Phenomics: An integrative approach to Comparative Developmental Physiology

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By

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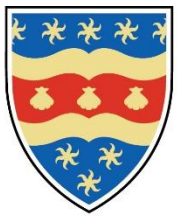

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Author's Declaration

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.

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Conferences

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Abstract

Phenomics, the high-throughput acquisition of phenotypic data at the scale of the whole organism, has led to considerable advancements in plant biology and medicine, yet applications within animal environmental physiology and evolutionary biology are rare. The main aim of this thesis is to understand what phenomics can contribute to our understanding of Comparative Developmental Physiology (CDP), in terms of how environmental and evolutionary change affects the development of physiological function in aquatic embryos. Additionally, I aim to understand the consequences of changes in high-dimensional phenotyping methods used throughout the thesis within the context of embryonic life history. To achieve this, I characterised evolutionary and temperature induced changes in high-dimensional phenotypic space in embryos of three species of freshwater snail with pre-established sequence heterochronies, evolutionary differences in the relative timings of developmental events. This was achieved through the use of a novel video based approach to phenomics in developing embryos termed 'Energy Proxy Traits' (EPTs) that integrate aspects of embryonic physiology and behaviour as a spectrum of energy. I found that evolutionary and intraspecific differences in developmental event timings were associated with high-dimensional phenotypic change, which may themselves act as objects of selection (Chapter 2). Additionally, EPTs revealed interspecific differences in whole-embryo sensitivities to chronic elevated temperature regimes, and considerable differences in responses between

different physiological windows of development. EPTs were transferable between species that vary greatly in their developmental itineraries, and physiological windows of development that vary in their observable phenotypes (Chapter 3). Finally, I quantified changes in EPTs alongside life history traits, following serial experimental manipulation of nutrient content of developing embryos, with the aim of understanding how induced changes in EPTs affect other aspects of embryonic development (Chapter 4). Increases in total energy and concomitant reductions in size and rates of growth following removal of nutrient content suggest a trade-off between these variables, and a potential re-allocation of energetic reserves following removal of nutrient content in these embryos. In summary, EPTs enabled the continuous acquisition of physiological time series', revealing evolutionary and environmentally induced changes in high-dimensional phenotypic space, changes which may have consequences for aspects of embryonic life history.

Contents

Chapter 2. Comparative phenomics as an approach to study heterochrony

Figures and Tables

Chapter 1

What can phenomics contribute to Comparative Developmental Physiology?

Understanding how evolutionary and environmental change influences physiological development is a central aim of Comparative Developmental Physiology (CDP), and an important consideration of Biology more broadly (Warburton *et al*., 2006). Early development is a period of considerable functional, spatial and temporal change, and developmental responses to environmental change are highly multifaceted, comprising a multitude of individual trait changes. Faced with such a complex array of phenotypic change, most researchers typically opt to quantify changes in small, but carefully chosen sets of observable traits with some pre-established functional significance (Forsman, 2015; Lürig *et al*., 2021). However, doing so can introduce 'elements of chance' into the selection of traits that ultimately contribute to our questions, biological endpoints of interest, and the conclusions we draw (Houle *et al*., 2010; Forsman, 2015).

Phenomics, the high-throughput acquisition of phenotypic data at the scale of the whole organism, is posited as a solution, incorporating a more systematic and global approach to the study of the phenotype, and it has led to significant advancements in fields in which it has been adopted. A key enabler of phenomics, most notable for medicine and plant biology, is the availability of technologies capable of measuring phenotypic change with high dimensionality, incorporating the collection of data with greater temporal, spatial and functional resolution, across greater sample sizes (Furbank, 2009; Furbank & Tester, 2011; Tardeiu *et al*., 2017; Zhang & Zhang, 2018). Phenomics technologies and approaches to date, have been highly specific to particular model systems and this has limited its applicability more broadly in biology, including key ecological and evolutionary questions surrounding the development of physiological function.

In this introduction, I first summarise the current state of phenomics as an approach, including its applications and current methodologies, before discussing what phenomics can contribute to CDP, and to the more recent convergence of Developmental Integrative Physiology.

1.1 Phenomics – an introduction

Understanding the overwhelming complexity of organismal biology has long resulted in biologists restricting phenotyping efforts to small numbers of observable traits. Whilst providing a tractable approach to understanding biological processes, restricting phenotyping efforts to single, or small numbers of traits is a gross oversimplification of organismal biology (Lürig *et al*., 2021). Understanding the relationships between phenotypes, genotypes, environmental changes, and processes at other levels of biological organisation has been carried out using single traits, but the task of measuring considerable numbers of observable traits is just beginning (Houle *et al*., 2010). Conceptualisation of the 'phenome' to describe the phenotype as a whole can be attributed to microbial physiologist Bernard D. Davis (1949), in which he and molecular geneticist Werner K. Maas coined the term to describe 'the sum of extragenic, non-autoreproductive portions of the cell, whether cytoplasmic or nuclear'. In essence, the phenome is described in this paper as a representation of the phenotype as a whole, and is analogous to how the genome represents the complete set of genetic information of an organism (Davis, 1949), a view that was solidified by Michael Soulé (1964) who described the phenome as 'the phenotypic analog of the genome'. The term 'phenomic' as a descriptive term also originates from the work of Davis (1949), in which the term 'phenomic lag' is used to describe delays in translation from the genome to phenome. However, coinage of the term 'phenomics' in reference to the study of the phenome is frequently attributed to Steven A. Garan in 1996 (Yu & Fang, 2009; Shi *et al*., 2014; Jin, 2021). Irrespective of its origins, phenomics is currently contextualised as an approach involving the acquisition of

phenotypic information at these genome wide scales, and has frequently been regarded as such in reviews and textbooks (Bilder *et al*., 2009; Furbank & Tester, 2009; Houle *et al*., 2010; Hancock, 2014; Tardeiu *et al*., 2017).

Evolutionary geneticist David Houle *et al.* (2010) highlighted that phenomics, whilst proposed by multiple authors as the natural complement to genomics (Schork *et al*., 1997; Freimer & Sabatti, 2003; Bilder *et al*., 2009), was initially met with indifference, implying quantification of small numbers of phenotypes relevant to the biological process or system of interest was deemed sufficient for the understanding of these processes. Similarly, funding of the Human Genome Project was initially met with opposition as it was argued that reductionist approaches involving selection of regions of interest of the genome were sufficient for its understanding (Houle *et al*., 2010; Moraes & Goés, 2016). However, the Human Genome Project led to considerable advancements including the discovery of genetic variants underlying various diseases, and stimulated major progression in bioinformatics and computational biology, outpacing the rate at which phenotypic information could be acquired at similar scales (Gibbs, 2020). Whilst selection of phenotypes of interest depends fundamentally on the biological question being asked, adoption of high-dimensional phenotyping approaches has led to significant advancements in linking genetic changes to biological endpoints such as disease (Denny *et al*., 2010; Pendergrass *et al*., 2011; 2013; Özdemir, 2020), and characterising responses to toxicants (Audira *et al*., 2020a; 2020b; 2021a; 2021b; Hussain *et al*., 2020), but also in assessing responses to environmental change, particularly within the plant biology literature (e.g. Warringer *et al*., 2003; Schnaubelt *et al*., 2013; Singh *et al*., 2018; Adhikari *et al*., 2019; Li *et al*., 2019; Marsh *et al*., 2021; Tills *et al*., 2021; 2022). Houle *et al.* (2010) argued that limiting the number of phenotypic traits measured to those with some pre-established functional significance can obscure the identification of potentially important traits implicit in the biological response or endpoint of interest, and that only by measuring large numbers of phenotypic traits at the scale of the whole organism can we identify the traits that really matter. The explosion of literature adopting high-dimensional phenotyping approaches in the plant

biology and medical literature within the last decade is testament to this. It remains the case however that the integration of phenomics into research areas outside of these disciplines, and to non-model organisms remains underdeveloped.

Initially, within fields which now see widespread adoption and successful application of high-dimensional phenotyping approaches, a major bottleneck to their application was a lack of transferable infrastructure and technologies capable of acquiring data at these scales (Bilder *et al*., 2009; Furbank & Tester, 2009; Tardeiu *et al*., 2017). The availability of such technologies that are capable of incorporating the sample sizes, number of phenotypes measured, precision of measurements, and in the case of longitudinal studies, the temporal resolution with which these measurements are made, was initially lacking (Furbank & Tester, 2009; Singh *et al*., 2014). Recent decades have seen considerable advancements and investment in such technologies, particularly within medicine and plant biology, and these enablers of phenomics have undoubtedly been key to the development of the approach in these fields. In medicine, transferable technologies are capable of whole body imaging, and phenome wide association studies have allowed biologists to uncover the genetic causations of complex traits and disease (Denny *et al*., 2010; Pendergrass *et al*., 2011; 2013; Özdemir, 2020). In plant biology, imaging technologies for high-throughput imaging on an industrial scale in both field and greenhouse environments allow for the quantification of multiple proxies of plant performance, function and growth in response to environmental stress and disease, thereby allowing for trait-based breeding for improving crop yield (Baker, 2008; Finkel, 2009; Rajendran *et al*., 2009; Furbank & Tester, 2011; Großkinsky *et al*., 2015; Neto & Borém, 2015; Gustavo *et al*., 2017; Underwood *et al*., 2017; Razzaq *et al*., 2021).

Usage of the term phenomics varies considerably within the literature. Phenomics is frequently regarded as a means of linking genes to phenotypic change (Schork, 1997; Bilder *et al*., 2009; Houle, 2010; Hancock 2014; Fuentes *et al*., 2018), however, defining phenomics within the context of linking genetic changes to phenotype is overly restrictive. For example, quantification of phenotypic change is necessary for understanding the relative performance

of an organism when subjected to environmental drivers. Applications are beginning to move away from this gene-centric view of phenomics, particularly within the ecotoxicological literature (e.g. Audira *et al*., 2020a; 2020b; 2021a; 2021b; Carvalho *et al*., 2020; Hussain *et al*., 2020; Rodrigues *et al*., 2021), and in assessing responses of commercially important crop species to environmental stress (e.g. Schnaubelt *et al*., 2013; Singh *et al*., 2018; Adhikari *et al*., 2019; Marsh *et al*., 2021). However, given these differences in definition, for the sake of consistency within this thesis I define phenomics as the high-throughput acquisition of phenotypic data at organism wide scales (*sensu* Houle *et al*., 2010), and I define the phenome as the phenotype of an organism as a whole, including the sum of its morphology, physiology and behaviour (*sensu* Keller *et al*., 1992).

1.2 Phenomics and its current applications

1.2.1 Identifying the genetic underpinnings of phenotypic traits

Over the last 20 years, the genomics revolution has facilitated rapid advancements in biology, and the advent of high-throughput sequencing technologies, and computational methods have enabled biologists to profile gene expression with unprecedented throughput and sensitivity (Mortazavi *et al*., 2008; Rapaport *et al*., 2013). The widespread implementation of 'genome-wide association studies' (GWAS) during the early 2000's lead to important discoveries of the functional significance of thousands of single nucleotide polymorphisms (SNPs). SNPs were often used as an approach in plant biology for identifying the genetic basis of traits related to crop yield (Eshed *et al*., 1996; Xue *et al*., 2013), stress resistance (Varshneya *et al*., 2012) and disease resistance (Gebhardt & Valkonen, 2001), and in medicine for understanding the genetic causation of complex diseases such as type II diabetes (Sladek *et al*., 2007), and intermediate phenotypes contributing to disease (Hindorff *et al*., 2009; Lou *et al*., 2009).

However, significant disparities between the wealth of acquired genetic information and an understanding of how genotype translates to functional and performance outcomes, were obvious, owing mainly to a lack of transferable technologies permitting the high-throughput acquisition of phenotypic data at sufficient scales (Furbank, 2009; Perez-Sanz *et al*., 2017; Tardieu *et al*., 2017). Over the last decade, 'phenome wide association studies' (PheWAS) have been (and continue to be) successfully applied as an alternative method for identifying new relationships between phenotypes and large amounts of acquired genetic information (e.g. Pendergrass *et al*., 2011; 2013; Alexandrov *et al*., 2016). GWAS typically focus on a specific phenotypic outcome alongside global genomic data, whereas in PheWAS approaches, this logic is reversed, instead characterising the associations between a small number of common genetic variants, and a large number of phenotypic traits (Hebbring, 2014). PheWAS approaches also allow geneticists to uncover pleiotropic effects, in which a gene influences two or more seemingly unrelated phenotypes (Pendergrass *et al*., 2013). PheWAS approaches have also been successful in advancing understanding of gene function, including in mammals (Meehan *et al*., 2017; Brown *et al*., 2018), and commercially important crops (Borrill *et al*., 2018). A frequently cited example showcasing the success of PheWAS is that of Alexandrov *et al*. (2016), in which machine learning was applied to >3000 behavioural traits in mice, revealing integrative phenotype signatures that accurately predicted genotypes associated with Huntington's disease.

1.2.2 Predicting biological outcomes

Adopting a similar approach to that of PheWAS, the phenotype can also be quantified alongside specific biological outcomes, including performance and fitness differences, as well as clinical endpoints such as morbidity and disease (Alexandrov *et al*., 2016; Davatzikos *et al*., 2018). Whilst an understanding of the genetic underpinnings of observable differences in

phenotype can allow biologists to determine the functional significance of particular genes, risk factors associated with disease, and select crop genotypes with desirable traits (Furbank, 2009; Lobos *et al*., 2017; York, 2019), understanding how phenotypes interact with their environment to produce observable differences in performance and fitness, is only resolved through direct quantification of phenotype. Selection acts on variations in fitness mediated through an individual's phenotype, and its interaction with the environment (Lande & Arnold, 1983; Laughlin & Messier, 2015) (Fig. 1.1). Predicting how these fitness outcomes are driven by differences in the phenotype provides insight into the functional significance of aspects of morphology, physiology and behaviour under different environments, the sensitivity of populations to environmental change, and the potential for individuals to adapt over relatively short time scales (Kültz *et al*., 2013; Moczek *et al*., 2011; Nettle & Bateson, 2015).

Measurement of multiple traits in a combinatorial fashion is necessary to understand the relative influences of traits on fitness outcomes, and is an approach aligned with the theory behind multivariate selection (Lande & Arnold, 1983; Kingsolver & Pfennig, 2007; Crean *et al*., 2011; Forsman, 2015; Fig. 1.1). Phenomics approaches are uniquely suited to investigate the relative contributions of multiple traits to aspects of organismal fitness. Traditionally, studies investigating the relationships between phenotypes and their outcomes quantify change in small numbers of phenotypes (Forsman, 2015). Whilst this may be adequate in targeting particular phenotypes to understand their functional significance, multiple authors argue that measurement of only a limited numbers of phenotypes introduces a significant element of chance over whether traits contributing to our biological responses of interest have been correctly identified, and the potential therefore for misinformed conclusions (Houle *et al*., 2010; Kültz *et al*., 2013).

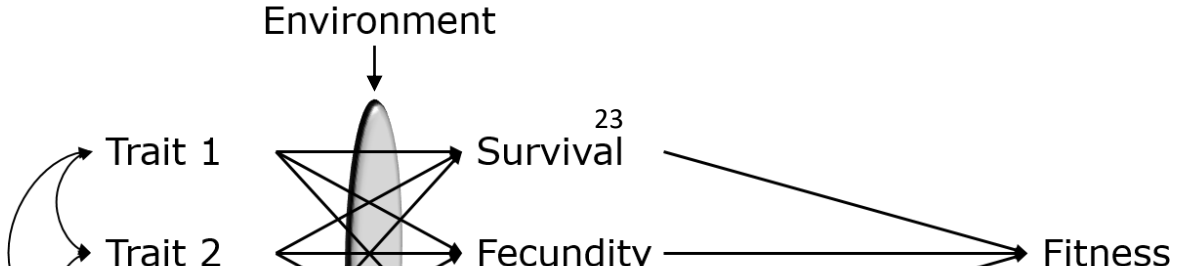

Figure 1.1. Simplified pathway model adapted from Arnold (1983) and Laughlin & Messier (2015) illustrating relationships between multiple phenotypic traits, and components of performance contributing directly to organismal fitness. Traits interact with the environment and each other to produce differences in performance that act as proximate drivers of fitness, expressed here as survival, fecundity and body size. Covariance is also present between multiple phenotypic traits, shown as curved arrows. Covariance between traits and how these interact with the environment to produce differences in fitness underpins a multitude of biological process including population viability and community dynamics.

More recently, phenomics approaches have been proposed as a method of linking phenotype to plant performance and fitness, through the emerging field of 'functional phenomics' (York & Lynch, 2015; York, 2019). Use of statistical techniques including multiple pairwise regression are then applied to acquired data allowing new relationships between phenotype and outcomes related to plant performance and fitness. Following statistical analyses, relationships can then be further investigated using targeted physiological studies, allowing for the selection of genotypes with desirable traits for breeding (York *et al*., 2013; York, 2019).

1.2.3 Measuring responses to environmental change

Responses to environmental change are typically composed of changes to a broad suite of phenotypes across various levels of biological organisation (Forsman, 2015; Perrichon *et al*., 2017; Iverson *et al*., 2020), yet due to methodological limitations, research typically opts for reductionist approaches, selecting small numbers of traits that are easily measurable and that provide broad indicators of organismal performance (Houle *et al*., 2010; Tills *et al*., 2021). In plant biology, phenomics is increasingly applied with the purpose of understanding how different environmental drivers, including increases in temperature, salinity and drought stress, influence the morphology and physiology of crop species (Jones *et al*., 2009; Costa *et al*., 2013). Methods for characterising these changes frequently rely on analysis of images of plant specimens through the use of computer vision methods, for example for the automated measurement of plant shape and size (Furbank & Tester, 2011; Zhang & Zhang, 2018), as well as other proxies of physiological performance, such as the use of thermography and fluorescence imaging in crop species, acting as indicators of physiological performance (Jones *et al*., 2009; Costa *et al*., 2013; Zia *et al*., 2013).

Phenomics is also beginning to extend to understanding the effects of forms of environmental stress on animal species, for example in understanding the effects of chronic and acute exposure to elevated temperature regimes in species of aquatic invertebrates (Tills *et al*., 2018; 2021), and interactive effects of salinity and temperature (Tills *et al*., 2022). However, application of phenomics approaches within the context of animal environmental physiology, as well as more broadly within ecological and evolutionary research is currently significantly underutilised (Lürig *et al*., 2021), despite acquisition of high-dimensional phenotyping data arguably needed for uncovering the causal links between environmental factors, genotypes and phenotype (Houle *et al*., 2010).

Finally, phenomics is beginning to be utilised in aquatic toxicology for characterising the effects of environmentally relevant toxicants on model species (Kalueff *et al*., 2016). In particular,

development of behavioural phenomics for model species such as the zebrafish *Danio rerio* has allowed biologists to investigate the neurotoxic effects of various environmental contaminants such as lead (Li *et al*., 2019), silica nano-particles (Li *et al*., 2020; 2021) and others (Audira *et al*., 2020; 2021; Hussain *et al*., 2020).

1.3 Acquisition of high dimensional phenotypic data

Phenomics is often referred to as being both complimentary and analogous to genomics, and having the potential to facilitate similarly rapid advances in our understanding of whole-organismal biology (Houle *et al.*, 2010). However, whilst a genome can be sequenced relatively cheaply and quickly, and relies on technologies that are transferable between species and experimental designs, the challenges faced by biologists in characterising the phenome are formidable. Advancements in technologies capable of capturing phenotypic data at these scales have been made over recent years, particularly in application to model species (*Danio rerio* – Xu *et al*., 2010; Pelkowski *et al*., 2011; Peravali *et al*., 2011; Spomer *et al*., 2012, *Caenorhabditis elegans* – White *et al*., 2010; Olmedo *et al*., 2015, *Drosophila melanogaster* – Dagani *et al*., 2007; Chung *et al*., 2010; Levario, 2016). These typically involve the use of non-invasive bioimaging platforms, allowing measurement of a range of phenotypes with high temporal resolution (Furbank & Tester, 2011).

1.3.1 Bioimaging for phenomics

Non-invasive bioimaging platforms allow for the measurement of a multitude of morphological, physiological and behavioural phenotypes with high levels of temporal and spatial resolution (Truong & Sapatto, 2011; Perez-Sanz *et al*., 2017; Pound *et al*., 2017; Zhang & Zhang, 2018). Bioimaging approaches have seen particularly widespread application to plant systems (Furbank & Tester, 2011), and model animal species (Chung *et al*., 2010; Pelkowski *et al*., 2011; Olmedo *et al*., 2015), and follow the generalised workflow outlined below.

Specimens are first prepared and mounted onto suitable platforms from which they can be imaged. Preparation of specimens within suitable platforms for imaging is often the factor that most greatly limits throughput, as these stages are typically carried out manually by a practised user. However, there are a number of methods for sorting and immobilisation of embryos and early life stages of these models in an automated fashion, and these increase throughput, notably through the use of microfluidic and automated flow through arraying platforms (Chung *et al*., 2008; Pardo-Martin *et al*., 2010; Cornaglia *et al*., 2015). An advantage to flow through culture methods is the ability to manipulate physical parameters *in vivo,* including temperature (Luchetta *et al*., 2005; Dagani *et al*., 2007) and oxygen gradients (Levario *et al*., 2016; Wang *et al*., 2017), and concentrations of teratogens (Yang *et al*., 2011; Akagi *et al*., 2012).

Following sample preparation, image data are then acquired, for which a multitude of visible light camera systems (i.e. video data in the visible spectrum), multispectral and hyperspectral imaging, stereo vision, optical fluorescence microscopy and confocal microscopy systems have been developed (Krig, 2014; Li *et al*., 2014; Perez-Sanz *et al*., 2017; Zhang & Zhang, 2018; Lürig *et al*., 2020). From these video data, phenotypic traits of interest are then measured (Truong & Sapatto, 2011; Perez-Sanz *et al*., 2017). Images and video first undergo pre-processing in which objects or regions of interest are enhanced, to increase rates of successful feature computation downstream (Krig, 2014; Perez-Sanz *et al*., 2017). Images are then often, but not always, segmented, during which objects of interest are isolated from the background, determined by the similarity in pixel intensities, shape and statistics (Narkhede

2013; Krig, 2014). Segmentation is supported with threshold algorithms, where groups of pixels are either retained or removed based on their intensities (Otsu, 1979), or using Artificial Intelligence (AI) methods whereby a model is trained to identify regions of interest (Perez-Sanz *et al*., 2017). Finally, feature computation methods, in which quantitative values are calculated from segmented objects including object size, shape and intensity, as well as rates and directionality of movement, thereby extracting biologically meaningful measures from segmented images (Krig, 2014).

Motion analysis methods can be used to measure movement parameters including velocity, distance travelled, and direction of movement (Amsler *et al*., 2006; Williams *et al*., 2012; Tinevez *et al*., 2013), however application of these approaches to study systems such as the early developmental stages of organisms is problematic, as individuals exhibit often subtle and complex movements (Rudin-Bitterli *et al*., 2014; Tills *et al*., 2018; 2021; 2022), and exhibit considerable variability in form and function between stages of development. Acquisition of video data allows for the measurement of phenotypic information that spans combinatorial signals from a multitude of physiological, behavioural and morphological traits (Kültz *et al*., 2013; Fuentes *et al*., 2018; Walls *et al*., 2019). Furthermore, 'proxy traits', technology enabled measurements with no direct manual equivalents, can also be measured from video data. Proxy traits have seen particularly successful application in plant phenomics, for example, the use of thermography and fluorescence imaging in crop species, acting as indicators of physiological performance in response to forms of environmental stress (Jones *et al*., 2009; Costa *et al*., 2013; Zia *et al*., 2013; Zhang & Zhang, 2018) and disease (Pérez-Bueno *et al*., 2016; Mahlein, 2016). Proxy traits can also be measured from visible light video data, for example the measurement of fluctuations in pixel intensities to infer physiological and behavioural movement in animal specimens (Tills *et al*., 2013; 2018; Rudin-Bitterli *et al*., 2014; Tills *et al*., 2018; 2021; 2022; Ibbini *et al*., 2022). Responses to environmental change are highly multifaceted, and comprise a multitude of individual trait changes, and responses can vary considerably between different life stages (Forsman, 2015; Pandori & Sorte, 2019).

Consequently, utilising computer vision methods that integrate the complexity of biological responses during early development can provide useful indicators of whole-organism responses to environmental change (Kültz *et al*., 2013).

1.3.2 Analysing phenomic datasets

Phenomics produces high-dimensional datasets, with potentially thousands of predictor-outcome variable relationships (Houle *et al*., 2010; Furbank & Tester, 2009). Furthermore, a lack of transferable approaches and standards means that the analysis and management of phenomics datasets presents a significant challenge, and the progression of phenomics as a discipline may be as limited by our ability to analyse and handle data at such scales as the collection of data themselves (Tardieu *et al*., 2017). When presented with large numbers of predictor variables, dimensionality reduction techniques including principal component analysis (PCA), t-Distributed Stochastic Neighbour Embedding (tSNE), principal component regression (PCR) and Least Absolute Shrinkage Selector Operator (LASSO) regression are typically applied, thereby reducing the numbers of predictor variables *a priori*, and allowing changes to a multitude of response variables to be investigated in a combinatorial manor (Wold *et al*., 1987; Fan & Li, 2006; Tills *et al*., 2021). Such methods also allow for estimation of the contribution of each variable to the overall variance in the dataset. Phenotypes are often also highly correlated with each other (Gillooly *et al*., 2001; Angilletta *et al*., 2004; Marshall & Bolton, 2007), and another advantage to the statistical approaches highlighted above are their ability to maintain performance in the presence of high multicollinearity.

An added challenge of working with large datasets, such as is the case with phenomics, is that multiple hypothesis testing leads to inflated false positive discovery rates when conducting multiple analyses on the same dependent variable (Shear & Zumbo, 2013). To account for this, a number of corrective thresholds are typically applied to reduce the occurrence of Type

I error (Armstrong, 2014; Verma & Ritchie, 2017). However, as well as increasing the prevalence of Type II error, categorising large numbers of predictor variables as having significant or non-significant effects tells us nothing about the magnitude with which predictor variables contribute to our biological response/outcome of interest (Armstrong, 2014; Houle *et al.*, 2010). Additionally to this, false positive corrections typically assume that all tests are independent, however in the case of multiple phenotype measures, phenotypes often interact to produce observable differences in fitness or performance (Angilletta *et al*., 2004; Marshall & Bolton, 2007; Gilooly *et al*., 2001, Hebbring, 2014).

1.4 What can phenomics contribute to the study of physiological development?

Comparative Developmental Physiology (CDP) (Burggren & Warburton, 2005; Warburton *et al*., 2006), and the more recent interdisciplinary convergence of Developmental Integrative Physiology, have at their centre the integration of development, physiology and evolution (Mueller *et al*., 2015a; Spicer *et al*., 2018). Comparative developmental physiologists use biological diversity to investigate an array of important biological questions, including the significance of variation in the timings of development, the role of developmental plasticity in producing evolutionary change (Gould, 1977; 1982; McKinney, 1988; West-Eberhard, 2003; 2005; Warburton *et al*., 2006; Spicer & Rundle, 2007; Spicer *et al*., 2018; Pfennig, 2021), the significance of critical windows during early development (Burggren & Reyna, 2011; Burggren & Mueller, 2015; Burggren, 2020), and the role of epigenetics in the formation of phenotype during early development (Burggren 2014; Burggren & Crews, 2014). Central to the advancement of CDP as a discipline and in improving our understanding of these fundamental questions, is the continued advancement of experimental designs, methodologies, and conceptual frameworks (Mueller *et al*., 2015a; Burggen, 2021a).

1.4.1 Heterochrony – establishing links between development and evolution

Understanding the link between development and evolution is central goal of Comparative Developmental Physiology (Warburton *et al*., 2006). Changes in the timings of developmental processes between ancestors and their descendants termed 'heterochronies', are frequently regarded as the main mechanism linking development to evolution (Gould, 1977; 1982; McKinney, 1988; Smith, 2001; 2003). Research investigating the evolutionary significance of the timings of developmental events has a rich history, commencing with the work of Haeckel and his 'Biogenic law' (Haeckel, 1866). Amongst considerable theoretical objections to this law (Sedgewick, 1909; McMurrich, 1912; Garstang, 1922), the perceived significance of heterochrony in evolution grew following the work of de Beer (1958). Historically, many studies have focussed on rates of growth of morphological structures, as size was frequently used as a proxy for age (Gould, 1977; McKinney, 1988; Spicer & Gaston, 1999; Smith, 2001; 2003), however, more recent research measures changes in the relative timings of developmental events between closely related species (Smith, 2001; Jeffery *et al*., 2002a; 2002b; Bininda-Edmonds *et al*., 2003; Smirthwaite *et al*., 2007). Furthermore, calls for the integration of physiological developmental events shifts the emphasis of event timings on to functional developmental events, which are ultimately the object of selection (Spicer, 2006; Spicer & Rundle, 2006). Currently, heterochrony is regarded as one of the key drivers of evolutionary change (Gould, 1977; 1982; McKinney, 1988; Smith, 2001; 2003).

Early development is frequently regarded as a series of discrete events, a conceptual framework that ultimately act as a mental construct with which to measure changes in the timings of development between closely related species (Cooper & Jaiswal, 2016; Walls *et al*., 2019; Burggren, 2021a). Such developmental events rely on the concept of idealisation and discussion of their limitation has a long and controversial history (Hopwood, 2005). In his recent review, Burggren (2021) argues that we should be moving towards approaches that treat physiological development for what it is – a continuous process rather than a set of

discrete events. However, continuous quantification of physiological change during early development presents a number of difficulties. Methods for acquiring physiological data during this period of massive structural and functional upheaval are limited given the often time consuming and laborious methods employed (Burggren, 1987). Additionally to this, methods used to measure physiological change can be inherently disruptive to the developing organism. For example, quantification of rates of oxygen consumption as a proxy for metabolic rate frequently employs closed chamber respirometry, in which changes in oxygen are recorded over time (Steffensen, 1989; Keller & Steltzer, 2008; Nelson, 2016). These reductions in oxygen levels may in themselves induce trait changes at later periods of development, thereby preventing the reliable acquisition of a continuous physiological time series for an individual organism. Consequently, intra-individual changes in developmental physiology are, owing to the approaches used, frequently inferred from inter-individual comparisons (Spicer & Gaston, 1999; Spicer & Burggren, 2003). One solution to these difficulties is to adopt methodologies based on the visual quantification of developmental phenotypes through the use of video based data.

Earlier in this chapter I discussed the application of bioimaging approaches for the measurement of phenotypic change during early development. Bioimaging approaches allow for the continuous non-invasive observation of early development and have seen successful application in characterising physiological development through a multitude of imaging methodologies (Chambers *et al*., 1995; Phoon & Turnbull, 2003; Schwerte & Fritsche, 2003; Phoon, 2006; Turnbull & Mori, 2007; Boss *et al*., 2008; Bradley *et al*., 2008; Gu *et al*., 2011; Raghunathan *et al*., 2016). For example, computed tomography (CT) scans have been used to describe the *in ovo* development of blood vessels and skeletal structures in embryos of the Ostrich *Struthio camelus* (Winkens *et al*., 2021), and biomagnetism in the form of magnetoencephalography used in the detection of onset of cardiovascular function in embryos of the same species (Freesmeyer *et al*., 2022). Video-based imaging of physiological development is also possible in transparent species, or in those that develop inside

transparent egg cases (for example, Schwerte & Fritsche, 2003; Burggren & Blank, 2009; Shin *et al*., 2010; de Luca *et al*., 2014; Gierten *et al*., 2020; Ibbini *et al*., 2022). For example, Bagatto & Burggren (2005) utilised video-based microscopy to characterise the development of cardiovascular function and vessel development in larval zebrafish *Danio rerio* in 3-dimensions, by immobilising larvae in a rotating agarose cylinder, and imaging the specimen from multiple angles using a video-microscope. Similarly, ontogeny of cardiac physiology was studied throughout stages of embryonic and larval development in the crayfish *Procambarus clarkii*, through the use of video based microscopy (Harper & Reiber, 2004). These approaches are frequently based on acquisition of single or small numbers of traits, however, recent research has begun to characterise developmental transitions in high-dimensional phenotypic space in embryos of freshwater snails (Tills *et al*., 2021). Ultimately, automated bioimaging systems allow for the acquisition of video data at high temporal resolutions during embryonic development, and may provide a powerful means by which to measure evolutionary changes in the timings of development of physiological function (Truong & Supatto, 2011; Tills *et al*., 2013; Burggren *et al*., 2021a).

1.4.2 Measuring developmental responses to environmental change – a role for phenomics?

Recent decades have seen an explosion of literature centred on understanding the impacts of future climate change projections on individual organisms, however, research frequently focuses on mature organisms, rather than their developmental counterparts (Burggren, 2018; 2021). At the centre of the integration of comparative animal physiology with development and evolution, and the subsequent establishment of CDP and Developmental Integrative Physiology (Burggren, 1998; 2000; Burggren & Warburton, 2005; Warburton *et al*., 2006; Mueller *et al*., 2015a), is the investigation of developmental plasticity - the interspecific and intraspecific variation in the developmental phenotype associated with exposure to changes

in environmental conditions (Spicer *et al*., 2018). Phenotypic responses to environmental change during early development often comprise complex changes to multiple aspects of morphology, physiology and behaviour, often necessitating the measurement of broad indicators of organismal performance and fitness (Hochachka & Somero, 2002; Forsman, 2015; Iverson *et al*., 2020). Whilst these are invaluable in providing measures of organismal performance and fitness under future climate change projections, there is inherent value in 'breaking down' these broad scale measures of organismal performance. A developing organism is a functionally integrated unit, and a genotype when interacting with an environment may show plasticity in suites of traits, whereas others show little or no response. Similarly, absence of plasticity in the observed traits in response to an environmental variable may be compensated for by plasticity in some other physiological or behavioural mechanism (Pigliucci and Preston, 2004; Houle, 2007; Valladares *et al*., 2007; Whitman and Agrawal, 2009). Additionally, species exhibit plasticity in different suites of traits in response to different environmental stressors. For example, Rundle *et al*., (2010) showed that when exposed to predatory cues, embryos of the pulmonate gastropod *Radix balthica* showed significant differences in the timings of both initiation of mantle muscle flexing and crawling. However, in embryos of the same species exposed to salinity stress, differences were observed in the timings of the first heartbeat, eye spot formation and foot attachment to the wall of the egg capsule (Tills *et al*., 2010). Consequently, the complexity of organismal responses to environmental change arguably make adoption of a phenome-level perspective necessary if we are to make robust estimates of the responses of individuals to environmental change (Forsman, 2015).

Current high-dimensional phenotyping efforts are largely limited to small numbers of model species, however to broaden our understanding of the consequences of global environmental change on animals occupying marine and freshwater environments, we urgently need phenotyping approaches that are applicable to the early life stages of a range of species (Burggren, 2021a; b). The use of model organisms operates on the assumption that these

study organisms can provide insight into the biology of many other organisms. If our questions are generalizable, then utilisation of model species will likely be sufficient (Feder, 2006). However, within the context of understanding how development is influenced by global environmental change, the utilisation of model species may fail to provide sufficient insight into how other species of interest respond to environmental change, given that responses will be largely dependent on the biology of the animal, i.e. how it develops, as well as the ecology of the animal, i.e. where it develops. Furthermore, substantial variation exists between even closely related species in how development progresses, for example, in the relative timings of developmental events (Smith, 2002; Bininda-Emonds *et al*., 2007; Smirthwaite *et al*., 2007; Keyte & Smith, 2014). Consequently, high-dimensional phenotyping approaches must be readily transferable despite this variation. Transferability to a range of non-model organisms of interest is a key strength of many molecular omics approaches, application of which has led to considerable advancements in our understanding of responses to environmental change in many non-model species (Todgham & Hofmann, 2009; Meyer *et al*., 2015; Clark *et al*., 2017; Collins *et al*., 2017). A lack of transferable technologies capable of integrating the complexity of phenotypic responses to environmental change during early development, and that are readily applicable to a range of non-model species therefore poses a major limitation to widening our understanding of how future climate change projections will influence marine and freshwater ecosystems.

1.5 Thesis outline

Consequently, the central aim of this thesis is to understand what phenomics can contribute to our understanding of Comparative Developmental Physiology, in terms of how

environmental and evolutionary change affects the development of physiological function in aquatic embryos. Additionally to this, I aim to understand the consequences of changes in high-dimensional phenotyping methods used throughout the thesis within the context of embryonic life history. This will be achieved through completion of the following chapters:

In Chapter 2, I characterise high-dimensional phenotypic changes in embryonic physiological development in three closely related freshwater gastropod species with pre-established sequence heterochronies (evolutionary differences in the timings of events in their developmental sequences) (Section 1.6). This is with the aim of understanding whether heterochronic differences are reflected in or are predicable of changes in high-dimensional phenotypic space.

In Chapter 3, I characterise interspecific differences in the integrated developmental response of gastropod models (Section 1.6) to chronic elevated temperatures. This is with the aim of understanding how high-dimensional physiological responses to elevated temperatures change between species that have different developmental pathways, and between physiological windows of development that vary in their observable phenotypes.

In Chapter 4, I aim to determine if there is any relationship between the high-dimensional phenotyping methods used throughout this thesis (Section 1.7.2) and life history traits, specifically size at hatch and rates of growth and development, through serial experimental manipulation of the nutrient content of developing embryos.

Finally, in Chapter 5 I provide a general discussion on the findings of this thesis, discuss any emergent findings from these works, consider the broader implications of the thesis for the field of CDP, and I discuss potential future directions for these fields within a high-dimensional organismal phenotyping framework.

In the remaining sections of this introductory chapter, I will i) provide justification for the model species used throughout this thesis (Section 1.6) and ii) introduce the EmbryoPhenomics platform used for the acquisition of timelapse video of developing embryos, from which

phenomic data are extracted in the form of 'Energy Proxy Traits' (EPTs) (Section 1.7), that will be used to address the aims outlined above.

1.6 Freshwater pulmonate gastropod molluscs as model species

Gastropod molluscs have long been used as a model species for studying the patterns of embryonic development, largely owing to the qualitative description of their morphogenesis (Raven, 1966) and physiological development (Wilbur & Yonge, 1966). Embryos develop inside transparent egg masses, making longitudinal observation of their embryonic development tractable (Cumin, 1972). Furthermore, individual eggs dissected from their egg masses continue to develop normally, allowing for internal observation under varying environmental regimes. Eggs also develop (i.e. fertilisation to hatching) over relatively short time periods, usually within approximately 14 days (Smirthwaite *et al*., 2007).

Freshwater pulmonates have provided effective model species for the study of variation in event timings both within evolutionary contexts (Smirthwaite *et al*., 2007) and between conspecifics (Tills *et al*., 2010; 2011; 2013a). Previous research has established sequence heterochronies in a number of major behavioural and physiological events in a clade of freshwater gastropods comprising the Lymnaeidae, Physidae and Planorbidae (Smirthwaite *et al*., 2007). Additionally to this, previous research has also identified considerable variation in the timings of morphological and physiological events between conspecifics of the freshwater pulmonate *Radix balthica* (Tills *et al*, 2013a), variability which has shown to be heritable (Tills *et al*., 2013b) and have a genetic basis (Tills *et al*., 2011). Freshwater pulmonates therefore provide tractable model species with which to investigate the consequences of variation in physiological event timings in high-dimensional phenotypic space.

Following removal from the egg mass, embryos of freshwater pulmonates can be easily cultured and viewed under different environmental regimes. Within these species much of the literature has centred around plasticity in life history traits or the development of different shell morphologies (for example, Crowl & Covich, 1990; DeWitt, 1998; DeWitt *et al*., 1998; Rundle *et al*., 2004; Hollander *et al*., 2006; Edgell & Neufeld, 2008; Bourdeau, 2012; Melatunan *et al*., 2013). A meta-analysis reports that in studies on phenotypic plasticity in freshwater pulmonates, studies outside of growth/life history measures, and differences in shell morphology account for approximately 3% of the literature (Bourdeau *et al*., 2015). Furthermore, a significant bias is evident in the environmental/biotic variables under which such plasticity is observed, with 78% of the literature documenting predator induced phenotypic plasticity. Research into plasticity under variation in abiotic factors in gastropods is significantly lacking, despite these species inhabiting extremely temporally variable habitats where environmental heterogeneity is high (Brown *et al*., 1998; Strong *et al*., 2008).

Eggs of freshwater pulmonates are amenable to direct manipulation of nutrient content. Eggs of the lymnaeid *Lymnaea stagnalis* are approximately 1 - 4 mm in diameter with intra-capsular volumes ranging from 0.6 - 1 µL (Taylor, 1973). The relatively large size of these eggs makes removal of intra-capsular fluid (the main source of nutritive fluid during embryonic development post-gastrulation (Raven, 1946; Taylor, 1973; Wijsman and van Wijck-Batenburg, 1987)), tractable through the use of micro-injection, similar to methods well-established in application to the zebrafish *Danio rerio* (e.g. Jardine & Litvak, 2003). As far as I am aware, no studies have attempted to directly manipulate the volume of intra-capsular fluid in embryos of *Lymnaea stagnalis*. Whilst previous studies have cultured de-capsulated embryos in a number of nutritive media, culture of developing embryos *in vitro* can only be achieved for relatively short periods of developmental time (Taylor, 1973; Arambašic *et al*., 1989; Dickinson & Croll, 2001).

Finally, recent studies have shown the applicability of HDOP approaches to a species of freshwater pulmonate gastropod (*Radix balthica*). Embryos were reared under multiple

temperatures, and aspects of embryo morphology, physiology and behaviour were quantified using computer vision methods of analyses. Specifically, embryonic size, rates of growth and rates of cumulative movement (i.e. displacement of the centre of mass of the embryo within the egg capsule) were measured, as well as 'proxy' traits, representing combinatorial measures of aspects of embryo physiology and behaviour (Tills *et al*., 2018; 2021). Such an approach has yet to be extended to other species of gastropod mollusc. Given the considerable interspecific and intraspecific variation in the relative timings of development, and the ease of imaging of developing embryos, species within this clade provide a tractable model system with which to address the aims of this thesis.

1.7 The *EmbryoPhenomics* platform

Application of technologies for the high-throughput phenotyping of embryonic development have seen significant advancements in recent years (Xu *et al*., 2010; Pelkowski *et al*., 2011; Spomer *et al*., 2012; Olmedo *et al*., 2015), and these are often purpose built such that transferability to other species remains limited. This PhD will utilise *EmbryoPhenomics*, a high throughput platform for the phenomics of aquatic embryos (Tills *et al*., 2018). The *EmbryoPhenomics* platform comprises two major components, an Open-source Video Microscope ('OpenVIM') for culturing and continuous imaging of developing embryos (Fig. 1.3), and a Python package 'Embryo Computer Vision' (EmbryoCV) for quantification of high-dimensional phenotypic change from video of developing embryos.

1.7.1 OpenVIM

OpenVIM, is an open source video microscope designed for the longitudinal observation of large numbers of developing embryos, under varying environmental regimes. Embryos are placed within individual wells of multiwell plates (Nunc™, Microwell™, 96 wells, 350µL well^{-1}), which are placed into incubation chambers (H101-K-Frame, Okolab™, Italy) (Fig. 1.2a). Temperature within the incubation chambers is manipulated through circulation of water through the chamber, supplied by a temperature bath (temperature: minimum = 10-15ºC below ambient, maximum = 60ºC, H101-CRYO-BL, Okolab™, Italy). Although not employed in the present studies, gas concentrations and rates of air flow within incubation chambers can be controlled using a CO_2-O_2 unit (CO_2-O_2 Unit-BL [0-20; 1-95], Okolab™, Italy), and gas entering incubation chambers is pre-humidified using a humidity module (Okolab™, Italy) to minimise evaporation within wells of multiwell plates. Air is supplied into the incubation chambers using an air pump (OKO AP, Okolab™, Italy) (Fig. 2b). Temperature and gas concentrations are controlled using a touch screen interface (OKO-TOUCH, Okolab™, Italy). Incubation chambers are housed on a motorised XY stage (SCAN 130x85, Märzhäuser Wetzlar™, Germany), which are both mounted onto an aluminium frame, the position of which are controlled using a stepper motor controller (TANGO Desktop, Marthauser Wetzlar™, Germany). An inverted lens (magnification: 20-200x, VH-720R, Keyence™, UK) attached to a monochrome Charged Coupled Device camera (resolution: 2048x2048 pixels, Pike F421B, Allied Vision™, Germany) is then mounted beneath the incubation chamber and motorised XY stage. Dark field illumination of embryos is achieved using an LED ring light (LDR2-42-SW2, CCS, UK) placed above the incubation chamber and lens, and controlled using a digital control unit (PD3-3024-3-EI, CCS Inc., UK) (Fig. 1.2a). The entire apparatus described above is mounted on a gas pressurised table (Technical Manufacturing Corporation, MA, USA) to reduce vibrations in multiplate wells.

The digital camera is controlled using the open source ImageJ plugin µManager (Edelstein *et al*., 2010; Tills *et al*., 2018), allowing the user to control the spatial and temporal resolution of image acquisitions. Image acquisitions are carried out using the multi-dimensional acquisition

function in µManager. Image sequences for each embryo are acquired sequentially for each time point at a frame rate enabling real time physiological quantification, initiated using a BeanShell script (Supplementary information 20) within which the time interval between acquisition periods, and the number of time points to be acquired are specified. Images are stored as sequences of 8bit .tif files with their accompanying metadata, and are hierarchically stored (experiment > treatment > time point > embryo > .tif files + metadata) onto 2TB hard drives (Barracuda XT, Seagate, Ireland) within a hard drive enclosure (eBOX TeSu, DATOptic, USA).

1.7.2 EmbryoCV and Energy Proxy Traits (EPTs)

Imaging of developing embryos using the automated OpenVIMs described above produces large video data sets encompassing timelapse video that extends across the entirety of embryonic development, but also real time video at every time point, enabling visualisation of real time physiological and behavioural phenotypes. This high temporal resolution, combined with high spatial resolutions, requires methods of image analysis that are capable of operating at such resolutions. Automated methods of image analysis using computer vision methods have seen successful application to imaging datasets of developing embryos (Truong & Supatto, 2011). However, these toolkits are typically purpose built for model species of interest, and quantify specific aspects of the developing animals physiology, morphology or behaviour (for example, Schwerte *et al*., 2003; Dagani *et al*., 2007; Chung *et al*., 2008; Vogt *et al*., 2009; White *et al*., 2010; Xu *et al*., 2010; Pelkowski *et al*., 2011; Spomer *et al*., 2012; de Luca *et al*., 2014; Zulueta-Coarasa *et al*., 2014; Corngalia *et al*., 2015; Olmedo *et al*., 2015; Levario *et al*., 2016; Fuentes *et al*., 2018; Peravali *et al*., 2018; Gierten *et al*., 2020), and consequently their transferability is limited. Energy Proxy Traits (EPTs) are a new approach to measuring biological responses in developing embryos. Rather than targeting specific

aspects of an embryos physiology or behaviour, EPTs integrate all observable movement present in video data in the form of pixel value fluctuations, and convert these into levels of energy within discrete temporal frequency bins. For experimental work throughout this thesis, EPTs were calculated using the methods and algorithms within the Python package EmbryoCV (Tills *et al*., 2018). For Chapters 2 and 3, EmbryoCV was used for the automated segmentation of the embryos, with associated measures of size, shape and movement.

Imaging of developing embryos produces individual videos corresponding with a single embryo at a single time point. The EmbryoCV image analysis workflow begins with attempting to locate eggs in individual images of these video, and creates a bounding box surrounding the egg capsule of the embryo (Fig. 1.3a). Within these bounding boxes, the embryo is segmented, following which a number of traits are quantified, including embryo size, shape and position of the centre of mass. Embryo size and shape are used to calculate growth rates, and embryo position across frames, from which cumulative rates of movement within each timepoint are also calculated. To calculate EPTs, mean pixel values for each frame of the video are first calculated across the whole area of the bounding box (Fig. 1.3b). These are then used to construct time series of mean pixel values across frames of each video. From these time series, signal decomposition is applied using Welch's method (Welch, 1967). Welch's method is a form of Fourier transformation in which the time series is first divided into successive overlapping segments, forming a periodogram for each of these segments. A periodogram is used to identify dominant frequencies in a time series. These periodograms are subsequently averaged to produce an estimate of the power spectral density, a measure of a signal's power content vs frequency. The end result of this analysis is a spectrum of power measurements representing the amplitude of the signal (energy) across various temporal frequency bins (Fig. 1.3a). In summary, the constituent frequencies are calculated for a time series of changes in pixel values within a video, thereby integrating aspects of physiological and behavioural movement into a spectrum of energy values across frequencies. EmbryoCV produces data in the form of a hierarchical multi-dimensional HDF5 file for each embryo. From

this file, EPT data are extracted using a number built Python functions (link to repository: https://github.com/jcsmccoy/ECV-Analysis). This produces .csv files for individual embryos containing frequency x energy data for each time point, for the duration of the experiment (Fig. 1.3b). Additionally to this, these scripts contain functions for summarising data in formats that will be used throughout the thesis. In addition to an overall energy spectrum for each video, the sum of all energy within the frequency spectrum is calculated in these scripts to produce a measure of 'total energy'. Given that this is a sum of energy values across all frequencies, total energy integrates all observable forms of movement present in video data, thereby providing a holistic measure of rates of embryonic physiology and behaviour (Tills *et al*., 2018). Faced with the overwhelming complexity of behavioural and physiological responses to environmental change through the extremely dynamic period of embryonic development, EPTs were developed with the premise of extracting as much biologically informative information from video of developing embryos as possible. Given that movement in video is represented simply as changes in pixel values, an advantage to the use of these proxy traits is the capacity to integrate all aspects of embryonic movement into a single measure. This is of particular importance for embryos of gastropod molluscs which exhibit complex and subtle movements including rotation within the egg capsule, muscular flexing, digestive movements, heart beating and function of the radula (Rudin-Bittlerli *et al*., 2014).

A

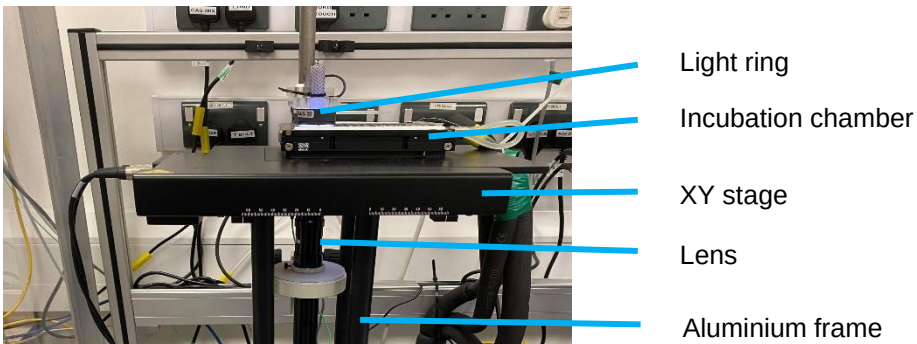

Figure 1.2. Annotated photographs of: **A)** Constituent parts of OpenVIM system **B)** Unit for control of environmental variables within incubation chambers.

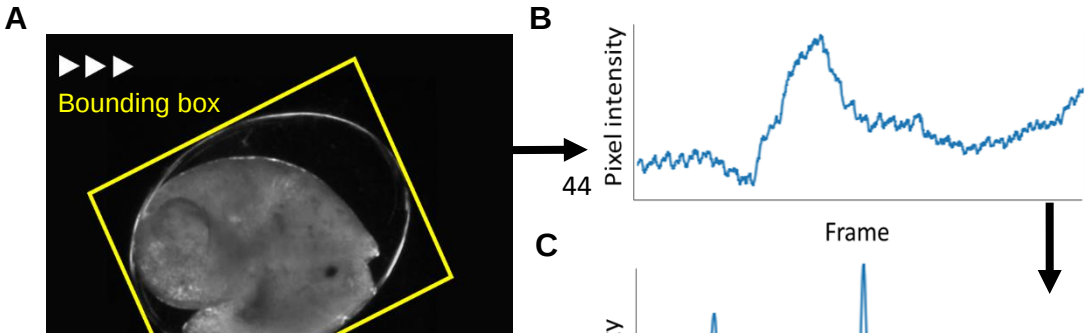

Figure 1.3. Analytical workflow for calculation of Energy Proxy Traits (EPTs), and extraction of data. **A)** Video of developing embryos is recorded using an Open Video Microscope (OpenVIM)**,** mean pixel values are calculated within the bounding box of each frame, and spectral decomposition using Welch's method (Welch, 1967) is applied to mean pixel value time series to calculate constituent frequencies. **B)** HDF5 is file produced by analysis of timelapse video by EmbryoCV, thereby producing timepoint specific energy x frequency data for each embryo.

Chapter 2

Comparative phenomics as an approach to study heterochrony

2.1 Abstract

Understanding the links between development and evolution is a central challenge of biology. Evolutionary alterations in developmental event timings termed ‘heterochronies’ are posited as a key mechanism of evolutionary change, however they are a gross, and until now necessary, simplification of the process of organismal development. How changes in event timings influence development more broadly remains poorly understood. Here, I use a new phenotyping approach termed ‘Energy Proxy Traits’ (EPTS) to quantify high-dimensional phenotypic ontogenetic change alongside previously identified heterochronies in three freshwater pulmonate molluscs (*Lymnaea stagnalis*, *Radix balthica*, and *Physella acuta*). EPTs were calculated from time lapse video of the entire period of embryonic development to construct a continuous functional time series. Transitions in these time series aligned with major sequence heterochronies. Finally, differences in event timings between conspecifics were associated with changes in high-dimensional phenotypic space. EPTs may provide a more powerful approach to investigating the evolutionary significance of differences in developmental event timings through the continuous quantification of changes in high-dimensional phenotypic space, rather than simply measurement of the timings of discrete events in developmental time.

2.2 Introduction

Heterochronies, defined as changes in the timings of developmental processes between ancestors and their descendants, are proposed as an important mechanism of evolutionary change, and are frequently regarded as the main process linking development to evolution (Gould, 1977; 1982; McKinney, 1988; Smith, 2001; 2003). Heterochrony research typically involves comparing the timings of development between extant, closely related taxa, and subsequently inferring evolutionary change by mapping these changes to a phylogeny (Smith, 2003). A relatively recent advancement has been the use of relative timings of developmental events, and changes in their sequence relative to one another (Smith, 2001; Jeffery *et al*., 2002a; Bininda-Edmonds *et al*., 2003). Historically, such studies have focussed on the rates of growth of morphological structures, primarily because of the reliance on morphology by palaeontologists studying heterochrony, who are often forced to use size as a proxy for age (Gould, 1977; McKinney, 1988; Spicer & Gaston, 1999; Smith, 2001; 2003). More recently there have been calls for more integrated approaches to the investigation of developmental event timings, via the inclusion of both functional and behavioural developmental characters (Spicer, 2006; Spicer & Rundle, 2006). Incorporation of functional developmental event timings into heterochrony research has enabled changes in functional events to be examined within the evolutionary context of heterochrony (Spicer & Rundle, 2006).

Measuring the timings of discrete developmental events enables direct comparison of developmental itineraries between species, but this comes at the expense of reducing complex and dynamic developmental processes down to a single point (Burggren, 2021b). Furthermore, the selection of developmental events from a vast number of candidates is; i) reliant on their occurrence in the study species of interest, and therefore must be identified *a priori* (Walls *et al*., 2019), and ii) potentially introduces a significant element of chance to selection of developmental events related to biological processes of interest (Houle *et al*., 2010; Forsman, 2015). Consequently, our understanding of the links between development and evolution *via* heterochrony must be limited by the current frameworks and methodologies used to investigate it.

The capacity to use bioimaging to continuously measure high-dimensional phenotypic change in developing animals offers new opportunities for interrogating the lines between heterochrony as a pattern, and its role as a process in macro-evolutionary change (Burggren, 2021a). Bioimaging enables researchers to apply new computer-vision approaches to measuring phenotypic change, using methods with no manual equivalents. Energy Proxy Traits (EPTs) are a measure of fluctuations in pixel intensities quantified as a spectrum of energies across different temporal frequencies (Welch, 1967) and are proving a valuable approach to measuring complex phenotypes in developing embryos (Tills *et al*., 2018; 2021; 2022). EPTs, rather than selecting specific aspects of an organism's morphology, physiology or behaviour, are indiscriminate measures of the phenotype applicable to different species and experimental designs that can be followed continuously during the course of development. As a method of quantifying features of developing embryos they therefore overcome the limitations associated with measuring discrete points in development time. EPTs are effective at capturing developmental transitions in embryos of an aquatic invertebrate, where traditional phenotypic measures are largely ineffective or non-transferable between stages of development. There is also evidence to suggest that they are indicative of biochemical energy turnover at the biochemical level (Tills *et al*., 2021). However, so far EPTs have not been used to compare species with different developmental itineraries, to investigate high-dimensional phenotypic change associated with evolutionary differences in the timings of development.

Consequently, our aim was to investigate the extent to which evolutionary differences in the timings of developmental events (heterochronies) are associated with high-dimensional phenotypic change, using EPTs. We hypothesise that the onset of developmental events are associated with changes in EPT spectra, and that evolutionary differences in the timings of developmental events between species would be reflected in time series of EPT spectra. To do this, I measured interspecific differences in EPTs in encapsulated embryos of species within a well-resolved phylogeny. Species of freshwater pulmonate snail exhibit sequence heterochronies at the familial level (Smirthwaite *et al*., 2007), thereby providing a tractable

model system. Smirthwaite *et al*. (2007) investigated event timings in 13 species across three families and detected significant sequence heterochronies in embryos of the Lymnaeidae and Physidae. Embryos of the physid *Physella acuta* exhibit sequence heterochronies in the timings of muscular crawling and cardiovascular function, relative to embryos of the lymnaeids *Lymnaea stagnalis* and *Radix balthica*, specifically an earlier onset of cardiovascular function relative to muscular crawling in the two lymnaeids. Therefore, EPTs were used to characterise high-dimensional changes in observable phenotype throughout embryonic development in these species, alongside measuring the timings of major functional developmental events: i) the onset of ciliary driven rotation; ii) the onset of cardiovascular function; iii) attachment to the wall of the egg capsule and the transition to muscular crawling; iv) the onset of radula function (referred to throughout as rotation, heart, crawling and radula respectively) (Smirthwaite *et al*., 2007).

2.3 Materials and Methods

2.3.1 Animal collection and maintenance

Adult snails *Lymnaea stagnalis* and *Physella acuta* were collected using a sweep net (1mm mesh) from Exeter canal, Devon, UK (50°41'57.8"N 3°30'43.7"W). Adult *Radix balthica* were collected using the same method from drainage canals, Middle Furlong Rhynie Bridgwater, UK (51°11'23.9"N 2°52'47.9"W). Snails were immediately transferred to the laboratory in plastic containers containing water and pondweed within 24 h of collection. Upon arrival snails were divided between a number of plastic containers (volume = 12 L) each filled with continuously aerated artificial pond water (APW) ($CaSO_4$ – 120 mg L^{-1}, $MgSO_4$ – 245 mg L^{-1}, $NaHCO_3$ – 192 mg L^{-1}, KCl – 8 mg L^{-1}), and maintained at T = 15°C. Approximately 45 adults of each species were maintained across 6 containers (n = 15 per container) and during this

time adults were fed spinach and lettuce *ad libitum*. Snails were acclimated to laboratory conditions for a minimum of 1 week prior to experimentation under a 12 h light/12 h dark light regime, and with weekly water changes.

2.3.2 Embryo collection

Snails regularly deposited egg masses onto the walls and floor of rearing aquaria. These masses (*L. stagnalis* n = 3, *R. balthica* n = 6, *P. acuta* n = 3) were carefully removed using a piece of thin laminate plastic within 24 h of deposition. When viewed under low power magnification (10-40x) any eggs that had not developed past the 4-cell stage were removed. Embryos from a minimum of 3 egg masses were used for each species in order to account for any brood variation, and a total of 48 embryos were used for each species. Individual embryos were carefully removed from their egg masses and transferred into individual wells of microtitre plates containing APW (Nunc, Microwell, 96 wells, 350 µL per well).

2.3.3 Bioimaging

Embryonic development from the 4-cell stage to hatching was recorded using an Open Video Microscope (OpenVIM), enabling long term repeated video imaging of aquatic embryos (Tills *et al.*, 2018). Microtitre plates containing embryos were placed into incubation chambers (H101-K-Frame, Okolab^{TM}, Italy), and reared for the duration of their embryonic development at 20°C. Control of physical parameters within incubation chambers, and acquisition of video data is detailed in section 1.7.1. There were mortalities of 33.3%, 16.7% and 10.4% in developing embryos of *L. stagnalis, R. balthica* and *P. acuta* respectively. These embryos

were excluded from analyses given that these embryos did not undergo all developmental events used in this study.

Raw video data, some of which was published in Tills *et al*. (2018; 2021), was used for *R. balthica*. Image sequences were acquired hourly for 30 s at between 30 frames s^{-1} (*R. balthica*) and 48 frames s^{-1} (*P. acuta* and *L. stagnalis*) for the duration of embryonic development, using the open source ImageJ plugin µManager (Edelstien *et al*., 2010). A resolution of 512 x 512 pixels was used for embryos of *P. acuta* and *L. stagnalis*, and 1048 x 1048 pixels for embryos of *R. balthica*.

2.3.4 Image analysis

Energy Proxy Traits (EPTs) were calculated for each 30 s video timepoint, for each embryo, using the method of Tills *et al*. (2021), detailed in section 1.7.2. In brief, mean-pixel values of the region containing the embryo in each frame were extracted as a time series. Signal decomposition using Welch's method (Welch, 1967) was then used to decompose mean pixel value signals into the temporal domain, thereby providing a spectra of energy values at different temporal frequencies (Fig. 1.3).

For each embryo, the timings of onset of a number of key developmental events were quantified from video *via* manual analysis for each species. These were i) the onset of ciliary driven rotation, ii) the onset of cardiovascular function (determined by the first visible heart beat), iii) attachment to the wall of the egg capsule and transition to muscular crawling (the point at which there is a clear attachment of the foot, rather than the embryo simply 'resting' on the wall of the egg capsule), and iv) the onset of radula function, the last developmental stage in all three species used (Smirthwaite *et al*., 2007) (Table. 2.1, Fig. 2.1). Heterochronies in the timing of muscular crawling and cardiovascular function exist between *P. acuta, L. stagnalis* and *R. balthica*. *P. acuta* exhibits a significantly earlier onset of attachment and

crawling on the wall of the egg capsule, relative to the onset of cardiovascular function, and relative to the timing of this event in *L. stagnalis* and *R. balthica* (Smirthwaite *et al*., 2007).

Table. 2.1. Descriptions of developmental events used in this study (after Smirthwaite *et al*., 2007). Each developmental event was recorded when it was first observed during observations of hourly timepoints recorded for each embryo.

Developmental event	Description
Rotation	Onset of ciliary driven rotation of the embryo.
Attachment and crawling	Attachment of the foot to the wall of the egg capsule, and onset of muscular crawling.
Cardiovascular function	First observable heartbeat.
Radula	Onset of radula function, located in the head.

To visualise developmental differences in EPTs between species, the sum of energy across all frequencies of the EPT spectra (hereafter referred to as total energy) were calculated, normalised within each individual (0-1), and expressed as a time series. The duration of embryonic development (i.e. the time taken to hatch) varies between individuals and species, therefore to generate a standardised developmental rate that would allow for comparison between species and individuals, and be invariant to differences in overall rates of development, the absolute timings from the 4-cell stage to hatching were converted to relative time (0-1).

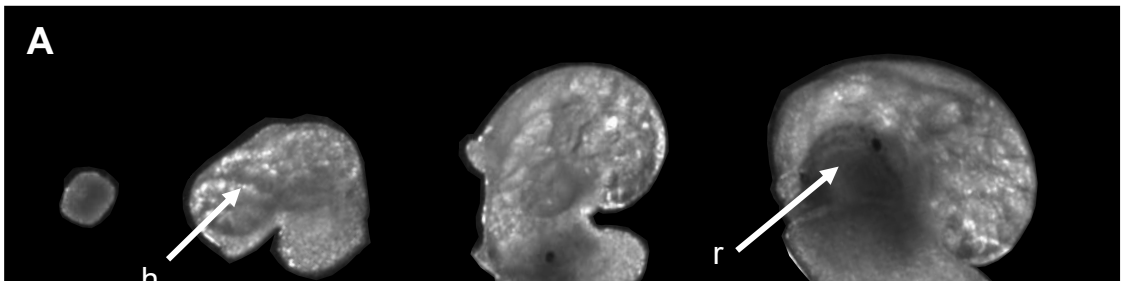

Figure 2.1. Developmental events used in this study (1 = onset of ciliary driven rotation, 2 = onset of cardiovascular function, 3 = foot attachment and onset of muscular crawling, 4 = onset of radula function). A) *Lymnaea stagnalis* B) *Radix balthica* C) *Physella acuta*. Locations of developmental events recorded are indicated: h = cardiovascular function, f = foot attachment, r = radula function. In *Physella acuta,* attachment of the foot on the wall of the egg capsule occurs before the onset of cardiovascular function.

2.3.5 Dimensionality reduction

EPT spectra are high-dimensional representations of observable movement, and therefore to compare interspecific, and development stage specific differences in combinatorial signals across frequency spectra, dimensionality reduction in the form of principal component analysis (PCA) was used. Temporal frequency data were binned to 0.1 Hz increments (0.03 – 6.0 Hz, 60 frequency bins). Frequency data were restricted to 6.0 Hz as the upper limit at which biologically meaningful signals would be expected. Mean energy within each frequency bin for each normalised time point (0-1) was calculated and log transformed. PCA (prcomp, package 'stats', v4.0.3) was applied to these data and the resulting eigenvectors were used to determine whether the onset of developmental events were associated with changes in high dimensional phenotypic space.

2.3.6 Statistical analysis

All data were analysed in R v4.0.3 (R Core Team, 2020). To determine whether time series of total energy data were different between species, a repeated measures analysis of variance (ANOVA) was applied to total energy data. Additionally, Bayesian structural time series (BSTS) model using the R package CausalImpact (Brodersen *et al*., 2014) was applied to determine whether the onset of developmental events were associated with changes in total energy time series. To determine whether the onset of developmental events was associated with frequency specific differences in EPTs, a Kruskal-Wallis test was applied to mean energy values within each of the 60 frequency bands of 5 time point values preceding and proceeding the onset of each developmental event. Multiple testing correction was applied using the Bonferroni method.

2.4 Results

2.4.1 Interspecific differences in developmental EPTs

There were clear differences in EPT profiles between species (repeated measures ANOVA, $F_{2, 199} = 54.52$, $P < 0.0001$, Fig. 2.2), and the onset of functional developmental events were also associated with pronounced differences in EPTs within each species (Table. 2.2). The time of onset of rotation was associated with a marked increase in total energy during the first quartile of development (Table. 2.2), and followed by reductions in total energy preceding the onset of the heart and crawling approximately midway during development (Fig. 2.2, Table. 2.2). This reduction in total energy appeared to occur earlier in *Physella acuta* relative to *Lymnaea stagnalis* and *Radix balthica*.

Additionally, this period of total energy decline preceding the onset of crawling was compressed in *P. acuta* relative to *L. stagnalis* and *R. balthica*. In *L. stagnalis* and *R. balthica*, embryos remain free swimming during the development of the heart and other functions. Before crawling, embryos will intermittently rest with increasing frequency on the wall of the egg capsule, which is evident *via* the gradual reduction in total energy preceding crawling. However, in *P. acuta* this free swimming stage is absent which likely explains the comparatively compressed period of total energy decline prior to crawling. Finally, the generally later onset of each developmental event in *R. balthica* was associated with a shift of the entire time series later into relative developmental time (Fig. 2.2).

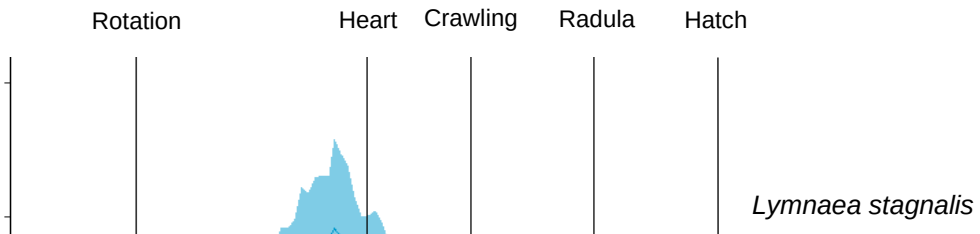

Figure 2.2. Developmental time series of normalised (0-1) total energy in embryos of *Lymnaea stagnalis* (N = 28), *Radix balthica* (N = 40) and *Physella acuta* (N = 43), across normalised developmental time (0-100%) (mean ± sd). Median values of developmental event timings represented by vertical lines (Supplementary information 1). These include: i) rotation (the onset of ciliary driven rotation); ii) heart (the onset of cardiovascular function); iii) crawling (attachment to the wall of the egg capsule and the onset of ciliary driven rotation); iv) radula (the onset of radula function); v) hatch (emergence from the egg capsule).

Table 2.2. Effect of the onset of developmental events on time series of total energy through application of a Bayesian structural time series (BSTS) model using the R package CausalImpact. Bracketed values indicate confidence intervals for absolute and relative effects (Brodersen *et al*., 2014).

Species	Event	Actual value	Predicted value	Absolute effect	Relative effect	Posterior tail-area probability (P)	Posterior probability of causal effect (%)
Lymnaea stagnalis	Rotation	26631.02	107.8	26523.22 (29257.96, 26527.96)	246.4 (246.08, 245.99)	**0.001**	99.90
	Heart	35968.13	26581.52	9386.62 (24814.87, -5117.48)	-0.35 (0.93, -0.19)	0.12	88
	Crawling	11201.04	37678.77	-26477.73 (-14668.26, -38047.04)	-0.70 (-0.38, -1.01)	**0.001**	99.90
	Radula	6480.77	11538.94	-5058.32 (-3917.99, -6262.28)	-0.43 (-0.34, -0.54)	**0.001**	99.90
Radix balthica	Rotation	30577.35	94.01	30483.34 (30587.88, 30378.51)	324.36 (325.37, 323.14)	**0.001**	99.90
	Heart	41150.14	30476.69	10673.45 (37004.12, -6126.12)	0.35 (0.87, -0.20)	0.11	89
	Crawling	9957.89	42632.19	-32674.29 (-24544.44, -41431.51)	-0.77 (-0.58, -0.97)	**0.001**	99.89
	Radula	1704.99	10389.89	-8684.90 (-5566.04, -11619.42)	-0.84 (-0.54, -1.12)	**0.001**	99.89
Physella acuta	Rotation	21995.84	729.63	21266.21 (21489.20, 21020.03)	29.15 (29.45, 28.81)	**0.001**	99.90
	Crawling	17465.63	21645.32	-4179.69 (5091.05, -14801.30)	-0.19 (0.27, -0.68)	0.20	80
	Heart	10011.76	17011.78	-7000.02 (-2763.39, -11586.88)	-0.41 (-0.16, -0.68)	**0.002**	99.79
	Radula	5910.40	10086.95	-7000.02 (-3442.49, -4974.14)	-0.41 (-0.34, -0.49)	**0.001**	99.90

2.4.2 Dimensionality reduction for EPT spectra differentiation

To determine whether the onset of developmental events was associated with high-dimensional phenotypic change, PCA was applied to multivariate EPT time series. Data collected from individuals for each species were first averaged (mean) by normalised time point (0-1). Following this, levels of energy within each frequency band were normalised (0-1) across relative developmental time.

Principle components 1 and 2 incorporated 97.2%, 95.2% and 93.5% of the variance in the EPT spectrum for *L. stagnalis* (PC1: 85.5%, PC2: 11.7%) (Fig. 2.3a), *R. balthica* (PC1: 75.2%, PC2: 20%) (Fig. 2.3b), and *P. acuta* (PC1: 69.5%, PC2: 24%) (Fig. 2.3c) respectively. The onset of some developmental events were associated with distinct separation of points, notably the onset of muscular crawling and radula function in *L. stagnalis* and *R. balthica*. In *L. stagnalis* there were clear separation of points along the axis of PC1 between the onset of cardiovascular function and muscular crawling. Variation along the axis of PC1 was predominantly driven by frequencies ranging from 1.7 - 3.7 Hz (Fig. 2.3a). Additionally, frequencies up to 0.9 Hz were predominantly driving variation along the axis of PC2, suggesting that differences in embryos between the onset of crawling and radula function were driven predominantly by frequencies within this range (Fig. 2.3a) (Supplementary information 2).

2.4.3 High-dimensional phenotypic change associated with the onset of developmental events

EPT are a spectra of energy values across different temporal frequencies. Therefore, to determine whether the onset of the developmental events used in this study were associated with composition differences of EPT spectra across temporal frequency bands, pairwise

comparisons of energy preceding and proceeding the onset of each developmental event were carried out for binned frequencies. In all species, the onset of ciliary driven rotation was associated with a significant increase in the levels of energy in all temporal frequency bands (Kruskal-Wallis, $P < 0.0001$, Fig. 2.4, Supplementary information 3). The onset of cardiovascular function was associated with a significant increase in levels of energy up to 0.7 Hz and 1.6 - 1.8 Hz in *L. stagnalis*. In *R. balthica* and *P. acuta* the onset of cardiovascular function coincided with a significant increase in energy within the range of 1.2 - 1.6 Hz and 1.6 - 4.0 Hz respectively. (Kruskal-Wallis, $P < 0.0001$, Fig. 2.4). The onset of muscular crawling on the wall of the egg capsule was associated with a significant reduction in energy within all temporal frequency bands for *P. acuta* (Kruskal-Wallis, $P < 0.0001$, Fig. 2.4). Finally, following the onset of radula function, there were significant reductions in the levels of energy up to 0.5 Hz in *P. acuta* (Kruskal-Wallis, $P < 0.0001$, Fig. 2.4).

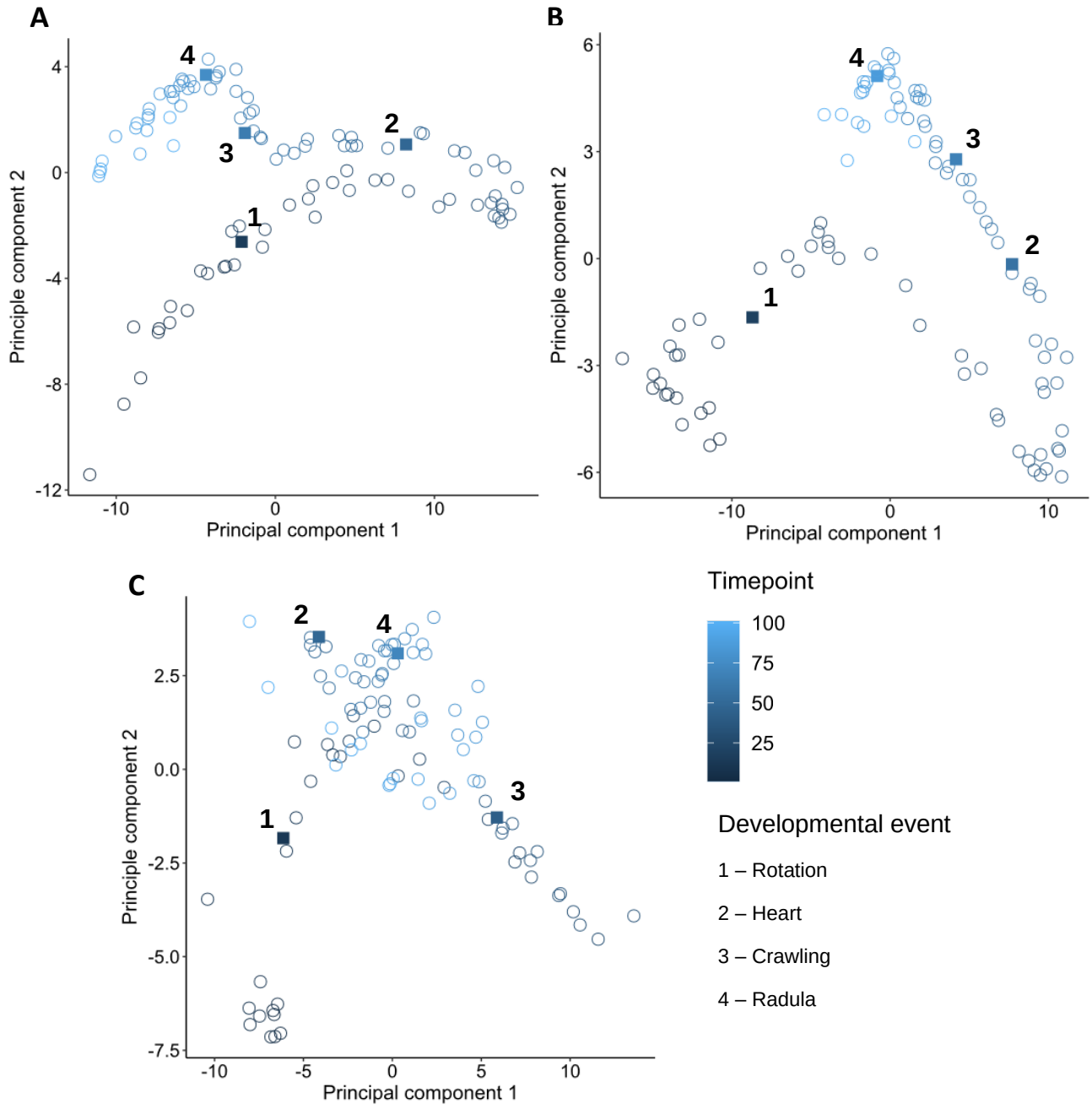

Figure 2.3. Principal component analysis (PCA) of mean levels of energy within 60 temporal frequency bins across normalised time points (0-1). **A)** *Lymnaea stagnalis* **B)** *Radix balthica* **C)** *Physella acuta***.** Square filled points and adjacent numbers indicate mean time point of onset of developmental events: 1 = Rotation (onset of ciliary driven rotation); 2 = Heart (onset cardiovascular function); 3 = Crawling (attachment to the egg capsule and onset of muscular crawling); 4 = Radula (onset of radula function).

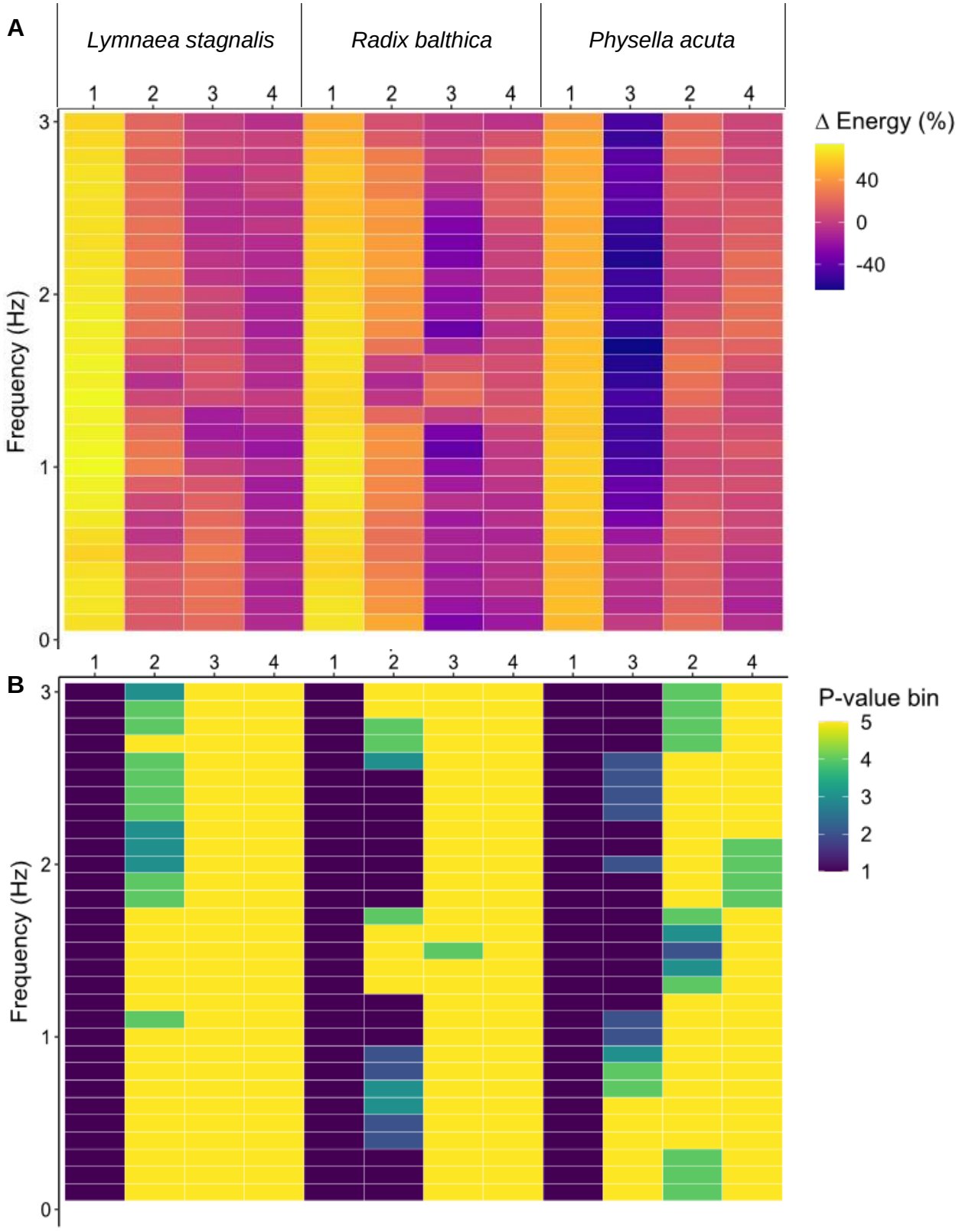

Figure 2.4. **A)** Percentage change in energy values between each developmental event across frequencies in *Lymnaea stagnalis*, *Radix balthica* and *Physella acuta*. 1 = onset of ciliary driven rotation, 2 = onset of cardiovascular function, 3 = attachment to the wall of the egg capsule and onset of muscular

crawling, 4 = onset of radula function. Colour indicates magnitude of change. **B)** Results of Kruskal-Wallis test (Bonferroni adjusted, P = 0.00167) and statistical significance indicated by colour. P-value bins: 1 = 0-0.000001, 2 = 0.000001-0.00001, 3 = 0.00001-0.0001, 4 = 0.0001-0.00167, 5 = 0.00167-1 (not significant).

2.5 Discussion

Our aim was to determine the extent to which evolutionary differences in the timings of developmental events (heterochronies) were associated with high-dimensional phenotypic change, using Energy Proxy Traits (EPTs), a new approach to spectral phenotyping. We hypothesised that the onset of developmental events used in this study are associated with changes in EPT spectra, and that evolutionary differences in the timings of developmental events would be associated with changes in time series of EPT spectra between species. Causal impact analyses and PCA revealed that the onset of developmental events were associated with changes in EPT spectra. Furthermore, results indicate that evolutionary differences in the timings of developmental events are associated with pronounced changes in high-dimensional phenotypic space. Energy Proxy Traits (EPTs) measure complex phenotypes as a spectrum of energy and application of these to the complete embryonic development of three closely related snail species *Lymnaea stagnalis*, *Radix balthica* and *Physella acuta* revealed interspecific differences in these spectra, tightly associated with previously described sequence heterochronies. Furthermore, investigation of EPT time series revealed functional changes preceding and proceeding the onset of developmental events, but also striking differences between species for the same developmental events. Combinatorial analysis of EPTs using dimensionality reduction revealed distinct separation of points between developmental events, highlighting transitions in the functional phenotype of embryos in high-dimensional space. This suggests that developmental events, while a useful

approach to comparative studies of development, may bely complex differences in functional phenotypes that could themselves be the object of natural selection.

2.5.1 Interspecific differences in EPTs coincide with pre-established sequence heterochronies

Time series of EPTs reflected interspecific differences in developmental event timings. Firstly, differences in the relative timing of onset of ciliary driven rotation was evident from time series of total energy between species. Ciliary driven rotation occurred earliest in *Physella acuta* and this is evident as an earlier increase in total energy in relative developmental time. Following the onset of rotation, levels of total energy gradually increased in all species. Following this, embryos of all species showed clear reductions in total energy leading up to the transition to muscular crawling on the wall of the egg capsule, the timing of which reflected evolutionary differences between these species. In *P. acuta,* the onset of muscular crawling is brought forward significantly relative to the timing of cardiovascular function, and the onset of this event in *L. stagnalis* and *R. balthica* (Smirthwaite *et al*., 2007; Supplementary information 1). From time series of EPT data, a marked reduction in total energy can be observed occurring at approximately 37% of relative developmental time in *P. acuta* whereas this pronounced reduction in energy occurs at approximately 41% and 52% of relative developmental time in *L. stagnalis* and *R. balthica* respectively (Fig. 2.2).

The observed changes in energy both before and after the timings of discrete developmental events used in this study showcase potential shortcomings in current approaches used to quantify the timings of development. Burggren (2021) observed that current research often focusses on development as a series of discrete events, when development should be seen as a continuum. Experimental protocols should gather data at multiple points of the developmental continuum, thereby allowing phenotypic measurements to be put into the context of the entirety of development. Here, I demonstrated considerable phenotypic change

both preceding and proceeding the onset of discrete developmental events, including the onset of ciliary driven rotation, and attachment to the wall of the egg capsule. The gradual increases and decreases in total energy likely reflect the continuous nature with which major multi-faceted developmental transitions take place in these embryos.

Firstly, the gradual increases in total energy with time co-occurs with increases in rates of rotation as development progresses (Fig. 2.2). Without generalising to the other two species tested, previous research showed that embryos of *L. stagnalis* exhibited greater rates of rotation at the hippo stage (equivalent to approx. 40% developmental time) relative to the veliger stage (approx. 30% developmental time), which likely explains the gradual increases in total energy following the onset of rotation. Byrne *et al*. (2009) found that in embryos of *L. stagnalis* rates of rotation at the hippo stage were approximately 1.3 - 1.6 times greater than that of the veliger stage, and suggested that such differences in rotation rate between these two stages may be due to increases in anatomical complexity at the hippo stage.

Secondly, there were gradual decreases in total energy prior to attachment to the wall of the egg capsule and the onset of muscular crawling. Before this developmental event, embryos of *R. balthica* and *L. stagnalis* remain free swimming in the egg capsule (Meshcheryakov, 1990; Smirthwaite *et al*., 2007). During this period, embryos will increase the frequency at which they intermittently 'rest' their head on the wall of the egg capsule, before firmly attaching with the foot and commencing muscular crawling, which likely explains the gradual reductions in energy (and therefore to some extent overall rates of movement) proceeding the onset of this developmental event. When viewed as a discrete developmental event, the considerable phenotypic changes the developing embryo undergoes prior to its onset becomes masked, and arguably the event itself becomes increasingly arbitrary.

Additionally to this, there were features to both total energy time series and time series PCA that cannot be explained by the onset of, or changes in the timings of the developmental events used in this study. For example, in each of the total energy time series, there is a distinct peak in total energy occurring at approximately 38%, 50% and 33% relative

developmental time for *L. stagnalis*, *R. balthica* and *P. acuta* respectively (Fig. 2.2). This peak occurred prior to the onset of cardiovascular function in *L. stagnalis* and *R. balthica* and muscular crawling in *P. acuta*, yet did not coincide with the onset of any of the developmental events used in this study. This highlights a potential limitation with EPTs. Given that EPTs are a novel approach to whole-organism phenotyping, in this study I have used a number of known developmental events as a 'scaffold' with which to interpret the results used here, however without these manually determined event timing data, changes in these time series become difficult to interpret. However, future investigation can be directed towards attempting to understand what is driving these unexplained phenomena in EPT time series, through observation of video coinciding with these time points. Furthermore, measurement of this change in energy as a continuum also reveals evolutionary differences in developmental phenotype preceding the onset of attachment and muscular crawling, rather than just a difference in the timing as a discrete event in developmental time. The period of total energy decline in *P. acuta* prior to the onset of muscular crawling is compressed relative to *L. stagnalis* and *R. balthica* (Fig. 2.2). In *P. acuta*, there is an absence of a free swimming stage, with embryos attaching to the egg capsule and commencing muscular crawling following the trochophore stage (Smirthwaite, 2007), thereby producing the relatively condensed period of total energy decline observed in the total energy time series (Fig. 2.2). Consequently, by viewing development as a continuum of phenotypic change as carried out in this chapter, it was possible to identify evolutionary differences in high-dimensional phenotypic space before, during and after the onset of discrete developmental events.

2.5.2 A high-dimensional phenotyping approach to physiological heterochrony

Development is an inherently complex phenomenon characterised by massive functional and spatial variation, variation that may act as a source of evolutionary change and innovation

(West-Eberhard, 2003). There has been a historical emphasis on heterochrony as the main mechanism linking development to evolution (Gould, 1982; Smith, 2001; 2003). Whilst measurement of the timings of developmental events or stages in investigations of heterochrony facilitates direct interspecific comparison (Walls *et al*., 2019), quantification of the timings of developmental events which are in themselves discrete points in time, fails to capture any notion of development as a continuous and dynamic process. Furthermore, quantifying high-dimensional phenotypic change alongside the timings of discrete developmental events may provide a means of investigating the evolutionary significance of variation in developmental event timings, by linking the phenotype, which is the ultimate object of selection, to the timings of developmental events. Most biologists currently confine phenotyping efforts to a small number of observable traits, given the often overwhelming complexity of organismal biology and the challenge of quantifying it in a discrete and reproducible way. However, selection typically does not act on single traits, rather on multiple traits simultaneously (Lande & Arnold, 1983; Phillips & Arnold, 1999). When presented with high-dimensional phenotypic datasets, dimensionality reduction allows for the visualisation of structure and cumulative drivers in high-dimensional phenotypic space. In the current study, there was considerable separation of points associated with different points in relative developmental time from principal component analysis (PCA). Additionally, PCA of time series data revealed distinct clustering of points following the onset of various developmental events, but also revealed considerable variation between these developmental events (Fig. 2.3). Results from PCA of time series analysis further demonstrates how developmental events are only a snapshot in developmental time, and that focussing on these discrete events occludes high-dimensional phenotypic change during the periods of development surrounding them. Given what we know about how selection operates on multiple traits simultaneously, expanding the scale at which we capture phenotypic information during periods when phenotypic complexity is at its greatest will provide greater insight into the developmental mechanisms driving evolutionary change (Blows, 2007).

Despite this, application of high-dimensional phenotyping approaches are rarely expanded to species and systems that provide effective models for investigating the evolutionary significance of changes in the timings of development. In areas of research where high-dimensional phenotyping approaches are most developed, for example in plant and medical phenomics, there are already well-established and standardised phenotyping approaches that are applicable to model species of interest (Houle *et al*., 2010; Furbank & Tester, 2011; Alexandrov *et al*., 2016; Tardieu *et al*., 2017). A potential shortcoming in the field of comparative phenomics, and across evolutionary developmental biology more generally is an absence of phenotyping approaches that are readily transferable to non-model species of interest (Tills *et al*., 2018). One of the cornerstones of comparative developmental physiology is the selection of species which are best suited to answer a particular biological question (Krogh, 1929), yet approaches to high-dimensional organismal phenotyping remain constrained to model animals of interest, particularly the zebrafish *Danio rerio* (Pelkowski *et al*., 2011; Kalueff *et al*., 2016; Peralvi *et al*., 2018), nematode worm *Caenorhabditis elegans* (Yemini *et al*., 2013; Cornaglia *et al*., 2015; Olmedo *et al*., 2015), and the fruit fly *Drosophila melanogaster* (Chung *et al*., 2010; Levario *et al*., 2016). Here, EPTs were effective at characterising high-dimensional functional change in embryos of three species of freshwater gastropod, despite significant differences in their patterns of development. EPTs provide an effective and transferable phenotyping approach to quantifying phenotypic change in early life stages of a range of non-model species.

I suggest that evolutionary differences in the timings of developmental events are associated with differences in high-dimensional phenotypic space. As well as detecting interspecific differences in the timings of development between embryos of each species, there were also differences in the timings of EPTs between conspecifics. Previous research has shown that considerable standing variation exists in developmental event timings between conspecifics, which may provide the raw variation on which heterochronies are formed (Reilly *et al*., 1997; Mabee *et al*., 2000; Schmidt & Starck, 2004; de Jong *et al*., 2009; Kawajiri *et al*., 2009; Rundle

et al., 2011; Tills *et al*., 2013; Rager *et al*., 2014). In addition to the observed interspecific differences in EPTs, individual level EPT data indicate considerable variation in EPTs between conspecifics. Comparison of developmental event timings alongside EPT data indicates that this variation corresponds with intraspecific differences in the timings of developmental events (Supplementary Fig. 1, 2, 3; Supplementary information 6). The consequences of such variation are not currently understood but provide interesting lines of enquiry with which to investigate the performance and fitness implications of variation in the timings of developmental events between conspecifics. From previous research, EPTs appear to be related to some components of organismal fitness. Tills *et al*. (2021) showed that higher levels of total energy (reported in Fig. 2.2) were associated with a faster growth rate in *R. balthica*, and posited that EPTs may in themselves be indicative of biochemical energy turnover, given that rates of biochemical energy turnover (metabolism) have been positively correlated with growth rates in a number of studies (Metcalfe, 1998; Yamamoto *et al*., 1998; Nylin & Gotthard, 1998) (although this is not always the case as such relationships are often context dependent, particularly between wild and laboratory reared specimens: Álvarez & Nicieza, 2005; Burton *et al*., 2011). Consequently, differences in the timings of developmental events may ultimately manifest as differences in biochemical energy turnover in developing embryos. Rates of energy expenditure are a significant object of selection (Bartheld *et al*., 2015), however, our understanding of the extent to which EPTs are linked with biochemical energetic turnover is currently poorly understood, and so is our understanding of how variation in EPTs ultimately influence components of organismal performance and fitness post-hatch. Further research is now needed to understand: i) the extent to which EPTs are related to biochemical energy turnover in developing embryos; and ii) the consequences of ontogenetic variation in EPTs for aspects of organismal performance and fitness.

2.6 Summary and conclusions

Evolutionary biologists have long sought to establish mechanistic links between development and evolution. Current research frames heterochrony, alterations in the timings of development as the main mechanism by which development leads to evolutionary change. However, the current focus of heterochrony as the timings of discrete points in development may preclude quantification of phenotypic change associated with differences in the timings of these events. Here, through the application of a novel spectral phenotyping approach (EPTs), I captured a continuous functional time series of the embryonic development of three freshwater snails. Analysis of these time series provided evidence that evolutionary differences in the timings of development are associated with high-dimensional phenotypic change. Additionally to this, this chapter provides preliminary evidence that intraspecific differences in EPTs coincide with differences in developmental event timings between conspecifics. EPTs may provide an alternative approach to investigating the evolutionary significance of variation in the timings of development by allowing for the continuous quantification of high-dimensional phenotypic change associated with intraspecific and evolutionary differences in the timings of development.

Chapter 3

A phenomics approach reveals interspecific differences in the integrated developmental response to chronic elevated temperatures

3.1 Abstract

Developmental responses to elevated temperatures are multifaceted and consist of a multitude of individual trait changes, effective quantification of which is constrained by manual methods. 'Energy Proxy Traits' (EPTs) quantify biological responses in complex and dynamic developing organisms, and are a novel approach to phenomics. EPTs capture fluctuations in pixels in video and measure these as levels of energy across temporal frequencies. This method has proven effective in providing integrative measures of acute and chronic responses to environmental stress for individual species. However, EPTs remain untested in comparing the environmental sensitivity between different species, a significant precursor to establishing their utility as a comparative approach to assessing responses to global change drivers. Here, EPTs were applied with the aim of understanding the relative thermal sensitivities of embryos of three species of freshwater snail with markedly different developmental itineraries, i.e. the timings of their developmental events. Inherent within this aim is establishing the capability of EPTs to quantify responses between species, and different physiological windows (periods of development corresponding with the onset of functional developmental events) in development. Changes in the levels of energy across the EPT spectra revealed marked differences in thermal sensitivities between species, and temperature induced changes in the relative timings of major physiological events, notably ciliary driven rotation and muscular crawling, at 25°C by embryos *Lymnaea stagnalis* and *Radix balthica*. Furthermore, changes in the response of EPTs between physiological windows reflected ontogenetic changes in

observable embryonic phenotypes. Gaining a robust understanding of how climatic changes impact early life stages of marine and freshwater animals requires phenotyping approaches that are applicable to a broad range of non-model organisms. Such approaches should be capable of capturing the widespread phenotypic change associated with responses to global change drivers. Here I show marked differences in the thermal sensitivity of EPT spectra across development in species with different developmental itineraries, and is of importance in considering how best to measure biological sensitivity during dynamic periods of life.

3.2 Introduction

Assessing the thermal sensitivity of the phenotype during periods of early development is central to predicting how species will respond to future climate change projections (Burggren, 2018; 2021). Chronic elevated temperatures affect processes at every level of biological organisation (Hochachka & Somero, 2002; Iverson *et al*., 2020) resulting in changes in the absolute and relative timings of organismal development (Johnston, 1993; Gillooly *et al*., 2002; Gomez-Mestre & Buchholz, 2006; Klimogianni *et al*., 2004), rates of various aspects of organismal physiology (Birchard & Reiber. 1996; Styf *et al*., 2013; Du & Shine, 2015; Adrianov *et al*., 2019), behaviour (Oppenheim & Levin, 1975; Peterson & Robichaud, 1983; Peterson *et al*., 2004; Du & Shine, 2015; Tills *et al*., 2018), and sizes at hatch (Pepin *et al*., 1997; Angiletta & Dunham, 2003; Mitz *et al*., 2019). Despite such broad-scale changes, current approaches to measuring the response of the phenotype typically relies on reductionist approaches involving the measurement of single or a small numbers traits with some pre-established functional significance, or in some cases gross indicators of organismal performance are used (e.g. metabolic rate, developmental rate, size at hatch).

Measurement of small numbers of traits may lead to erroneous conclusions over the significance of an environmental stressor in influencing the sensitivity of a developing animal,

given that a lack of plasticity in an observed trait, can be compensated for by plasticity in another, unobserved trait (Pigliucci and Preston, 2004; Houle, 2007; Valladares *et al*., 2007; Whitman and Agrawal, 2009). Whilst such approaches are important in providing indications of organismal performance and fitness, understanding the physiological mechanisms underlying such broad scale organismal changes requires approaches that are capable of quantifying high-dimensional phenotypic change (Forsman, 2015). High-dimensional approaches to organismal biology are well established within the molecular omics, and have led to considerable advancements across the biological sciences, including our understanding of responses to climatic change in non-model species of interest (Todgham & Hofmann, 2009; Meyer *et al*., 2015; Clark *et al*., 2017; Collins *et al*., 2017). However, within these contexts, quantifying phenotypic change at scales in any way resembling that of the molecular omics is rarely undertaken.

Measuring phenotypic responses during early development presents a number of key challenges. Firstly, periods of early development are characterised by high degrees of functional and spatial change, requiring either a focus on a small period of development, or using phenotyping approaches that are transferable between stages of development that vary greatly in their observable phenotypes. Secondly, responses to elevated temperature regimes in early life stages typically involve a large number of individual trait plasticities (Hochachka & Somero, 2002; Iverson *et al*., 2020). Finally, substantial variation often exists in the timings of physiological development between closely related species (Smith, 2002; Bininda-Emonds *et al*., 2007; Smirthwaite *et al*., 2007; Keyte & Smith, 2014), requiring the selection of equivalent stages of development when comparing responses between species (Fuiman *et al*., 1998). Current solutions to the above points include use of standardised indicators of developmental stages such as ontologies when comparing interspecific responses at various stages of development (Robinson & Webber, 2014; Oellrich *et al*., 2015 ; Walls *et al*., 2019), and the use of equivalent developmental events when comparing between species, as is seen developmental event timing research (e.g. Smirthwaite *et al*., 2007). However these

approaches to phenotyping require identification and dedicated construction of stages or events that are conserved between species of interest. Furthermore, they rely on the reduction of the high-dimensional continuum of biological development into a simplified framework, ultimately reducing transferability to other non-model species of interest.

'Energy Proxy Traits' (EPTs) are emerging as a tractable approach to high-dimensional organismal phenotyping (Tills *et al*., 2018; 2021). EPTs are a spectrum of levels of energy within different temporal frequencies in the pixel brightness fluctuations in videos of developing embryos. Rather than targeting specific aspects of physiology or behaviour, EPTs integrate all biological sources of pixel fluctuations present in video data and quantifies these as a spectrum of energy within discrete temporal frequency bins. Although EPTs have proven effective at characterising acute and chronic responses to environmental stress in embryos of aquatic invertebrates (Tills *et al*., 2018; 2021), they remain untested in comparing responses between species, a major prerequisite to establishing their utility as a comparative approach to assessing species-specific thermal sensitivity.

Consequently, I applied EPTS with the aim of understanding the thermal sensitivities of embryos of three species with markedly different developmental itineraries. Associated with this aim is establishing the capability of EPTs in quantifying responses of different species with comparable, yet different physiological windows of development that vary greatly in their observable phenotypes. I hypothesise that the magnitude of change in energy within particular frequencies will reflect the thermal sensitivity of various species and physiological windows in development, with a greater magnitude of change suggesting a higher thermal sensitivity. To address these aims, I applied EPTs to test the relative sensitivities of three species of freshwater gastropods (*Lymnaea stagnalis, Radix balthica* and *Physella acuta*) to chronic elevated temperatures. Freshwater pulmonate gastropods can occur in highly thermally variable habitats, and whilst data are available on acute responses of adults (Hoefnagel & Verberk, 2017; Johansson & Laurila, 2017), integrated developmental responses to different chronic temperatures remains limited. Based on thermal tolerance data on adults of each of

these species, embryos of *P. acuta* could be hypothesised to be the least thermally sensitive, given that adults of this species show a comparatively higher thermal tolerance (41.7˚C, Koopman *et al*., 2016), relative to embryos of *L. stagnalis* (40.5˚C, Hoefnagel & Verberk, 2017) and *R. balthica* (36.7-38.5˚C, Johansson & Laurila, 2017). Additionally to this, species within this clade have markedly different embryonic developments, displaying evolutionary differences in the relative timings of physiological events during their development (heterochronies, Gould, 1977). Species within the family Physidae (*Physella acuta*), exhibit a significantly earlier onset of attachment to the wall of the egg capsule and commencement of muscular crawling, relative to the onset of cardiovascular function, and a number of other physiological events. Conversely, in the Lymnaeidae (*Lymnaea stagnalis* and *Radix balthica*), embryos develop cardiovascular function during a free swimming stage prior to this attachment and onset of muscular crawling (Smirthwaite *et al*., 2007). Consequently, these species provide excellent models for testing the transferability of EPTs in assessing responses between species and stages of development.

3.3 Materials and Methods

3.3.1 Embryo collection, temperature exposure and bioimaging

Adult *Lymnaea stagnalis, Physella acuta* and *Radix balthica* were collected and maintained under laboratory conditions as documented in Chapter 2 (Section 2.3.1). Eggs from each egg mass were evenly distributed between two microtitre plates (Nunc, Microwell, 96 wells, 350 µl well^{-1}), with each microtitre plate corresponding with a different temperature (T = 20 or 25°C).

An open-source autonomous video microscope (OpenVIM) (Tills *et al*., 2018; Section 1.7.1) was used to record embryonic development from the 4-cell stage to hatching. Two microtitre

plates containing embryos from each of the egg masses were placed into incubation chambers (H101-K-Frame, Okolab^{TM}, Italy) of two separate imaging systems, each corresponding with a different rearing temperature (T = 20 or 25 ± 0.2°C). Video data of *R. balthica* acquired from and published in Tills *et al*. (2021) was taken from experiments in which embryos of *R. balthica* were reared at 20˚C, 25˚C and 30˚C for the duration of their embryonic development. Consequently, to standardise these with the current experiments, a control of 20˚C and an elevated temperature treatment of 25˚C was used for embryos of *L. stagnalis* and *P. acuta*. A total of 96 embryos was used for each species (N = 48 per temperature treatment). Control of physical parameters inside incubation chambers is outlined in section 1.7.1.

Acquisition of video is outlined in section 1.7.1 and 2.3.3, the only difference in the current chapter is the utilisation of two OpenVIM systems, to enable the use of two rearing temperatures. There were mortalities in *L. stagnalis* (20˚C = 33.3%, 25˚C = 45.8%), *R. balthica* (20˚C = 16.7%, 25˚C =22.9%) and *P. acuta* (20˚C = 10.4%, 25˚C = 12.5%), and these embryos were excluded from analyses as they did not pass through all physiological windows used in this study.

3.3.2 Image analysis

Manual analysis of the video time series for each developing embryo (N = 48 for each species and temperature) was used to ascertain the timings of a number of key physiological developmental events, outlined in section 2.3.4. These developmental events were used to mark major ‘physiological windows’ of developing embryos, periods of development between the onset of i) ciliary driven rotation (rotation), ii) cardiovascular function (heart), iii) attachment to the egg capsule and onset of muscular crawling (crawling), and iv) the onset of radula function (radula).

Energy Proxy Traits (EPTs) were calculated using an open source Python package Embryo Computer Vision (EmbryoCV) (Tills *et al*., 2018), outlined in section 1.7.2. Temporal frequency data were binned (0.1 Hz intervals to a maximum frequency of 6 Hz) producing a total of 60 frequency bands. Total energy was calculated to produce a proxy for gross rates of embryonic physiology and behaviour (Tills *et al*., 2018; 2021). To standardise rates of development and enable direct comparisons between species and temperatures, the absolute timings from the 4-cell stage to hatching were converted to relative time (0-1).

3.3.3 Dimensionality reduction and statistical analyses

All data were analysed in R v4.0.3 (R Core Team, 2020). Interspecific differences in the developmental response to chronic elevated temperatures were investigated using a repeat measures ANOVA to time series of total energy data. Posthoc analyses using a TukeyHSD was used to test for pairwise differences between temperature treatments in total energy at each point in relative developmental time. To investigate differences in response to chronic elevated temperatures between different physiological windows in development, principal component analysis (PCA) was applied using the R function prcomp() (package stats, v4.0.3). Mean values of energy within each frequency band were calculated at 4 key stages of development outlined above (rotation, heart, crawling, radula), for each temperature (20°C and 25°C). PCA was applied to logged EPT data and eigenvectors were used to investigate combinatorial signals from EPTs at different temperatures and physiological windows of development.

To understand how responses to chronic elevated temperatures change between different physiological windows, pairwise differences in the levels of energy within discrete temporal frequency bands between temperatures within each physiological window were analysed

using a multivariate Kruskal-Wallis test. To minimise false discovery rates a Bonferroni correction was applied (P = 0.00083).

3.4 Results

3.4.1 Interspecific differences in the developmental response to chronic elevated temperatures

Time series of total energy at each hourly timepoint revealed differences in the magnitude of response to chronic elevated temperatures between embryos of each species (Fig. 3.1d). Total energy is the sum of energy within all frequency bands, and may be linked to biochemical energy turnover through physiological and behavioural movement in developing embryos (Tills *et al*., 2018; 2021). Consequently, a greater thermal sensitivity was predicted to result in a greater magnitude of change in total energy in embryos reared at 25˚C. Total energy showed the greatest magnitude of change in *Radix balthica*, which was significantly increased at 25°C relative to 20°C (repeat measures ANOVA, $F_{1, 99} = 67.94$, $P < 0.0001$). Embryos at 26-62% of relative developmental time exhibited a significant increase in total energy at 25°C (TukeyHSD, P < 0.0059, Supplementary information 7, Fig. 3.1b). Conversely in *Physella acuta* a considerably lower magnitude of change was observed, and increases in total energy at far fewer points in relative developmental time (repeat measures ANOVA, $F_{1, 99} = 3.16$, $P < 0.0001$) (22-36% relative developmental time, TukeyHSD, P = 0.034, Supplementary information 7) (Fig. 3.1c). In *Lymnaea stagnalis* there were both significant increases and decreases in total energy at 25°C relative to 20°C ($F_{1, 99} = 21.16$, $P < 0.0001$). At approx. 22-30% relative developmental time there was a decrease in total energy (TukeyHSD, P < 0.042, Supplementary information 7), whereas at 38-58% relative developmental time, total energy was increased (TukeyHSD, P < 0.0001, Supplementary information 1) (Fig. 3.1a).

Additional to the observed differences in the magnitude of response to chronic elevated temperature regimes, time series of total energy revealed temperature-related differences in the relative timings of development. For *L. stagnalis* this was evident as an earlier occurrence in relative developmental time at 25°C of the overall time series observed at 20°C. Manual quantification of the timings of major developmental events used in this study showed that all were accelerated at 25°C relative to 20°C (Kruskal-Wallis, $P < 0.001$, Supplementary information 8). This was also observed in *R. balthica*, and manual quantification of events revealed an acceleration of the onset of muscular crawling and cardiovascular function ($P < 0.001$, Kruskal-Wallis, Supplementary information 8).

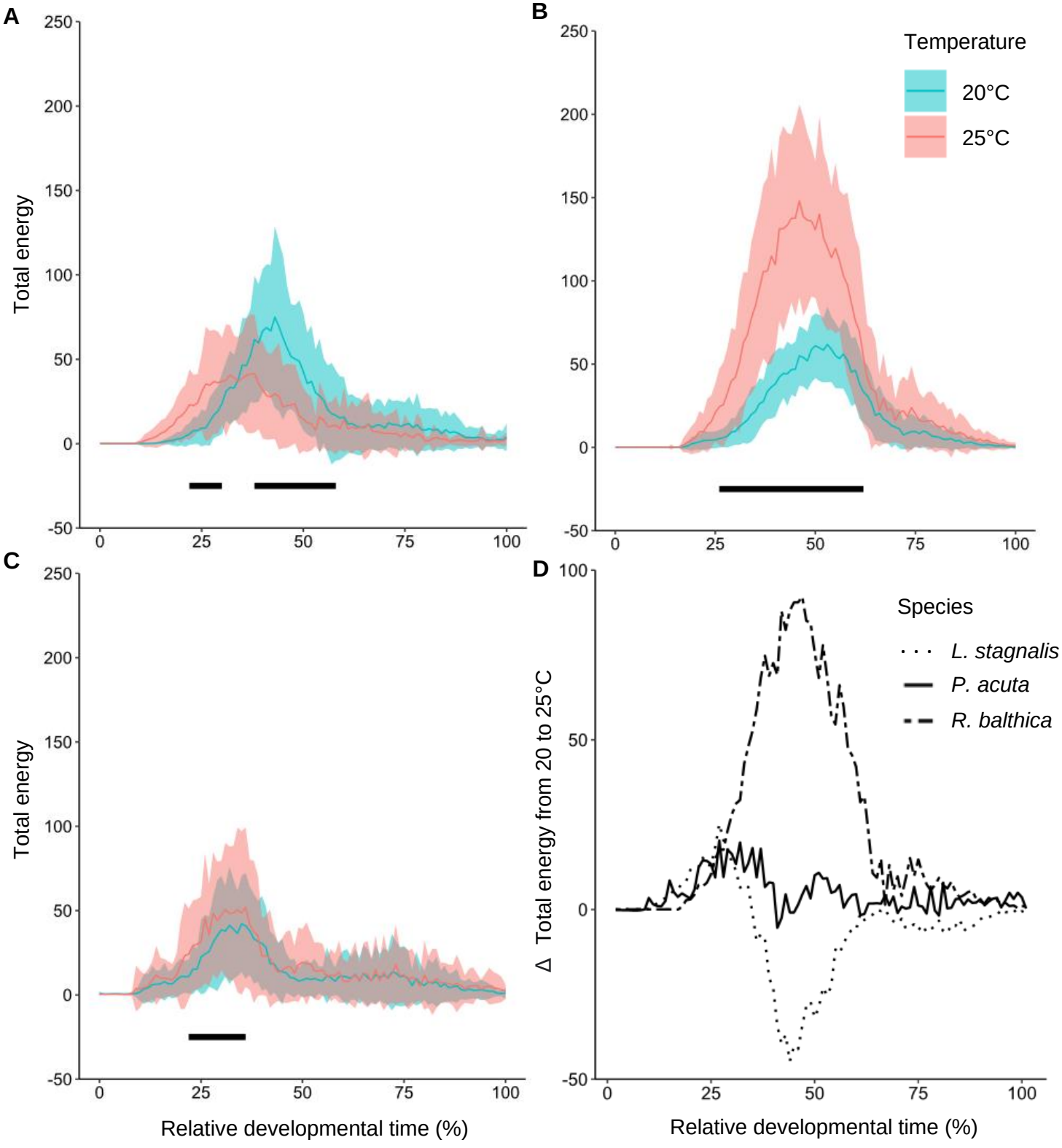

Figure 3.1. Developmental time series of total energy in embryos of **A)** *Lymnaea stagnalis* (20°C: n = 32, 25°C: n = 26), **B)** *Radix balthica* (20°C: n = 40, 25°C: n = 37) and **C)** *Physella acuta* (20°C: n = 43, 25°C: n = 41) (mean/1000 ± sd), and **D)** change in total energy between 20 and 25°C (mean/1000) for each species. Time is normalised (0-1) between the 4-cell stage and hatching. Black lines indicate

regions of relative developmental time in which total energy is significantly different between temperature treatments (TukeyHSD, $P < 0.05$).

3.4.2 Differences in thermal responses between physiological windows in development

Multivariate analysis of EPT data revealed high-dimensional differences in the response to chronic elevated temperatures in different windows of physiological development by embryos of *L. stagnalis*, *R. balthica* and *P. acuta*. In *L. stagnalis* there were no significant differences in the levels of energy between temperature treatments during both ciliary driven rotation and cardiovascular function. However, during muscular crawling (i.e. when the embryo had attached to the wall of the egg capsule and commenced muscular crawling) there were significant increases in the levels of energy at 25°C relative to 20°C in frequencies within the ranges of 0.6 - 0.9 Hz and 1.8 - 2.1 Hz. Additionally, significant increases in energy was observed during the onset of radula function in embryos reared at 25°C for frequencies within the range of 1.8 - 2.1 Hz (Kruskal-Wallis, $P < 0.00083$, Fig. 3.2, Supplementary information 9).

R. balthica embryos showed significant increases in energy at 25°C relative to 20°C within a broad range of frequency bands throughout development. Embryos during ciliary driven rotation exhibited a significant increase in energy in frequencies within the range of 0.1 - 1.7 Hz, 4.1 - 4.7 Hz and 5.9 - 6.0 Hz. During cardiovascular function there was a significant increase in energy at 25°C in frequency bands ranging from 0.4 - 1.8 Hz and 2.6 - 6.0 Hz. During muscular crawling, energy of frequencies within the range of 0.1 - 2.7 Hz and 3.2 - 6.0 Hz was significantly increased at 25°C. Finally, following the onset of radula function embryos showed a significant increase in energy at 25°C at frequencies ranging from 0.3 - 1.1 Hz, 1.8 - 2.5 Hz and 3.4 - 6.0 Hz (Kruskal-Wallis, $P < 0.00083$, Fig. 3.2, Supplementary information 9).

Finally, in *P. acuta* during ciliary rotation embryos exhibited a significant increase in energy at 25°C relative to 20°C at frequencies ranging from 0.1 - 0.4 Hz and 4.4 - 6.0 Hz. During muscular crawling, temperature effects were limited to significant increases in energy at 0.5 Hz and 4.6 - 5.0 Hz. However, after the appearance of cardiovascular function there were significant increases in the levels of energy at 25°C within frequencies ranging from 0.4 - 1.0 Hz and 1.8 - 2.5 Hz. During radula function, energy was significantly greater at 25°C than 20°C in the frequencies 0.5 Hz and 2.0 - 2.7 Hz (Kruskal-Wallis, $P < 0.00083$, Fig. 3.2, Supplementary information 9).

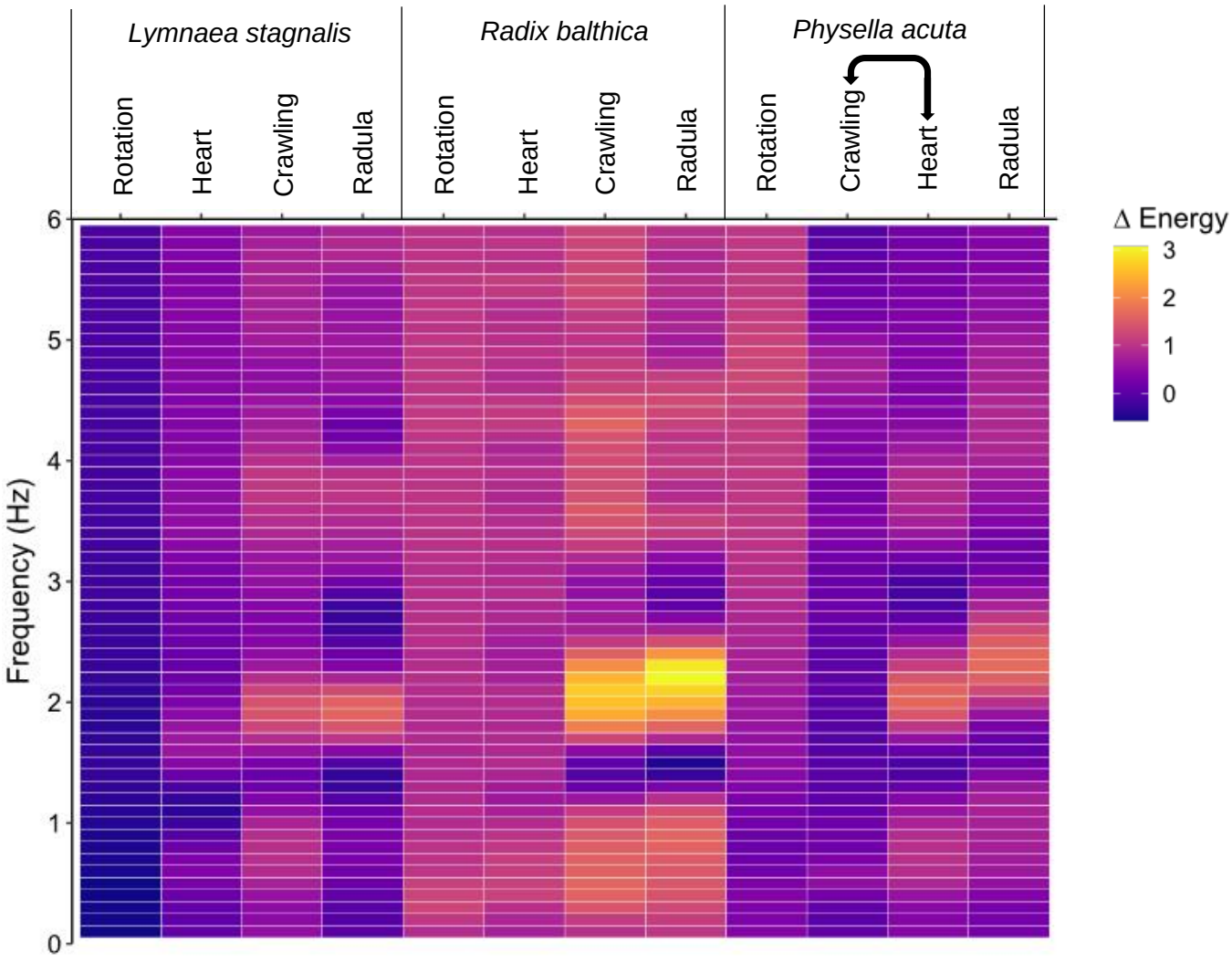

Figure 3.2. Magnitude of change in energy values across 60 temporal frequency bands at 4 key physiological windows in development, between embryos of *Lymnaea stagnalis*, *Radix balthica* and

Physella acuta reared at 20 and 25°C. Rotation = onset of ciliary driven rotation, Heart = onset of cardiovascular function, Crawling = attachment to the wall of the egg capsule and onset of muscular crawling, Radula = onset of radula function. Arrow represents sequence heterochrony between crawling and heart function in *Physella acuta* (Smirthwaite *et al*., 2007).

3.4.3 Combinatorial analysis of EPTs

Reduction of EPT data (0 – 6.0 Hz) at each physiological window in development to three dimensions using PCA, revealed distinct clustering based on temperature and developmental stage. The first 3 principle components of the PCA cumulatively explained 93.15%, 87.30% and 90.50% of the variance for *L. stagnalis*, *R. balthica* and *P. acuta* respectively. For *L. stagnalis*, separation of points between temperatures during ciliary driven rotation and muscular crawling was predominantly along the axis of PC2 (Fig. 3.3a). The dominant frequencies driving treatment separation along PC2 were 0.03 - 1.0 Hz, suggesting that temperature differences evident during ciliary driven rotation and muscular crawling were driven mainly by differences in these lower frequency ranges (Supplementary information 10). Furthermore, during radula function embryos from 20°C and 25°C were mainly separated along the axis of PC3. Treatment separation along the axis of PC3 was predominantly driven by frequencies in the range of 1.8 - 2.1 Hz (Supplementary information 10), suggesting that these frequencies were predominantly driving temperature differences during radula function (Fig. 3.3a).

In *Radix balthica* during ciliary driven rotation and after the onset of cardiovascular function, embryos at different temperatures were principally separated along the axis of PC1. Frequencies in the range of 1.8 - 2.5 Hz predominantly drove separation of points along the axis of PC1, suggesting that frequencies within this range were driving differences between temperature groups during these physiological windows. During muscular crawling, separation

of embryos at different temperatures was distributed fairly evenly across the axes of both PC1 and PC2. Separation along the axis of PC2 was driven mainly by frequencies in the range of 0.03 - 0.8 Hz and 1.9-2.3 Hz, therefore differences between embryos at 20°C and 25°C during muscular crawling are likely driven by frequencies within the range of 0.03 – 0.8 Hz and 1.8 - 2.5 Hz (PC1 and PC2). Additionally, frequencies in the range of 0.1 - 0.6 Hz, 1.5 - 1.7 Hz and 3.0 - 3.2 Hz were predominantly driving separation of points along the axis of PC3 (Supplementary information 10). After the onset of radula function, there was clear separation of points between temperature groups along the axis of PC3. Frequencies ranging from 0.1 - 0.6 Hz, 1.5 - 1.7 Hz and 3.0 - 3.2 Hz were driving separation of points along this axis suggesting that frequencies in this range were driving differences between embryos at 20°C and 25°C (Fig. 3.3b).

Finally, in *P. acuta*, during ciliary driven rotation, embryos at different temperatures were separated along the axis of PC1. Frequencies ranging from 2.0 - 4.0 Hz were driving differences along PC1 indicating that temperature differences during this physiological window were driven by frequencies within these ranges. During cardiovascular and radula function embryos at different temperatures were separated along the axis of PC1 and PC2. Additionally, there was a clear separation of points along the axis of PC3. PC2 and PC3 were driven predominantly by frequencies within the range of 0.03 - 1.0 Hz and 2.0 - 2.4 Hz, suggesting that differences between embryos at these stages were driven by frequencies within these ranges. (Fig. 3.3c, Supplementary information 10).

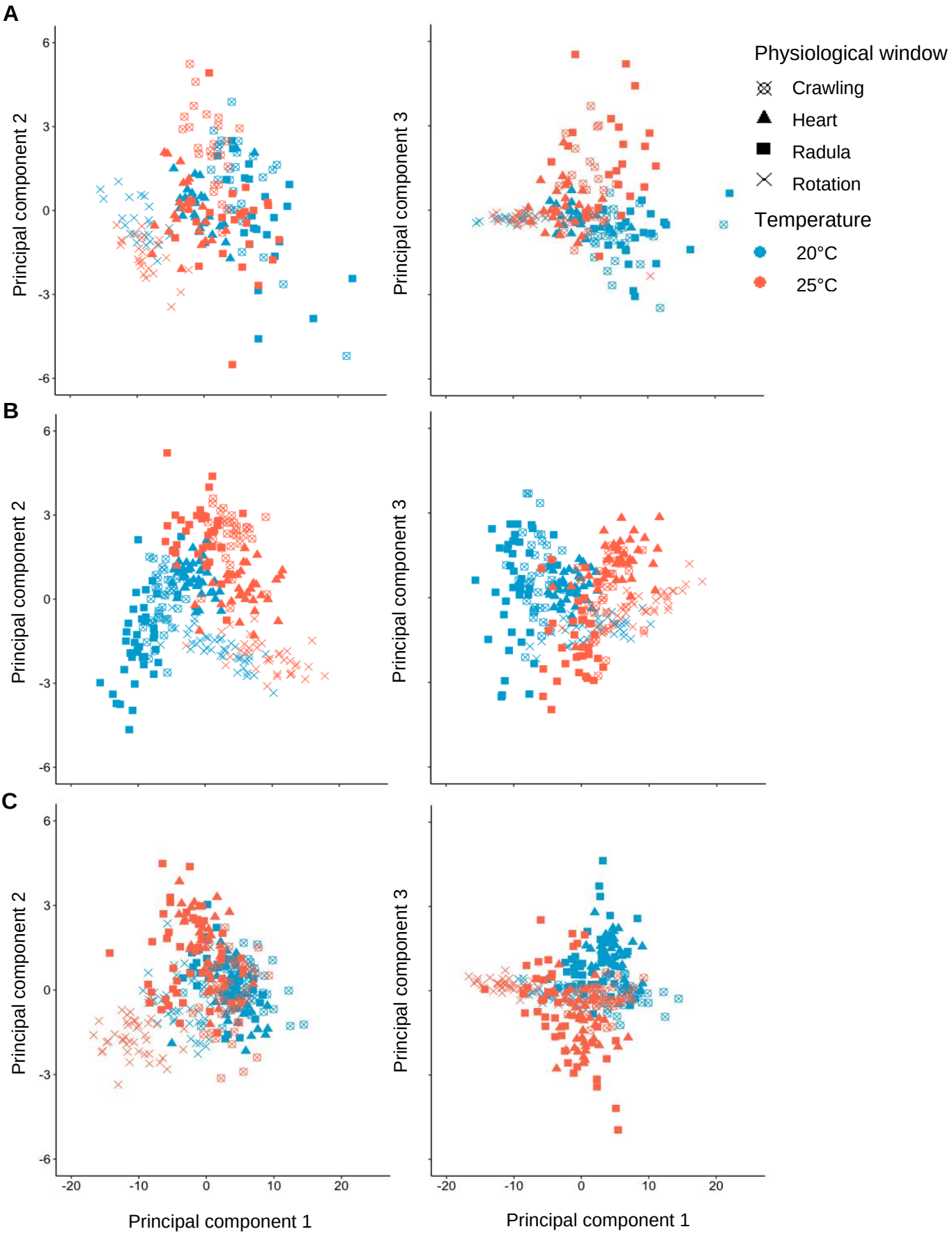

Figure 3.3. Principal component analysis (PCA) of the mean levels of energy within 60 temporal frequency bins at two temperatures (20°C and 25°C) at 4 physiological windows in development

(rotation, crawling, heart, radula) during the embryonic development of **A)** *Lymnaea stagnalis*, **B)** *Radix balthica* and **C)** *Physella acuta*.

3.5 Discussion

This study aimed to understand the extent to which a phenomics approach can be used to compare interspecific differences in the developmental response to environmental change. Additionally, it sought to determine the extent to which phenomics approaches captured responses between physiological windows in development that differ in their observable phenotypes. To do this I tested the relative thermal sensitivities of embryos of three species of freshwater snail with pre-resolved sequence heterochronies thereby providing us with a scaffold from which to interrogate EPT data of embryos at different physiological windows. EPTs revealed interspecific differences in relative sensitivities to chronic elevated temperatures, and differences in thermal responses between physiological windows in development, with embryos of *P. acuta* predicted to show the lowest magnitude of change in energy under chronic elevated temperatures, owing to their comparatively lower predicted thermal sensitivities. I suggest that temperature differences in *Lymnaea stagnalis* embryos largely manifest as an increase in the magnitude of energy at frequencies associated with cardiovascular function following the onset of muscular crawling and radula function, and an earlier occurrence in relative developmental time of major physiological events, evident in EPT spectra. In *Radix balthica* embryos, energy within a broad range of frequency bands increased at 25°C, during all physiological windows assessed in this study, indicating an increase in the rates of many observable embryonic physiologies and behaviours throughout its embryonic development. In *Physella acuta*, temperature induced increases in rates of organismal physiology and behaviour were largely limited to frequencies corresponding with cardiovascular function whilst there was little change in the magnitude of energy at other

frequencies. In summary, EPTs exhibited marked differences in the magnitude and direction of thermal effect between species and physiological windows in development.

3.5.1 EPTs reveal interspecific differences in the developmental response to chronic elevated temperatures

Investigation of total energy time series revealed major differences in the magnitude of response between chronic elevated temperatures. Total energy is the sum of energy across all frequencies for each hourly 30 s video, and it integrates all sources of biological movement present in video. Given that all forms of movement incur energetic costs, total energy is likely linked to overall rates of biochemical energetic turnover through observable movement in developing embryos, and recent work has shown that this appears to be the case (Tills *et al*., 2021). *Radix balthica* showed the greatest magnitude of change in total energy from 20°C to 25°C compared with *Lymnaea stagnalis* and *Physella acuta* (Fig. 3.1). Temperature affects rates of processes at every level of biological organisation, and any increase in total energy under chronically elevated temperatures could indicate heightened sensitivity to relatively moderate increases in temperature over extended periods of time in embryos of *R. balthica* (Hochachka & Somero, 2017; Iverson *et al*., 2020). Conversely, there were considerably lower magnitudes of change in total energy in *P. acuta* and *L. stagnalis*, and even a reduction in total energy at one point in relative developmental time in *L. stagnalis* (Fig. 3.1a, c, d). The comparatively lower thermal sensitivity in total energy in embryos of *L. stagnalis* and *P. acuta*, either indicate a) considerably lower thermal sensitivity of rates of observable embryonic physiology and behaviour at these stages of development, unlikely given the obvious effects on energy at frequencies associated with cardiovascular function (Fig. 3.2, 3.3; sections 3.4.2, 3.4.3), or b) a decoupling of levels of overall embryo activity, and maintenance of basic physiological function under chronically elevated temperatures (Pörtner, 2010). Additionally,

the observed reduction in total energy in embryos of *L. stagnalis* at 25°C may suggest some form of limitation on these embryos, thereby reducing energy allocated to gross rates of physiology and behaviour. Rates of many observable organismal physiologies and behaviours continue to increase until a thermal optimum is reached, beyond which rates rapidly decline (Huey & Kingsolver, 1989; 1998; Angiletta, 2006). In species that can behaviourally thermoregulate or that experience relatively mild variations in temperature in their environments, behavioural thermal ranges are generally assumed to match physiological tolerance limits (Hernández & Bückle, 2002; Monaco *et al*., 2017). However, when behavioural thermoregulation is not an option, rates of activity can decline at lower temperatures than those of physiological function. For example, Monaco *et al*. (2017) showed that in six species of intertidal gastropod, the CT_{max} of crawling speed was less than that of heart rate. Furthermore, species occupying greater shore heights and therefore greater temperature extremes, generally exhibited a greater degree of decoupling between these traits. Previous research applying EPTs to the embryonic development of *R. balthica* showed that development at 30°C resulted in a major reduction in energy across the whole period of embryonic development, indicating depressed rates of organismal movement, despite an increase in heart rate (Tills *et al*., 2018). This also highlights a limitation of the methodology used in this study. Given that two temperatures were used (20˚C and 25˚C), I was unable to identify specific temperatures at which physiological performance begins to decline, or construct a thermal performance curve for each of these species (Angilletta, 2006). Consequently, whether the comparatively low magnitude of change in total energy at 25˚C in *L. stagnalis* and *P. acuta* is due to a reduction in physiological performance is difficult to determine. Consequently, future research could be directed towards establishing thermal performance curves for EPTs for these species. Given that frequencies within energy spectra correspond with different observable physiologies and behaviours, EPTs may provide an effective means with which to construct thermal performance curves for whole-organism aspects of physiology and behaviour.

Shifts in the relative timings of development between embryos reared at 20°C and 25°C were also apparent from interrogation of the total energy time series. In *L. stagnalis* increases in the levels of total energy associated with the onset of ciliary driven rotation, as well as decreases in energy associated with the onset of intermittent resting behaviours (McCoy, unpublished obs.), both commenced earlier in relative developmental time, reflecting an acceleration of the timings of these events under chronic elevated temperatures (Fig. 3.1). Additionally to this, were was a decoupling of these two events in *R. balthica*, where the onset of ciliary-driven rotation remained unchanged, with the timing of onset of intermittent resting being accelerated at 25°C. Manual quantification of the timing of onset of ciliary driven rotation and muscular crawling confirmed that these differences mirrored these transitions evident in total energy time series (Supplementary information 8). Acceleration of the relative timings of developmental events have been demonstrated in a number of species, and so too has uncoupling of the timings of different developmental events. For example, following an increase in developmental temperature of 5°C in larvae of *Dicentrarchus labrax*, the relative timings of development of the larval yolk sac and formation of pectoral fins were accelerated, when standardised against total length (Koumoundouros *et al*., 2001). In embryos of herring, *Clupea harengus* exposed to elevated temperatures, the timings of various developmental events exhibited different thermal sensitivities. Increased developmental temperature resulted in differences to the relative timing of organogenesis (spinal cord, pectoral fin buds and myotomal muscle fibres), whilst the timings of tissue differentiation remained almost unchanged (Johnston, 1993). EPT spectral time series indicate that whilst for *L. stagnalis* growth at a higher temperature results in an acceleration of the majority of the developmental itinerary, for *R. balthica* (and *P. acuta*) there is a decoupling of these major developmental transitions in relative developmental time. This is of particular interest as plasticity in the timings of development may act as a driver of evolutionary change (Spicer & Rundle, 2007; Spicer *et al*., 2018). Here, shifts in total energy time series in relative developmental time at 25°C corresponded with differences in manually determined timings of physiological events, and have previously found that standing intraspecific variation in the timings of developmental

events correspond with intraspecific differences in EPT time series (J. McCoy, unpublished obs., Chapter 2). Selection typically acts on multiple traits simultaneously (Lande & Arnold, 1983; Phillips & Arnold, 1999) and given that EPTs integrate a number of observable embryonic physiologies and behaviours, it is not unreasonable to question whether EPTs, and temperature induced changes in the timings of total energy, may act as objects of multivariate selection.

3.5.2 Differences in thermal responses between physiological windows in development correspond with ontogenetic changes to observable phenotype

In *L. stagnalis* embryos, frequencies corresponding with observable heart beating (1.8 - 2.1 Hz) (Voronezhskaya *et al*., 2007) were found to be driving differences between embryos reared at 20°C and 25°C during radula function. This was also associated with a significant increase in energy at these frequencies during radula function at 25°C. However, during muscular crawling, temperature differences were predominantly driven by frequencies in the range of 0.6 - 0.9 Hz. During muscular crawling, embryos of *L. stagnalis* exhibit a number of other observable physiologies, notably body flexing and mantle muscle control (Meshcheryakov, 1990; Smirthwaite *et al*., 2007), changes in the rates of which may be contributing to the observed differences in energy ranging from 0.6 - 0.9 Hz. Conversely in *R. balthica* there was an increase in energy across a broad range of frequency bands across the whole period of development. This is suggesting that in embryos of *R. balthica* rates of a considerable number of observable embryonic movements are increased under chronic elevated temperatures. Temperature affects the rates of processes at every level of biological organisation, and consequently increases in levels of energy across the EPT spectrum may indicate a greater thermal sensitivity in the embryonic development of *R. balthica*. Finally, in *P. acuta* and in common with *L. stagnalis* there was a significant temperature induced increase

in energy in frequencies associated with changes to heart rate after the onset of cardiovascular (1.8 - 2.5 Hz) and radula functions (2.0 - 2.7 Hz) in *P. acuta* (Seeland *et al*., 2013), as well increases in energy at 0.4 - 1.0 Hz during cardiovascular function. Following the onset of cardiovascular function *P. acuta* embryos also exhibit body flexing and mantle muscle control. These physiologies exhibit lower rates than that of observable heart beating (J. McCoy, pers. obs.), and therefore increases in energy within the 0.4 – 1.0 Hz range likely correspond with increases in the occurrence of these observable physiologies. In summary, differences in the thermal response of EPT spectra between physiological windows of development in embryos of each species, largely correspond with ontogenetic changes in observable phenotype.

3.5.3 Implications

Research aimed at understanding the broad implications of climatic change on animals inhabiting marine and freshwater habitats typically centres on sexually mature adults, often ignoring early life stages. This is despite periods of early development showing equivalent if not greater sensitivities to forms of environmental change (Burggren, 2018; 2021). However current approaches to phenotyping periods of early development are often not transferable between species that vary in their relative timings of development as they a) fail to integrate the considerable structural and functional changes associated with embryonic development, and b) utilise reductionist approaches centring on small numbers of observable phenotypes rather than integrating the widespread changes to observable phenotypes typically associated with the response to chronic elevated temperatures (Burggren, 1987; Spicer & Burggren *et al*., 2003; Forsman, 2015). In the current study, EPTs revealed major differences in the relative sensitivities of embryos of *R. balthica*, *L. stagnalis* and *P. acuta* to chronic elevated temperatures, as well as differences in thermal sensitivity between various physiological windows in these embryos. Whilst high-dimensional phenotyping approaches are well

established for early developmental stages of model species such as the zebrafish *Danio rerio* (Xu *et al*., 2010; Peravali *et al*., 2011; Spomer *et al*., 2012), the nematode *Caenorhabditis elegans* (White *et al*., 2010; Olmedo *et al*., 2015) and the fruitfly *Drosophila melanogaster* (Chung *et al*., 2010; Levario *et al*., 2016), approaches that are transferable between non-model species of interest are lacking. The indiscriminate nature by which embryonic movements comprising observable physiologies and behaviours are captured by EPTs facilitates transferability between non-model species of interest. Expansion of animal models beyond common model species, to support biological research across a greater breadth of diversity, would improve the evidence base for the effects of climatic change on early development (*sensu* Krogh, 1929; Burggren, 2021b). However, there are still limitations associated with using this approach as a method for quantifying environmental sensitivity. Whilst EPTs enable transferability between species and physiological windows of development, interpretation of their responses in a *de novo* sense (i.e. when applied to a new species) may present difficulties. In the current study, the use of known physiological windows in development allows for the interpretation of results within the scaffold of a set of known embryonic physiologies and behaviours, thereby allowing for the interpretation of observed changes in EPTs under chronic elevated temperatures. Without these, physiological windows and developmental events being known *a priori*, interpretation of these results becomes difficult. However, the capacity to integrate all observable forms of embryonic movement and analyse these in a combinatorial fashion can still be achieved in application to multiple species of interest, for example in the detection of stress responses to various environmental toxicants (Rudin-Bitterli *et al*., 2014), and EPTs can be used in the detection of known aspects of organismal physiology (Ibbini *et al*., 2022).

EPTs also enable quantification of phenotypic change continuously across multiple physiological windows in development despite major transitions in observable phenotype. Whilst in the current study EPT data were also presented and analysed as discrete sections in relative developmental time, these were chosen to reflect major differences in the

progression of physiological development of embryos, enabling robust linkage of EPT spectra to known differences in observable embryonic phenotype. Embryonic development is a period of unrivalled levels of structural and functional change, rendering the continuous quantification of environmental effects on developmental phenotype problematic (Burggren, 1987; Spicer & Burggren *et al*., 2003). Assessment of phenotypic responses throughout embryonic development often necessitates quantification of changes in specific traits or broad scale indicators of organismal performance, for example rates of oxygen consumption (Pörtner *et al*., 2011) and tolerance limits to forms of environmental stress (Kuramoto, 1978; Hammond & Hofmann, 2010; Storch *et al*., 2011; Truebano *et al*., 2018), at discrete times or stages in developmental time. In his recent review, Burggren (2021) highlighted that a significant limitation of physiological measurements at discrete points in development may be inaccurate, and that by considering development as a continuum, physiological measurements can be put into the context of an organism's entire development. Continuous quantification of phenotypic change rather than at discrete points in developmental time may provide us with more complete measurements of developmental responses to climatic change (Burggren, 2021a).

Finally, the observed changes in EPTs under elevated temperatures stimulates the idea that such changes in EPT spectra may have implications for organismal performance and fitness. Previous relationships were established between EPTs and a developmental outcome (growth rate), suggesting that EPTs may provide a visual proxy for rates of biochemical energy turnover in developing embryos (Tills *et al*., 2021). Total rates of biochemical energetic turnover (metabolic rate) during early development and the time taken to develop multiply to form the cost of development. The optimal temperature at which development takes place, is hypothesised to be the temperature at which developmental cost is minimised, and any deviation from that optimum will result in an increase in the cost of development, thereby reducing energy reserves at the onset of nutritional independency, likely having impacts on organismal performance (Marshall *et al*., 2020). Here, there were differences in both the magnitude of change in total energy, and shifts in EPT time series in relative developmental

time following exposure to chronic elevated temperatures. Allocation of energetic reserves to various behavioural and physiological functions is hypothesised to be a careful trade off based on the environmental conditions under which an organism finds itself (Brafield & Llewellyn, 1982). If EPTs are directly related to biochemical energy turnover in developing embryos, visual quantification of levels of biochemical energy turnover within different temporal frequencies could provide useful proxies for how energy is allocated into various processes throughout the whole period of development, rather than characterising energetic turnover at discrete points in development (Attard & Hudon, 1987; Rombough, 1994; Stackley *et al*., 2011). Such a proxy may provide useful insights into the effects of climatic change on performance and fitness of developing embryos, *via* quantification of biochemical energy turnover.

3.6 Summary and conclusions

Assessing phenotypic responses to elevated temperatures during early development is crucial for predicting how species will respond to climatic change (Burggren, 2018). EPTs revealed interspecific differences in relative sensitivities to chronic elevated temperatures, temperature induced changes in the relative timings of development, and differences in thermal responses between physiological windows in development that largely coincide with ontogenetic differences in observable phenotypes. Crucially, EPTs provided an approach to high-dimensional organismal phenotyping that is transferable between species that vary in their early development, and between physiological windows in development that vary in their observable phenotypes. Furthermore, the indiscriminate nature with which EPTs capture observable embryonic phenotypes allowed for the integration of all observable forms of embryonic movement, and analysis of this data in a combinatorial fashion, rather than focussing on small numbers of observable embryonic phenotypes. Understanding the broader

implications of climatic change on early life stages of marine and freshwater animals requires phenotyping approaches that are applicable to non-model species favoured by the Krogh principle, and to assess phenotypic change continuously through early development, rather than simplifying the dynamic process of embryonic development into small sets of discrete developmental stages.

Chapter 4

Effects of experimentally reducing biochemical energetic content on energy proxy, and life history traits

4.1 Abstract

Understanding evolutionary and environmental drivers of physiological development is crucial for understanding evolutionary changes and responses to novel environmental regimes, however measurement of high-dimensional phenotypic changes is limited by current methodologies. Energy Proxy Traits (EPTs) are a novel approach to phenotyping during early development that quantify changes in pixel brightness as levels of energy across temporal frequencies in videos of developing embryos. However, the consequences of changes in EPTs in relation to an organism's overall development and performance is poorly understood. Consequently, I aimed to determine if there was any relationship between EPTs and aspects of organismal development. This was achieved by measuring changes in EPTs and a number of life history traits (development time, growth rate and size at hatch) following experimental reduction of the 'energy' of developing embryos of *Lymnaea stagnalis* using a micro injection system. Removal of 50% of the perivitelline fluid resulted in a ~125% increase in total EPT spectra energy. This increase in total energy may in part be driven by altered viscosity of the remaining perivitelline fluid post removal. Additionally to this I observed a decrease in rates of growth and size upon removal of 50% of perivitelline fluid, however this change was considerably lower than predicted. This may indicate that eggs of *L. stagnalis* are overprovisioned with nutrient at laying. Taken together, the observed increases in total energy accompanying reduced growth and size indicate a negative relationship between these two variables under nutrient limited conditions, potentially suggesting a trade-off between energy

invested into growth and gross rates of movement. Future research should be directed towards establishing correlative links between EPTs, and rates of biochemical energetic turnover during embryonic development.

4.2 Introduction

Evolutionary and environmentally induced changes to developmental phenotype are complex and multifaceted, and effectively measuring these changes is significantly limited by current phenotyping technologies (Truong & Supatto, 2011; Forsman, 2015; Lürig *et al*., 2021). These limitations can be addressed through the use of non-invasive video based proxies, which provide scalable approaches to measuring high-dimensional phenotypic change (Tardieu *et al*., 2017; Lürig *et al*., 2021). Energy Proxy Traits (EPTs) are a novel approach to measuring developmental responses in video of developing embryos. Rather than targeting specific aspects of an organism's physiology or behaviour, EPTs measure the levels of energy within specific temporal frequencies calculated from fluctuations in pixel brightness in videos of developing embryos, and therefore encompass a range of observable phenotypes in a developing organism (Tills *et al*., 2022). EPTs have proven effective at quantifying both lethal and sub-lethal thermal responses in species of freshwater gastropod (Tills *et al*., 2021; Chapter 3), amphipod (Tills *et al*., 2018) and decapod crustacean (Tills *et al*., 2022). Additionally to this, EPTs are effective at characterising high-dimensional phenotypic changes associated with evolutionary differences in the timings of developmental events (heterochronies), as well as differences in event timings between conspecifics (Chapter 2). Despite this, progress in understanding the significance of EPTs within the context of an organism's performance and overall development is still in its infancy.

Tills *et al*. (2021) suggested that EPTs may be related to aspects of an organism's life history. They showed that the levels of total energy i.e. the sum of the spectrum of energy values

across frequencies, was positively related to the growth rate of developing embryos, suggesting that EPTs may in themselves be related to the turnover of biochemical energy during growth. Whilst an interesting and potentially useful notion, studies are still required to test or validate this idea. In particular, gaining a robust understanding of the significance of observed changes in EPTs in terms of an organism's development and physiological performance requires measurement of EPTs alongside a wider range of life history traits. Life history traits are frequently used as indicators of developmental change following exposure to forms of environmental stress, and measures including size at hatch, development time and growth rate are effective as broad scale indicators of the performance of developing animals (Nylin & Gotthard, 1998; Dmitriew, 2011; Chaparro-Pedraza & de Roos, 2018). As a result, characterising the relationship between these traits and EPTs will provide some understanding of the significance of changes in EPTs for an organism's development and performance.

Understanding how changes in EPTs relate to other measures of developmental and physiological performance can either be achieved through the use of correlative approaches, in which EPTs are quantified alongside life history traits under control conditions, or through the use of treatment based designs, in which the 'normal' developmental trajectory of an individual is perturbed, and the relationship between EPTs and life history traits is characterised in each treatment. Alterations to the levels of biochemical energy available to a developing animal such as the quantity of yolk, is known to elicit broad scale changes to the development of an individual, and this has been achieved in a range of animal species. Removal of biochemical energy has been shown to impact development time (Alquati *et al*., 2007), size at hatch (Finkler *et al*., 1998; Jardine & Litvak, 2003), and can influence the performance of animals post hatch (Sinervo, 1990; Emlet & Hoegh-Guldberg, 1997; Willems *et al*., 2014; 2015; Peña-Villalobos *et al*., 2017). Consequently, quantification of EPTs alongside various life history traits following alteration to the biochemical energetic content of a developing embryo may provide an effective means with which to understand how changes in EPTs relate to an organism's development and performance.

Here I aimed to determine if there was any relationship between EPTs and aspects of organismal development. I predict that a reduction in biochemical energy content of the developing embryo will result in a reduction in total energy, an indicator of gross rates of embryonic movement, and a concomitant reduction in rates of organismal development, and size at hatch. To achieve this, I measured EPTs and a number of life history traits including development time, growth rate and size at hatching following serial experimental manipulation of egg nutrient content in the pond snail *Lymnaea stagnalis,* an emerging model for developmental biology. Embryos of *Lymnaea stagnalis* develop inside transparent egg capsules, allowing for the direct observation of rates of embryonic movement originating from physiological and behavioural processes (Morrill, 1982; Meshcheryakov, 1990; Smirthwaite *et al*., 2007). Following gastrulation, embryos exogenously feed on perivitelline fluid within the egg capsule which is primarily composed of protein (~90%) for growth and galactogen (~10%) for maintenance of function (Taylor, 1973; 1977). Crucially, developing embryos of *L. stagnalis* are a closed nutrient system with a fixed content of nutrient available at the start of development, making embryos of *L. stagnalis* a tractable model species for this experiment.

4.3 Materials and methods

4.3.1 Animal collection and maintenance

Adult *Lymnaea stagnalis* were collected using an FBA (Freshwater Biological Association) net from south drain in Somerset levels (51°10'50.5"N 2°52'49.9"W). Snails were transported in plastic containers containing water and pondweed from the site of collection to the laboratory within 12 h of collection. There, adults were placed into 12 L plastic containers and maintained for a minimum of 2 weeks at T = 15°C in artificial pond water (APW) ($CaSO_4$ – 120mg L^{-1}, $MgSO_4$ – 245 mg L^{-1}, $NaHCO_3$ – 192 mg L^{-1}, KCl – 8 mg L^{-1}) aerated using an air stone. A 12h

light/12 h dark regime was used and snails were fed spinach and lettuce *ad libitium*. Water was completely changed within rearing aquaria weekly.

4.3.2 Embryo collection and egg volume calculation

Snails regularly laid egg masses onto the walls and floor of rearing aquaria. 48 hours prior to experimentation water was completely changed in rearing aquaria and all egg masses removed. Following this, once new egg masses were laid they were removed using a thin piece of laminate plastic. Preliminary trials indicated that manipulation of nutrient content of the egg capsule at the 4-cell division to blastula stage resulted in death of the embryo within the following 12 h. As a result, egg masses were viewed under low power magnification (10-40x) and eggs at the gastrula stage, identified by formation of the blastopore at the vegetal pole, were removed.

Embryos were divided between 4 treatments: 50% (removal of half of the perivitelline fluid volume); 25% (removal of a quarter of the perivitelline fluid volume); sham control (embryos that were punctured but had no perivitelline fluid removed); and control embryos (subjected to no manipulation other than that of general experimental handling). A total of 96 eggs were used from 3 egg masses (24 per treatment, 8 per egg mass within each treatment). Prior to experimentation, mean volumes were calculated for a sample of eggs from the source population. Eggs of *L. stagnalis* can be modelled as prolate spheroids (Taylor, 1973), and as a result volumes were calculated as:

$$(1)\ V = \frac{1}{6}\pi\ a\ b^2$$

Where *V* = volume, *a* = distance from the oblate spheroids centre along the longest axis, and *b* = distance from the oblate spheroids centre along the shortest axis (Taylor, 1973). Subsequent volumes were then used to calculate removal quantities for both the 50% and 25% treatment. From these findings eggs with a mean volume 840 ± 62 nL were subject to a removal of 210 nL (25% treatment) and 420 nL (50% treatment) of perivitelline fluid.

There were mortalities of 58.3%, 45.8% and 12.5% in 25%, 50% and control embryos respectively. These embryos were removed from analyses as they failed to hatch, and therefore measurements of hatch size, development time and growth rate could not be established from these embryos.

4.3.3 Perivitelline fluid removal

Needles for extracting perivitelline fluid were pulled from glass capillary tubes (diameter = 1.2 mm, WPI, United States) using a micropipette puller (PUL-1, WPI, United States). The tips of pulled glass capillaries were then broken under low power magnification, producing tips with an aperture of ~40 µm. Needle tips were inserted into a microsyringe (10 µL, Mikrolitерspritzen, Innovative Labor Systeme, Germany) back filled with mineral oil (Thermo Fisher Scientific, United Kingdom).

For removal of nutrient content, eggs were first transferred to a Petri dish containing agar jelly using a micro-pipettor (1000µL, Sartorius, Germany). Furrows within the agar jelly were made to contain the egg. Eggs were first gently blotted with filter paper to remove any excess inter-capsular fluid (Taylor, 1973). Fine scale movement of the microsyringe and injection into the egg was achieved using a micromanipulator (MMJR, WPI, United States). An UltraMicroPump

II (WPI, United States) was used to draw the perivitelline fluid into the needle tip at a rate of 25 nL s^{-1}, using a microprocessor (Micro4^{TM}, WPI, United States). Needle tips were then slowly removed from the egg, and the perivitelline fluid was discarded. Following removal of perivitelline fluid, water was pipetted onto punctured eggs to prevent desiccation, following which eggs were transferred to individual wells of a microtitre plate (Nunc, Microwell, 96 wells, 350µL per well) using a micro-pipettor (1000µL, Sartorius, Germany). Control eggs were exposed to the same protocols detailed above with the exception of puncturing of the egg capsule. The sham control incorporated all of these steps, but with no removal of perivitelline fluid.

4.3.4 Sham controls

For sham control embryos, the needle tip was inserted as described above, but slowly removed after ~15 s. For the main experiment, there was significant leakage of perivitelline fluid in a number of eggs from the sham treatment following removal of the needle tip, and in the days following its removal (Fig. 4.1b). Consequently, this control treatment was deemed to be a poor control for the effect of needle tip insertion alone. As a result, I repeated this treatment and a comparison with control eggs (i.e. eggs that had not been manipulated at all) using eggs from a laboratory stock population of *L. stagnalis*, to determine whether the control eggs from our initial experiment were an effective baseline of comparison with 50% and 25% treatments. Embryos were taken at approximately the late gastrula stage determined by the formation of functional cilia in the prototroch and foot region, and the slow rotation of the embryo (Meshcheryakov, 1989). Protocols were followed as described above, however to minimise leakage needle tips were removed gradually in approximately 10 increments over a period of ~45 s, and little to no leakage of perivitelline fluid was observed. I observed no mortalities in the sham control treatment, however mortality of 13.3% in control embryos.

These embryos were excluded from analyses as measurements of hatch size, development time and growth rate could not be established.

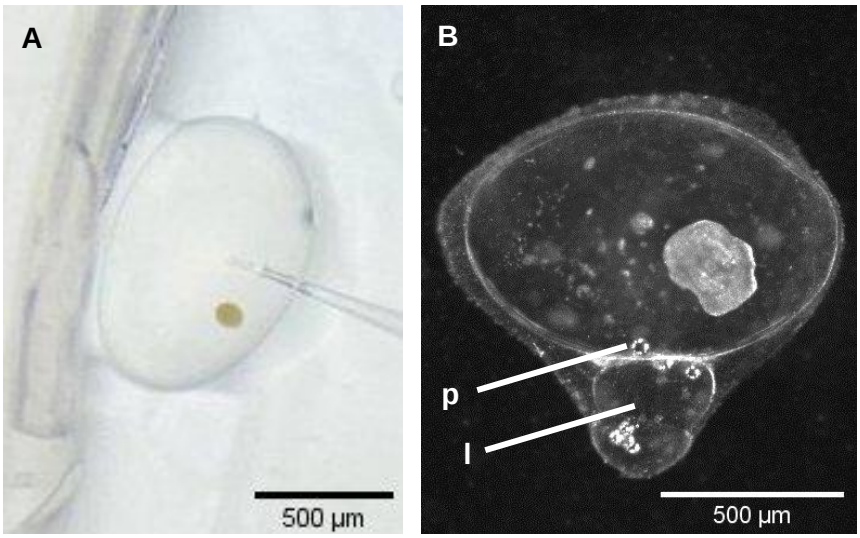

Figure 4.1. *Lymnaea stagnalis* embryo **A)** pierced by needle tip prior to extraction of perivitelline fluid. **B)** with sham control displaying large leakage of perivitelline fluid following puncturing by needle tip, p = site of puncture, l = leaked perivitelline fluid.

4.3.5 Embryo maintenance and bioimaging

An autonomous open-source video microscope (OpenVIM, Tills *et al*., 2018) was used to record embryonic development from the gastrula stage until hatching. Embryos within a microtitre plate were placed into an incubation chamber at 20°C (H101-K-Frame, Okolab™, Italy), the temperature of which was controlled by circulation of water through the chamber by a temperature bath (H101-CRYO-BL, Okolab™, Italy). A constant supply of pre-humidified air was provided using a humidity module (Okolab™, Italy). The position of the incubation chamber was controlled using a motorised XY stage (SCAN 130x85, Märzhäuser Wetzlar™, Germany), which was mounted on to an aluminium frame.

Image sequences of developing embryos were acquired using a Charged Couple Device digital camera (resolution: 2048 x 2048 pixels, Pike F421B, Allied Vision™, Germany) attached to an inverted lens at 200x magnification (VH-720R, Keyence™, UK). Dark field illumination was achieved using an LED light ring placed above the incubation chamber and lens (LDR2-42-SW2, CCS, UK). Image sequences of developing embryos were acquired hourly for 30 s at 30 frames s^{-1}, at a resolution of 1024 x 1024 pixels for the duration of their embryonic development, using the open source ImageJ plugin µManager (Edelstien *et al*., 2010).

4.3.6 Image analysis

Prior to calculation of EPTs, an AI model was used to identify and segment embryos (Supplementary information 22, 23). Following removal of nutrient content, a large number of eggs increased in opacity, likely due to biological growth on the outside of the egg capsule, thereby reducing contrast and considerably lowering the success of segmentation based on pixel values alone. As a result, in order to isolate a bounding box surrounding the egg for the calculation of EPTs, and segment embryos for measurement of size throughout development, transfer learning using a Mask R-CNN was applied using TensorFlow (Abadi *et al*., 2016) (Supplementary information 21). This model was trained using approximately 500 images of embryos at various stages of development, and with varying degrees of biological growth on the egg capsule. Once a region of interest was isolated around the egg, EPTs were calculated as outlined in section 1.7.2. Frequencies were binned into 0.1 Hz increments, to a maximum of 6 Hz, producing a total of 60 frequency bins. Embryo area (μm^2) was recorded throughout development using an AI model. Size at hatching was recorded as the area of the embryo prior to hatching (mm^2), and the time taken to hatch (h) defined as emergence from the egg capsule. Growth rates ($mm^2\ h^{-1}$) were subsequently calculated from area and development time data.

4.3.7 Statistical analysis

All data were analysed in R v4.0.3 (R Core Team, 2020). Gross rates of embryonic physiology and behaviour were established through the calculation of total energy, the sum of energy values across all frequency bands. Total energy was calculated first for the lifetime of each individual, to provide a gross indicator of how total energy trades off with rates of growth and size under varying nutrient content levels. A one-way ANOVA ($P < 0.05$) was used to test for differences between treatments, coupled with a TukeyHSD to test for pairwise differences. Temporal changes in total energy were also investigated by binning total energy data into 5 relative developmental time increments (0 - 20%, 20 - 40%, 40 - 60%, 60 - 80%, 80 - 100%). A two-way ANOVA ($P < 0.05$) was applied to the log of these data, followed by a TukeyHSD. PCA was also applied to logged EPT data and eigenvectors were used to investigate combinatorial signals from EPTs at different treatments and relative developmental time increments. Differences in hatch size, developmental time and growth rate between treatments were analysed using a one-way ANOVA ($P < 0.05$), again, followed by a TukeyHSD.

4.4 Results

4.4.1 Sham controls

Following the initial experiment, sham control embryos in which the needle tip was removed more delicately as to minimise leakage of perivitelline fluid, were imaged alongside control embryos. The hatch time of sham control embryos was not significantly different from control embryos (ANOVA, $F_{1, 50} = 0.629$, $P = 0.431$), and neither was the size of hatchlings (ANOVA,

$F_{1, 49}$ = 1.875, P = 0.164). Furthermore, total energy showed no significant differences between treatments (ANOVA, $F_{1, 42}$ = 3.019, P = 0.0889). As a result, given that puncturing of the egg capsule resulted in no statistically significant differences in two major life history traits, as well as total energy in developing embryos, control embryos that had not been punctured from the initial experiment were used as the baseline of comparison between embryos in which perivitelline fluid had been removed.

4.4.2 Effects of nutrient content manipulation on EPTs

Removal of perivitelline fluid volume influenced the levels of total energy across the lifetime of developing embryos (ANOVA, $F_{2, 41}$ = 19.04, P < 0.0001). Total energy was significantly increased following removal of 50% of perivitelline fluid volume (Tukey HSD, P < 0.0001), and were also greater than those of embryos in which 25% of the perivitelline fluid was removed (Tukey HSD, P = 0.0003) (Fig. 4.2a). Time series showed an increase in total energy from approximately 25 - 50% of relative developmental time following removal of 50% of perivitelline fluid volume (Fig. 4.2b).

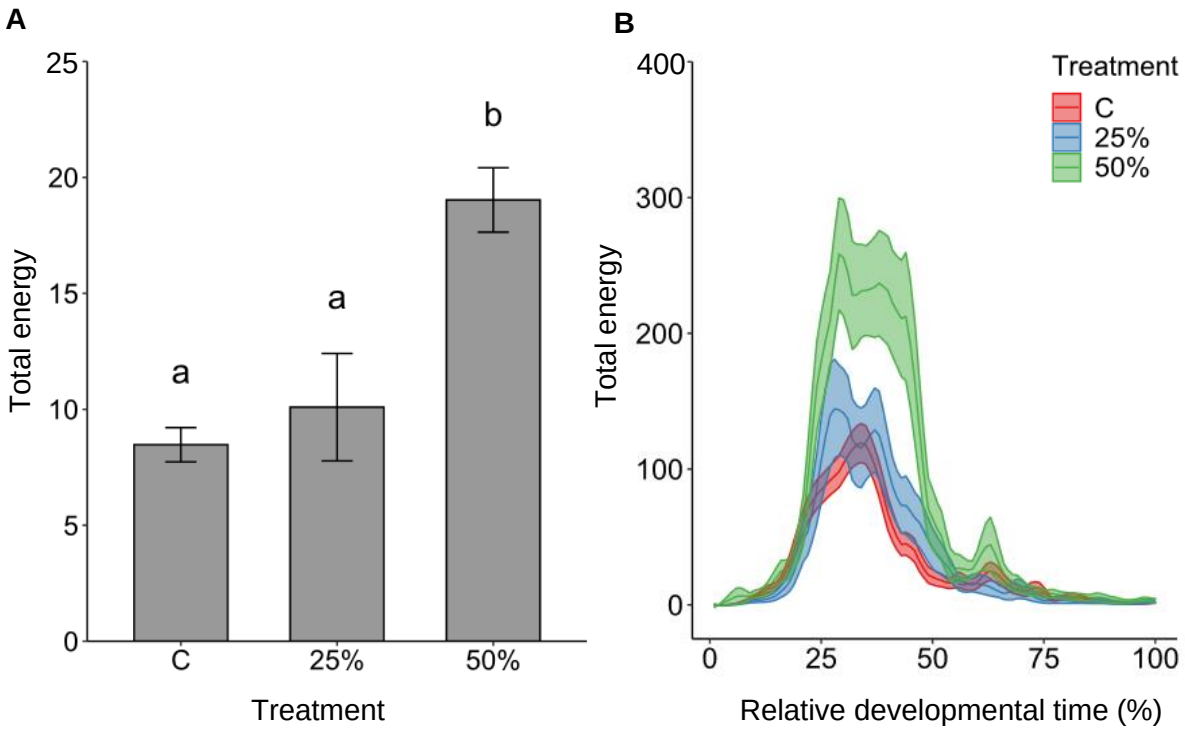

Figure 4.2. **A)** Sum of total energy across the life time of embryos following removal of 25% (N = 10) and 50% (N = 13) of perivitelline fluid volume, relative to control (C) embryos (N = 21) (mean/1000 ± se). **B)** Time series of total energy in embryos of *Lymnaea stagnalis* following removal of 25% and 50% of perivitelline fluid volume, relative to control embryos (mean ± se). Data are smoothed with a 10% span (LOESS).

To determine at which points during early development total energy was influenced by the experimental manipulation of egg nutrient content, data were binned into five periods. Two-way analysis of variance (ANOVA) detected a significant interaction between total energy and relative developmental time increments ($F_{7, 182}$ = 2.489, P = 0.0138). Removal of 50% of perivitelline fluid resulted in an increase in total energy relative to control embryos at 20-40% (TukeyHSD, P < 0.0001) and 40-60% relative developmental time (TukeyHSD, P < 0.0001). Additionally to this, total energy was greater in 50% relative to 25% from 20-60% relative developmental time (20-40%, TukeyHSD, P < 0.0001, 40-60% relative developmental time, TukeyHSD, P = 0.0417) (Fig. 4.3).

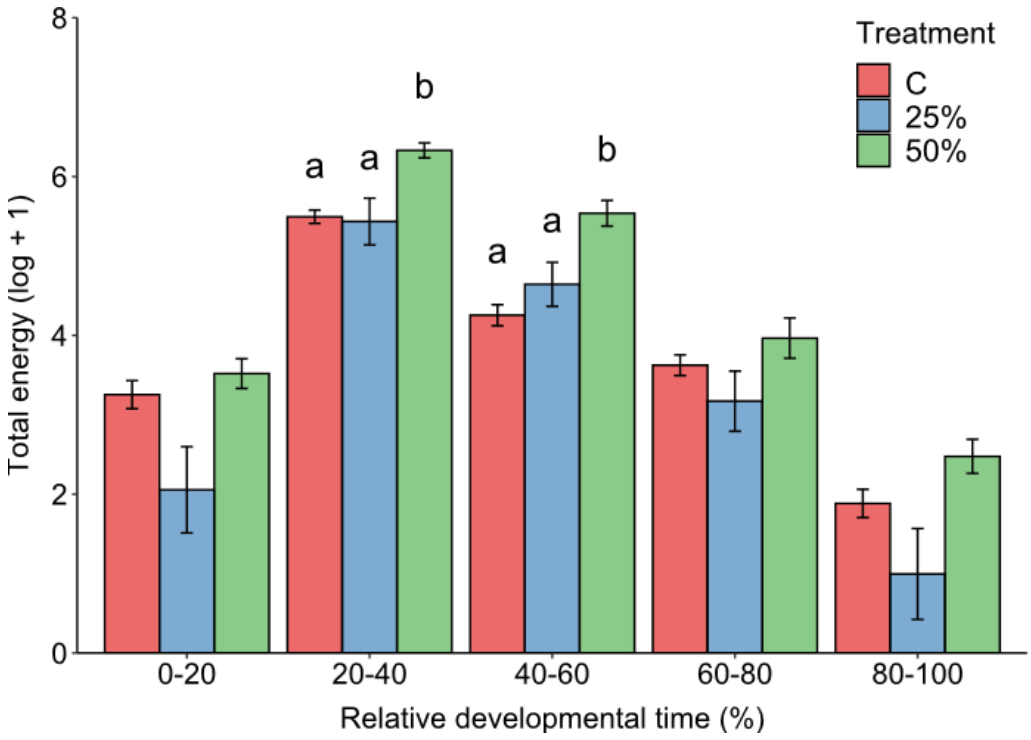

Figure 4.3. Total energy (log + 1) of developing embryos following experimental removal of 25% and 50% of total perivitelline fluid volume relative to control embryos (C), across 5 relative developmental time bins (mean ± se). Pairwise significant differences (two-way ANOVA, $P < 0.05$) within developmental time bins indicated by lower case letters above treatments.

4.4.3 Dimensionality reduction

To investigate combinatorial signals of frequency specific EPT data at different points in development following removal of perivitelline fluid, principal component analysis (PCA) was applied to data binned into discrete time intervals. PCA revealed distinct clustering based on developmental time interval and treatment. Separation of points between treatments was most pronounced at time intervals corresponding with 0 - 20%, 20 - 40% and 40 - 60% of relative developmental time. Control and 50% embryos were predominantly separated across the axis of PC2 at 0 - 20% and 20 - 40% relative developmental time (Fig. 4.4). Separation of points

along this axis was predominantly driven by frequencies in the range of 0.03 - 1.2 Hz incorporating responses including whole body muscular contractions and flexing, compared to PC2 for which the frequencies 4.1 - 4.7 Hz were driving separation of points, likely incorporating responses including cardiac function and ciliary activity.

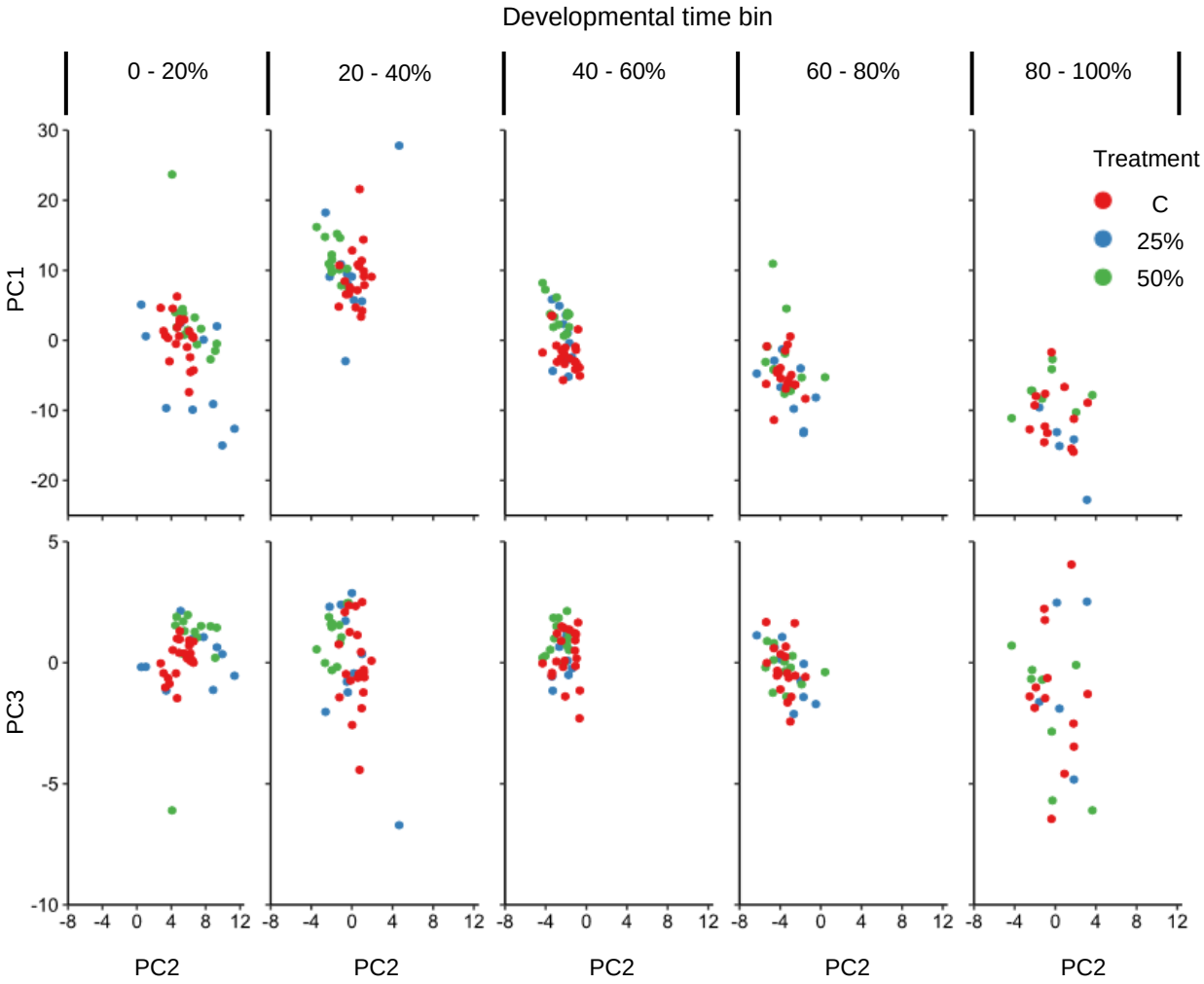

Figure 4.4. Principal component analysis (PCA) of the levels of energy within 60 temporal frequency bins at discrete time intervals in embryos of *Lymnaea stagnalis* under control (C) conditions, and following removal of 25% and 50% of perivitelline fluid volume.

Additionally to this, separation of control and 50% embryos was distributed predominantly across the axis of PC1 at 40 - 60% relative developmental time (Fig. 4.4). Frequencies in the range of 0.03 - 0.2 Hz were driving separation of points along PC1. Finally, there was also

strong separation of control and 50% embryos across the axis of PC3 at 0 - 20% relative developmental time (Fig. 4.4), and frequencies in the range of 0.03 - 0.3 Hz, 1.1 - 1.3 Hz, 2.2 - 2.5 Hz, 3.5 - 3.6 Hz and 4.2 - 4.5 Hz, were predominantly driving differences along the axis of PC3 (Supplementary information 14).

4.4.4 Growth

There was no significant effect of perivitelline removal fluid on developmental time (ANOVA, $F_{2, 39} = 1.694$, $P = 0.196$) although, size at hatch was significantly influenced by removal of perivitelline fluid (ANOVA, $F_{2, 34} = 9.835$, $P = 0.000158$). Removal of 25% of perivitelline fluid resulted in embryos that were ~7% smaller than control individuals, although this difference was not significant (TukeyHSD, $P = 0.0901$), whereas removal of 50% of perivitelline fluid resulted in embryos that were ~15% smaller than control individuals (TukeyHSD, $P < 0.0001$) (Fig. 4.5a). Additionally, growth rates of developing embryos were influenced by experimental manipulation of perivitelline fluid volume ($F_{1, 36} = 12.36$, $P < 0.0001$). Removal of 25% and 50% of nutrient resulted in reduced growth rates relative to control individuals (Tukey HSD, 25%: $P = 0.0143$, 50%: $P < 0.0001$) (Fig. 4.5b).

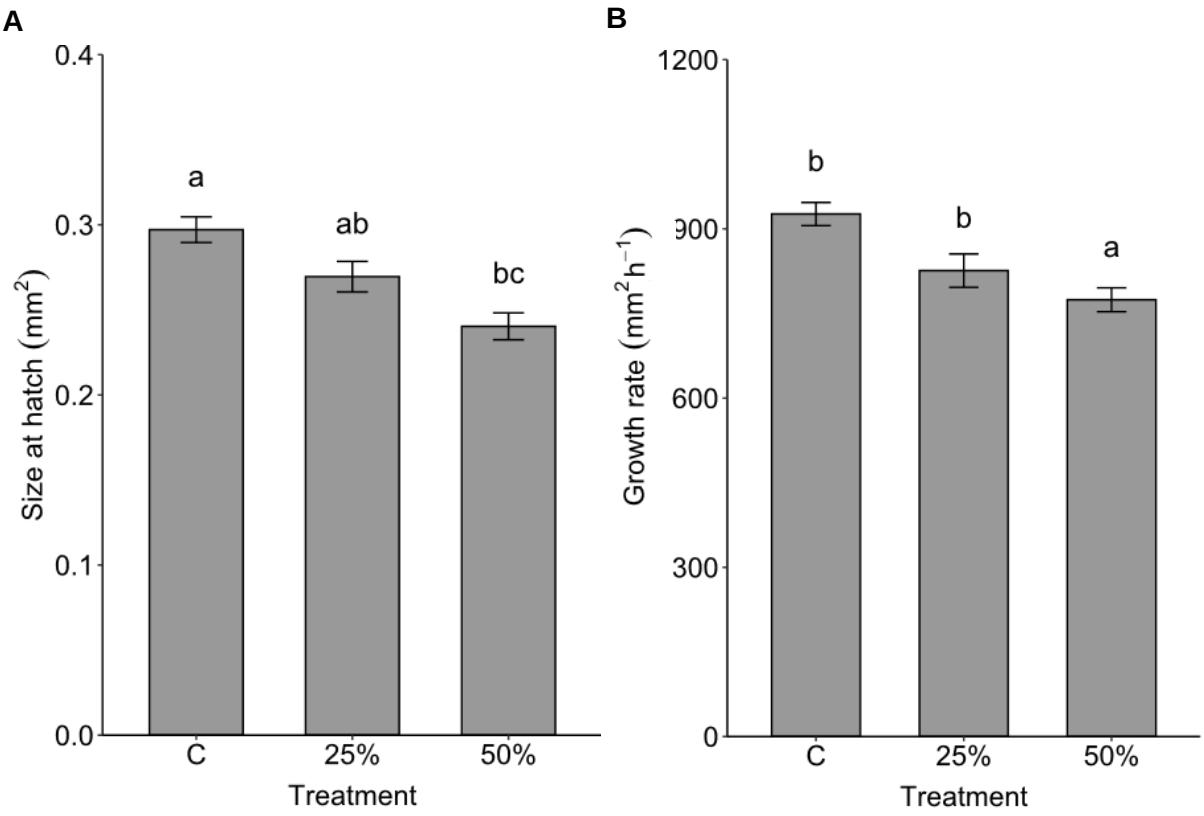

Figure 4.5. Size at hatch and growth rate for embryos of *Lymnaea stagnalis* following removal of, 25% (N = 10) and 50% (N = 11) of perivitelline fluid, relative to control embryos (C) (N = 17) (mean ± se). **A)** Size at hatch (mm^2) **B)** Growth rate (mm^2 h^{-1}). Lower case letters denote statistically significant differences (Tukey HSD, $P < 0.05$).

4.5 Discussion

The aim of this study was to determine if there is a relationship between EPTs and aspects of organismal development, through the experimental manipulation of embryonic energetic content in eggs of *Lymnaea stagnalis*. Removal of 50% perivitelline fluid resulted in a significant increase in the levels of total energy across the lifetime of these embryos, thereby representing increases in gross rates of embryonic physiology and behaviour. Increases in total energy in the 50% treatment appeared to be limited to periods of embryonic development corresponding with 20 - 60% relative developmental time, approximating to the free swimming

stage in these embryos. Additionally to this, dimensionality reduction of EPT data revealed frequency specific drivers of differences in combinatorial signals associated with responses to removal of 25% and 50% of total nutrient content. Size at hatch and rates of growth were both reduced following removal of perivitelline fluid volume, however the magnitude of observed decreases in hatchling sizes was lower than expected. Together, these results suggest a trade-off between EPTs and life history traits in these embryos under nutrient limiting conditions. As the amount of energy available to embryos at the start of development decreases, gross rates of embryonic physiology and behaviour increases during the free swimming stage, whilst rates of growth and sizes at hatch decrease, potentially suggesting a re-allocation of energetic reserves under reduced nutrient availability.

4.5.1 Influence of nutrient reduction on EPTs

Removal of 50% of the perivitelline fluid of eggs of *L. stagnalis* resulted in a significant increase in the levels of total energy across the lifetime of embryos of *L. stagnalis* (Fig. 4.2). Total energy is the sum of energy values across the EPT spectra, and consequently represents gross rates of embryonic physiologies and behaviours (Tills *et al*., 2018; 2021). These increases in total energy were limited to periods of embryonic development corresponding with 20 - 60% of relative developmental time (Fig. 4.3). This period of development is associated with the onset of a number of physiological functions, notably the onset of ciliary driven rotation and free swimming stages, as well as the onset of cardiovascular function, body flexing and mantle muscle control (Meshcheryakov, 1990; Smirthwaite *et al*., 2007; Chapter 2). Whilst it could be predicted that a reduction in the energy available to a developing embryo would reduce total energy, observed increases in total energy during these periods of development following removal of 50% of perivitelline fluid are suggesting an increase in gross rates of embryonic physiology and behaviour. The perivitelline fluid of these developing

embryos is a viscous fluid composed predominantly of protein (90%), as well as galactogen (~10%) (Taylor, 1973; 1977). Following removal of this perivitelline fluid, there was an immediate decrease in egg volume as the wall of the egg capsule compressed. However, in the hours following this removal after the eggs were placed into multiwell plates containing artificial pond water, these eggs ‘re-inflated’ likely due to the resorption of water back into the egg capsule (J. McCoy, pers. obs.). Consequently, the influx of water into the egg capsule likely reduced the viscosity of the fluid inside the egg capsule. The free swimming embryo, now with this viscosity limitation partially removed, is able to move with less resistance inside the egg capsule, and therefore is likely explaining the observed increase in total energy at 20 - 60% relative developmental time. Whilst there was an observed increase in total energy levels, determination of whether this equates to changes to the level of biochemical energy expended is problematic. As the physical characteristics of the perivitelline fluid are altered, the 'work needed’ to move the embryo through the intracapsular fluid is also changed. Consequently, an increase in total energy, resulting from an increase in gross rates of movement related to embryonic physiology and behaviour, may not necessarily reflect alterations to the rate at which biochemical energy is expended. This highlights another limitation with the approach used here. Given that I here have measured movement related to aspects of embryonic physiology and behaviour, it was not possible to quantify rates of biochemical energy expenditure related to other biological processes, for example intracellular processes such as DNA repair or production of antioxidant enzymes. Consequently, any future research directed towards establishing correlative links between EPTs and rates of biochemical energy turnover must be limited within the context of energy expended *via* rates of organismal movement.

Dimensionality reduction also revealed differences in combinatorial signals between treatments. From 0 - 20% relative developmental time there was separation of control and 50% embryos along the axis of PC2 and PC3. Variation along these axes was driven by a broad range of frequencies, however as development progressed from 20 - 60% relative

developmental time, frequency drivers of differences between treatments were progressively lower, with differences at 40 - 60% relative developmental time only being driven by frequencies in the range of 0.03 - 0.2 Hz. 0 - 20% relative developmental time corresponds roughly with the period of ciliary driven rotation, as a result, the broad range of frequencies driving variation in the PCA may be as a result of the movement of the entire embryo. In Chapter 2, the onset of ciliary driven rotation was associated with significant increases in energy across virtually all frequency bands in *L. stagnalis* (Fig. 2.4b), consequently, it is unsurprising that treatment differences in this developmental time range is driven by such a broad frequency range. Differences between control and 50% embryos at 20-60% relative developmental time were driven by progressively lower ranges of frequencies. This period of development corresponds approximately with the free swimming veliger and hippo stages, but is also associated with gradual increases in the frequency of intermittent resting on the wall of the egg capsule (Fig. 2.2), prior to the onset of muscular crawling. This gradual 'slowing' of observable embryonic movements as the embryo progresses towards muscular crawling, likely explains the lower frequency drivers of treatment differences at later relative developmental time bins.

4.5.2 Size and growth rate

As well as changes in total energy, there were differences in rates of growth and sizes at hatch following removal of perivitelline fluid (Fig. 4.5). Firstly, size at hatch was significantly reduced following removal of perivitelline fluid volume, and so were rates of growth. Following removal of 25% and 50% perivitelline fluid volume, there was a ~7% and ~15% reduction in size at hatch respectively. Whilst reductions in hatch size have been observed following experimental reductions in egg volume or nutrient levels (Finkler *et al*., 1998; Jardine & Litvak, 2003; Allen *et* al., 2006; Pernet *et al*., 2012; Landberg, 2014), the magnitude of change observed here

was considerably lower than expected. This discrepancy between the amount of perivitelline fluid removed and the magnitude of change in size stimulates interesting questions surrounding the utilisation of perivitelline fluid during embryonic development in these species. Firstly, exogenous uptake of perivitelline fluid, according to Raven (Raven, 1970), does not commence until just prior to the mid-blastula stage (Meshcheryakov, 1990). At this stage, all cells of the blastula begin to uptake perivitelline fluid, *via* pinocytosis, with extracellular digestion of the capsular fluid commencing at the trochophore stage (Ami, 1974). Prior to the onset of exogenous uptake of perivitelline fluid, embryos are provisioned with cytoplasmic yolk granules (Raven, 1946). Before the early trochophore stage and the onset of extracellular digestion of perivitelline fluid, these yolk granules are consumed and gradually reduce in size (Raven, 1945). As far as I am aware, the relative contributions of this intracellular yolk and exogenous perivitelline fluid to the biomass of the embryo at hatch are not known. Given that perivitelline fluid was only removed at the gastrula stage, it is reasonable to attribute the relatively small magnitude of effect on size at hatching to the presence of cytoplasmic yolk. An alternative explanation is that embryos of *L. stagnalis* used in this study were provisioned with an excess of perivitelline fluid at the start of development. Therefore, fluid removal resulted in a considerably lower magnitude of effect on size at hatch. Adults of *L. stagnalis* used here were sourced from a wild population and kept in laboratory conditions for at least two weeks prior to experimentation, during which they were fed *ad libitium*. Consequently, in the absence of any food limitation, eggs may have been provisioned with more than enough nutrient content for completion of embryonic development. Maternal food supply is a major predictor of egg size in a range of species (Glazier *et al*., 1992; Karell *et al*., 2008; Vijendravarma *et al*., 2010; Iguchi, 2012), and substantial variation in egg size can exist between field and laboratory reared populations (Kaplan & King, 1997; Fox, 2000). Without measurement and knowledge of rates or quantities of perivitelline fluid uptake in these embryos, this discrepancy between magnitudes of effect on embryo size, and quantity of perivitelline fluid removed may be in part due to the points highlighted above.

4.5.3 Implications

Increases in total energy following experimental removal of 50% perivitelline fluid volume were associated with reductions in rates of growth and size at hatch. Whilst correlative data at the level of the individual are not possible from this experimental design, this concomitant reduction in mean rates of growth with increases in gross rates of embryonic physiology and behaviour may indicate a relationship between these two variables. I suggest there may be a negative relationship between rates of growth and total energy, the opposite direction to that described in previous research. In embryos of *Radix balthica*, under various temperatures the rates of growth are positively correlated with levels of total energy (Tills *et al*., 2021), however these data are a) based on another species, b) based on correlations at the individual level, and c) were established in embryos in which nutrient supply has not been limited. Relationships between energy turnover (i.e. metabolic rate) and growth/size have been previously established for a number of species (Steyermark, 2002; Alvarez & Nicieza 2005; Burton *et al*., 2011; Auer *et al*., 2015a; 2015b; Rosenfield *et al*., 2015). More importantly, these relationships can be largely dependent on the environment/conditions under which an organism finds itself (McCarthy, 2000; Alvarez & Nicieza 2005; Burton *et al*., 2011; Norin & Malte, 2011; Robertsen et al. 2014; Auer *et al*., 2015a; 2015b). For example, growth rate was found to be positively correlated with resting metabolic rate in juvenile Chinese crucian carp (*Carassius auratus*) when individuals were fed *ad libitum*, however when food was restricted this relationship dissolved (Zeng *et al*., 2017). Similarly, in the zebra finch *Taeniopygia guttata*, when fed *ad libitum* a positive correlation was found between rates of growth and resting metabolic rates, but under low food conditions, this relationship was again not present (Mathot *et al*., 2009). The apparent negative 'relationship' between gross rates of embryonic physiology and behaviour and rates of growth/size at hatching observed in the current study may be as a result of reduced nutrient availability, following experimental removal of 50% of the perivitelline fluid. Given that embryos of *L. stagnalis* are provisioned with a fixed volume

of nutrient at the start of development, the increased rates of organismal physiologies and behaviours may incur a greater energetic cost, and could be reducing the amount of energy invested into the production of biomass. Consequently, changes in EPTs in the form of total energy may elicit effects on other aspects of embryonic development that are predictors of performance and fitness post hatch, including size at hatch (Peters, 1983; Schmidt-Nielsen, 1984; Perrin, 1998; Wikelski & Romero, 2003; Kingsolver & Huey, 2008).

Direct manipulation of the energetic content of developing embryos resulted in considerable differences to EPTs, specifically levels of total energy. Previous research has also shown EPTs to be positively correlated with rates of growth in embryos of the pond snail *Radix balthica* (Tills *et al*., 2021). Tills *et al.* (2021) suggest that EPTs may in themselves be indicative of biochemical energetic turnover in developing embryos. Whilst in the current study I did not directly measure rates of biochemical energetic turnover in these embryos, the observed alterations to EPTs following experimental manipulation of energy available to developing embryos coupled with previous evidence (Tills *et al*., 2021), stimulates interesting questions surrounding the relationship between EPTs, and rates of biochemical energetic turnover. Given this, there are a number of avenues for experimentation that can contribute towards answering this question. First, is the utilisation of alternative model species such as the zebrafish *Danio rerio*. Embryos of *D. rerio* contain a large yolk cell which supplies the developing embryo with nutrients prior to absorption of exogenous food sources after approximately 5 days (Quinlivan & Farber, 2017). The size of this yolk sac is easily quantifiable from video data, allowing for the simultaneous measurement of gross rates of biochemical energy turnover in the form of changes in the size of the yolk sac, and calculation of EPTs from video data. Furthermore, motion analysis has been successfully applied to quantify gross movements in embryos of *D. rerio* following exposure to various concentrations of ethanol (Rudin-Bitterli *et al*., 2014). This species may therefore provide an effective model species with which to establish correlative relationships between EPTs and rates of energetic turnover. Alternatively, measurement of EPTs alongside rates of oxygen consumption would allow direct

correlative data to be established between EPTs and rates of metabolism. Achieving this is difficult for such small specimens, however, application of microelectrodes has allowed for the quantification of oxygen levels and rates of oxygen consumption surrounding embryos of the rainbow trout *Onchorynchus mykiss* (Miller *et al*., 2008), inside egg capsules of the gastropod mollusc *Chorus giganteus* (Cancino *et al*., 2011), and inside egg masses of the nudibranch *Tritonia diomedea* (Moran & Woods, 2007). Whilst application of these approaches may limit measurement continuously through embryonic development, measurement of rates of oxygen consumption at discrete points in the developmental itinerary, with concomitant video recording and calculation of EPTs may provide an alternative correlative approach to understanding the relationship between EPTs and rates of biochemical energy turnover in developing embryos.

Finally, as far as I am aware, this study is the first instance of experimental manipulation of nutrient content in eggs of *L. stagnalis*. *L. stagnalis* is a widely used model species of major interest to fields of evolutionary biology, neuroscience, ecotoxicology, bio-mineralisation and understanding the origins of chirality during early development (Kuroda & Abe, 2020). Microinjection of the eggs and embryos with various substances including mRNAs with marked dies (Kuroda & Abe, 2020) has been utilised for a number of research purposes including understanding determinants of chirality during early cellular divisions (Kuroda *et al*., 2009; 2015; Abe *et al*., 2009), synapse formation (Getz *et al*., 2018) and characterisation of inter-cellular communication pathways (Serras *et al*., 1990). However, this chapter demonstrates the first instance of physical removal of nutrient content from eggs of this species. This opens up a number of interesting lines of enquiry around the consequences of nutrient content on aspects of organismal development either under control conditions, or during exposure to changing environmental regimes. For example, temperature is known to reduce offspring size in a number of species (Ernsting & Isaaks, 1997; Atkinson *et al*., 2001; Fischer *et al*., 2003; Beacham & Murray, 1990; 2011; Pettersen *et al*., 2020). Understanding the consequences of this reduction in egg size on offspring development under elevated

temperature regimes is best achieved through experimental manipulation of egg size, given that exploiting standing variation in egg size is associated with a number of other uncontrollable epigenetic/maternal effects (Pernet *et al*., 2012).

4.6 Summary and conclusions

EPTs are a novel approach to phenotyping during early development, however the significance of evolutionary and environmentally driven changes in EPTs for organismal development and performance is poorly understood. Here, I aimed to determine if there was any relationship between EPTs and aspects of organismal development, specifically life history traits including size at hatch, development time and growth rate, through the serial removal of egg nutrient content in eggs of *L. stagnalis*. Removal of 50% of perivitelline fluid resulted in a significant increase in total energy, alongside a significant reduction in rates of growth and sizes at hatch. Whilst increases in total energy may be due to reductions in viscosity of the perivitelline fluid following influx of water into the egg capsule, the relatively low magnitude of effect on growth and size, and the apparent relationship between these variables and total energy present interesting lines of future inquiry into the energetic significance of EPTs for developing embryos, and their relationship between growth and size under varying food/nutrient conditions. The apparent trade-off between levels of total energy, representing gross rates of physiology and behaviour, and life history traits may suggest differential allocation of energetic reserves into these processes. Future research should be aimed towards establishing direct correlative relationships between EPTs and rates of metabolism/biochemical energetic turnover in developing embryos.

Chapter 5

Conclusion

5.1 Introduction

The main aim of this thesis was to understand what phenomics can contribute to our understanding of Comparative Developmental Physiology, in terms of how environmental and evolutionary change affects the development of physiological function in aquatic embryos. Additionally to this, I aimed to understand the significance of changes in high-dimensional phenotyping methods used throughout the thesis within the context of embryonic life history. Underpinning this aim has been the application of a novel phenotyping measure, Energy Proxy Traits (EPTs), to understand how i) differences in the timings of developmental events between species (heterochronies) and conspecifics are associated with differences in high-dimensional phenotypic space; ii) the extent to which phenomics can be used to compare interspecific differences in developmental responses to environmental change and iii) whether there is any relationship between EPTs and life history traits, specifically size at hatch and rates of growth and development. In this final discursive chapter, I consider how each of these chapters contributes to the overarching aims of this thesis, discuss emergent findings from each of these works, consider the broader implications of the thesis to Comparative Developmental Physiology, and suggest future avenues for the integration of phenomics into this field.

5.2 Phenomics as an integrative approach to Comparative Developmental Physiology

5.2.1 A phenomics approach to assessing environmental sensitivity during early development

Assessing responses to environmental change during early development is crucial for predicting how biodiversity of marine and freshwater systems will be impacted by future climate change projections (Pottier *et al*., 2022; Burggren, 2018; 2021). Neglecting measurement of phenotypic changes during early development is a major oversight given that these life stages can exhibit heightened and considerable variability in their sensitivities to environmental change (Burggren, 2018; Truebano *et al*., 2018; Madeira *et al*., 2020; Pottier *et al*., 2022), and exposure to environmental stress during early development can have significant carry-over effects to later life history stages (Harms, 1992; Spicer & El-Gamal, 1999; Pechenik, 2006; Diedrich *et al*., 2011; Hettinger, 2012; Li & Chiu, 2013; Gobler *et al*., 2014; Segura *et al*., 2014; Vanderplankce *et al*., 2015). Responses to components of global environmental change, particularly temperature, can encompass changes to a range of phenotypes and consequently, current research frequently measures broad indicators of organismal performance that are the sum of a number of morphological, behavioural and physiological traits (Hochachka & Somero, 2002; Forsman, 2015; Iverson *et al*., 2020). Furthermore, development is extremely dynamic, meaning that phenotypes that are observable and measurable actually change considerably during ontogeny, necessitating the application of phenotyping approaches that are transferable between morphologically, behaviourally and physiologically distinct periods of early development. Finally, considerable variation exists in the relative timings of development between even closely related species, as well as between conspecifics (Reilly *et al*., 1997; Mabee *et al*., 2000; Schmidt & Starck, 2004; de Jong *et al*., 2009; Kawajiri *et al*., 2009; Rundle *et al*., 2011; Tills *et al*., 2013; Rager *et al*., 2014) making effective comparison of the developmental effects of environmental stress

problematic (Fiuman, 1998; Smith, 2002; Bininda-Emonds *et al*., 2007; Smirthwaite *et al*., 2007; Keyte & Smith, 2014).

Energy Proxy Traits (EPTs), measures of the levels of energy within discrete temporal frequencies from pixel value fluctuations, provided an approach with which to quantify temperature induced high-dimensional phenotypic change continuously through the embryonic development of three species of freshwater snail, and revealed both interspecific, and ontogenetic differences in the thermal sensitivities of these species. In Chapter 3, I assessed thermal responses in both gross rates of embryonic physiology and behaviour throughout relative developmental time in the form of total energy in the EPT spectrum, and levels of energy within specific frequencies across a number of discrete physiological windows. Total energy time series revealed differences in the magnitude of response between species, as well as across developmental time within each species (Fig. 3.1, 3.2). The large magnitude of thermally-driven increases in total energy in embryos of *Radix balthica* seen in Chapter 3 indicate relatively high sensitivities of these embryos to an increase in developmental temperature of 5°C, whereas in *Physella acuta* total energy exhibited a considerably lower magnitude of change, suggesting comparatively low sensitivities to chronic elevated temperatures. However, in *Lymnaea stagnalis*, total energy was even reduced at early stages of development, potentially reflecting a reduction in the gross rates of observable behaviours and physiologies including free swimming within the egg capsule (Tills *et al*., 2018). *P. acuta* is an invasive generalist, and adults of this species have been shown to have high acute thermal tolerance (~41.7°C, Koopman *et al*., 2016). Conversely, previous research indicates that thermal tolerance in adults of *L. stagnalis* (40.5°C, Hoefnagel & Verberk, 2017) and *R. balthica* (36.7-38.5°C, Johansson & Laurila, 2017) is comparatively lower. Whilst acknowledging obvious differences in local thermal adaptation, rates of thermal ramping, experimental acclimation temperatures, and the nature of temperature exposure (acute shock in the cited studies in contrast to more moderate chronic elevated temperatures in Chapter 3), these studies highlighted differences in the thermal tolerance of adults of each of these

species. Consequently, it would not be unreasonable to posit that the comparatively low thermal sensitivity of embryos of *P. acuta* to chronic elevated temperatures may be in part driven by their comparatively high thermal tolerance. Unrelated to the main aims of Chapter 3, as well as revealing differences in the magnitude of change, comparison of total energy time series between temperatures revealed changes in the timings of development in *L. stagnalis* and *R. balthica*. Specifically, these changes in total energy time series between temperature treatments reflected acceleration of the timings of onset of ciliary driven rotation (*L. stagnalis*) and muscular crawling (*L. stagnalis* and *R. balthica*) (3.1), which was confirmed by manual quantification of the timings of these events from video (Supplementary Information 1). Here, temperature induced plasticity in the timings of these major physiological events/transitions is associated with high-dimensional phenotypic change. These temperature induced high-dimensional phenotypic changes may act as significant objects of selection under novel environmental regimes, consequently, future research should be directed towards understanding the fitness and performance implications of these changes in total energy time series.

A key strength of EPTs is their granularity associated with measuring not just overall energy in fluctuating pixel values, but responses of individual frequencies (Tills *et al*., 2018; 2021). In Chapter 3, frequency specific data enabled measurement of changes in thermal responses between major physiological windows of embryos of *L. stagnalis, R. balthica* and *P. acuta*. The frequencies in which significant increases in energy were observed corresponded with ontogenetic changes in physiological window specific phenotype, and these drove clustering in multivariate PCA plots (3.2, 3.3). This included increases in energy within frequency bands ranging from 1.8 – 2.5 Hz during crawling and cardiovascular function in *L. stagnalis* and *P. acuta*, frequencies that are most strongly associated with heart function (Voronezhskaya *et al*., 2007; Seeland *et al*., 2013; Tills *et al*., 2018). Additionally, during muscular crawling and cardiovascular function in *L. stagnalis* and *P. acuta* respectively there were increases in energy within frequencies associated with body flexing, the first muscular contractions

involving the embryo flexing through the ventral/dorsal axis, and mantle muscle control, initiation of movement of the muscle group used to retract the shell over the head in later life stages (Smirthwaite *et al*., 2007). Multivariate analysis of EPT data also revealed differences in combinatorial responses between species. Whilst responses in *L. stagnalis* and *P. acuta* were primarily limited to the frequency bands associated with physiological rates highlighted above, *R. balthica* showed increases in energy across most frequency bands following exposure to chronic elevated temperatures, suggesting increases in the rates of all observable embryonic physiologies and behaviours. Measurement of small numbers of phenotypes can lead to erroneous conclusions over the significance of an environmental stressor in driving our phenotype of choice (Forsman, 2015; Laughlin & Messier, 2015; Morel-Journel *et al*., 2020). An absence of change in one trait, can ultimately be compensated for by plasticity in another trait. Therefore, understanding how the phenotype contributes to differences in observable performance and fitness is best achieved through quantification of the phenotype at the scale of the whole organism (Pigliucci and Preston, 2004; Houle, 2007; Valladares *et al*., 2007; Whitman and Agrawal, 2009; Laughlin & Messier, 2015). Comparative developmental animal physiologists and environmental physiologists should capitalise on recent advancements in technologies permitting acquisition of phenotypic data at these scales.

5.2.2 Flags on a shifting landscape

Whilst not outlined in the main aims of this thesis, both Chapter 2 and Chapter 3 highlighted limitations in current frameworks used to assess evolutionary and environmentally driven changes in physiological development. Development is frequently regarded as a series of discrete events or stages, however development of morphology and physiological function are continuous processes (Burggren, 2021a), and research investigating the effects of environmental change on early development frequently measures changes in phenotype at

discrete points in developmental time. Whilst these approaches have provided valuable insight into the relative sensitivities of various life stages during early development (e.g. Laurence & Rogers, 1976; Anger, 1991; Krebs & Loeschcke, 1995; Mills & Barnhart, 1999; Jantrarotai *et al.,* 2002; Bowler & Terblanche, 2008; Fawcett & Frankenhuis, 2015; Turriago *et al*., 2015; Tills *et al*., 2018; 2022; Truebano *et al*., 2018; Dwane, 2021), such an approach is in essence attempting to measure a complex landscape of continuous multivariate phenotypic change, by placing 'flags' at discrete points in development, and inferring environmental sensitivities from only biological responses measured at these positions. This approach largely negates phenotypic change in between these discrete 'flags' during development and may therefore omit considerable amounts of phenotypic change around these points (Burggren, 2021a; Fig. 5.1b). Below I discuss emergent findings from this thesis within this context, and discuss areas of research within Comparative Developmental Physiology (CDP) that will benefit from the adoption of more continuous approaches to measuring developmental phenotypic change.

Investigation of the evolutionary significance of variation in the timings of development between species (heterochrony), and between congeners, frequently measures the timings of discrete developmental events, such as the onset of morphological characters or physiological and behavioural functions (e.g. Spicer & El-Gamal, 1999; Warkentin, 2000; 2002; Jeffery *et al*., 2002a; Spicer & Burggen, 2003; Smirthwaite *et al*., 2007; Mitgusch *et al*., 2009; Tills *et al*., 2010; 2011; Fabrezi, 2011; Rundle *et al*., 2011). However, as highlighted above, simplification of development into a set of discrete stages omits considerable amounts of phenotypic change occurring both before and after the chosen point in developmental time (Burggren, 2021a; Fig. 5.1a). In Chapter 2, there were obvious changes in high-dimensional phenotypic space preceding and proceeding the onset of functional developmental events, evident in examination of total energy time series, and also the clustering evident from dimensionality reduction of EPT data (Fig. 2.2, 2.3). The lymnaeids *Lymnaea stagnalis* and *Radix balthica* both exhibited a gradual reduction in total energy prior to the onset of muscular crawling (Fig. 2.2), during which embryos increase the frequency with which they intermittently rest their

head on the wall of the capsule, before firmly attaching the foot (Smirthwaite *et al*., 2007). In these instances, characterising variation in the timing of this transition as a single 'event timing' is both methodologically challenging and highly reductionist. Here, the continuous measurement of phenotypic change becomes more appropriate, and in doing so I was able to show that both evolutionary and intraspecific differences in the timings of major physiological events were associated with changes in high-dimensional phenotypic space, potentially acting as objects of selection during early development. In this case, EPTs may provide a powerful alternative to investigating the evolutionary significance of differences in physiological event timings through measurement of the onset of phenotypes in a continuous, rather than discrete fashion.

Additionally to this, research into the investigation of developmental critical windows may benefit from adoption of more continuous phenotyping methods. Developmental critical windows are a well-established component of CDP (Burggren & Mueller, 2015), and represent periods of development during which a phenotype is particularly sensitive, i.e. the degree of plasticity is high, in response to a change in the external (or internal) environment (Burggren & Reyna, 2011; Eme *et al*., 2015; Mueller *et al*., 2015a; 2015b; Tate *et al*., 2015). High-dimensional phenotyping approaches such as EPTs can provide an effective means for the detection of developmental critical windows, allowing for the continuous quantification of rates of embryonic physiology and behaviour during and surrounding the established critical window of interest. Furthermore, continuous measurement of phenotypic change also allows for the determination of any 'repair' capabilities of the individual, i.e. establishing whether the individual can return to a 'normal' developmental itinerary following exposure during a window of development (Burggren & Mueller, 2015). Additionally to this, the dimensionality at which critical windows are detected is also currently limited. Understanding how all components of a developing organism are affected by exposure during a critical window can only be resolved through quantification of high-dimensional phenotypic change (Burggren & Mueller, 2015).

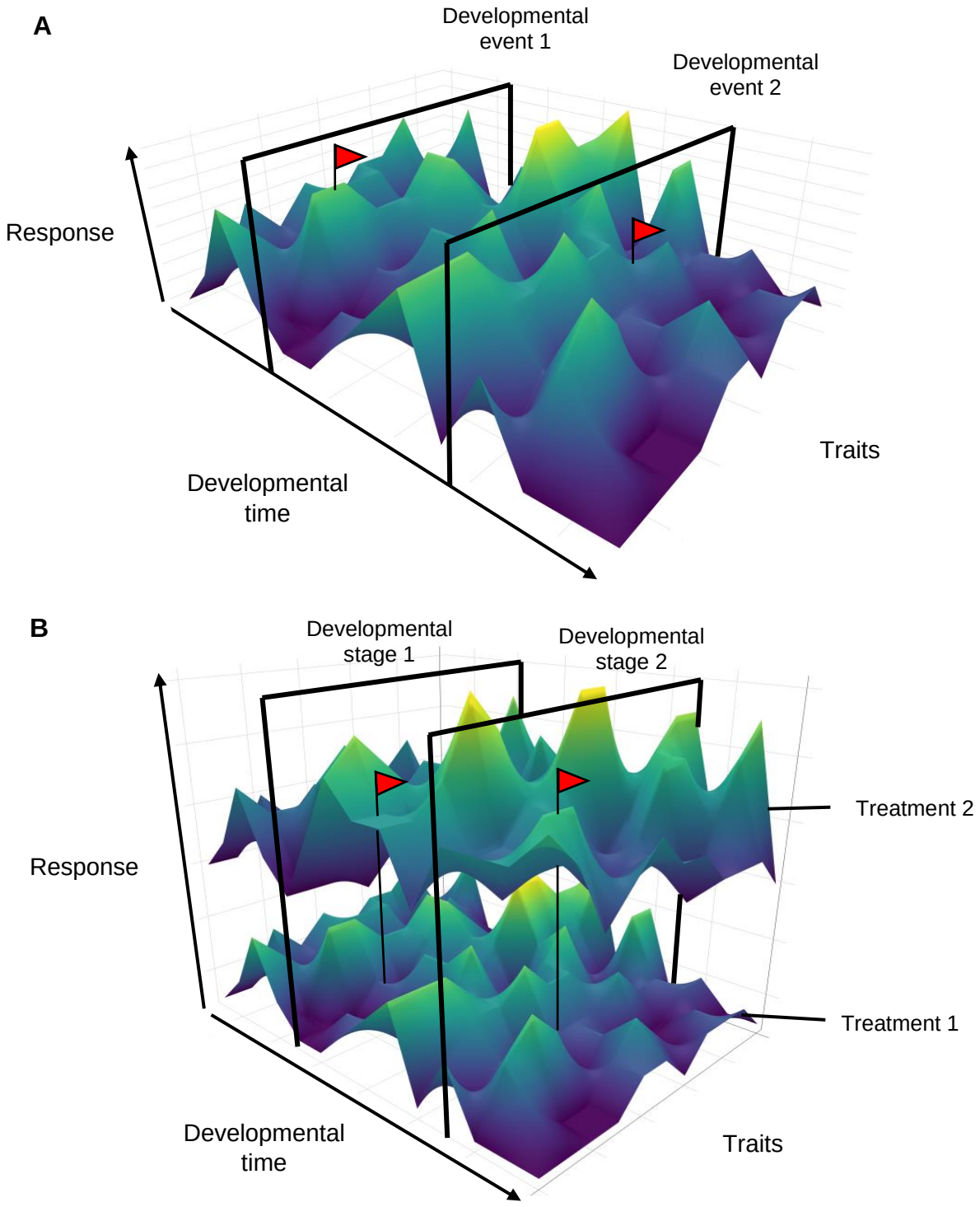

Figure 5.1. Flags on a shifting landscape. **A)** A multivariate phenotypic response is measured over the course of relative developmental time, rather than as the timings of discrete points corresponding with the onset of developmental events. Considerable phenotypic changes proceed and precede the onset of developmental events. Consequently, measurement of a single phenotypic trait (indicated as a flag) misses considerable change in both the X (time) and Y (trait) dimensions. **B)** Exposure to environmental

drivers transforms the response x time x trait phenotypic landscape. Quantification of individual phenotypic traits (indicated as flags) at specific points during development misses considerable phenotypic change associated with complex environmentally driven responses, potentially leading to inaccurate conclusions over the sensitivity of an individual to environmental stress (Burggren, 2021a).

Continuous acquisition of video data of developing animals in bioimaging systems that allow for fine temporal control of environmental conditions such as temperature, oxygen and salinity, as is the case for the OpenVIM systems (Tills *et al*., 2018) used in this thesis, may address some of the limitations highlighted above within the context of developmental critical windows. In summary, application of automated bioimaging approaches coupled with video based phenotyping methods such as EPTs, enables the interrogation of developmental responses to environmental stress within the context of an organism's entire development, rather than as discrete 'flags' on a shifting phenotypic landscape.

5.2.3 EPTs – relationships with embryonic life history

EPTs exhibited considerable evolutionary and environmentally induced changes (Tills *et al*., 2018; 2021; 2022; Chapter 2; Chapter 3), however the significance of these changes in relation to organismal development and performance is still poorly understood. To address this, I aimed to determine if there was any relationship between EPTs and aspects of organismal development by experimentally removing 25% and 50% total perivitelline (intracapsular) fluid volume of developing embryos of *Lymnaea stagnalis*, and quantifying changes in EPTs alongside life history traits related to size and growth. Removal of 50% of perivitelline fluid volume resulted in a significant increase in total energy, likely as a result of the reduction in intracapsular viscosity following removal of perivitelline fluid, and the

subsequent influx of water into the egg capsule. However, size at hatch and rates of growth were decreased following removal of 50% perivitelline fluid volume. Whilst these are not direct individual level correlative data and are instead means of treatment level responses, these data still indicate a trade-off between total energy, a measure of gross rates of embryonic physiology and behaviour, and growth. Increases in gross rates of embryonic physiology and behaviour may be limiting energy available for investment into growth under nutrient limiting conditions (50% perivitelline fluid removal).

Given the apparent trade-off between growth and EPTs under nutrient limiting conditions, and results from previous studies showing EPTs are correlated with rates of growth in embryos another freshwater snail (Tills *et al*., 2021), there was some basis for believing that EPTs themselves are indicative of biochemical energetic turnover in developing embryos, providing potentially fruitful lines of future enquiry into the significance of EPTs within the context of biochemical energy turnover. Rates of biochemical energetic turnover can be driven by differences in the external environment (Diez & Davenport, 1990; Oliva-Teles & Kaushik, 1990; Swanson, 1996; Barrionuevo & Burggen, 1999; Gillooly *et al*., 2001; García-Guerrero, 2010; Brown *et al*., 2012; Lambret *et al*., 2021), and sensitivities to increases in environmental temperatures can be in part driven by associated effects on rates of energy consumption (Zuo *et al*., 2012; Pettersen *et al*., 2020). For example, increased rates of energy consumption influence the quantity of nutrient available at hatch in a number of species (Rosa *et al*., 2012; Rodriguez-Montes de Oca *et al*., 2015; Ituarte *et al*., 2020), and relative quantities of nutrient at hatch are strong predictors of organismal performance and viability post-hatch (Kamler, 1992; Emlet & Hoegh-Guldberg, 1997; Jardine & Litvak, 2003). Consequently, knowledge of how rates of biochemical energetic turnover are influenced by changes in environmental conditions can provide insight into the mechanisms underlying reductions in performance and fitness at hatch, and at later stages of life. Current approaches to quantifying biochemical energetic turnover use indirect calorimetry methodologies including measurement of aerobic respiration *via* rates of oxygen consumption as a proxy for metabolic rate (Battley, 1995;

Treberg *et al*., 2016), or using bomb calorimetry in which the nutrient content at the start and end of development is directly quantified (Bouchaud, 1991; Pearson *et al*., 2002; Walsberg & Hoffman, 2005; Ghosh *et al*., 2023). However these are difficult to achieve continuously, as measurement of rates of oxygen consumption can alter the treatment conditions under which an individual is maintained, eliciting phenotypic changes later in development (Spicer & Gaston, 1999). Non-invasive, video based proxies like EPTs could provide scalable alternatives with which to continuously quantify rates of biochemical energetic turnover. Therefore, an important next step is to direct research efforts to better understanding the relationship between EPTs and rates of biochemical energetic turnover at the biochemical level. I propose two means with which to achieve this below:

Firstly, this can be achieved through the use of alternative model species. Embryos of *D. rerio* rely on absorption of a yolk sac attached to the embryo, which is absorbed over the first 5 days of embryonic development (Quinlivan & Farber, 2017). The size of this yolk sac is easily quantifiable, allowing rates of energy consumption to be easily quantified over discrete periods in development. Furthermore, embryos of *D. rerio* develop in transparent egg capsules over relatively short periods of time, hatching approximately 3 – 4 days post fertilisation in laboratory strains (Singleman & Holtzman, 2014). Quantification of EPTs alongside measurement of changes in yolk sac area in embryos of *D. rerio* may therefore provide an effective model system with which to establish direct correlative relationships between EPTs and rates of biochemical energetic turnover.

Alternatively, biochemical energy turnover in the form of standard and active metabolic rates can also be quantified through measurement of rates of oxygen decline using closed chamber or flow-through respirometry systems (Rosewarne *et al*., 2016; Lighton *et al*., 2018; Tomlinson *et al*., 2018; Killen *et al*., 2021). However this is methodologically challenging, and declines in oxygen levels risk impacting the biology of developing embryos. Determining metabolic rate in developing embryos or larvae is further complicated by the small size of individual specimens, often necessitating the measurement of oxygen decline in small volumes with high

numbers of replicates (Sprung, 1984; Hoegh-Guldberg & Manahan, 1995; Spicer & El-Gamal, 1999). Consequently, measurement of oxygen decline in closed volumes may introduce stress in embryos/larvae resulting in alterations to metabolic rates (Hoegh-Guldberg & Manahan, 1995). A promising alternative is the use of microelectrodes for the quantification of oxygen levels in small specimens, an approach that has proven successful in measuring oxygen levels in embryos of gastropod molluscs and fish (Moran & Woods, 2007; Miller *et al*., 2008; Cancino *et al*., 2011). Application of microelectrodes does not require the measurement of oxygen levels in closed systems, and would perhaps more easily accommodate the simultaneous acquisition of video data alongside measurement of embryonic oxygen levels. Video data can subsequently be used to calculate EPTs, thereby allowing correlative relationships to be established between levels of energy across the EPT spectrum of an embryo, and the levels of oxygen consumed during this same period.

5.3 Future directions and final conclusion

Technology-enabled high-dimensional phenotyping approaches are an extremely valuable resource for improving our understandings of how phenotype interacts with the environment to produce variations in fitness and performance, and may provide a means with which to predict survival and performance at the level of the individual (Houle *et al*., 2010). A current application of such approaches include understanding which individuals, genotypes and populations will be the ‘winners’ of climate change to support projections, but also conservation management (Hamer, 2010; Somero, 2010; Watson, 2018), and they may therefore provide information critical to the implementation of mitigation strategies in particularly vulnerable populations and ecosystems (Boyd *et al*., 2018). Application of artificial intelligence has allowed the combination of considerable numbers of phenotypes to be used to predict biological outcomes of interest, again, particularly within the context of trait based

breeding (Harfouche *et al*., 2019; Parmley *et al*., 2019; Nabwire *et al*., 2021; Pérez-Encisco *et al*., 2021; Xu *et al*., 2022), and predicting clinical outcomes (Alexandrov *et al*., 2016; Richesson *et al*., 2016; Davatzikos *et al*., 2018). The extent to which EPTs can be used in a similarly predictive capacity is poorly understood, however given that they integrate all observable forms of embryonic physiology and behaviour, directing future research efforts towards establishing EPTs as a predictive tool may provide a robust means with which to link embryonic phenotype to observable differences in fitness and performance.

Understanding the broader implications of global environmental change on early development requires the application of phenotyping approaches that are transferable between a range of non-model species of interest. August Krogh stated that for every physiological question there is an animal with which to answer it (Krogh, 1929). Within the context of understanding high-dimensional phenotypic changes associated with the response to differences in developmental environmental conditions, implementing this principle, which is so fundamental to how modern Comparative Developmental Physiology operates, becomes particularly challenging. High-dimensional phenotyping approaches are often limited to a number of model species of interest, likely due to the considerable resources associated with developing computational tools that can measure such change (Lussier & Liu, 2007; Chung *et al*., 2010; White *et al*., 2010; Xu *et al*., 2010; Peravali *et al*., 2011; Spomer *et al*., 2012; Olmedo *et al*., 2015; Levario *et al*., 2016; Lürig *et al*., 2021). Video based phenotyping methods, may provide a means with which to address this. Previous research has shown EPTs to be applicable to embryos of a freshwater snail (Tills *et al*., 2018; 2021; Ibbini *et al*., 2022), amphipod and decapod crustaceans (Tills *et al*., 2018; 2022; Ibbini *et al*., 2022), and in the current thesis, between embryos of three species of freshwater snail that vary in their developmental itineraries (Chapter 2, 3). Each of these studies revealed considerable alterations to the phenotype of these developing animals, providing novel insight into how periods of early development are shaped by environmental change. Underpinning the utilisation of any model species is the assumption that it can provide sufficient insight into the biology of others (Feder,

2006). Burggren (2021) highlights that comparative developmental physiologists need to expand the animal models used in their research, given that alternatives could be better suited to answering our biological questions. EPTs are centred around the theoretically simple quantification of changes in pixel values present in video data, rather than focussing on specific phenotypes or regions of interest in a developing animal. I suggest that adoption of EPTs and similarly transferable approaches to high-dimensional organismal phenotyping will provide an important first step for the integration of phenomics within the scope of the Krogh principle.

Phenomics, the high-dimensional acquisition of phenotypic data at the scale of the whole organism (Houle *et al*., 2010), has the potential to facilitate significant advancements in our understanding of how environmental and evolutionary change influences the development of physiological function (Burggren, 2018; 2021; Lürig *et al*., 2021). Here, application of EPTs as an approach to phenomics enabled quantification of high-dimensional phenotypic changes that were transferable between species that vary in their developmental itineraries, and between physiological windows of development that vary in their observable phenotypes. I suggest that future efforts within the confines of Comparative Developmental Physiology and Developmental Integrative Physiology will greatly benefit from the integration of phenomics into their research agendas. The phenotype is the ultimate object of selection, and evolutionary changes in development and responses to environmental change constitute changes to a massive number of individual phenotypes (Forsman 2015). Capturing this complexity of change through the application of technology enabled approaches, at scales that have long been adopted by the molecular omics, I believe, will lead to considerable advancements in our understanding of how development drives evolutionary change, and how predicted global environmental change will impact the early development of vulnerable populations.

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