to be maintained and the magnitude of those differences to increase within a small population. I was unable to detect any relationship between the topographical distance of eggs collected and either their genetic or developmental dissimilarity which is perhaps not surprising given this species' mating system and population structure. Moreover, as most of the genetic variation in our study was at the level of the individual I am confident that maternal effects (i.e. differences between eggs masses) were highly unlikely to have driven the main findings of this study.

The genesis of sequence change and the fact that that through time the magnitude of the change came to mirror genetic dissimilarity, I suggest is evidence for the role of intraspecific developmental sequence variability in the formation of heterochrony. The next step in linking heterochrony at the intra- to inter-specific level will be to demonstrate a direct genetic link underlying intra- and inter-specific variation in developmental timing. A genetic basis for both the overall number of developmental events with dissimilarity in their time of onset, and in event-specific differences in time of onset, suggest that intraspecific

variation in the timing and sequence of developmental events, which might have historically been treated as experimental noise, may be a target for natural selection. If so, the differences reported here are highly significant in evolutionary terms and take us a step closer to understanding at least one of the origins of heterochrony. Once the link between inter- and intra-specific heterochrony is revealed and if it can be shown that intra-specific heterochrony has fitness consequences, this will represent a major advance in our understanding of heterochrony as an evolutionary process.

Having identified a potential genetic basis for intraspecific altered timing within a population, in Chapter 3 I will extend my focus to investigate how variance in event timing is partitioned at different biological levels when considering populations from across *R. balthica*'s geographical range.

## CHAPTER 3

### EFFECTS OF BIOLOGICAL ORGANISATION AND MATERNAL PROVISIONING ON VARIANCE IN GASTROPOD EMBRYONIC DEVELOPMENTAL EVENT TIMING

#### 3.1 Abstract

Intraspecific variation in developmental event timing is becoming increasingly apparent and is believed to be the raw material from which heterochronies (altered timing of developmental events between ancestors and descendants) arise. However, our understanding of how variance in intraspecific developmental event timing is distributed at different biological levels is extremely poor. Here I used high (temporal and spatial) resolution imaging of the entire embryonic development of *Radix balthica*, a pond snail, to investigate variance partitioning between the biological levels of population, egg mass and individual, in: the relative timing of four key developmental events (four cell division, a discrete heart beat, capsule rupture and hatching); egg volume (a measure of maternal investment); and hatchling size and shape (to compare embryonic with post-hatch variance). I found that the timing of developmental events other than four cell division plus all measures of hatchling size and shape had most variance partitioned at the individual level, and variance at the population level was surprisingly low despite sampling populations from across *R. balthica*'s range. This pattern raises important questions regarding variation in developmental event timing in evolutionary ecology.

### 3.2 Introduction

Variation is the raw material required for natural selection to operate (Darwin 1859) and understanding the nature of variation in physiological, morphological, developmental and behavioural traits is key to our knowledge of their ecology and evolution (Spicer and Gaston 1999; Arthur 2011). To understand the biological significance of variation the pattern this variation takes in nature should be documented (Spicer and Gaston 1999; Chown and Gaston 2008). However, given that this process of quantifying variance in a particular trait at different biological levels is relatively simple compared to investigating the processes underlying variation, it is surprising that this key step is often omitted in research of trait function, ecology and evolution. Although, there are studies documenting variance partitioning (e.g. Heatwole et al. 1965; Chown et al. 1999; Bagatto 2000), they are rare, particularly for lower biological levels (e.g. population, clutch, inter-individual).

Chown et al. (1999) investigated variance partitioning at the levels of genus, species, population and individual, in traits related to desiccation resistance in African keratin beetles. Variance in physiological traits, which were strongly influenced by body size, was partitioned mostly at the genus level with the lowest levels of variance in these traits measured at the population and individual levels. Once the effects of body size had been accounted for, however, variance in survival time and the rate of maximum water loss were partitioned mostly at the species level, whereas maximum tolerable water loss and lipid and water content exhibited most variance at the individual level. As this study demonstrated, variation at the inter-individual level can dominate even when considering higher biological levels, but a major barrier to

investigating variation at this level is both the inability to replicate the individual (Spicer and Gaston 1999) and that variation at this level has often been regarded as developmental and experimental noise (Spicer and Gaston 1999). Therefore, our understanding of variation among individuals is limited. However, within the field of evolutionary biology a growing body of evidence is revealing significant variation in the timing of developmental events at the inter-individual level.

Heterochrony, defined as altered timing of developmental events between ancestors and descendants, is a well documented macroevolutionary pattern (Bhuller et al. 2012, McNamara 1996, Spicer 2006, Spicer et al. 2011 – Appendix 1) which was proposed by Gould as being the main mechanism of evolutionary innovation (Gould 1977). Most study of heterochrony has concentrated on documenting differences in developmental event timing between species, with the reasoning that any within species variation will be minor, and probably not appropriate to addressing questions relating to this macroevolutionary pattern. However, the body of evidence for intraspecific differences in developmental timing has grown substantially recently, including the study detailed in the previous chapter, which has hinted that this inter-individual variation could have a genetic basis (Chapter 2; Tills et al. 2011 – Appendix 3). Tills et al. (2011) discovered using embryos of *Radix balthica*, a pond snail, that within a single population there was a positive relationship between inter-individual genetic distance and the degree of dissimilarity in embryonic developmental event timing; suggesting there to be a genetic basis for inter-individual variation, even within a single population. Given the recent highlighting of intraspecific variation in developmental timing understanding how

this variation is partitioned at different biological levels and therefore its potential significance in the formation of heterochronies is timely.

Here, I used *Radix balthica* to investigate how variance in the timing of five key embryonic developmental events, which exhibit variation at both the inter- and intra-specific levels (Smirthwaite et al. 2007; Tills et al. 2010 – Appendix 2; Tills et al. 2011 – Appendix 3), was partitioned at the levels of population, egg mass and inter-individual. Given the potential influence of maternal investment on development (Ho and Burggren 2010) I investigated variance partitioning in egg volume and the effect of including egg volume as a covariate on the partitioning of variance in the other traits we examine. Finally, I measured variance partitioning in hatchling size and shape to investigate whether variance was distributed in the same manner as for embryonic traits. My overall aim was to understand how variation in these early developmental traits was partitioned at different biological levels and the factors which might be generating it, a key step in understanding what intraspecific variation in developmental event timing means for heterochrony.

### **3.3 Material and Methods**

#### 3.3.1 Embryo culture

Second generation stock populations of *Radix balthica* from each of four European populations spanning this species' latitudinal range (North Sweden – 66.428, 19.683; South-west England – 50.439, -3.690; Bavaria, Germany – 50.007 9.156; South France – 44.053, 4.784) (Pfenninger et al. 2011) were maintained at 15°C under a 12:12 light-dark regime. Snails were cultured in aquaria (vol. = 15 l; stocking density = 15) containing Artificial Pond Water

(Rundle et al. 2004) and fed lettuce and spinach *ad libitum*. Egg masses were harvested from aquaria and examined under low power (x 10) and if eggs had not developed past the first cell division, they were used in the experiments described below.

### 3.3.2 Bio-imaging

Eggs were dissected from egg masses under low power (x 10) and six eggs were selected haphazardly from along the length of the egg mass (France - 7 egg masses; Sweden - 4 egg masses; Germany - 3 egg masses; England - 6 egg masses) and cultured at 20°C in a 384 well microtitre plate (well vol. = 84 µl). The entire development of embryos was recorded using an automated imaging system designed in our laboratory for imaging aquatic animals (Sect. 1.7.1). This system comprises a 4 megapixel shutterless monochrome camera (Pike 421 B, Allied Vision Technologies, Germany) connected to a zooming lens system (Zoom 70 XL, Optem, Luxembourg), inverted beneath a motorised XY stage (Optiscan XY stage, Prior Scientific, England). Transmitted, dark-field, cold lighting was provided by light emitting diodes (LEDs). The camera and XY motorised stage were controlled using the open-source software MicroManager 1.3 (Edelstein et al. 2010) running on a Mac Pro using OS X. The first four days of development were captured by recording a single image of each embryo every 30 min. After day four, image sequences were acquired at 7.5 frames sec<sup>-1</sup> for 20 sec, every 60 min. Artificial pond water (Rundle et al. 2004) in each well of the multi-well plate was replaced daily.



### 3.3.3 Image analysis

Following image acquisition, image sequences were analysed by manual observation of the sequences using Image J (Abramoff, et al. 2004). The time of onset of six embryonic developmental events; two cell division, four cell division, a discrete heart beat, radula ontogeny, egg capsule rupture and hatching, were recorded for each embryo. The events used, have all been shown to vary in the timing of their appearance both between and within species (Smirthwaite et al. 2007; Tills et al. 2010 – Appendix 2; Tills et al. 2011 – Appendix 3). The time period between two cell division and radula ontogeny was used to standardise the timing of the remaining four developmental events (four cell division, a discrete heart beat, egg capsule rupture and hatching) to provide a measure of relative event timing and therefore control for differences in the overall rate of development between embryos. As a result of this standardisation 2 cell division and radula ontogeny had no variance in relative timing and so were excluded from subsequent analyses. Radula ontogeny was chosen as the second developmental event with which to standardise developmental timing, rather than capsule rupture or hatching, as radula ontogeny exhibits far less variation in timing (pers. obs.) and so its use produces a more consistent measure of relative developmental event timing. The resultant values for the relative timing of four cell division, a discrete heart beat, egg capsule rupture and hatching were used in subsequent statistical analyses.

Egg volume was calculated using the formula  $1/6\pi lw^2$  (Taylor 1973) from measurements of egg length (l) and width (w) obtained from images acquired at the beginning of development. The shell parameters length, width, aperture length, aperture width and aperture height were measured immediately after

hatching. Aspect ratio (length:width), aperture ratio (aperture length:aperture width) and the ratio of length: aperture height were calculated to provide measures of shell shape (Tills et al. 2010 – Appendix 2).

#### 3.3.4 Data analysis

A nested analysis of variance, using the Satterthwaite approximation (Sokal and Rohlf 1995) for unequal sample sizes, was performed to partition variance in the relative timing of the four developmental events (four cell division, a discrete heart beat, capsule rupture, hatching), egg volume and hatchling shell size and shape parameters between the levels of population, egg mass and individual (including the error term).

### **3.4 Results**

#### 3.4.1 Egg volume

Egg volume was significantly different between egg masses ( $F_{17, 63} = 16.94$ ,  $P \leq 0.001$ ), but not populations ( $F_{3, 63} = 1.69$ ,  $P = 0.207$ ). Most variation in egg volume was partitioned at the egg mass level (72%) and the least at the population level (11%) (Fig. 3.1). Egg volume had significant effects on both the relative time of onset of developmental events (Fig. 3.2 and Table 3.2) and the size of hatchlings (Fig. 3.3) and, therefore, analysis of variance was performed, both with (ANCOVA) and without egg volume (ANOVA) as a covariate.

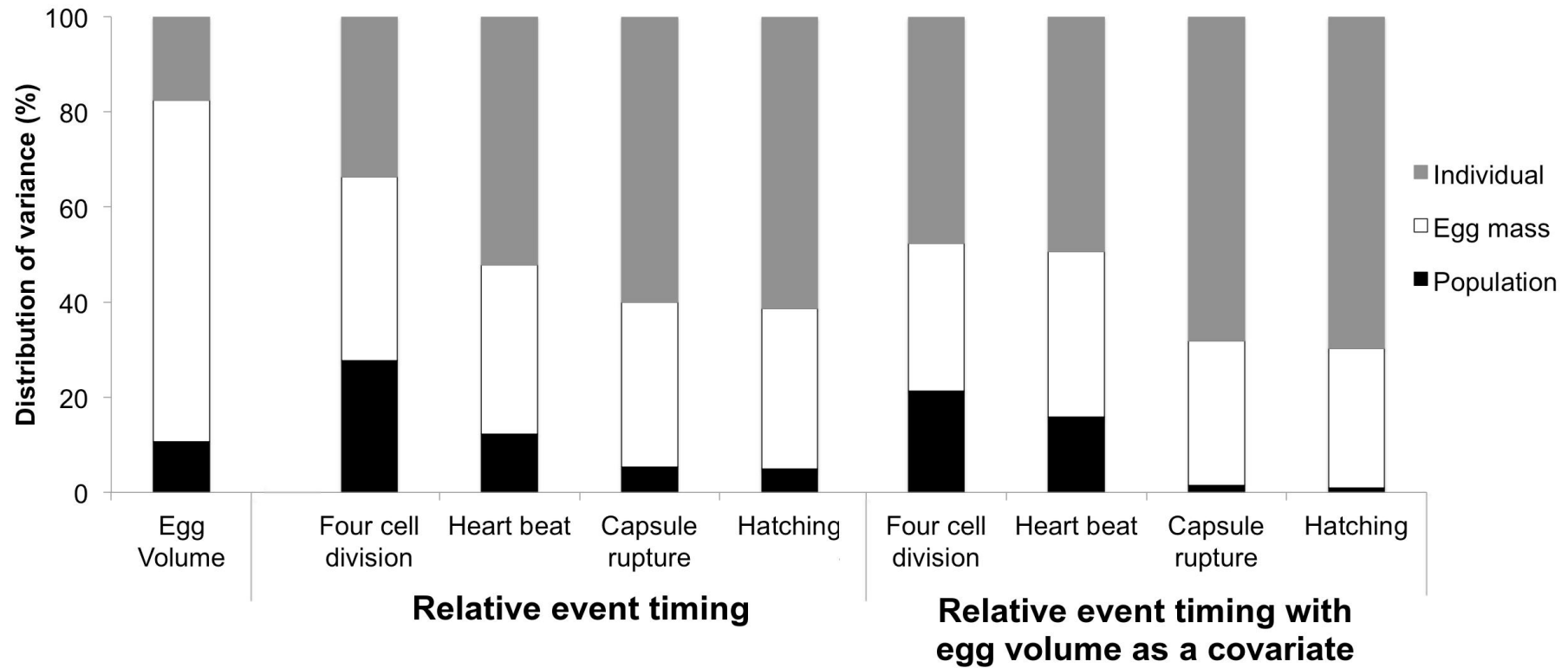
#### 3.4.2 Developmental event timing

Variance in the relative timing of all four measured developmental events was lowest at the population level, both with and without (Fig. 3.1) egg volume factored into the analyses. With egg volume as a covariate in the ANOVA model, most variation in the timing of developmental events was partitioned at the

individual level, however, ANOVA without egg volume as a covariate revealed the timing of four cell division had more variance partitioned at the egg mass (39%) than individual level (34%). This relationship between egg volume and the relative timing of four cell division was confirmed by a significant, negative regression between these two parameters (Fig. 3.2a). Regression analysis also revealed the relative timing of a discrete heart beat to be significantly influenced by egg volume (Fig. 3.2b).

<b>Factor</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>	
<b>Four cell division</b>						
Population	3	0.0001802	0.0000601	3.88	0.028	*
Egg Mass	17	0.0002626	0.0000154	5.46	≤ 0.001	**
Error	63	0.0001784	0.0000028			
<b>A discrete heart beat</b>						
Population	3	0.038403	0.012801	2.23	0.122	ns
Egg Mass	17	0.097698	0.005747	3.67	≤ 0.001	**
Error	63	0.098787	0.001568			
<b>Capsule rupture</b>						
Population	3	0.40546	0.13515	1.53	0.242	ns
Egg Mass	17	1.49737	0.08808	3.24	≤ 0.001	**
Error	63	1.71081	0.02716			
<b>Hatching</b>						
Population	3	0.37815	0.12605	1.49	0.253	ns
Egg Mass	17	1.43682	0.08452	3.14	≤ 0.001	**
Error	63	1.69404	0.02689			

**Table 3.1.** Results of ANOVA testing for differences in the relative time of onset of key embryonic developmental events between populations and egg masses in the pond snail, *Radix balthica*. <sup>ns</sup> –  $p > 0.05$ ; \* -  $p < 0.05$ ; \*\* -  $p \leq 0.001$ .



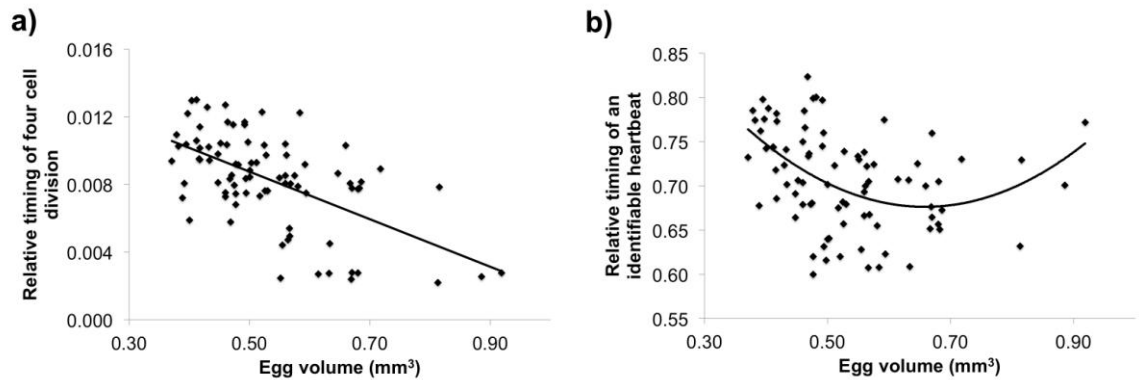
**Figure 3.1** Variance in egg volume (%) and the relative time of onset of developmental events partitioned at the population, egg mass and individual level in the pond snail, *Radix balthica*. Percentages are calculated both with and without egg volume included as a covariate.

<b>Factor</b>	<b>df</b>	<b>SS</b>	<b>MS</b>	<b>F</b>	<b>P</b>	
<b>Four cell division</b>						
Egg volume	1	0.0000002	0.0000002	0.08	0.7785	ns
Population	3	0.0000870	0.0000290	3.24	0.046	*
Egg Mass	17	0.00001698	0.0000100	3.48	≤ 0.001	**
Error	62	0.0001781	0.0000029			
<b>A discrete heart beat</b>						
Egg volume	1	0.001846	0.001846	1.18	0.281	ns
Population	3	0.038546	0.012849	2.57	0.085	ns
Egg Mass	17	0.094898	0.005582	3.57	≤ 0.001	**
Error	62	0.096941	0.001564			
<b>Capsule rupture</b>						
Egg volume	1	0.00313	0.00313	0.11	0.737	ns
Population	3	0.22627	0.07542	1.14	0.357	ns
Egg Mass	17	1.23417	0.07260	2.64	0.003	*
Error	62	1.70767	0.02754			
<b>Hatching</b>						
Egg volume	1	0.00223	0.00223	0.08	0.776	ns
Population	3	0.20813	0.06938	1.10	0.375	ns
Egg Mass	17	1.18079	0.06946	2.55	0.004	*
Error	62	1.69181	0.02729			

**Table 3.2** Results of an ANCOVA testing for differences in the absolute time of onset of key embryonic developmental events between populations and egg masses in the pond snail *Radix balthica*, with egg volume as a covariate. <sup>ns</sup> –  $p > 0.05$ ; \* -  $p < 0.05$ ; \*\* -  $p \leq 0.001$ .

The partitioning of variance at the population level decreased as embryonic development progressed (i.e. four cell division > heart beat > capsule rupture > radula > hatching) whereas the partitioning of variance at the individual level increased (Fig. 3.1). The relative timing of four cell division, the earliest developmental event analysed here, was significantly different between both populations and egg masses, whereas the timing of an identifiable heart beat,

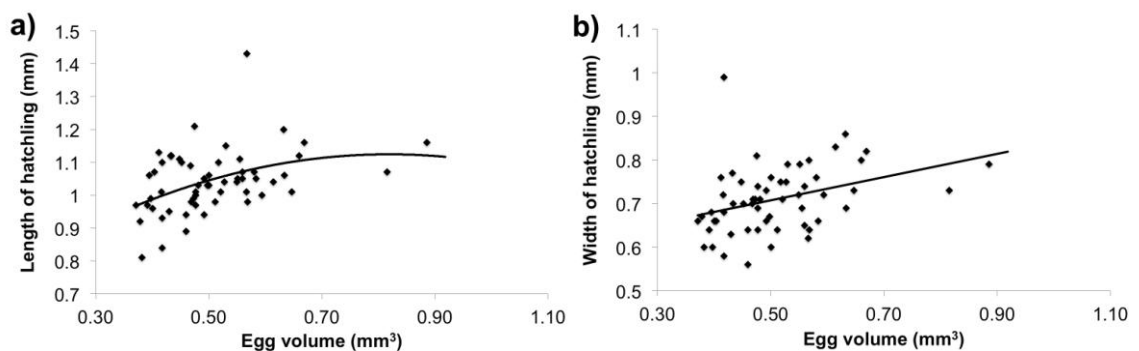
capsule rupture and hatching were significantly different between egg masses, but not populations (Tables 3.1 and 3.2).



**Figure 3.2 (a)** Regression analysis of the effect of egg volume on the relative timing of four cell division ( $R^2 = 33.6$ ,  $F_{1, 82} = 42.94$ ,  $P \leq 0.001$ :  $y = 0.16 - 0.14 x$ ) and **(b)** a discrete heart beat ( $R^2 = 19.0$ ,  $F_{2, 81} = 10.7$ ,  $P \leq 0.001$ :  $y = 0.78 - 0.13 x$ ) in the pond snail, *Radix balthica*. Models are those of best fit.

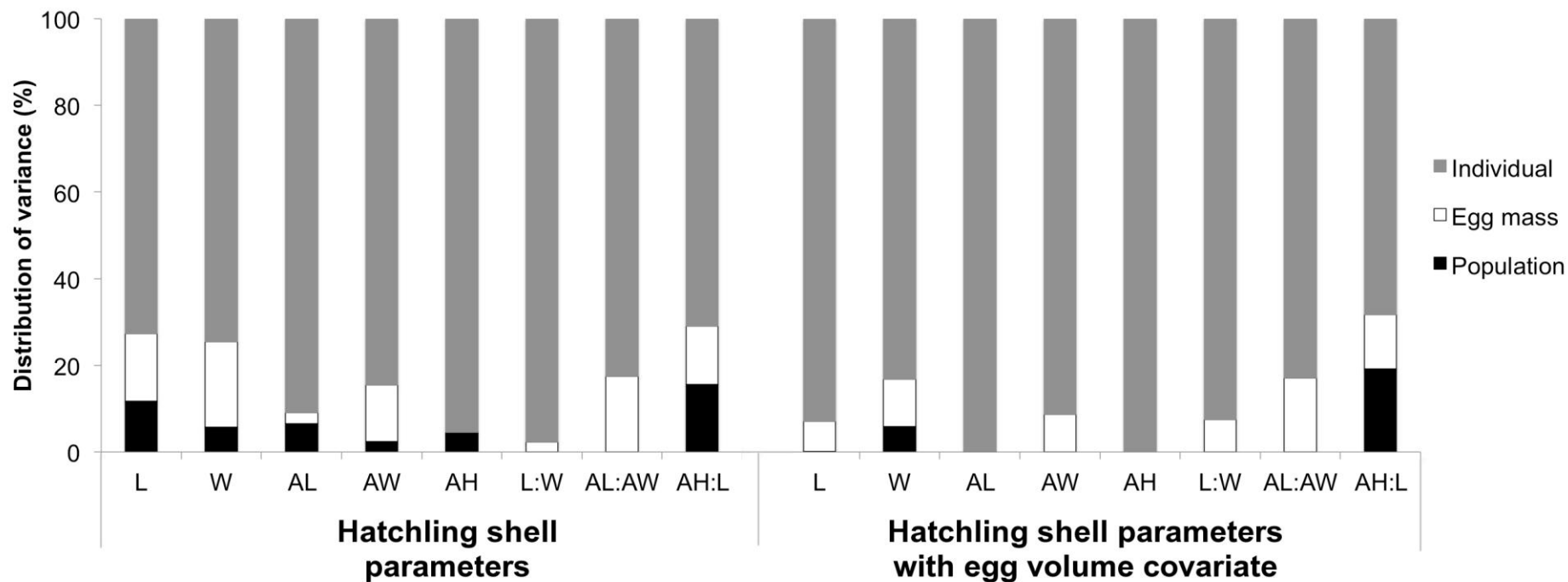
### 3.4.3 Hatchling size and shape

Most variation in size and shape parameters was partitioned at the individual level, both with (68% - 100%, Fig. 3.4) and without (71% - 98%, Fig. 3.4) egg volume as a covariate. There were no significant differences between either populations or egg masses in any of the size and shape parameters measured.



**Figure 3.3** Regression analysis of the effect of egg volume on the length ( $R^2 = 14.9$ ,  $F_{1, 54} = 5.9$ ,  $P = 0.005$ :  $y = 0.6 + 1.28 x - 0.78 x^2$ ) and width ( $R^2 = 11.0$ ,  $F_{1, 55} = 7.9$ ,  $P = 0.007$ :  $y = 0.57 + 0.27 x$ ) of hatchlings, in the pond snail, *Radix balthica*. Models were those of best fit.

Egg volume was a significant predictor of both length and width of hatchling snails (Fig. 3.3). This relationship appears to have been driven by both a prolonged overall duration of embryonic development in larger eggs and a prolonged relative period of development between the radula becoming functional and hatching.



**Figure 3.4** Variance (%) in hatchling shell parameters (L – length, W – width, AL – aperture length, AW – aperture width, AH – aperture height, L:W [aspect ratio], AL: [aperture ratio], AH:L – ratio of aperture height to length) partitioned at the levels of population, egg mass and individual level in the pond snail, *Radix balthica*. Percentages are calculated both with and without egg volume included as a covariate.



### 3.5 Discussion

In this chapter I investigated variance partitioning in the relative timing of four key embryonic traits, plus egg volume and hatchling size and shape, between the levels of population egg mass and individual in *R. balthica*. Variance partitioning in the timing of all embryonic developmental events occurring after the four cell division stage was greater at the individual level (52 – 61%) than at either egg mass (36 – 34%) or population levels (12 – 5%) (Fig. 3.1). Egg volume had most variance (72%) partitioned at the egg mass level and was also a predictor of the relative timing of four cell division and first heart beat (Fig. 3.2) and the size of hatchlings (Fig. 3.3). Egg volume when included as a covariate in the analysis decreased variance partitioning in developmental traits at both population and egg mass levels whereas hatchling size and shape parameters had most variance partitioned at the individual level regardless of whether egg volume was included as a covariate (Figs. 3.1 and 3.4).

An assumption in many studies of heterochrony has been that intraspecific variation in developmental event timing is both low and inappropriate for addressing questions relating to this macro-evolutionary pattern. Here, I show not just the prevalence of intraspecific variation in developmental event timing, but that the partitioning of this variation at different hierarchical levels (greater inter-individual than population variance partitioning) is not as might have been predicted and provides some insight into the factors that could contribute to producing such variance. The predominance of individual level variation in the traits studied here could either have a genetic basis or be attributable to variation in environmental conditions during development (Spicer and Gaston 1999). In Chapter 2 I studied variation in embryonic developmental event timing

within a population of *R. balthica* and demonstrated a positive inter-individual relationship between developmental timing dissimilarity and genetic distance (Tills et al. 2011). This previous study suggests there may be a genetic basis for inter-individual differences in embryonic developmental event timing and therefore presents the possibility that some portion of the variance partitioned at the individual level in the current study might result from genetic differences. Chapter 2 also showed that embryos from within a single egg mass were no more closely related to each other than to embryos from other egg masses within their population. A proposed explanation for this pattern of relatedness is that *R. balthica* is a mixed mating, simultaneous hermaphrodite. Thus a single egg mass can contain eggs produced using sperm from more than one father (Jarne and Delay 1990). If some portion of the variance uncovered here has a genetic component, previous evidence of an absence of genetic differentiation between egg masses in a *R. balthica* population may explain why there is not more partitioning of this variance between egg masses.

*Radix balthica* embryos have been shown to exhibit plasticity in the timing of developmental events in response to both biotic (Rundle et al. 2011) and abiotic (Tills et al. 2010 – Appendix 2) stressors. No changes were intentionally made to the environmental conditions in which either parents or embryos were cultured. However differences in egg volume may have contributed to some of the variance recorded. When egg volume was included as a covariate in the ANOVA model, variance in developmental event timing at the egg mass level decreased, suggesting that egg volume was driving some of the variance partitioning at the egg mass level. For example, the distribution of variance in the relative timing of four cell division was strongly influenced by egg volume.

Without egg volume as a covariate, four cell division had most variance at the egg mass level (39%). When egg volume was included as a covariate, however, most variance was partitioned at the individual level (48%). Here, embryos were harvested from F2 stock populations in which variation in maternal age might exist, and this could have contributed both to variation in egg volume between egg masses and subsequent variation in embryonic developmental traits. Egg size has been shown to be affected by factors including: maternal age (both positive [Bingham et al. 2004; Chester 1996; George 1994] and negative [Ito 1997; Qian and Chia 1992] relationships), nutrition (Bertram and Strathmann 1998) and exposure to toxicants (Lardies et al. 2008). Egg size has also been observed to have effects on offspring development (discussed in Ho and Burggren 2010).

The potential influence of maternal provisioning was evident in the fact that egg volume was a significant predictor of both length and width of hatchling snails with larger snails hatching from larger eggs (Fig. 3.3). Despite significant relationships between egg volume and hatchling size, and differences in egg volume between egg masses, variation in hatchling length and width was partitioned mostly at the individual level (length – 73%, width – 74%) with little variation being explained by egg mass (length – 15%, width – 20%), or population (length – 12%, width – 6%). This suggests that either: (i) egg volume may be a poor indicator of maternal investment; (ii) hatchlings do not make complete use of maternal investment; or (iii) that hatchling size is not a complete indicator of the maternal investment to the offspring occurring during its embryonic development. Baur (1994) showed that eggs of the land snail *Arianta arbustorum* had less variable nutrient content than egg volume and that

both nutrient content (nitrogen and carbon) and egg volume were more variable between, than within, egg masses. Baur (1994) also reported that only 61% of egg masses contained eggs with levels of nitrogen correlated with their volume. Here, while egg volume can be used as an indicator of maternal investment, it is unlikely to provide a complete measure. Egg size has also been shown to be heritable in the serpulid polychaete, *Hydroides elegans*, (Miles et al. 2007) in response to artificial selection, using a half-sib breeding analysis. In the current study egg volume affected both the size of hatchlings and the timing of several developmental events and, therefore, if some component of egg volume has a genetic component this may have important developmental and ecological consequences.

The least variation in all of the developmental events measured here was partitioned at the population level despite the use of populations from the extremes of *R. balthica*'s geographical range (North Sweden – South France) (Pfenninger et al. 2011). This is surprising because if a genetic basis for developmental differences can arise within a single population (Tills et al. 2011 – Appendix 3), it might be expected that such differences would also be evident between populations and therefore that a significant portion of variation would be distributed at this level. However, Pfenninger et al. (2011) demonstrated across *R. balthica*'s range that there was no significant relationship between the geographical distance between populations and their genetic distance. A key dispersal mechanism for *R. balthica* is believed to be transport via waterfowl and therefore the magnitude of dispersal can be fairly independent of distance. Pfenninger et al. (2011) attributed the absence of a relationship between geographical and genetic distance to the genetic variability which results from

bird mediated transport. Although the findings of Pfenninger et al. (2011) help to explain why variation at the population level might be lower than otherwise expected, it is still surprising given the results of Tills et al. 2011 which demonstrated developmental timing differences related to genetic distance within a single population (from a 10 m stretch of shore) that there was not more variance partitioning between populations.

Variance at the level of population was greatest early in development (i.e. in the timing of four cell division) and subsequently decreased as development progressed. Differences between populations in the timing of early development, which are subsequently replaced by variation mainly at the individual level in later development, is interesting as it suggests some mechanism by which inter-individual variation increases as development progresses.

De Jong et al. (2009) studied 82 developmental characters within *Haplochromis piceatus*, a species of Lake Victoria cichlid using Ontogenetic Sequence Analysis (Colbert and Rowe 2008). Data from 261 embryos revealed 26,880 equally parsimonious developmental sequences, which is an incredible complexity of intraspecific developmental variation. Here we employed relatively few embryonic developmental events. Whilst studying more traits may have revealed different patterns of variance distribution between groups of traits, the prevalence of variation in the traits we did use being largely partitioned at the individual level raises important questions regarding variance partitioning in developmental timing and what this may mean for the link between intraspecific variance in developmental timing and heterochrony.

Intraspecific variation in developmental event timing is a possible source of heterochrony. Hence, understanding the distribution of this intraspecific variance (both hierarchically and geographically) is of paramount importance to understanding the ecology and evolution of event timing, and ultimately to understanding how heterochronies occur. Inter-individual variation in embryonic developmental timing appears to not only be prevalent in this species and to have a genetic basis, but to still be the predominant level of variation, even when studying populations from across the species' geographical range. The implications of the variance partitioning reported here on the relationship between developmental event timing at the inter- and intra-specific levels is unclear, but highlights the need for us to address how variation in developmental event timing between these two levels is related.

Chapters 2 and 3 have focussed on altered event timing within an ecological context. Owing to the ecology of *R. balthica* these studies have revealed some unexpected distributions of variation in altered timing – a genetic underpinning to developmental differences within a population (Chapter 2) and inter-individual differences being predominant when considering populations spanning *R. balthica*'s range. In Chapter 4, I move on, from the ecological context of the previous two Chapters, to using a laboratory breeding study to compare the degree of cross-generational similarity in event timing.

## CHAPTER 4

### PARENT-OFFSPRING SIMILARITY IN THE TIMING OF DEVELOPMENTAL EVENTS COULD PROVIDE A MISSING LINK BETWEEN ONTOGENY AND PHYLOGENY

#### 4.1 Summary

Understanding the link between ontogeny (development) and phylogeny (evolution) remains one of the key aims of Biology. Heterochrony, the altered timing of developmental events between ancestors and descendants, could provide such a link, although the processes responsible for this macro-evolutionary pattern are still unclear. A candidate process is that intraspecific variation in developmental event timing provides the raw material from which heterochronies originate and evidence for such variation is growing. In Chapter 2 I showed that, in the pond snail, *Radix balthica*, variation in embryonic developmental event timing has a genetic basis. However, in order for intraspecific differences in developmental event timing to be the source of heterochronies, this variation must also be heritable. Consequently, I used high resolution (temporal and spatial) imaging of the entire embryonic development of *R. balthica*, to perform a parent-offspring comparison of the timing of a suite of twelve, physiological and morphological, developmental events. I show that between-parent differences in the timing of all twelve embryonic developmental events investigated are good predictors of such differences between their

offspring, and heritability was demonstrated for two of these developmental events (foot attachment and crawling). Such heritable intraspecific variation in developmental event timing could provide the raw material for speciation events and, therefore provide a fundamental link between ontogeny and phylogeny, *via* heterochrony.

## **4.2 Introduction**

How ontogeny (development) and phylogeny (evolution) are linked has been, and remains, one of the key questions in biology. In fact, heterochrony, the altered timing of developmental events between ancestors and descendants, has been suggested as the main driver of evolutionary change (deBeer 1958; Gould 1977). This suggestion has been re-enforced by the fact that heterochrony has been documented as an evolutionary pattern in several animal groups and for a diverse array of morphological (McNamara 1995; Raff 1996; Ji et al. 2009; Bhuller et al. 2012) and physiological (Spicer 2006; Spicer et al. 2011 – Appendix 1) traits. Despite the pervasiveness of heterochrony as a macroevolutionary pattern, however, we still know relatively little about the processes through which this pattern forms. An obvious candidate process is that intraspecific variation in developmental event timing provides raw material on which selection, either during or post development, could act. However, studies of heterochrony have focused on between-species comparisons, with an assumption that intraspecific developmental event timing is largely invariant or insignificant, despite variation being required for evolutionary change. Consequently we know far less about intraspecific differences in developmental event timing, despite variation at this level being a probable source of answers to questions regarding the formation of heterochronies. However, an increasing



number of studies have reported widespread intraspecific variation in developmental event timing (Cubbage and Mabee 1996; Mabee and Trendler 1996; Mabee et al. 2000; Schmidt and Starck 2004; Sheil and Greenbaum 2005; de Jong et al. 2009; Kawajiri et al. 2009). For example, de Jong (2009) showed, using 82 developmental characters in 261 embryos of the cichlid, *Haplochromis piceatus*, that embryos could follow 26,680 different developmental sequences (de Jong et al. 2009). This magnitude (and complexity) of intraspecific variation is astounding and demonstrates that intraspecific developmental timing is far from invariant.

It is clear that intraspecific variation in developmental event timing exists, however, its source and relationship to between-species differences in developmental event timing, are not. For intraspecific variation in developmental event timing to be the raw material from which heterochronies arise, requires it to both have a genetic basis and to be heritable. Chapter 2 revealed greater inter-individual magnitudes of difference in developmental timing with increasing genetic distance, indicating the presence of a genetic basis for intraspecific variation in developmental timing, but did not extend to investigating heritability (Tills et al. 2011 – Appendix 3).

Consequently, in this chapter I used *R. balthica* to perform a direct parent-offspring comparison to investigate whether cross-generational similarity in developmental event timing was evident, and whether this similarity extends to the level of heritability. Parents were first harvested, as embryos, from a stock population and their development was recorded. Once hatched, these snails were cultured in isolation through to reproduction and, because *R. balthica* is a

simultaneous hermaphrodite, offspring were produced sexually via selfing (Jarne and Delay 1990; Coutellec-Vreto et al. 1997). The embryonic development of these offspring were then recorded in the same manner as for their parents. A limitation of direct parent-offspring comparison is the inability to control for maternal effects (Falconer and Mackay 1990) and therefore here I used egg size as a measure of maternal investment (Sinervo 1990; Bernado 1996; Rutkowska and Cichoń 2002) and incorporated this measure into analyses to estimate its contribution to the observed relationships and to investigate relationships with its effect removed.

### **4.3 Materials and Methods**

#### 4.3.1 Animal culture

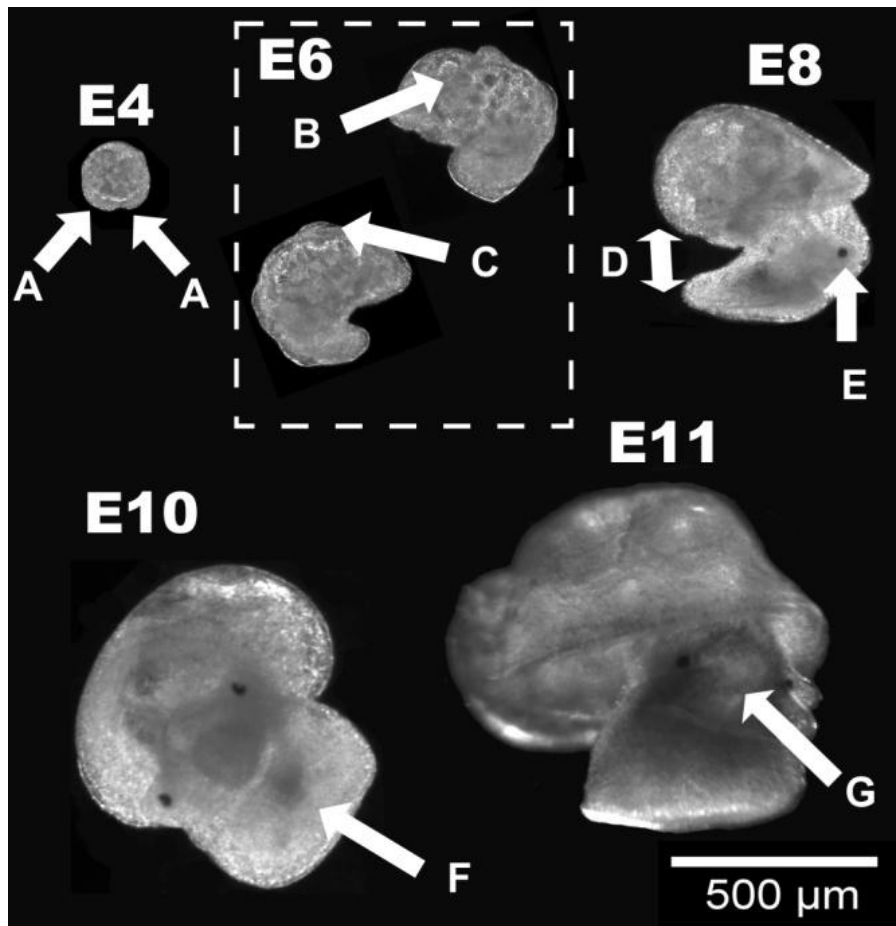
To begin the parent-offspring comparison, eggs were harvested from an F2 laboratory stock population of *Radix balthica* originating from the River Dart in Totnes, Devon, UK (50°26'19'N, 3°41'24'W). Eggs were examined under low power magnification (x 10) and those which had not developed past the two cell division stage were dissected from their egg masses and placed individually within a multiwell plate (384 well plate, vol. per well = 70 µl) containing Artificial Pond Water (APW; Rundle et al. 2004). This multiwell plate was placed into a custom system, designed for time lapse inverted imaging of aquatic embryos (Sect 1.7.1), housed in a 20°C controlled temperature facility. Water changes were performed every two days and each well was checked daily to identify hatchlings.

Hatchlings were cultured in glass jars (vol. = 40 ml) containing APW (vol. = 35 ml) and a 5 cm length of *Elodea densa*. Jars were placed on shelving positioned

in a west facing window (in locations chosen at random and moved every 21 days) in a 15°C controlled temperature facility. Snails were provided with washed lettuce discs (diam. = 4 mm) every two weeks. Mortality in the three months following hatching was 50%, reducing the number of snails cultured from 28 to 14. Three months after hatching, snails were transferred to larger jars (vol. = 500 ml) containing APW (vol. = 450 ml) and two 15 cm lengths of *Elodea*. Water was changed in these jars every 21 days and snails were fed discs of washed lettuce (diam. = 10 mm) every 14 days. When snails reached maturity, jars were checked regularly for eggs and if present these were examined under low power and if embryos had not developed past the 2 cell division stage their entire embryonic development was imaged using the same method as for their parents' embryonic development. Ten out of the fourteen snails that grew to a mature size reached sexual maturity and produced viable eggs. However, one of these snails only produced a single egg and therefore for statistical reasons its developmental data are not used here. The nine remaining snails produced between 3 and 19 viable embryos.

#### 4.3.2 Image acquisition

A 150 frame image sequence of each embryo was acquired (at 7.5 frames per second) every 2 h until hatching, using a custom built bio-imaging system (Sect. 1.7.1). Briefly, this imaging system comprises an aluminum frame housing an XY motorised stage (Prior Optiscan) mounted above a 4 megapixel monochrome camera (Allied Vision Technology Pike 421 B) connected to a zooming lens (Keyence VHZ20R) with illumination provided by a light emitting diode (LED) array. The motorized stage and camera were controlled and synchronized using Micromanager 1.3 (Edelstein et al. 2010) run on a Mac Pro.



**Figure 4.1** *Radix balthica* at different embryonic developmental stages (E4, E6, E8, E10, E11 (based on Cumin 1972) illustrating some of the developmental events used here. A – velar lobes are visible on either side of embryo, distinguishing the veliger; B – location of first, discrete heart beat; C – the shell begins forming as a ridge on top of mantle; D – body flexing occurs, during which the mantle is brought closer to foot and *vice versa*; E – eye spots form on either side of the head; F – snail attaches to the egg wall using its foot and begins crawling; G –radula movement detectable.

#### 4.3.3 Image analysis

The time of onset, from four-cell division, of twelve developmental events (Table 4.1) was determined by manual observation of the image sequences recorded for both parental and offspring embryonic development. Egg length and width was measured from the images at the time of four-cell division and from these values egg volume was calculated and incorporated into our analysis to

investigate the contribution of egg volume to observed relationships (Falcolner and Mackay 1990).

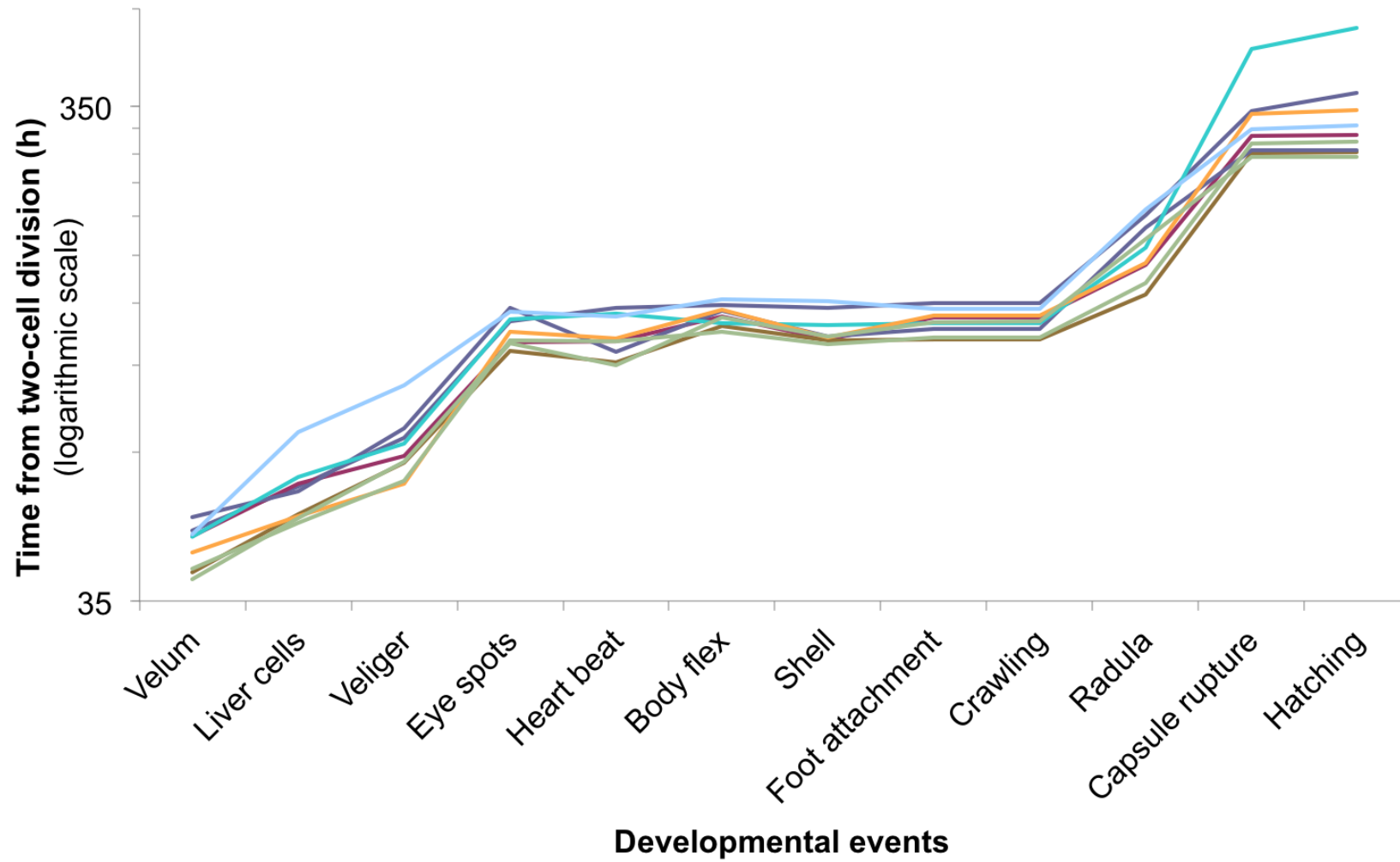
Developmental event	Description of event	Mean event timing (from four cell division) $\pm$ 1 S.E. (h)
Four cell division	Four individual cells visible as a cross.	
Velum	Opaque appearance of the embryo is broken by a semi-transparent, helmet-like velum that extends further outwards than the rest of the embryo at this stage.	43 $\pm$ 0.72
Liver cells	Liver cells visible as individual globular structures forming a cuplike layer running around the endodermal lumen.	55.4 $\pm$ 1.2
Veliger	Two lobes at either end of the embryo have developed and extend to transform the embryo into a "crescent moon" like shape.	68.26 $\pm$ 1.5
Eye spots	Onset of eye spot pigmentation.	119.9 $\pm$ 1.4
Discrete heart beat	First visible heart beat. This begins as an irregular beat but as development progresses becomes more regular.	115.4 $\pm$ 1.8
Body flexing	First muscular contraction causing the body of the embryo to flex resulting in the mantle and foot moving closer together. This movement is similar to that seen in adult snails when they retract their shell.	130.2 $\pm$ 1.2
Shell formation	Front edge of the shell becomes visible as a ridge extending around the mantle and this later in development extends down towards the head of the embryo.	121.1 $\pm$ 1.7
Foot attachment	Embryo uses muscular foot to attach to egg capsule wall, often only for very short periods before reverting back to gliding around the egg using cilia.	126.9 $\pm$ 1.4
Crawling	Embryo uses its muscular foot after attachment to the egg capsule wall to crawl. Crawling is initially usually for only very short periods.	127.1 $\pm$ 1.5
Use of radula	Radula begins functioning and its movement is visible within the embryo before it is extended outside of the head to use for rasping against the egg capsule wall.	173.9 $\pm$ 4
Capsule rupture	Embryo uses radula to rupture the egg capsule wall.	307.9 $\pm$ 9.4
Hatch	Snail emerges from egg capsule.	314.3 $\pm$ 10.97

**Table 4.1.** Descriptions of the embryonic developmental events used (based on Cumin 1972; Smirthwaite et al. 1997).

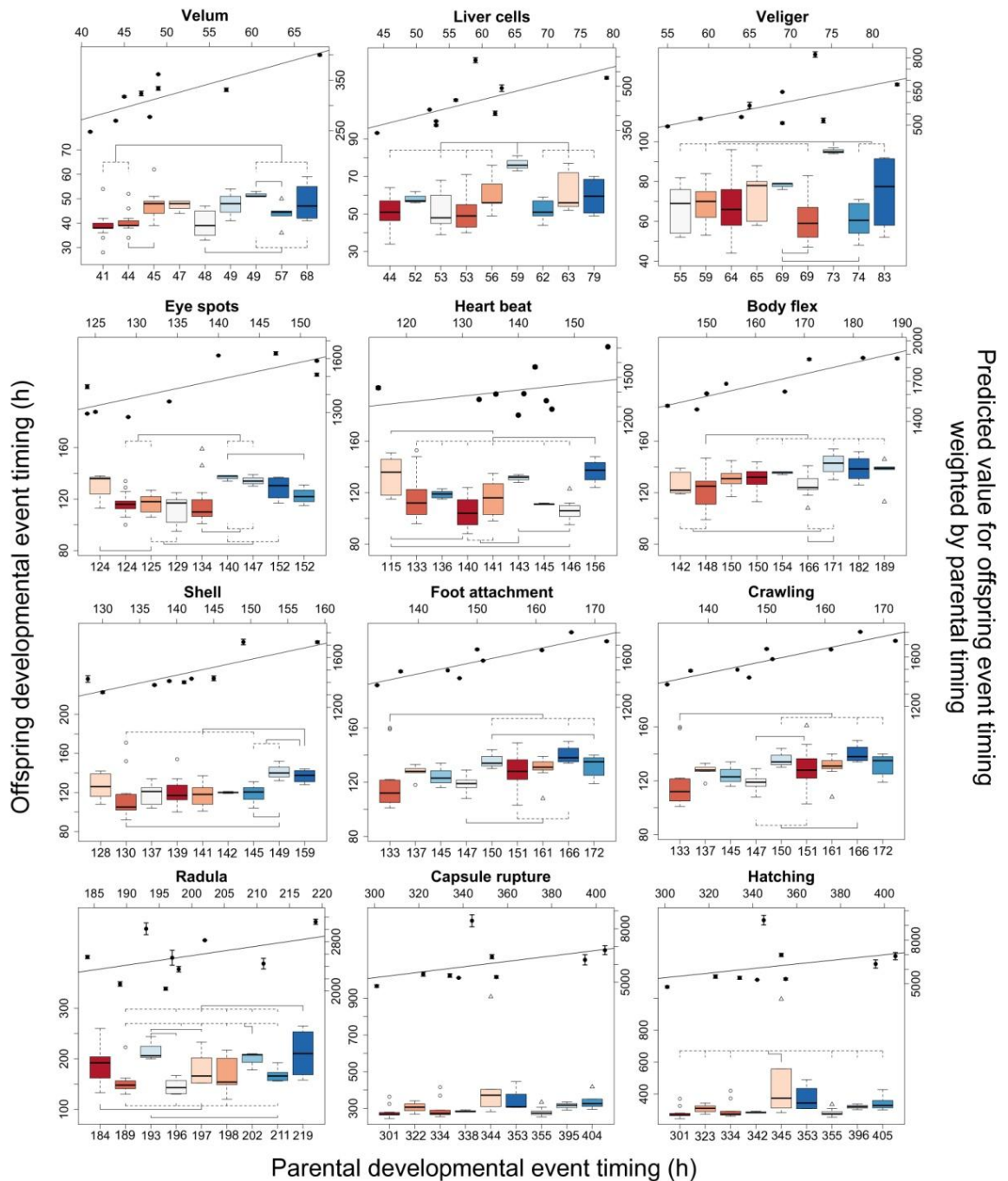
#### 4.4 Results

There was significant variation in the timing of all twelve developmental events used here (Fig. 4.2). To test for differences in developmental event timing between the offspring from different parents and to determine if there was a relationship between parents and their offspring, I performed an ANOVA with

parents weighted by their own event timing and egg volume included as a covariate (Table 4.2). The timings of all developmental events, with the exception of capsule rupture, were significantly different between the offspring from different parents. Predicted values for offspring timing of each developmental event, produced by the ANOVA, were related positively with parental timing, demonstrating parent-offspring similarity in the timing of all twelve of the developmental events used here (Fig. 4.1).



**Figure 4.2.** Mean developmental event timings for embryos produced from different parents.



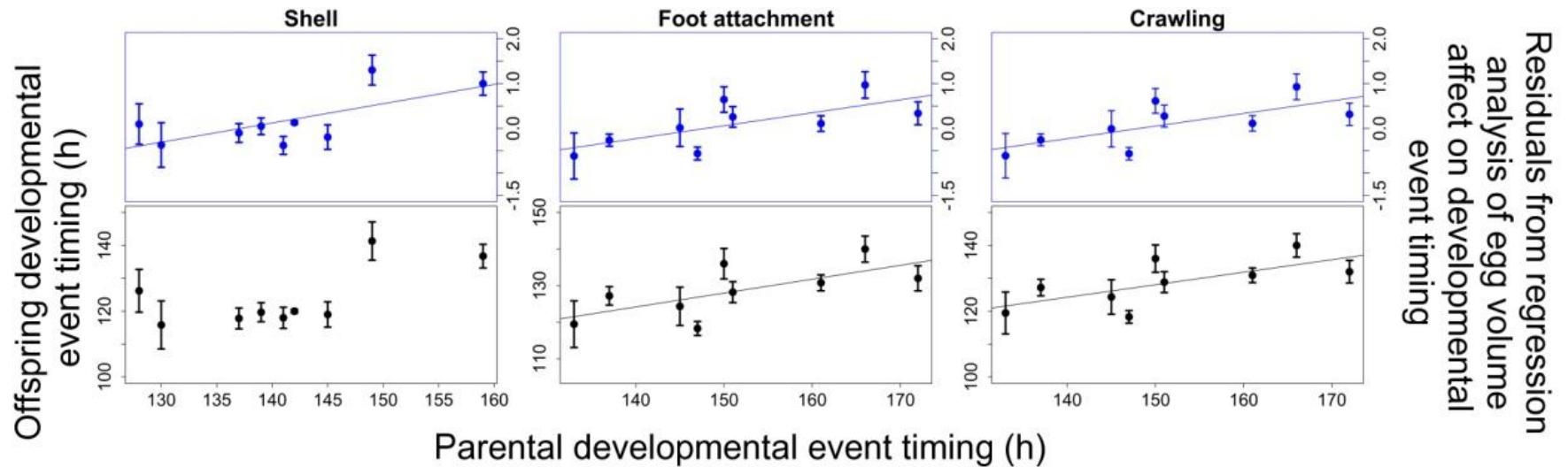
**Figure 4.3** Boxplots showing developmental event timing for offspring produced from different parents (indicated by different colours) arranged in increasing order of their event timing. Significant differences in developmental event timing between offspring produced from different parents are indicated by horizontal bars above box plots (determined by ANOVA between parents weighted with parental developmental timing using weighted least squares and with egg volume included as a covariate). Plots of developmental event timing (mean  $\pm$  1 S.E.) for offspring produced from different parents, predicted from the ANOVA model, are shown for each developmental event above the box plots.



Factor	df	SS	MS	F	P	
<b>Velum</b>						
Corrected model	9	61243.9	16804.9	5.45	≤ 0.001	***
Parent	8	37860.7	4732.6	3.79	0.001	***
Egg volume	1	1950.4	1950.4	1.56	0.216	ns
Error	65	81213	1249.4			
<b>Liver cells</b>						
Corrected model	9	167265.1	18585	4.63	≤ 0.001	***
Parent	8	107681.4	13460.2	3.79	0.003	**
Egg volume	1	10247.8	10247.8	1.56	0.115	ns
Error	65	261157.5	4017.8			
<b>Veliger</b>						
Corrected model	9	287564	31951.6	3.35	0.002	**
Parent	8	271444.4	33930.6	3.56	0.002	**
Egg volume	1	19261.9	19261.9	2.02	0.160	ns
Error	65	619614.4	9532.5			
<b>Eye spots</b>						
Corrected model	9	478403.1	53155.9	3.65	0.001	***
Parent	8	432102.8	54012.8	3.71	0.001	***
Egg volume	1	11627.3	11627.3	2.02	0.799	ns
Error	65	946437.1	14560.6			
<b>Discrete heart beat</b>						
Corrected model	9	1037396.2	115266.2	5.48	≤ 0.001	***
Parent	8	525374.6	65671.8	3.12	0.005	*
Egg volume	1	16229.6	16229.6	0.772	0.383	ns
Error	65	1366857.8	21028.6			
<b>Body flex</b>						
Corrected model	9	328270.9	36474.5	2.25	0.03	*
Parent	8	319486.9	39935.9	2.46	0.022	*
Egg volume	1	6374.3	6374.3	0.393	0.533	ns
Error	65	1055521.7	16238.8			
<b>Shell formation</b>						
Corrected model	9	507075.2	56341.7	2.16	0.037	*
Parent	8	441685.8	55210.7	2.11	0.047	*
Egg volume	1	68642.2	68642.2	2.63	0.110	ns
Error	65	1697490.3	26115.2			
<b>Foot attachment</b>						
Corrected model	9	421915.4	46879.5	2.28	0.027	*
Parent	8	358474.9	44809.4	2.18	0.04	*
Egg volume	1	4747.4	4747.4	0.231	0.632	ns
Error	65	1335904.7	20552.4			
<b>Crawling</b>						
Corrected model	9	428375.7	47597.3	2.15	0.037	*
Parent	8	370787.7	46348.5	2.1	0.049	*
Egg volume	1	4745.7	4745.7	0.215	0.645	ns
Error	65	1437098.1	22109.2			
<b>Radula</b>						
Corrected model	9	7437847.4	826427.5	5.41	≤ 0.001	***
Parent	8	7183402	897925.3	5.89	≤ 0.001	***
Egg volume	1	1339976.2	1339976.2	8.78	0.004	**
Error	65	9918989.2	152599.8			
<b>Capsule rupture</b>						
Corrected model	9	61794033.4	6866003.7	4.18	≤ 0.001	***
Parent	8	25015605.1	3126950.6	1.9	0.074	ns
Egg volume	1	10384373	10384373	6.32	0.014	*
Error	65	106700000	1642215.2			
<b>Hatching</b>						
Corrected model	9	93306401	10367377.9	4.87	≤ 0.001	***
Parent	8	44064875.8	5508109.5	2.59	0.016	*
Egg volume	1	10060779.1	10060779.1	4.72	0.033	*
Error	65	138500000	2130282			

**Table 4.2.** Results of an ANOVA testing for differences in the timing of developmental events (Table 4.1) between offspring produced from different parents with parents weighted by their own developmental timing using weighted least squares and egg volume included as a covariate. \*\*\* -  $\leq 0.001$ , \*\* -  $\leq 0.005$ , \* -  $\leq 0.05$ , <sup>ns</sup> – not significant.

Significant heritabilities of event timings, calculated by regressing the average timing of each event in offspring on that in their parents (Falconer and Mackay 1990), were found for foot attachment ( $h^2 = 0.380$ ) and crawling ( $h^2 = 0.381$ ) (Fig. 3). An obvious limitation of direct parent-offspring comparisons is the inability to control for maternal effects. To assess if relationships between parents and their offspring in the timing of foot attachment and crawling were being driven by differences in egg volume, I performed the same analysis, but replaced means for offspring developmental event timing with means of the residuals from regressions testing for the effect of egg volume on the timing of these developmental events. This procedure effectively removes any effect of egg volume on offspring developmental timing. This analysis resulted in no significant change to the relationships between parents and their offspring in the timing of foot attachment and crawling, and again demonstrated heritability for these events, indicating that egg volume was not a driver of these relationships. In fact, the relationship between the timing of shell formation in parents and their offspring was only significant when the effect of egg volume was removed, which suggests that egg volume was actually masking this relationship (Table 4.1).



**Figure 4.4** Comparison of parental event timing with; (i) mean ( $\pm 1$  S.E.) offspring timing, to test for heritability (Foot attachment -  $R^2 = 0.47$ ,  $F_{1,7} = 6.28$ ,  $P = 0.041$ ; regression coefficient ( $h^2$ ) = 0.380. Crawling -  $R^2 = 0.44$ ,  $F_{1,7} = 6.22$ ,  $P = 0.041$ ; regression coefficient ( $h^2$ ) = 0.381), (ii) mean ( $\pm 1$  S.E.) offspring residuals, from a regression analysis testing the effect of egg volume on event timing (blue plots in top panel), to examine whether relationships which indicate heritability are still present with the effect of egg volume on developmental timing removed (Shell (residuals) -  $R^2 = 0.39$ ,  $F_{1,7} = 6.28$ ,  $P = 0.041$ ; regression coefficient = 0.043. Foot attachment (residuals) -  $R^2 = 0.43$ ,  $F_{1,7} = 7.06$ ,  $P = 0.033$ ; regression coefficient = 0.028. Crawling (residuals) -  $R^2 = 0.43$ ,  $F_{1,7} = 7.08$ ,  $P = 0.032$ ; regression coefficient = 0.029).

## 4.5 Discussion

In embryonic *Radix balthica* there was a positive relationship in the timing of a suite of twelve physiological and morphological developmental events between parents and their offspring. Further, heritability was evident in the timing of foot attachment and crawling in this comparison of parental and offspring developmental event timing. A limitation of direct parent-offspring comparisons is the inability to control for maternal effects. However, even when I factored egg volume into these analyses there remained a detectable parent-offspring similarity.

Smirthwaite et al. (2007) found that the timing of events including, eye spot formation, heart ontogeny, body flexing, foot attachment and crawling had all exhibited heterochrony within the group of pond snails, to which *R. balthica* belongs. Here I find the timing of many of the same developmental events exhibit significant parent-offspring similarity (Smirthwaite et al. 2007) and for two of these events (foot attachment and crawling) this similarity extends to heritability. For the events other than foot attachment and crawling the evidence of parent-offspring is consistent with the timing of these events also being heritable in the presence of more statistical power (i.e. larger numbers of embryos per parent). The parent-offspring similarity reported here is further supported by previous work with *R. balthica* embryos freshly collected from the field which revealed increasing inter-individual differences in developmental timing with increasing genetic distance, suggesting that there could be a genetic component to this inter-individual variation (Chapter 2; Tills et al. 2011 – Appendix 3). Our findings substantiate the idea that intraspecific and even inter-

individual, differences in developmental event timing can be the raw material from which heterochronies arise.

I have shown that within a single generation similarities in developmental timing between parents and their offspring are present and heritable and therefore the notion of heterochrony as a macroevolutionary pattern is too narrow. Historically, agreeing on a definition of heterochrony has been controversial (Spicer and Rundle 2006); however “altered developmental timing between ancestors and their descendants” is now a widely accepted usage of the term (Spicer et al. 2011 – Appendix 1). Thus, the findings presented here show that altered timing between ancestors and descendants (heterochrony) is not only interspecific, but can also be observed at the intraspecific level and therefore this is a fundamental link between ontogeny and phylogeny, but what do these heritable timing differences mean at the organismal level?

Outcomes of altered timing were categorised by Richardson et al. (2009), in a review of vertebrate limb development, and they categorised these as (i) functional changes in the adult; (ii) functional changes in the embryo (without change in the adult); and (iii) changes not related to selection for an adaptive trait. These authors warn of the danger of creating adaptive scenarios for heterochrony based on examples of timing shifts that seem to correlate with either changes to development or environment.

I found significant variation in the timing of twelve developmental events, including functional features such as an identifiable heartbeat, foot attachment, crawling and radula activity. Identifying whether altered timing of these events translates to difference in function in either the embryo or adult will be an

important future step in revealing how selection might act on variation in the timing of developmental events. In the study that documented heterochrony in this group of pond snails, Smirthwaite et al. (2007) discussed that in the two physid species they studied, foot attachment and crawling occurred early, relative to other species (Smirthwaite et al. 2007). Work using adult physids has revealed this group have a pronounced ability to use crawling as an escape response from predation (Rundle and Brönmark 2001). Early crawling in embryonic physids could perhaps therefore lead to improved crawling function in adults, scenario (i) described by Richardson et al. (2009). Work with embryonic *Lymnaea stagnalis*, another species of snail from this group, revealed increased spinning frequency in response to hypoxia (Byrne et al. 2009). These authors suggest this increased spinning activity improves mixing of capsular fluid thereby maintaining a diffusive gradient for diffusion of oxygen into the egg, providing an adaptation to the embryo for surviving hypoxic environments and this is in line with scenario (ii) outlined by Richardson et al. (2009).

The variation observed here is in a diverse array of developmental events and therefore it is conceivable that altered timing of particular events might result in any of the three functional outcomes outlined by Richardson et al. (2009). Given the potential functional consequences to altered timing described above, it also seems likely that the functional consequences of these events might be very environment-specific. The current study did not investigate environmentally cued altered timing, which has been previously demonstrated in the development of embryonic *R. balthica* (Tills et al. 2010; Rundle et al 2011). A potentially fruitful, future research direction for work investigating intraspecific

altered timing could be to consider the implications of both background and environmentally induced, altered timing and how these contribute to the event timing embryos undergo *in situ*. Research in this direction would allow us to understand heritable heterochrony within an ecological context and therefore begin to grasp the effect of environment on ontogeny and how this then translates to phylogeny.

Whereas Chapters 2-4 have primarily focussed on the time of onset of developmental events, Chapter 5 investigates the ontogeny of function in the cardiovascular system, during the period of embryonic development. In Chapter 5 I use the development of cardiovascular function as a model to investigate the origins, pattern and implications, of variation during ontogeny.

## CHAPTER 5

### ONTOGENY OF CARDIOVASCULAR FUNCTION IN THE POND SNAIL *RADIX BALTHICA* AS A MODEL TO STUDY THE ORIGINS, PATTERNS, AND IMPLICATIONS OF VARIATION WITHIN AND BETWEEN GENERATIONS

#### 5.1 Summary

Our knowledge of ontogeny of invertebrate cardiovascular (CV) function is poor, with most of our understanding deriving from studies on crustaceans. Development of CV function in other groups, including molluscs, has received very little attention. Consequently, using the pond snail *Radix balthica*, I firstly described the 'normal' pattern of CV ontogeny from the appearance of a functional heart to hatching, before going on to explore how variation in this pattern is distributed between populations and egg masses. I then present the results of a parent-offspring comparison of CV development and, finally, investigate how differences in the ontogeny of CV function correlate with various life history characteristics. The first heartbeat commenced between 129 and 159 h after first cell division and increased rapidly over the first 66 h of CV function. However, the ontogeny of CV function then appeared to follow one of two paths: either a steady decrease in HR with time (Trajectory 1) or continued increase, but at a less rapid rate (Trajectory 2). These trajectories occurred with equal frequency and those embryos following Trajectory 1 were significantly larger at hatching than those adopting Trajectory 2, suggesting a fitness cost of the latter developmental path. For embryos following Trajectory 1 during Phase



2, there were significant between-population differences, in both the rate of increase of heart rate during Phase 1 and the rate of decrease during Phase 2. Between egg masses the relative timing of the breakpoint between Phases 1 and 2 of CV development was significantly different. Moreover, in a comparison of parent-offspring CV development the timing of the breakpoint was also a trait exhibiting significant cross-generational similarity. Together these findings suggest intraspecific variation in CV development appears to have both functional and ecological consequences for this species.

## **5.2 Introduction**

### 5.2.1 The development of cardiovascular function as a model for studying variation

The cardiovascular system (CV) is one of the first physiological systems to appear in the developing human embryo (Chen 1996) and has been noted in many early comparative studies, most notably that of Harvey where he investigated not just humans but other vertebrates and even a number of invertebrate groups (Harvey, 1889). Knowledge of a species' basic pattern of cardiovascular (CV) development is crucial to making any sense of the form and function of its CV system and most physiological texts attempt to present a 'normal' or 'standardised' pattern of CV development. However, it has long been recognized that physiological variation is both pervasive and widespread (Spicer and Gaston 1999). Establishing the "basic pattern" is a necessary first step to studying development of a CV system, but is only the start of a framework with which to work from. Using this framework it is then possible to understand how, why, and the consequences for, individuals deviating during

their ontogeny from this 'basic pattern'. Although the significance of variation in physiological traits is recognized (e.g. Spicer and Morrill 1996; Spicer and Gaston 1999), our knowledge of such variation in CV development and how this variation partitions different levels of biological information is scarce, with a few noticeable exceptions. Bagatto et al. (2000) investigated within- and between-litter variation in banded armadillos using a number of physiological parameters including heart rate. This species produces monozygous quadruplets and with this mode of reproduction, genetic variation within litters is negligible. However variation in heart rate, although lower than between litters, was still evident. Spicer and Morrill (1996) also revealed that something other than genetic differentiation was responsible for variation in heart rate in *Daphnia magna*, as levels of variation in heart rate were the same in cloned and wild type populations.

Perhaps surprisingly, the life-history consequences of variation in CV function have also received little attention. The most notable exceptions here are the studies of beat-to-beat variation within the medical literature, that have led to variation in this trait being used in medicine as a good predictor, and indicator, of adult heart disease (e.g. Appel et al 1989; Tsuji et al 1996). Decreased beat-to-beat variation, i.e. a more metronomic heartbeat, is a key indicator of the level of risk posed to a patient by serious ventricular arrhythmias (Malik et al 1990; Malik 2006). This link between beat-to-beat variability and human pathology demonstrates the importance of understanding variation in the development of CV function to understanding organism function and life history.

In conclusion the CV system seems an ideal model system to study physiological variation during development. It shows variation at a number of different hierarchical levels, and this variation is pervasive. Cardiac function is also relatively easy to observe and quantify in embryos, and if invertebrates are used, comparatively large numbers of individuals may be employed in experiments. The question then is, given the variation catalogued and examined in previous chapters (Chapters 2, 3 and 4) is the CV system of gastropod molluscs a good model system to investigate variation during development, both within and between generations?

#### 5.2.2 Cardiovascular function in invertebrates

While much is known of the ontogeny of CV function in higher, and to a lesser extent lower, vertebrates (Burggren and Warburton 1994) this is not so for even the most well-studied invertebrate groups (McMahon, et al. 1997). Nearly all of the research on CV function in invertebrates has focused on crustacean hearts (McMahon et al. 1997), and: i) how cardiac function (mainly rate of beating) changes through time (Spicer 1994; Spicer and Morritt 1996; McMahon et al. 1997; Spicer 2001; Reiber and Harper 2001); ii) how cardiac response to neurohormones appears and develops through ontogeny (Harper and Reiber, 2001, 2004); and iii) the effect of environmental factors such as temperature (Spicer 1994), trace metals (Spicer 1996) and hypoxia (Reiber 1997) on heart function. The picture that emerges of crustacean CV function is that: i) there is interspecific variation in the timing of when it appears during development; ii) cardiac activity increases in rate with increasing development and is initially myogenic and insensitive to environmental perturbation; and iii) subsequently, there is a shift and cardiac activity decreases in rate with development, and in

some, but not all species this shift co-occurs with a switch from myogenic to neurogenic control (Yamagishi and Hiroshi 1996; Yamagishi et al. 1997).

Despite beginning to understand CV functional development in crustaceans, our knowledge of other invertebrate groups is extremely poor. This is the case even for a group like the molluscs where our understanding of adult CV function is comparatively good (Yeoman et al, 1999; Bourne, et al. 1990; Buckett et al. 1990; Martin 1980; Jones 1983). Work describing the morphology (reviewed in Raven 1958) and physiology (reviewed in Hill and Welsh 1966) of the molluscan CV system was performed in the mid-nineteenth century<sup>1</sup> and the function of the adult CV system was later investigated by Bourne et al. (1990). Apart from a small number of sporadic studies, for example on the effect of temperature on cardiac activity in freshwater pulmonates (Bachrach and Cardot, 1923) and the development of the heart beat and its temperature sensitivity in a slug species (Crozier and Stier 1925), it is only recently that the ontogeny of embryonic CV function was described (Bitterli et al. 2012). Bitterli et al. (2012) focused on the effect of hypoxia on the rate of beating of the true and larval hearts (a transitory organ that beats alongside the true heart during early CV development) in the marine gastropod, *Littorina obtusata*. This study revealed that both larval and

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<sup>1</sup> Raven (1958) provided a comprehensive review of molluscan morphological development including the CV system. The heart in most molluscs (excluding the cephalopods – the only molluscan group with a closed CV system) arises from a pallial origin together with the kidney, pericardium and, often, reproductive organs. The heart begins as an ectomesodermal vesicle behind the velum on the right hand side of the animal and as the snail undergoes torsion the heart moves anteriorly and across from the right to the left hand side (Raven 1958). Gastropod molluscs have a two-chambered heart, consisting of an auricle which receives blood from the gills, kidney or venous sinuses, connected to a ventricle which pumps blood out through an aorta, positioned at the opposite end of the ventricle to the auricle (Hill and Welsh 1966). Mollusc hearts are myogenic (Krijgsman and Divaris 1955, Hill and Welsh 1966, Irisawa 1978), but, despite some disagreement, it is now evident that there is also some level of nervous innervation from the visceral nerves to both the auricle and ventricle (Carlson 1905; Divaris 1955 Krijgsman 1954; Ripplinger 1957). We also have some knowledge of the response of adult molluscan heart rate to temperature (Skarmlik 1929), hypoxia (Nomura 1950), neurohumor analogues (Loveland 1963) and various drugs (Krijgsman and Divaris 1955).

adult heart were sensitive to acute hypoxia, but at different times during their ontogenies and highlights interesting questions regarding the function of this larval organ during embryonic development. However, this study only investigated embryos during the period at which both hearts beat together (up to 25 days from first cell division) and so didn't provide a full description of the true heart's functional ontogeny.

### 5.2.3 This chapter

In summary, variability in CV development pervades all biological levels, but there has been little, systematic exploration of this variation. Whilst the mollusc heart may be a good model for studying developmental variation, our knowledge is lacking, and any attempt to use such a model must work from a basic description of the pattern of the development of CV function.

Consequently, in this chapter, I describe, with high temporal resolution, the basic pattern of the ontogeny of CV function in the pond snail, *Radix balthica*, focusing on the timing of a number of key events. Next, I use this basic pattern to explore how variation in ontogeny of CV function is distributed between egg masses and populations. To probe further the potential source of this variation I perform a direct parent-offspring comparison of CV ontogeny. Finally, I use embryonic growth rate and size at hatching as proxies for Darwinian fitness and relate these to variation in CV development in order to assess the biological implications of CV functional ontogeny.

## 5.3 Material and Methods

### 5.3.1 Embryo culture

*Radix balthica* from each of four second generation stock populations, founded from European populations, spanning this species latitudinal range (Swedish – 66.428, 19.683; English – 50.439, -3.690; German – 50.007 9.156; French – 44.053, 4.784; Pfenninger et al. 2011), were maintained at 15°C under a 12:12 light-dark regime. Snails were cultured in aquaria (vol. = 15 l; stocking density = 15) containing Artificial Pond Water (APW) (Rundle et al. 2004) and fed lettuce and spinach *ad libitum*. Egg masses were harvested from aquaria and examined under low power magnification (x 10). Only eggs that had not developed past two-cell division were used in observations.

### 5.3.2 Bio-imaging

Under low power magnification (x 10), six eggs were selected haphazardly from each egg mass (French population - 7 egg masses; Swedish - 4 egg masses; German - 3 egg masses; English- 6 egg masses), dissected from the mass and placed in random positions within a 384 well microtitre plate which was covered with a lid (cell vol. = 70 µl).

The entire development of these embryos was recorded using a purpose-made, automated bio-imaging system (Sect. 1.7.1), housed in a 20°C facility. This system comprised a 4 megapixel shutterless monochrome camera (Pike 421 B, Allied Vision Technologies, Germany) connected to a zooming lens system (Zoom 70 XL, Optem, Luxembourg), inverted beneath a motorised XY stage (Optiscan XY stage, Prior Scientific, England). Transmitted dark field cold lighting was provided by an array of light emitting diodes (LEDs). The camera

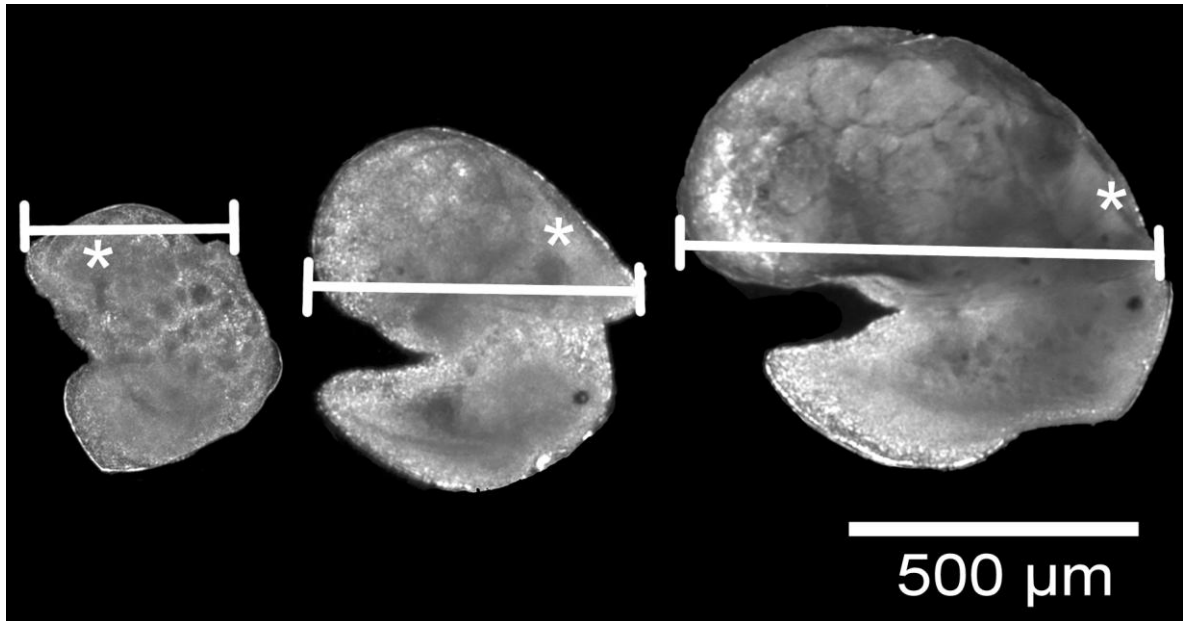
and XY motorised stage were controlled using the open-source software MicroManager 1.3 (Edelstein et al. 2010) running on a Mac Pro using OS X. Embryos were imaged every 1h by acquiring an image sequence at 7.5 frames  $\text{sec}^{-1}$  for 20 sec and these image sequences were written to mirrored hard drives, to guard against loss of data from hardware malfunction. Artificial pond water (Rundle et al. 2004) in each well of the multi-well plate was replaced daily, *via* manual pipetting.

### 5.3.3 Image analysis

Following image acquisition, image sequences were analysed manually. Firstly, the time of onset of the four developmental events - two cell division, first discrete heartbeat, radula function and hatching, were recorded for each embryo. The time period between two cell division and radula ontogeny was used to provide a standardised measure of developmental timing, hereafter referred to as relative timing. Radula ontogeny was chosen for use in standardization as it exhibits relatively little variation in its time of onset (pers. obs.). The next set of observations investigated the development of heart function. Heart rate was measured, *via* manual observation, of the 20 sec image sequences, from 15 h after the first discrete heartbeat, every 6 hours until hatching.

I also used animal size as a measure of ontogeny and this parameter was measured as the distance from the anterior to the posterior margins of the mantle (Fig. 5.1) from when the heart function was quantifiable, up to hatching. Hatchling size was measured immediately prior (i.e. within 2 h) to hatching (Fig.

5.1). Egg volume, at the beginning of development, was calculated using the formula  $1/6\pi lw^2$  (Taylor 1973), where  $l$  = egg length and  $w$  = egg width.



**Figure 5.1.** Micrograph illustrating embryos at various stages of development and where the measurement of mantle length from the recorded image sequences was made. This measurement was used as a metric of animal size from the first heart beat to hatching. Asterisks indicate the approximate position of the heart in embryos at these three different stages of development (left to right, E6, E9, E11; Cumin 1972).

#### 5.3.4 Data analysis

This is the first detailed examination of molluscan heart rate during development and, hence, an initial visual examination of heart rate time series for all individuals was used to identify fundamental patterns. This qualitative analysis of heart rate ontogeny suggested a biphasic pattern. A biphasic heart rate had also been observed in developing brine shrimp (*Artemia franciscana*) (Spicer 1994). These crustaceans showed an initial increase in heart rate followed by a decrease, which occurred close to the time at which the heart switched from differentiation to elongation. This pattern of heart rate development did not conform to a simple power-curve model and Spicer (1994) found that



segmented regression was an effective method for analysis of this ontogenetic trend. Given the apparent biphasic nature of heart rate in *R. balthica*, I too applied segmented regression analysis to explore the ontogeny of these two phases of heart rate (Fig. 5.2). This analysis was performed using the 'R' package 'segmented'. Three different measures of ontogeny were used: i) time from two cell division (absolute timing); ii) proportion of time between two cell division and radula (relative timing); and iii) size. The regression analysis was repeated for each of these measures of ontogeny.

Analysis of variance was performed to test for differences in aspects of cardiovascular development between embryos from different populations and egg masses. For these analyses egg mass was nested within population and both were classed as random factors. Egg volume was included in all analyses as a covariate to provide a measure of, and to control for, differences in maternal investment.

#### 5.3.5 Parent-offspring comparison

Comparison of CV functional ontogeny between five parents and 29 of their offspring (2 – 14 offspring per parent) was performed and these data are presented here. Parents were taken from a laboratory stock population, as embryos that had yet to develop past the first cell division, and their embryonic development was imaged using the method described in the section above. Once hatched these snails were cultured individually in glass vials (vol. = 40 ml), containing APW (vol. = 35 ml) and a 5 cm long piece of Canadian pondweed, *Elodea densa*. Jars were placed on shelving placed in a west-facing window, in a 15 ° C controlled temperature facility. Snails were fed lettuce discs (diam. = 4

mm) every two weeks and water was changed every three weeks, at which time position in the windows was also changed haphazardly. Three months after snails hatched they were transferred to larger volume jars (vol. = 500 ml; APW = 450 ml) containing two pieces of 15 cm *Elodea densa*. Water in these jars was changed every three weeks and snails continued to be fed lettuce discs (diam. = 10 mm). When snails reached sexual maturity their jars were checked for egg masses daily and, if present, and if they had not proceeded past the first cell division, their development was imaged using the same approach as adopted for their parents. Parent and offspring CV development was analysed using the same approach as detailed above. Comparison between parents and their offspring in aspects of CV development was performed using ANOVA to test for differences between embryos from different parents, with parents weighted by their own CV development (using Weighted Least Squares) and offspring egg volume included as a covariate.

## **5.4 Results**

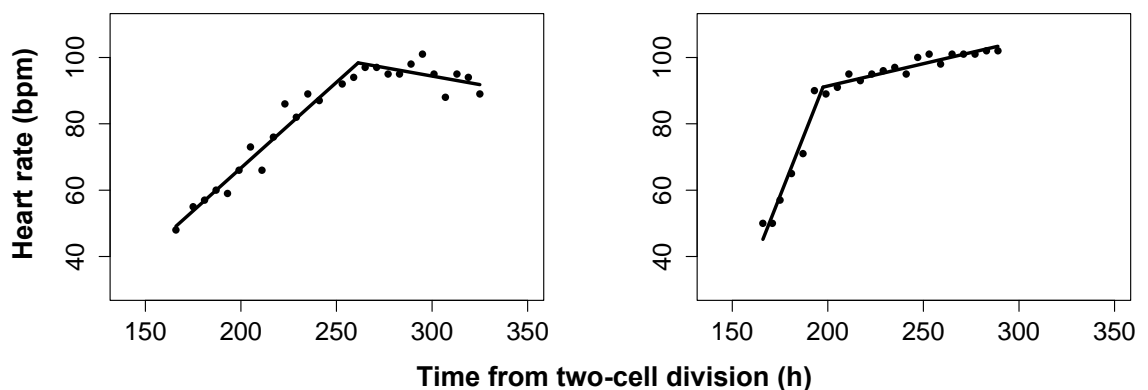
### **5.4.1 General description of the ontogeny of cardiac function**

The first discrete heartbeat occurred between 129 and 159 hours after the first cell division, across all individuals, with a mean value of occurrence of  $144 \pm 1$  h (mean  $\pm$  1 SE). The heartbeat remained faint and irregular for *circa* 15 hours following this first visual evidence of its ontogeny. After this time a regular heartbeat was apparent and quantifiable with a mean beat rate of  $44 \pm 2$  beats per minute (bpm). In most individuals, the ontogeny of heart rate then appeared to follow a biphasic trajectory. Heart rate first increased rapidly for

approximately 66 h to a mean value of  $84 \pm 1$  bpm around 81 h after the first discrete heartbeat (Figs. 5.3 and 5.4). Following this period of rapid increase the rate of change in heart rate slowed and then followed either a more gradual increase, or a decrease, until hatching (Fig. 5.2). Unlike the ontogeny of heart rate, embryonic growth exhibited a largely linear relationship with chronological time suggesting that the non-linear ontogeny of heart rate was not being driven by non-linear changes in embryonic growth.

#### 5.4.2 A model describing ontogeny of cardiac function

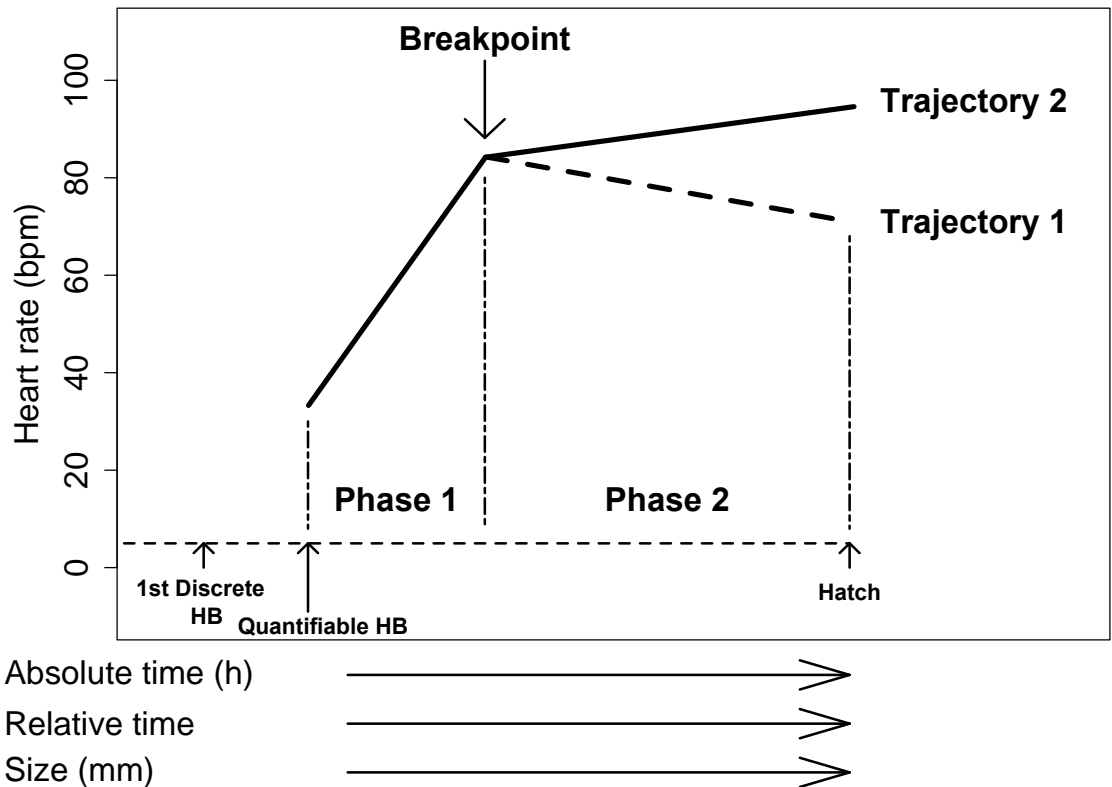
Segmented regression analysis confirmed a significant biphasic relationship in most (89%) of the embryos examined and provided a good fit for the ontogeny of heart rate in these embryos ( $R^2 = 0.81 \pm 0.019$ ,  $P = 0.0005 \pm 0.00024$ ) (Fig. 5.2). Of the 11% of embryos for which a segmented regression did not provide a good fit, 8% had no significant change in slope (i.e. no breakpoint) through their ontogeny and 3% had a highly variable heart rate to which regression was unable to fit a significant model.



**Figure 5.2.** The ontogeny of heart function in two individual embryos that differ in their trajectories. Line fitted to data is the result of segmented regression analysis.

All embryos exhibited increasing heart rate during the first part of their ontogeny of CV function (Phase 1 - Fig. 5.3). However, after this phase, embryos could be assigned to one of two different ontogenic trajectories depending on if their heart rate ontogeny showed: i) a negative (Trajectory 1), or ii) a positive (Trajectory 2), slope (Phase 2 - Fig. 5.3).

Embryos followed each trajectory with roughly equal frequency (Table 5.1) and there were some differences in heart rate ontogeny occurring prior to Phase 2 between these trajectories: embryos exhibiting a decreasing heart rate during Phase 2 (Trajectory 1) had both a later breakpoint (relative timing -  $F_{1,40} = 36$ ,  $P \leq 0.001$ ; absolute timing -  $F_{1,40} = 15.53$ ,  $P \leq 0.001$ ) and a less steep slope during Phase 1 (relative timing -  $F_{1,40} = 5.12$ ,  $P = 0.029$ , absolute timing -  $F_{1,40} = 6.49$ ,  $P = 0.015$ ) than embryos whose heart rate continued to increase during Phase 2 (Trajectory 2).

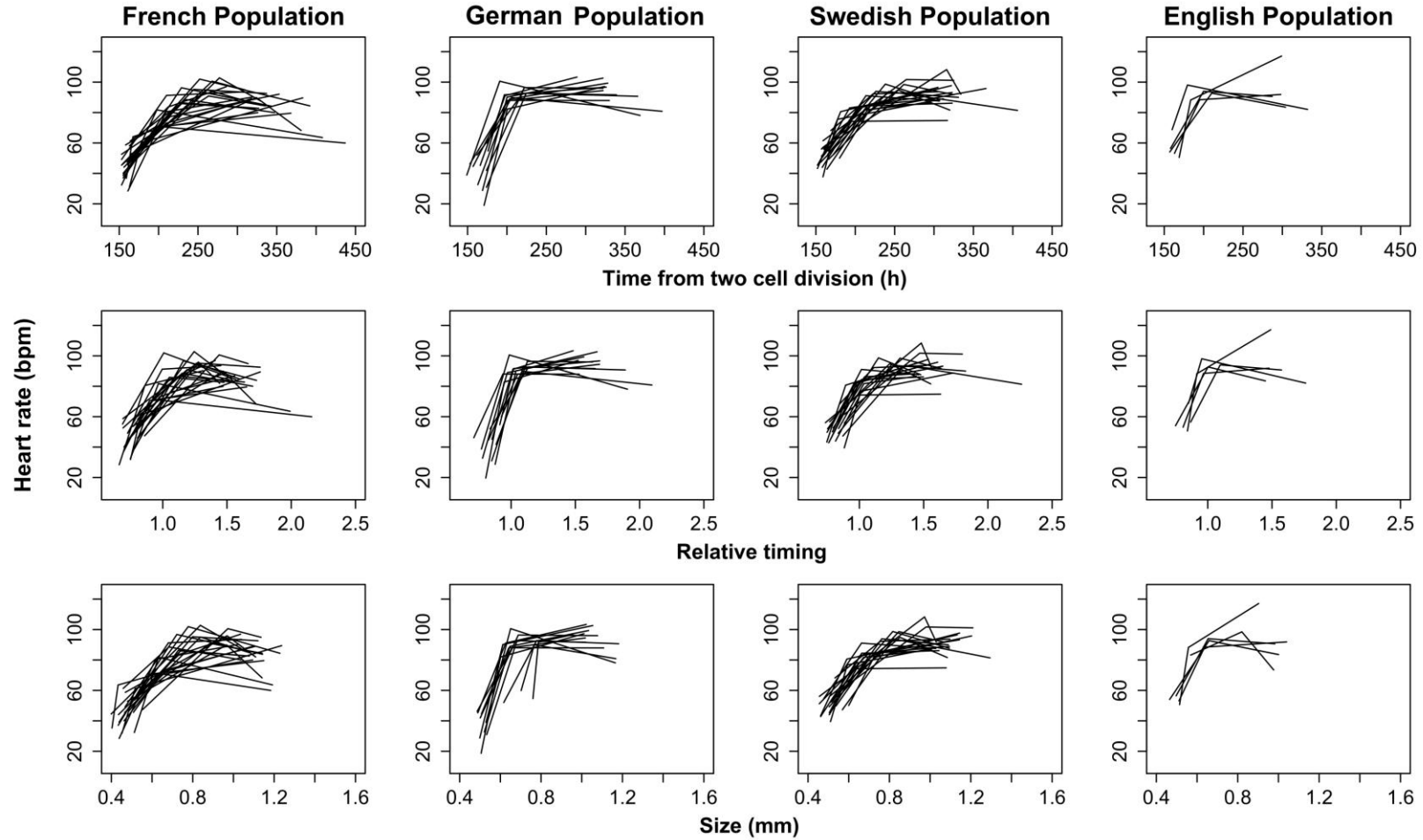


**Figure 5.3** Cartoon illustrating the components of CV functional development of *Radix balthica* used in analyses.

#### 5.4.3 Variation in the ontogeny of cardiac function

There were significant differences in the time to first heart function when ontogeny was measured as either absolute or relative timing, between embryos from different egg masses, but not populations (absolute timing –  $F_{12, 41} = 10.76$ ,  $P \leq 0.001$ ; relative timing –  $F_{12, 41} = 2.35$ ,  $P = 0.021$ ).

There were also noticeable differences in the pattern of the ontogeny of heart rate between embryos from different, populations (Fig. 5.4) and egg masses (Fig. 5.5).



**Figure 5.4.** Ontogeny of CV function in individual embryos from French, German, Swedish and English populations of *Radix balthica*. Each line show shows the results of segmented regression analysis of an individual's ontogenic trends in heart rate, with ontogeny measured as: i) time from two cell division; ii) relative timing (measured as proportion of time from two cell division to radula function); and iii) embryo size.

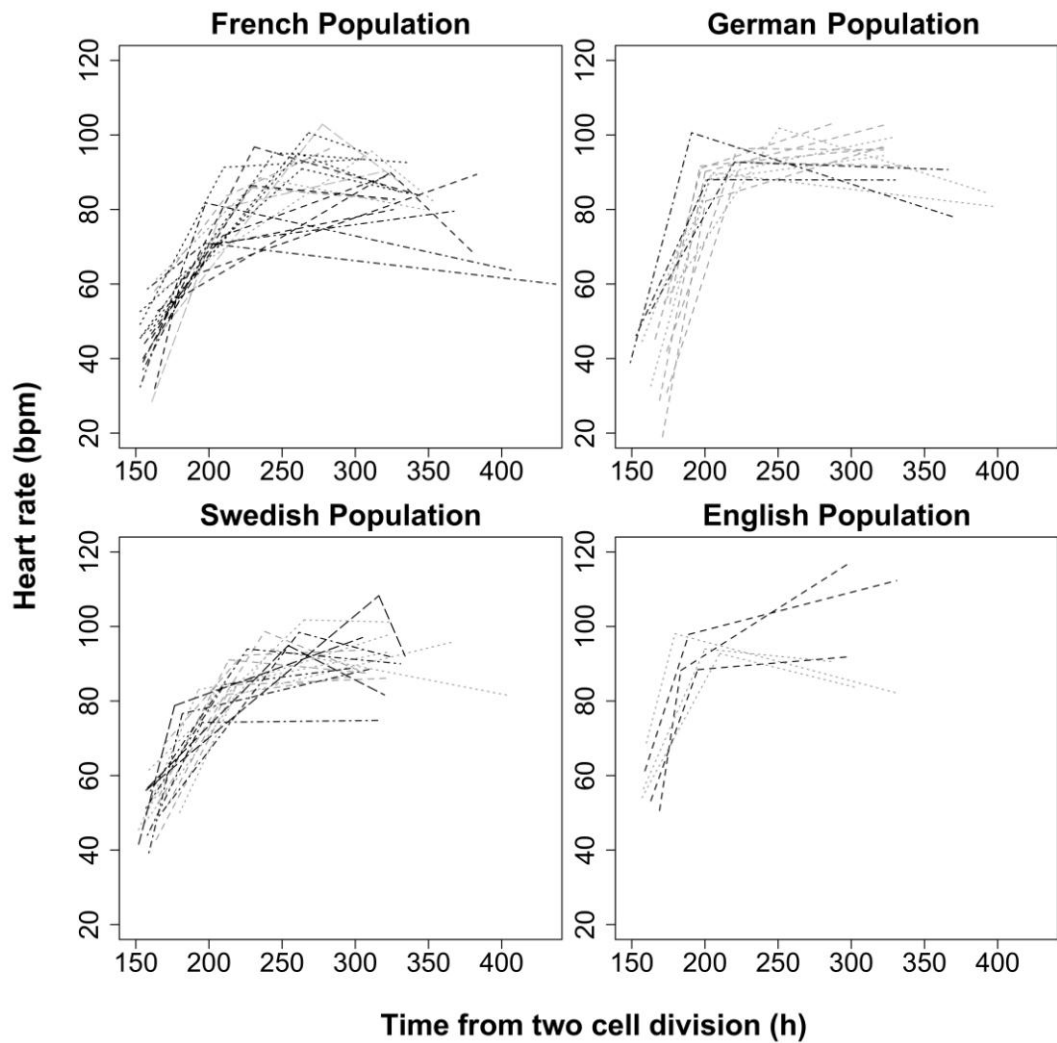
The ratio of embryos following Trajectories 1 or 2 was approximately equal within populations and egg masses (Table 5.1), although the small numbers of embryos obtained from each egg mass preclude any definitive statement on this ratio. Embryos from different populations whose heart rate followed Trajectory 1 displayed significant differences in their rate of increase in heart rate during the first Phase (relative timing -  $F_{3, 14} = 3.81$ ,  $P = 0.026$ ; absolute timing -  $F_{3, 14} = 3.24$ ,  $P = 0.045$ ), but there was no such difference in embryos following Trajectory 2, between populations. For embryos that followed Trajectory 1, the time during ontogeny at which the breakpoint (between Phases 1 and 2) in heart rate occurred also differed significantly between egg masses (relative timing -  $F_{13, 14} = 4.49$ ,  $P = 0.004$ ; absolute timing -  $F_{13, 14} = 4.28$ ,  $P = 0.005$ ). Further, the rate of decrease in heart rate in embryos following Trajectory 1 was different between populations ( $F_{13, 14} = 4.28$ ,  $P = 0.005$ ), but only when ontogeny was measured as relative, rather than absolute, time.

There were no significant differences between either egg masses or populations in growth trajectory during the embryonic period (Fig. 5.6) and egg volume was not a significant covariate of this trait.

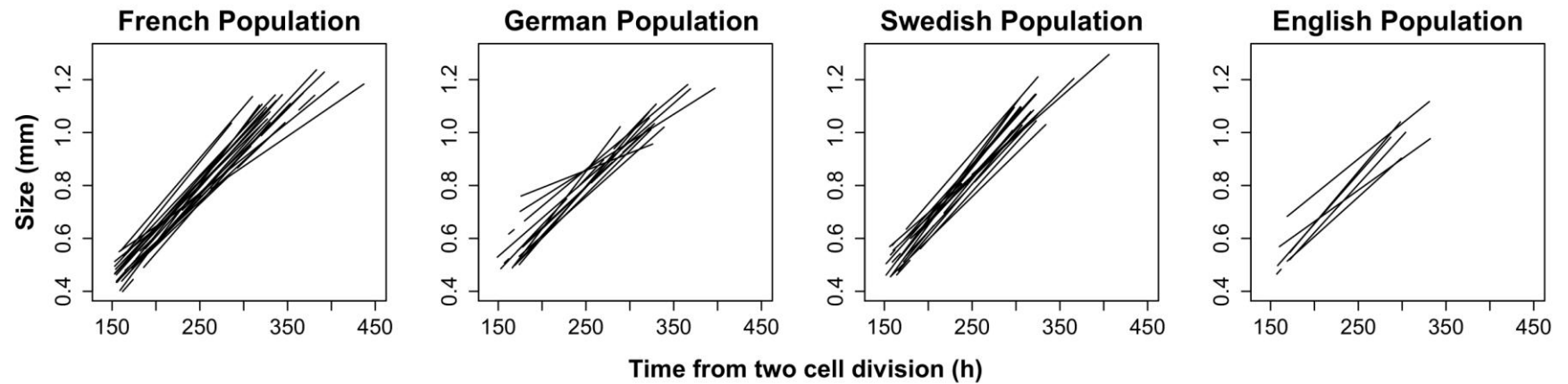
Population	Egg mass	Trajectory one (-ve)	Trajectory two (+ve)
French	a	1	1
	b	2	0
	c	2	1
	d	1	1
	e	2	4
	f	1	4
	g	1	
German	a	3	3
	b	2	2
	c	3	0
Swedish	a	2	3
	b	2	3
	c	2	3
	d	2	1
English	a	0	3
	b	3	0

**Table 5.1.** Numbers of embryos following Trajectories 1 or 2, from different egg masses and populations (Figs. 5.4 and 5.5).





**Figure 5.5.** Ontogenies of CV function in individual *Radix balthica* embryos from different populations and egg masses within these populations. Fitted lines were derived by segmented regression analysis and individuals from the same egg mass, within populations, are indicated by the same line style.



**Figure 5.6.** Growth trajectories of individual embryos from different populations of *Radix balthica*, determined by regression analysis. There were no significant differences in the rates of growth between embryos from either different egg masses or populations.

Factor	df	SS	MS	F	P	
<b>RELATIVE TIMING</b>						
<b>Breakpoint</b>						
<b>Trajectory One</b>						
Egg volume	1	0.00327	0.00001	0.54	0.476	ns
Population	3	0.02699	0.00442	1.1	0.383	ns
Egg Mass(Population)	13	0.02732	0.00487	4.49	0.004	*
Error	14	0.05888	0.00609			
<b>Trajectory Two</b>						
Egg volume	1	0.00001	0.00001	0	0.957	ns
Population	3	0.01326	0.00442	0.91	0.483	ns
Egg Mass(Population)	8	0.03893	0.00487	1.07	0.436	ns
Error	13	0.05888	0.00453			
<b>Slope 1</b>						
<b>Trajectory One</b>						
Egg volume	1	23	23	0.01	0.929	ns
Population	3	19115	6372	3.81	0.026	*
Egg Mass(Population)	13	19459	1497	0.52	0.874	ns
Error	14	40024	2859			
<b>Trajectory Two</b>						
Egg volume	1	22699	22699	0.46	0.509	ns
Population	3	82913	27638	1.98	0.22	ns
Egg Mass(Population)	8	123604	15450	0.31	0.947	ns
Error	13	638694	49130			
<b>Slope 2</b>						
<b>Trajectory One</b>						
Egg volume	1	2249	2249	0.69	0.42	ns
Population	3	6235	2078	0.58	0.636	ns
Egg Mass(Population)	13	47100	3623	1.11	0.42	ns
Error	14	45534	23252			
<b>Trajectory Two</b>						
Egg volume	1	53	53	0.44	0.521	ns
Population	3	255	85	0.7	0.581	ns
Egg Mass(Population)	8	976	122	0.99	0.486	ns
Error	13	1604	123			

**Table 5.2.** Results of an ANCOVA testing for differences in the development of CV function, with ontogeny quantified as relative timing, between embryos from different populations and egg masses, in the pond snail *Radix balthica*, <sup>ns</sup> – p > 0.05; \* - p < 0.05; \*\* - p ≤ 0.001.

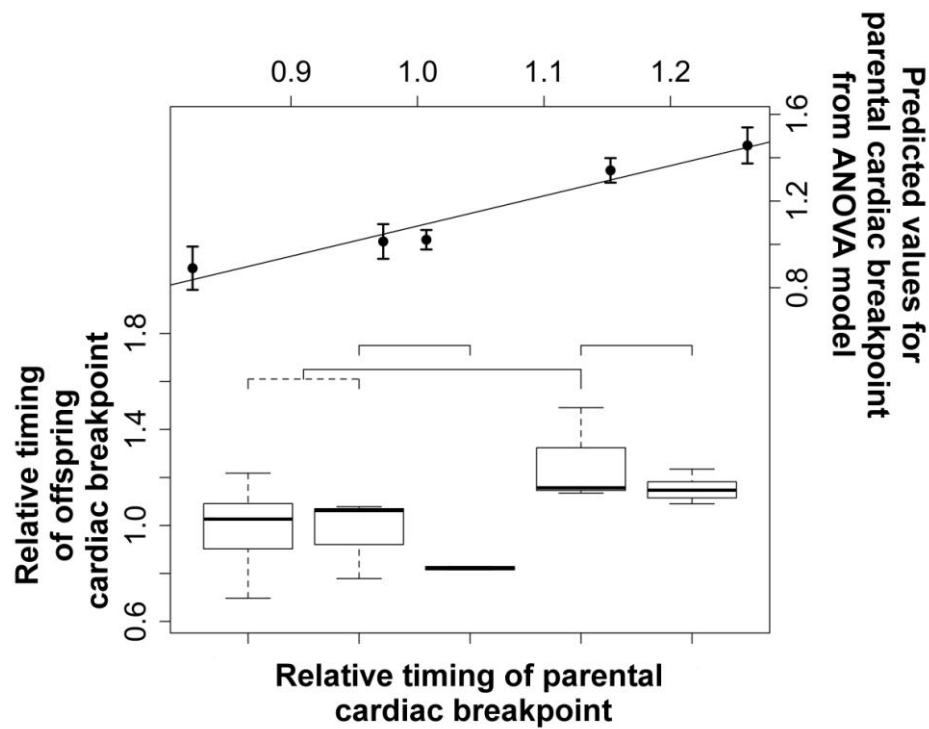
Factor	df	SS	MS	F	P	
<b>ABSOLUTE TIMING</b>						
<b>Breakpoint</b>						
<b>Trajectory One</b>						
Egg volume	1	323	323	0.85	0.373	ns
Population	3	5743	1914	1.3	0.313	ns
Egg Mass(Population)	13	21191	1630	4.28	0.005	*
Error	14	5329	381			
<b>Trajectory Two</b>						
Egg volume	1	122	122	0.14	0.719	ns
Population	3	2319	773	4.26	0.072	ns
Egg Mass(Population)	8	1696	222	0.24	0.976	ns
Error	13	11685	899			
<b>Slope 1</b>						
<b>Trajectory One</b>						
Egg volume	1	0.0002	0.00015	0	0.966	ns
Population	3	0.50975	0.16992	3.24	0.045	*
Egg Mass(Population)	13	0.63076	0.04852	0.62	0.803	ns
Error	14	1.09825	0.07845			
<b>Trajectory Two</b>						
Egg volume	1	0.1142	0.1142	0.41	0.532	ns
Population	3	0.741	0.247	1.41	0.319	*
Egg Mass(Population)	13	1.4404	0.1801	0.65	0.725	ns
Error	14	3.6046	0.2773			
<b>Slope 2</b>						
<b>Trajectory One</b>						
Egg volume	1	0.008782	0.008782	1.5	0.241	ns
Population	3	0.129686	0.043229	0.89	0.473	ns
Egg Mass(Population)	13	0.717397	0.055184	9.4	0.001	**
Error	14	0.08215	0.005868			
<b>Trajectory Two</b>						
Egg volume	1	0.0369	0.0369	0.21	0.657	ns
Population	3	0.7832	0.2611	737.4	0.946	ns
Egg Mass(Population)	8	0.0633	0.0079	0.04	1	ns
Error	13	2.3162	0.1782			

**Table 5.3.** Results of an ANCOVA testing for differences in the development of CV function, with ontogeny quantified as absolute timing, between embryos from different populations and egg masses, in the pond snail *Radix balthica*, <sup>ns</sup> –  $p > 0.05$ ; \* -  $p < 0.05$ ; \*\* -  $p \leq 0.001$ .

#### 5.4.4 Parent-offspring comparison of CV functional ontogeny

Comparison of CV functional ontogeny between parents and their offspring revealed that the relative timing of the breakpoint between Phase 1 and 2 was both significantly different between offspring from different parents and had significant similarity to the timing of their parent's CV breakpoint (Fig. 5.7; Table

5.4). None of the other heart rate parameters used here displayed significant cross-generational similarity, with the exception of the timing of a discrete heartbeat, which is analysed and discussed in Chapter 4 (using a larger dataset than in the current chapter).



**Figure 5.7.** Parent-offspring comparison of the relative timing of the breakpoint in the ontogeny of heart function in offspring produced from different parents. Offspring are weighted by parental breakpoint timing, and egg volume is included as a covariate in the model (Table 5.4). Significant differences between offspring produced from different parents are indicated by horizontal bars across box plots. The top plot shows developmental event timing (mean  $\pm$  1 S.E.) for offspring from different parents, as predicted from the ANOVA model.

Relative timing of breakpoint	df	SS	MS	F	P	
Corrected Model	5	0.455	0.091	4.11	0.001	***
Egg volume	1	0.026	0.026	1.16	0.293	ns
Parent	4	0.427	0.107	4.81	0.006	**
Error	22	0.488	0.022			

**Table 5.4.** Results of an ANOVA testing for differences in the relative timing of the breakpoint between the first and second phase of the ontogeny of CV function between embryos from different parents, with parents weighted by their own relative timing of breakpoint and egg volume factored as a covariate. <sup>ns</sup> –  $p > 0.05$ ; \* -  $p < 0.05$ ; \*\* -  $p \leq 0.001$ .

#### 5.4.5 Heart ontogeny, growth rate and hatchling size

Hatchling size was used to assess whether there were implications to individuals of differences in aspects of their CV development. This analysis revealed that embryos following Trajectory 1 hatched significantly larger than those following Trajectory 2 (Table 5.5), although there were no relationships detected between the other traits used here to describe heart ontogeny (Fig. 5.3) and hatching size. To investigate the origins of these size differences growth rates were calculated, during the periods from 24 h after first heart function to 72 h and from 72 h to 144 h. Embryos following Trajectory 1 exhibited faster growth from 72 h to 144 h than embryos following Trajectory 2, but not between 24 h and 72 h (Table 5.5). Hence, the differences in size at hatch between embryos following Trajectories 1 or 2 appears to originate from differences in growth rate during this late embryonic development, at approximately the same time at which they are displaying differences in heart rate trajectory.

There were also significant differences in size at hatch between embryos from different egg masses ( $F_{12, 40} = 2.47$ ,  $P = 0.016$ ) and differences in growth rate between 24 h and 72 h between populations.

Factor	df	SS	MS	F	P	
<b>Hatching size</b>						
Egg volume	1	0.000004	0.000004	0	0.967	ns
Trajectory 1 or 2	1	0.019859	0.019859	9.01	0.005	**
Population	3	0.023542	0.007847	1.62	0.228	ns
Egg Mass(Population)	12	0.065422	0.005452	2.47	0.016	*
Error	40	0.88120	0.002203			
<b>Growth from 24 h to 72 h after first heart function</b>						
Egg volume	1	0.000817	0.000817	0.7	0.408	ns
Trajectory 1 or 2	1	0.000210	0.000210	0.18	0.674	ns
Population	3	0.015092	0.005223	4.01	0.025	*
Egg Mass(Population)	12	0.015994	0.001333	1.14	0.357	ns
Error	40	0.046746	0.001169			
<b>Growth from 72 h to 144 h after first heart function</b>						
Egg volume	1	0.000163	0.000163	0.12	0.736	ns
Trajectory 1 or 2	1	0.009927	0.009927	7.02	0.012	*
Population	3	0.11260	0.003753	3.24	0.05	ns
Egg Mass(Population)	13	0.013856	0.001155	0.82	0.632	ns
Error	14	0.05230	0.001414			

**Table 5.5.** Results of an ANOVA testing for differences in the size and growth in embryos following different trajectories during phase two of the ontogeny of heart function. <sup>ns</sup> –  $p > 0.05$ ; \* -  $p < 0.05$ ; \*\* -  $p \leq 0.001$ .

## 5.5 Discussion

### 5.5.1 Ontogeny of CV function: pattern and timings

The ontogeny of cardiac function in *Radix balthica* follows a biphasic relationship between heart rate and developmental time. During Phase 1 heart rate increases until it reaches a breakpoint at which time two pathways are followed with apparent equal frequency – heart rate either, continues to increase (Trajectory 2), but at a reduced rate, or decreases (Trajectory 1), for the remainder of embryonic development. This is the first demonstration of a biphasic relationship in the development of CV function in molluscs and this

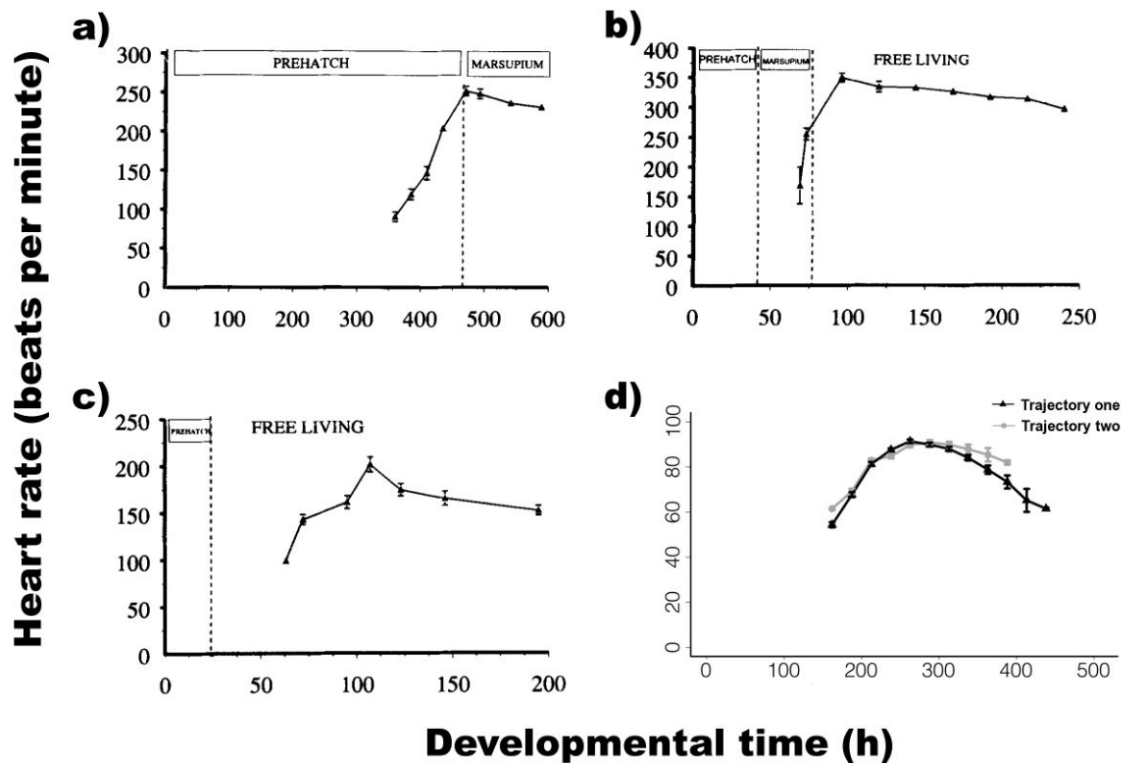
type of relationship appears to be similar to that reported for other invertebrates, such as the crustaceans (Spicer 2001) (Fig. 5.8).

In crustaceans the suggested mechanism underlying the breakpoint, from, a rapid increase to a decrease, in cardiac function, is the switch from myogenic to neurogenic cardiac innervation (Spicer, 2001). However, the heart of molluscs is myogenically controlled (Krijgsman and Divaris 1955; Hill and Welsh 1966; Irisawa 1978), presumably throughout development, and therefore, there must be another basis for the biphasic relationship in *R. balthica*. It is surprising, however, that a biphasic relationship is evident in both groups and perhaps suggests an, as yet, unidentified process might be responsible for the ontogenic patterns seen in the Crustacea other than a switch in mode of cardiac innervation (Spicer 2001). This said, it should be noted that mollusc hearts do possess some degree of neuronal control (Koester and Koch 1987), and so perhaps ruling out linking changes in cardiac control to this relationship is premature.

If the biphasic patterns of heart ontogeny in molluscs and crustaceans are not homologous this might go some way to explaining why molluscs exhibit two trajectories during Phase 2 (up or down) whereas crustaceans all appear to follow a downward trajectory during the second phase of this biphasic relationship. Figure 5.8 shows that for *R. balthica* when mean heart rate is plotted for embryos following Trajectories 1 and 2, both follow a downward trajectory during Phase 2, even though the embryos following Trajectory 2 all exhibit an increase in heart rate after the breakpoint. The averaging of data for embryos with different intercepts (breakpoint) and slopes during Phase 2 has



effectively masked the biologically relevant inter-individual variation. This plot highlights the problems inherent in presenting such data as mean values when studying such ontogenic trends. If individual embryos' ontogenies were not considered, the occurrence of embryos following both “up” and “down” trajectories during Phase 2 may well have been overlooked. The issue with being unable to replicate the individual is a problem highlighted by Spicer and Gaston (1999) and is illustrated here by much of the interesting variation in the ontogeny of heart function being lost when an average ontogenic path is considered.



**Figure 5.8.** Ontogeny of cardiac function, in (a) *Gammarus duebeni*, (b) *Daphnia magna* (clone), (c) *Artemia franciscana* and (d) *Radix balthica* (mean  $\pm$  1 SE). Plots (a) – (c) are adapted from Spicer and Morritt (1996). All data is for species cultured at 20°C.

### 5.5.2 Variation in ontogeny of CV function

When ontogeny was measured as relative time, embryos from different populations whose heart rate followed Trajectory 1 during Phase 2 had significant differences in both the rate of increase in heart rate during Phase 1, and the rate of decrease during Phase 2 (Table 5.1). Differences in the ontogeny of physiological traits between populations appear to be quite common (Stauber 1950; Prosser 1957; Vernberg 1964; Garland and Adolph 1994) and are often attributed to *in situ* environmental factors. Here, the population differences in the rates of change in heart rate during both Phases 1 and 2 could be caused by persistent physiological acclimatisation of differences *in situ* between populations, or alternatively are the result of genetic differences accompanying physiological differentiation (Spicer and Gaston 1999). Interestingly, population differences in the rates of change in heart rate were only present among individuals following Trajectory 2, suggesting that there might be different mechanisms underpinning these two trajectories.

There were also differences in the timing of CV developmental events between embryos from different egg masses, including the time of both the first discrete heartbeat and the breakpoint between Phases 1 and 2. In the comparison of parent-offspring CV development it was only the relative timing of the breakpoint that showed cross-generational similarity, suggesting that differences between egg masses, in this trait, might be caused by these egg masses originating from different parents, which is probable given that they were sourced from a stock tank. The parent-offspring comparison of CV development was performed using only a preliminary, small, dataset, but a parent-offspring similarity in the relative timing of the breakpoint was

nevertheless evident. It therefore seems that, as with the developmental events (including the timing of first heart function) used in the parent-offspring comparison in Chapter 4, a significant portion of the variation in the timing of the breakpoint has a parental origin.

Bagatto et al. (2000) studied variation in physiological traits, including heart rate, within and between Amadillo litters, which produce monozygous quadruplets with essentially zero genetic variation within-litters, and found that for all traits studied within-litter variability was always less than between-litter variability. These authors attribute this pattern of variability to genetic components that determine physiological characters. Similar patterns have been found in other studies (reviewed by Burggren 1999) and the consensus of authors seems to be that these CV differences between litters are a result of either difference in genetic regulation or maternal effects. However, to my knowledge, mine is the first study that has attempted to link differences in CV development between offspring from different parents back to the CV development of the parent. This parent-offspring comparison of CV development revealed cross-generational similarity, suggesting either genetic differences or perhaps maternal effects are the cause. Here, the analysis of parent-offspring similarity also incorporated egg volume as a measure of maternal investment and found this to not be a significant covariate and previous work with *R. balthica* (Chapter 2; Tills et al. 2011- Appendix 3) has hinted at a genetic basis for the timing of nine developmental events (not including the cardiac breakpoint described here). Therefore it would perhaps not be too surprising for a genetic underpinning to be responsible for variation in the timing of other developmental events. However, to truly understand the relative roles of genetic and epigenetic effects

on CV developmental event timing will require a more sophisticated breeding experiment than was performed here.

### 5.5.3 Effects of ontogeny of CV function on life history

Embryos whose heart rate followed Trajectory 2 (an increase) during Phase 2 were significantly smaller on hatching than those following Trajectory 1. This difference in size appeared to result from differential growth rates during the period of Phase 2, approximately 72 – 144 h after first heart function (Table 5.5). It therefore seems that during Phase 2 a trade-off exists between energy investment in growth and that invested in sustaining an elevated, and increasing, heart rate. That both trajectories occur with apparent equal frequency, and that Trajectory 2 appears to have negative life history effects, suggests that Trajectory 2 might have biological or ecological importance to justify the trade-off, of following this trajectory. Size at hatching has been shown in snails (Moran and Emler 2001; Spight 1976) and in other groups (e.g. turtles – Janzen (1993) and fish – Hutchings (1991)) to be a good predictor of survival, providing support for the hypothesis that here, the trade-off between growth and cardiac function during Phase 2 would result in reduced fitness for individuals, which as embryos, follow Trajectory 2.

It is also interesting to consider what effects these two Trajectories might have on embryos at different positions within the egg mass, or whether these trajectories might be linked with the position within the mass of the egg, perhaps via a maternal cue. Here, egg capsules were dissected from along the length of the egg mass and cultured separately and their original location within the mass was not recorded. However, it is known that developmental rate (Marois and

Croll 1991) and the microenvironment (Moran and Woods 2007) at different locations within an egg mass can differ. Understanding whether there are patterns to the location of embryos within egg masses following these alternative pathways might provide some insight as to whether these pathways provide different strategies for coping with environmental stress during the embryonic period (Cohen and Strathmann 1996). In other aquatic species it has been shown that embryos developing in the middle of gelatinous masses are close to the limit for adequate oxygen supply (Cohen and Strathmann 1996). For *R. balthica*, it could be predicted that embryos which follow Trajectory 2 might have higher Darwinian fitness in the middle of an egg mass, than embryos with a declining heartbeat, during this embryonic period. Or, perhaps these two trajectories do not exist in embryos cultured within the egg mass, but have resulted from their removal from the egg mass, and the associated environmental stressors, which might normally suppress this variation.

Studies of the implications of an organism's early development on later life history are quite rare, but crucial for understanding the implications to Darwinian fitness (or proxies of Darwinian fitness, i.e. size). Spicer and El-Gamal (1999) are a noticeable contribution in this area, having shown in brine shrimp that relatively small changes in the timing of respiratory regulation, driven by hypoxia, led to decreased Darwinian fitness (individual lifetime reproductive output). Here, I do not have life history data beyond size at hatching, so cannot be sure that these differences in CV functional development translates to differences in Darwinian fitness. However, the differences in hatching size do suggest a potential fitness cost of following Trajectory 2. It is also interesting to consider whether Trajectories 1 and 2 have any effects beyond hatching. Future work

must focus on whether these differences in heart rate extend beyond the embryonic stage and if so, whether this translates to differences in growth, behavior or other life history characteristics. Work with *Daphnia magna* revealed an inverse relationship between heart rate and longevity (MacArthur and Baillie 1929) and therefore here, if the differences between Trajectories 1 and 2 remain post-hatch, these might result in considerable differences to life history. *Radix balthica* hatchlings from the same egg mass can show considerable differences in growth rates (pers. obs.), but unfortunately I do not have post-hatch data for the embryos in this study so can only speculate on the implications for later life stages.

Here, I show that *Radix balthica*'s ontogeny of heart function appears similar to that of crustaceans, the best studied invertebrate group, in so far as the ontogeny follows a biphasic relationship. In crustaceans however, a switch from myogenic to neurogenic innervation was believed to be the cause of this switch and this cannot be the case for *R. balthica*. Further I find two alternative ontogenic pathways with embryos following Path 1 hatching smaller than those following Path 2. Finally, this study of events during ontogeny of heart function, as with other developmental events in *R. balthica* (Chapter 4), reveals that variation in the timing of cardiac functional ontogeny is prevalent and appears to have a parental origin. It appears that intraspecific variation in developmental event timing in this species' is not just "developmental noise" (Spicer and Gaston 1999), but also both functionally and ecologically relevant.

## CHAPTER 6

### CONCLUSION

#### 6.1 Introduction

The main aim of this thesis was to understand the extent to which intraspecific variation in developmental event timing might provide the raw material from which heterochronies may originate. In this final, discursive chapter, I revisit the three objectives of my thesis (Sect. 1.5) and consider, in turn, how each chapter has contributed to our understanding of the relationship between altered timing at the inter- and intra-specific levels, through the investigation of: i) patterns in altered event timing; ii) the genetic basis for altered event timing; and iii) fitness implications.

#### 6.2 Patterns in altered event timing

Variation in a trait is the raw material of natural selection (Darwin 1859) and therefore observing and describing the pattern of this variation, at the levels of both macro- and micro-evolution, is an important step in understanding evolutionary pattern and process (Arthur 2011). Altered timing at both the inter- and intra-specific level has been compared (e.g. Cabbage and Mabee 1996; Mabee and Trendler 1996; Mabee et al. 2000) and the importance of the relationship between these two levels has been discussed (e.g. Strauss 1990; Reilly et al. 1997; Mabee et al. 2000; Holtmeier 2001; Spicer et al. 2011 – Appendix 1), with Reilly et al. (1997) commenting that, “it is the interaction of intraspecific heterochrony [altered timing] and phylogenies that produces the interspecific patterns we observe”. However, studies focusing on how variation in developmental event timing is distributed at different, intraspecific biological

levels are extremely rare (Kawajiri et al. 2009), despite the fact that this distribution may be pivotal to the relationship between altered timing at the inter- and intra-specific levels. A notable exception is the study by Kawajiri et al. (2009), which compared fin development in a northern and southern Japanese population of an amphidromous medaka, *Oryzias latipes*. This study revealed differences between populations in the time of onset of fin development and growth rate relative to body size, with southern fish showing earlier and more rapid fin growth than northern fish. The authors suggest that delayed development in the northern population might be a trade-off for faster overall growth which has evolved as an adaptation to cope with a shorter growing season (Kawajiri et al. 2009).

Here, I found intraspecific variation in the developmental event timing of *R. balthica* embryos to be pervasive (Chapter 2-5) and that the distribution of this variation at different biological levels did not fit with previous predictions (see below; cf. Spicer and Gaston 1999 Ch. 4; Chown et al. 2002). In Chapter 3 I investigated how variation in embryonic event timing was partitioned in embryos from populations originating from across *R. balthica*'s latitudinal range (from the north of Sweden to the south of France). This study revealed that the timing of four cell division had most variance at the egg mass level, but that events later in development had more variance at the level of inter-individual, than egg mass, or population. The inclusion of egg volume in the ANOVA model also affected the partitioning of variance in event timing, increasing inter-individual and decreasing egg mass variance. Based on the findings of Chapter 2 (Tills et al. 2011), which suggested a genetic basis for altered developmental event timing, within a single population, it might have been predicted that variation would be



even greater at higher biological levels, but the findings of Chapter 3 indicate that this developmental divergence does not extend to the level of inter-population.

An explanation for this lack of scaling from within, to between, population differences in event timing might lie with examination of the population genetics of *R. balthica*. Recent work with *Radix balthica* has revealed that mediated transport, via migratory birds, has led to considerable mixing along a north-south trajectory in populations from across Europe, to the extent that there is no relationship between geographic and genetic distance in this species, even when comparing populations separated by over 2 500 km (Pfenninger et al. 2011; Haun et al. *in press*). These studies also showed that many of the most genetically distant populations were separated by only a few kilometers. Chapter 3 uses populations from along this migratory route and therefore considerable mixing between populations is a probable explanation for the low levels of population differentiation discovered.

The population genetics of *R. balthica* at smaller geographical scales is also surprising, with single, small, populations commonly containing distinct lineages which persist side-by-side, with limited inter-breeding, probably due to a mixed-mating structure (Jarne and Delay 1990; Jokela et al. 2006; Pfenninger et al. 2011; Haun et al. *in press*). Hence, it appears that this genetic divergence within a single population of *R. balthica* can be greater than that between populations separated by thousands of kilometers. This pattern could explain why, in this case, differences in developmental event timing did not extend to the population level (Chapter 3). The prevalence of variation at lower (inter-individual and egg

mass) rather than high (population) biological levels, highlights that future work aiming to link altered timing at the inter- and intra-specific levels should incorporate lower biological levels than might ordinarily be considered. It will, perhaps, only be through incorporating lower taxonomic levels that we can begin to understand how natural selection on intraspecific altered timing could lead to heterochrony.

Variation is prevalent at all levels in Biology and this variation is best resolved by increasing the resolution of biological data (discussed in Spicer and Gaston 1999). First identifiable heart function is an event used in Chapters 2-5, however heart rate is also visible beyond this event in the developing embryo and therefore in Chapter 5 I focused, with high temporal resolution, on how this cardiovascular (CV) function develops during embryonic development. I began by analysing the trends of individual embryos' ontogeny of CV function and discovered that this consisted of two Phases: during Phase 1 heart rate increased, but during Phase 2 heart rate either decreased (Trajectory 1) or continued to increase, all be it less rapidly than during Phase 1 (Trajectory 2) (Fig. 5.4). An analysis of these patterns of heart ontogeny revealed considerable variation, in both the timing of events and the rates of change in heart rate during each Phase. There were significant differences: i) between egg masses in the time during ontogeny at which the switch between Phases 1 and 2 occurred; and ii) between populations in the rate of decrease in heart rate during Phase 2, in embryos following Trajectory 1.

Despite the occurrence of two Trajectories (positive or negative) in Phase 2 of CV development, when mean values for embryos following each of these

different trajectories were plotted, both groups of embryo appear to show decreasing heart rate during Phase 2 (Fig. 5.8). The 'cancelling-out' of two different Trajectories when using mean values highlights the importance of analysing ontogenies of individuals, rather than groups of individuals (Spicer and Gaston 1999). The level of intraspecific variation reported here, in both developmental event timing and CV functional development, should perhaps serve as a warning to those comparing ontogenies of pooled groups of individuals, without first having a detailed understanding of the different ontogenic patterns individuals follow. In Chapter 5 this detailed study of individual ontogenies was fruitful, but would have, perhaps, been considerably less so had individual ontogenies not been analysed.

Variation in the development of *Radix balthica* appears to be predominantly at low biological levels. Given that this species has considerable mixing between populations across large geographic distances, yet isolated lineages within single populations, the distribution of variance reported here is perhaps not surprising. Future work with *R. balthica* aimed at understanding the importance of intraspecific variation in altered timing to the formation of heterochrony will require detailed knowledge of both the population and inter-individual genetics, as geographical distance cannot be relied on as a mode of isolation in this species. Pfenninger et al. (2006) investigated the taxonomy of the *Radix* genus within Europe and discovered that species identification based on morphological characters was unreliable due to continuous variation within and between species in traits such as shell shape. A genetic analysis of mitochondrial COI sequence showed that five Molecularly defined Taxonomic Units (MOTU) could be distinguished. Further work with *R. balthica* could

consider relationships between developmental event timing and genetic differentiation within and between the five MOTUs described for *Radix*. Such a research strategy will allow investigation of variation in altered timing from the inter-individual up to the inter-specific level and help to address the paucity of our understanding of the relationship between variation within and between these different biological levels.

### **6.3 A genetic basis for altered timing**

Variation is a requirement for natural selection to act (Darwin 1858), but a knowledge of the source of this variation is required in order to understand its evolutionary implications (Falcolner and Mackay 1996). Inter-individual variation in a trait can occur through: i) experimental error; ii) environmental influence; or iii) genetic variation (Spicer and Gaston 1999, p 98). For intraspecific variation to be the raw material from which heterochronies originate, such variation must have a genetic basis. Assessing whether there is a genetic basis for intraspecific altered developmental event timing is therefore of paramount importance for determining whether variation at the intraspecific level might be the raw material from which interspecific heterochronies form.

In this thesis I assessed whether intraspecific variation in event timing in *R. balthica* had a genetic basis using both a genetic analysis of a wild population (Chapter 2; Tills et al. 2011), and a laboratory breeding study (Chapters 4 and 5). Analysis of a wild population allowed me to assess whether a genetic basis for differences in developmental timing was present *in situ*, whereas, the laboratory breeding study enabled me to investigate cross-generational similarities in developmental event timing. Together these approaches let me

assess whether there was a genetic basis for altered timing within both an ecological (Chapter 2; Tills et al. 2011) and evolutionary (Chapters 4 and 5) context.

In Chapter 2 I investigated the relationship between inter-individual pairwise genetic distance and the magnitude of pair-wise difference in developmental event timing, using eggs collected from a small population of *R. balthica*. This study revealed a significant positive inter-individual relationship in the magnitude of difference between genetic similarity and the timing of developmental events, indicating the presence of a genetic basis for developmental event timing (Tills et al. 2011). Embryos with no difference in the sequence position, within their developmental itinerary, of the first identifiable heart beat, were also more genetically similar than embryos with a difference in the position of this event, suggesting that the sequence position of this event might also have a genetic underpinning. In Chapters 4 and 5 I used a laboratory breeding study to test whether this suggested genetic basis for event timing was detectable within a single generation, *via* a parent-offspring comparison of embryonic developmental event timing. These investigations revealed significant parent-offspring similarity in all twelve of the developmental events studied (Chapter 4) and in the timing of the breakpoint in cardiovascular functional development (Fig. 5.7) (Chapter 5). Further, in Chapter 4, for two developmental events (foot attachment and crawling) this similarity between parents and their offspring was sufficiently strong for the timing of these events to be classed heritable.

A parent-offspring comparison has the limitation that maternal effects (e.g. maternal investment) cannot be controlled for in the experimental design (Falconer and Mackay 1990), however in both Chapters 4 and 5 I factored egg volume into my analyses and the similarity between parents and their offspring was still present, suggesting, as in Chapter 2 (Tills et al. 2011), that intraspecific altered event timing has a genetic basis.

These findings from Chapters 4 and 5 highlight that the prevalent view of heterochrony (altered timing of developmental events between ancestors and descendants) as an interspecific phenomena is too restricted and that altered timing with a genetic basis can be observed at the very lowest evolutionary level of a parent (ancestor) and their offspring (descendant). Such heritable intraspecific developmental event timing might provide the raw material from which heterochronies arise, accompanying or perhaps even driving speciation events, and therefore this is a fundamental link between ontogeny and phylogeny (discussed in Spicer 2006). It is therefore perhaps at this level that we should investigate the evolvability (i.e. the ability to produce heritable phenotypic variation (Kirschner and Gerhart 1998)) of heterochrony (discussed in Minelli and Fusco 2012), and also the link between heterochrony and heterokairy (discussed in Spicer et al. 2011 – Appendix 1).

Experimental evolution, has the potential to be a powerful tool with which to observe heterochrony “in action”, in living animals (Garland and Rose 2009), and it will perhaps only be through observing the process of altered timing through successive generations that we will truly learn the role of heterochrony in evolution. Here, I performed a relatively simple parent-offspring comparison,

but more elaborately designed breeding studies should be a focus of future work. For example, a half-sib breeding design (comparison of siblings which share a single parent) would allow investigation of both maternal and paternal effects and therefore provide clearer evidence of the extent to which altered timing has a genetic basis (Lynch and Walsh 1998).

In this thesis, heritability was revealed using isolated *R. balthica* and this has demonstrated the possibility of culturing inbred lines, with differences in developmental event timing. Such lineages would be a valuable resource for addressing a multitude of questions relating to the genetic underpinning, heritability and implications of altered timing. Use of artificial selection with differences in developmental event timing would also provide the opportunity to address questions such as how variation in event timing compares between control and stress (heterokairy) conditions, and to what extent the magnitude and direction of heterokairy is influenced by the developmental timing observed under control conditions. Answers to these questions will be necessary for understanding how heterokairy fits with the findings presented in this thesis (see Spicer et al. 2011 – Appendix 1 for a fuller discussion).

#### **6.4 Implications**

Inter-individual variation must have implications for Darwinian fitness for evolution to proceed via natural selection (Darwin 1859). The step of linking variation in early development to Darwinian fitness, or proxies for Darwinian fitness such as size, is rarely performed, however it is important for understanding the implications of variation in early development. The effect of altered development on individuals is ultimately the deciding factor in whether a

particular pattern of development contributes an advantage, or disadvantage, to the developing, or developed, individual, and therefore whether it is adaptive. Richardson et al. (2009) is one of only a few studies to have considered how natural selection might have acted to produce heterochronies that are observable at macroevolutionary scales. This study investigated phylogenetic trends in tetrapod limb heterochrony and the developmental mechanisms underpinning limb formation and revealed that, despite a comprehensive knowledge of tetrapod limb evolution, current knowledge was insufficient for identifying developmental changes and selection pressures that might have resulted in limb heterochronies.

In Chapter 5 I focused on timing in the development of CV function and revealed two Trajectories during the second Phase of this development; with embryos having either an increasing (Trajectory 2) or a decreasing (Trajectory 1) heart rate. These different Trajectories were followed with approximately equal frequency within both populations and egg masses. Embryos following each Trajectory had no significant difference in growth rate from 24 – 72 h – the time during ontogeny that broadly coincided with Phase 1 of CV development. However, from 72 – 144 h, which was broadly when Phase 2 of CV development occurred, embryos that follow Trajectory 1 (decrease in heart rate) grew significantly faster and hatched larger, than those following Trajectory 2 (increase in heart rate). It therefore appears that slower growth rates in embryos following Trajectory 2 during late embryonic development may result from a trade-off between heart rate and higher levels of energy investment in growth.



Chapter 5 did not follow hatchling snails beyond the embryonic period and therefore it is not clear if embryos following Trajectory 1 would have had a higher Darwinian Fitness than those following Trajectory 2. *Radix balthica* are known to exhibit plasticity in growth rate (Lakowitz et al. 2008; personal obs.) and it is possible that snails hatching smaller could display catch up growth. *In situ*, *Radix balthica* would also usually develop within a gelatinous egg mass and previous work has revealed hatchlings can spend several days feeding on the algae which has grown on this mass (Smirthwaite et al. 2007). It would be interesting to investigate whether snails hatching at different sizes display differences in their post-hatch feeding behavior, or whether they have differences in physiology that persist after hatching.

Here, the occurrence of Trajectories 1 and 2 occurred without any intentional change to the embryonic environment, however these differences might be in response to different environmental conditions within the egg capsule, for example nutrient levels, oxygen availability or concentration of metabolites (Morrill 1982; Moran and Woods 2007). Differences in size at hatching for embryos following each of these trajectories demonstrates an effect of embryonic CV development on life history, however future work should try to reveal the implications on juvenile and adult development and ultimately whether this translates to affecting Darwinian fitness.

Most studies that have made the link between altered event timing and its implications to the organism, have been done so within the context of an environmental stressor and therefore have investigated implications of heterokairy, e.g. the effect of hypoxia on the onset of respiratory regulation

(Spicer and El-Gamal 1999) and effect of hatching plasticity to risk of predation (Gomez-Mestre et al. 2008). However, to my knowledge, no studies exist which investigate the implications of altered intraspecific developmental event timing in response not to an environmental stimulus, but due to genetic differentiation. A key next step in understanding the altered timing reported in this thesis will be to identify whether such intraspecific altered timing has implications to Darwinian fitness. To understand these implications, they will also need to be assessed in individuals cultured under environmentally relevant conditions, as subtle changes to an organism's development, through altered timing, might only prove adaptive within specific environments.

## **6.5 Final conclusion**

Altered timing of developmental events is evident in Biology at all evolutionary scales (discussed in Spicer et al. 2011 – Appendix 1), including both inter-specific (c.f. Jeffery et al. 2002; Smirthwaite et al. 2007) and intraspecific (c.f. Warkentin et al. 2005; Chapters 2-5). Only recently, however, has it been suggested that the study of intraspecific altered timing might prove a viable research vehicle for linking differences in event timing between these scales (Minelli et al. 2012). This thesis has adopted an integrative approach to the study of altered timing at the intraspecific level, incorporating both morphological and physiological developmental events, and has studied the timing of these events in the embryonic development of the pond snail *Radix balthica* with very high temporal resolution. This approach has revealed that variation in event timing appears to have a genetic basis and that the timing of several of these events are heritable. Heritable intraspecific event timing represents a fundamental link between ontogeny and phylogeny and this opens

up exciting new opportunities for understanding how the macroevolutionary pattern of heterochrony might develop.

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#### **APPENDIX 1**

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