The Evolution of Maturation in *Daphnia*

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by

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Abstract

Maturation is a key life history transition for many organisms, due to the importance of age and size at maturity in determining fitness. Understanding how maturation phenotypes evolve requires an appreciation of the underlying ontogenetic mechanisms, including the maturation threshold, which determines when an individual 'decides' to mature. Maturation thresholds are poorly understood, and little is known about how phenotypically plastic or genetically variable they are, or how variable thresholds influence fitness. In this thesis the evolution of maturation thresholds is investigated using the crustaceans *Daphnia magna* and *D. pulex*. A comprehensive approach to modelling the maturation process found that the maturation threshold was a developmentally plastic trait in response to variable resource availability, and more closely resembled a process with a rate than a discrete switch. The maturation threshold also differed between genotypes for both species, and these differences were more apparent in *D. magna* than *D. pulex*. A second study of maturation in *D. magna* identified clone-specific parental effects in the threshold. Furthermore, these parental effects influenced growth, and reaction norms for age and size at maturity were a product of interacting effects between both growth and maturation threshold. A microarray study of gene expression changes in *D. pulex* found that most gene expression changes during maturation were continuous, further supporting the idea that the threshold is better thought of as a rate than a switch. This study also identified increases in vitellogenin transcripts, indicating the allocation of resources towards reproduction, and potential mechanisms for epigenetic inheritance and endocrine control of maturation. Finally the fitness consequences of variation in the maturation threshold were investigated in *D. magna*. Genotypes with a smaller threshold had a higher intrinsic rate of population increase, but threshold size did not correlate well with competitive success when five clones were directly competed with each other, suggesting that interactions with other factors were influencing fitness. The findings of this thesis suggest that maturation thresholds are not based on a single fixed state, but are responsive to environmental variation. The presence of heritable variation and transgenerational effects in these developmentally plastic traits suggests an important and adaptive role for them in the evolution of age and size at maturity.

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Declaration of originality

I declare that all the work presented in this thesis is my own original research with the following acknowledgements:

Chapter 2: Tom Van Dooren (École Normale Supérieure, Paris) produced the statistical models and subsequent probabilistic maturation reaction norms. The resulting paper, published in *Evolution*, was a collaborative effort between Tom Van Dooren, Steve Paterson, Stew Plaistow and myself. A copy is attached as an additional appendix at the end of the thesis.

Chapters 2, 3, 4 & 5: Clones of *Daphnia magna* and *D. pulex* used throughout the thesis were lab strains brought to the University of Liverpool from Andrew Beckerman's lab at the University of Sheffield, and derived from field samples primarily caught by the Beckerman lab (*D. pulex*) or Dieter Ebert's lab (*D. magna*) at the University of Basel.

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Table of abbreviations

BLAST	Basic local alignment search tool
DS	Developmental stage
GLM	Generalised linear model
GO	Gene ontology
H3	Histone 3
H4	Histone 4
HPE	High-food parental effect
JH	Juvenile hormone
K	Population carrying capacity
LPE	Low-food parental effect
PE	Parental effect
PMRN	Probabilistic maturation reaction norm
r	Intrinsic rate of population increase
s	Selection coefficient
TOR	Target of rapamycin
SOD	Super-oxide dismutase
VTG	Vitellogenin

Contents

Chapter 1

Introduction

The natural world is replete with a huge diversity of organisms exhibiting an amazing variety of sizes, growth rates, fecundities and reproductive strategies (Darwin 1859). For example, the bamboo *Chusquea abietifolia* takes 32 years to grow and mature, and dies following a single flowering event (Seifriz 1920), whilst aphids such as *Acyrthosiphon pisum* may produce numerous clutches in the course of a year, and have telescoping generations, with individuals carrying not only their daughters, but their daughter's daughters (Kindlmann & Dixon 1989). A key question in evolutionary biology is therefore explaining how these myriad contrasting life histories arise and produce adaptive phenotypes: this is the goal of life history theory (Cole 1954; Stearns 1976; Roff 2002) . One phenotype of particular interest to life historians is age and size at maturity (McLaren 1966). Variation in age and size at maturity can carry prominent fitness consequences: individuals that mature at a young age are less likely to suffer mortality before reaching reproduction and will have a competitive advantage in expanding populations (Cole 1954). Maturing earlier, however, allows less time for growth and reduces adult body size (Stearns 1977), a trait often correlated with fecundity and mating success (Roff 2001). Early efforts to understand the evolution of age and size at maturity considered how the trade-off between them might limit phenotypic forms (Stearns 1976, 1977) and result in an 'optimal' age and size at maturity (Stearns & Crandall 1981). Although this early use of optimality models appeared to fit patterns from empirical data, it ignored the fact that optimal phenotype may change in different environments.

1.1 Developmental plasticity

The importance of environmental variation in shaping phenotypes had been recognised before the latter-half of the twentieth century (Baldwin 1896; Woltereck 1909; Johannsen 1911; Schmalhausen 1949), however, it was not until Bradshaw's review (1965) that mainstream evolutionary thinkers were reacquainted with the ecological consequences and evolutionary significance of phenotypic plasticity. Phenotypic plasticity can be defined as the ability of a genotype to alter its phenotype in response to the environment in which it develops (Pigliucci et al. 1996; Pigliucci 2001) and the variety of phenotypes that a genotype

can express across an environmental gradient is termed the reaction norm (Woltereck 1909; Schmalhausen 1949), and is central to conceptualising the disconnect between genotype and phenotype (Pigliucci 2001). Phenotypic effects are diverse (Via et al. 1995) and often adaptive (Dudley & Schmitt 1996), and understanding how organisms respond to variable environments requires an appreciation of both the proximate mechanisms that generate phenotypic plasticity during development, as well as the ultimate reasons for its existence (Callahan et al. 1997; Fusco 2001).

Developing phenotypes will respond to many environmental cues, including the non-genetically inherited factors transmitted by their parents (Kirkpatrick & Lande 1989; Mousseau & Dingle 1991; Bernardo 1996a; Mousseau & Fox 1998a). These 'parental effects' can take many forms, including the transmission of cytoplasmic components (nutrients, proteins, RNA), epigenetic states and the extra-organismal environment (for a review, see Bonduriansky & Day 2009). Although the effects of non-genetic inheritance have previously been characterised through their influence on static traits, from size at birth (e.g. Bernardo 1996b), to age and size at maturity (e.g. Marshall et al. 2003; Beckerman et al. 2006), it is during development that these factors are integrated into offspring phenotypes (Marshall & Uller 2007; Badyaev & Uller 2009), and any consideration of the evolution of life histories must recognise the important role that non-genetic inheritance can have on developing phenotypes (Badyaev 2011; Day & Bonduriansky 2011).

1.1.1 Plasticity in age and size at maturity

Variation in phenotypes arise because different aspects of development (i.e. processes related to differentiation and morphogenesis) respond to environmental cues such as resource availability, temperature, light, photoperiod and predation in numerous ways (Schlichting & Pigliucci 1995; Pigliucci et al. 1997; Dufty Jr et al. 2002). Life history phenotypes are no exception and display remarkable plasticity in response to environmental variation. For example, many insect species will adjust their development time in response to shortening days (Nylin et al. 1989), and most ectotherms mature at smaller sizes under higher temperatures (Atkinson 1994). Variation in the availability and quality of resources also results in plasticity in age and size at maturity, with limiting resources often resulting in delayed maturity at reduced sizes (Kennedy & Mitra 1963; Frisch 1978; McKenzie et al. 1983). In order to understand the evolution of plasticity in age and size at maturity, maturation reaction norms were modelled using optimality theory (Stearns & Koella 1986; Berrigan & Charnov 1994; Berrigan & Koella 1994; Sibly & Atkinson 1994). Although predominantly theoretical, this approach enjoyed some success in explaining empirical

patterns of variation that occur in age and size at maturity. Yet some of the assumptions contained within these models were biologically unrealistic. For example, growth was frequently modelled using the Von Bertalanffy equation (Von Bertalanffy 1957), despite the fact that it does not consider changing patterns of resource allocation that will occur during development (Day & Taylor 1997). Furthermore, these models use the intrinsic rate of population increase (*r*) as a fitness measure, which will be inappropriate in many environments (Mueller 1997; Brommer 2000). Perhaps most importantly, these models neglected a vitally important aspect of any reaction norm: the underlying developmental processes (Pigliucci & Schlichting 1995; Schlichting & Pigliucci 1995; Pigliucci et al. 1996).

1.1.2 Developmental thresholds and maturation thresholds

One of the first studies to explicitly consider how the underlying development can shape age and size at maturity was carried out by Wilbur and Collins (1973), following their observations that larvae of the salamander *Ambystoma maculatum* had to reach a minimum size 'threshold' before they could metamorphose, and appeared to delay metamorphosis in favourable conditions. Subsequent empirical studies documented minimum size thresholds for ontogenetic transitions in a wide range of taxa, including biennial plants (Werner 1975; Klinkhamer et al. 1987; Wesselingh & Klinkhamer 1996), crustaceans (Ebert 1992, 1994; Twombly 1996), insects (Nijhout & Williams 1974a; Bradshaw & Johnson 1995; De Moed et al. 1999), fish (Policansky 1983; Reznick 1990) and amphibians (Travis 1984; Denver 1997; Morey & Reznick 2000). The verbal model proposed by Wilbur and Collins (1973) also spawned a series of sub-models (Leips & Travis 1994; Bradshaw & Johnson 1995; Twombly 1996) that attempted to provide proximate (i.e. developmental) explanations for age and size at maturity. These models generally agreed upon the importance of a minimum threshold for ontogenetic transitions, but lacked consensus with regard to the timing of maturation or metamorphosis in favourable environments: specifically, should maturation be delayed when conditions are favourable (to increase size at maturity), or will the threshold result in the transition occurring earlier. This discrepancy was resolved by Day and Rowe (2002), who proposed that 'developmental thresholds' set a minimum size or state for the initiation of ontogenetic transitions, including maturation, and that reaction norms for age and size at maturity will be L-shaped, with age at maturity decreasing and size increasing as growth conditions improve. L-shaped reaction norms for age and size at maturity have subsequently been identified and ascribed to developmental thresholds, or 'maturation thresholds' following manipulation of resources (Plaistow et al. 2004; Hahn et al. 2008).

Others have failed to observe the strong negative relationship between age and size at maturity (Juliano et al. 2004; Etilé & Despland 2008), suggesting that the threshold model proposed by Day and Rowe (2002) in its current form cannot explain diverse patterns of age and size at maturity.

1.1.3 Deterministic versus probabilistic maturation reaction norms (PMRNs)

While optimality models can provide useful frameworks for exploring how patterns of age and size at maturity may evolve (e.g. Day & Rowe 2002), they are less effective when applied to the study of age and size at maturity in the real world, where maturation is a highly stochastic process (Bernardo 1993). This problem is particularly apparent when considering maturation schedules in fish stocks, and has led to the development of an alternative, probabilistic approach to understanding variation in maturation reaction norms. Age and size at maturity were seen to decline in many commercial fisheries during the 1980s (Beacham 1983a, 1983b, 1987) but it was unclear whether the response to harvesting was plastic or genetic (Rijnsdorp 1993; Trippel 1995). In an effort to disentangle phenotypic plasticity from genetic adaptation in fish stocks, Heino et al. (2002) developed the probabilistic maturation reaction norm (PMRN). By describing the probability of maturing as a function of age, size, and other variables such as growth history (Morita & Fukuwaka 2006), PMRNs can handle the stochasticity present in maturation data from fisheries. PMRNs have been used widely in fisheries data (Grift et al. 2003; Engelhard & Heino 2004; Ernande et al. 2004; Olsen et al. 2004), but are increasingly recognised as useful tools for studying maturation schedules under different ecological scenarios in a range of taxa (Van Dooren et al. 2005; Kuparinen et al. 2008; Uusi-Heikkilä et al. 2011). Furthermore, by treating maturation as a process (Van Dooren et al. 2005; Dieckmann & Heino 2007), PMRNs can provide some insight into the factors underlying age and size at maturity, such as the developmental threshold.

1.2 Mechanisms underlying developmental thresholds

Although developmental thresholds for maturation are widely observed, the physiological mechanisms that give rise to them are rarely understood (Berner & Blanckenhorn 2007). General life history approaches do not account for the complexity of maturation, which is comprised of numerous, often taxon-specific, developmental changes, each with their own neuroendocrinological mechanisms (Stern & Emlen 1999; Nijhout 2003). An ontogenetic

approach that considers the genetic and environmental components of developmental processes (including developmental thresholds) is necessary to understand evolutionary responses to the conflicting selective pressures that arise between age and size at maturity (Davidowitz et al. 2005; Nijhout et al. 2010). Currently, however, our understanding of the interactions between underlying physiological factors and environmental influences is limited to a few model insect (Layalle et al. 2008; Nijhout 2008) and amphibian (Boorse & Denver 2004) systems.

1.2.1 The control of developmental thresholds

The physiological basis for developmental thresholds is perhaps best understood in insects, largely due to work in the Lepidopteran *Manduca sexta* and Dipteran *Drosophila melanogaster*. In both species the final larval instar must achieve a critical weight before pupation can occur (Bakker 1959; Nijhout & Williams 1974a). Once *M. sexta* reach their critical weight, they cease production of the endocrine factor juvenile hormone (JH), and synthesise an enzyme that catabolises remaining JH. Following the clearance of JH, the moult hormone ecdysone is released and pupation occurs (Davidowitz & Nijhout 2004). The feedback mechanism between weight, nutrition and endocrine factors has been elucidated in *D. melanogaster*, where ecdysone release only occurs when the nutrient-dependent target of rapamycin (TOR) pathway is sufficiently upregulated (Layalle et al. 2008). However, although these mechanisms are likely to form the basis of maturation decisions in holometabolous insects, they may be modified in other holometabolous insects (Shafiei et al. 2001), and differ substantially in hemimetabolous species (Beckel & Friend 1964; Nijhout 2008). Furthermore, the environmentally dependent expression of these traits often differs substantially between species. Further empirical studies in alternative systems are required to improve our understanding of the complex interactions between development, genes and the environment.

1.2.2 The control of maturation in Daphnia

One of the few other organisms in which developmental thresholds have previously been studied to any degree is the crustacean *Daphnia* (McCauley et al. 1990a; Ebert 1991, 1992, 1994, 1997; Mc Kee & Ebert 1996). In *Daphnia*, initiation of maturation appears to be dependent on a developmental threshold, termed the maturation threshold (Ebert 1991). The maturation threshold appears to be based on size and is independent of the resources

available in the environment (Ebert 1992), but plastic with respect to temperature (Mc Kee & Ebert 1996). Although growth differences following the maturation threshold result in some variation in size at maturity (Ebert 1994), most variation is in age and instar number taken to reach maturity (Ebert 1997). Despite these results, a number of questions remain concerning the evolution of maturation in *Daphnia*, such as the impact of age on maturation thresholds, and the extent to which they vary between genotypes. In his analysis of the maturation threshold, Ebert (1992) made an a priori assumption that age did not influence the maturation threshold. Yet, through its effect on growth history, age may alter maturation reaction norms (Morita & Fukuwaka 2006). Furthermore, genotypic differences were hinted at but not fully investigated (Ebert 1994), suggesting that this may be a useful system for studying the evolution of maturation.

1.3 Thesis aims

The aim of this thesis is to explore the evolution of maturation, and in particular the maturation threshold, using the crustacean *Daphnia* as a model system. Currently little is known about why developmental thresholds, including maturation thresholds, have evolved. In the past they have been thought of primarily as a physiological constraint associated with costs of reproduction (Day & Rowe 2002) and a means of reducing variation in one state (e.g. size), at the expense of another (e.g. age) (Ebert 1997). A recent suggestion is that they are one of the targets for selection in the evolution of body size and development time (Nijhout et al. 2010). All of these possibilities may be true to some degree. Distinguishing between them requires a better understanding of variation in thresholds, as well as the proximate causes and evolutionary consequences of this variation. This thesis attempts to fill in some of the gaps by answering the following questions: to what extent is the maturation threshold variable? Are maturation thresholds fixed with respect to a certain state, such as size, or plastic in response to environmental variation? Do maturation thresholds vary between different genotypes and populations? Furthermore, if thresholds are genotypically and phenotypically variable, how does this variation impact upon fitness? And what are the deeper physiological mechanisms that generate the maturation threshold? The evolution of the threshold is considered from both an evolutionary and ontogenetic perspective: from the proximate mechanisms that underpin this important developmental process to the fitness consequences of genotypic variation and phenotypic plasticity in maturation. Taking such a broad view requires a multidisciplinary approach, and techniques ranging from life history assays and competition experiments to microarray analyses are adopted.

1.3.1 Thesis plan

Although age and size at maturity are commonly-studied life history traits (Roff 2001; Berner & Blanckenhorn 2007), quantifying these traits is not straightforward. Maturation reaction norms are stochastic and therefore a probabilistic approach is necessary (Heino et al. 2002); however, there is still some debate as to the best way to measure maturation (Van Dooren et al. 2005; Heino & Dieckmann 2008; Kuparinen et al. 2008). In **Chapter 2** I compared the utility of different statistical methods for fitting probabilistic maturation reaction norms (PMRNs), using three points in development as maturation indicators. The PMRNs are used to evaluate phenotypic and genotypic variation in PMRNs across a resource gradient in five clonal genotypes of *Daphnia magna* Straus and five of *D. pulex* Leydig, and to assess the relative importance of age and size in determining the probability of maturation.

As well as responding to variation in the current environment, it is possible that maturation is influenced by variation in the parental environment. Parental effects are often viewed as static influences on birth traits (Bernardo 1996b; Mousseau & Fox 1998b) or as modifying adult traits in response to natural selection (e.g. Agrawal et al. 1999; Donohue 2009; Duckworth 2009). Both these views fail to account for the expression of parental effects throughout ontogeny, even though they are increasingly seen as an important aspect of normal development (Galloway et al. 2009; Yanagi & Tuda 2010; Pascoal et al. 2012). In **Chapter 3** I directly compare the ontogeny of parental effects across an environmental gradient in three clones of *D. magna* by quantifying parental effects on neonate size, pre-maturation growth, maturation thresholds (measured as PMRNs), and post-maturation growth. Consequences for the adult phenotype are assessed by comparing age and size at maturity, and the number of offspring in the first clutch.

Although the maturation threshold is often viewed as a discrete switch, turning on the maturation process once a critical state has been exceeded (Ebert 1992; Day & Rowe 2002), the underlying mechanisms that control the expression of the threshold are poorly understood outside of a small number of model systems (Denver 1997; Davidowitz & Nijhout 2004; Layalle et al. 2008; Nijhout 2008). However, appreciation of these mechanisms is critical to understanding how developmental plasticity in maturation arises (Callahan et al. 1997; Dufty Jr et al. 2002). **Chapter 4** uses a microarray study to investigate whether gene expression changes in developmental stages either side of the maturation threshold of *D. pulex* are indicative of a discrete switch or a gradual shift towards reproductive function.

The maturation threshold is an important aspect of development with clear consequences for age and size at maturity (Van Dooren et al. 2005; Berner & Blanckenhorn 2007). Genotypic variation in the threshold suggests that they are adaptive (Piché et al. 2008; Skilbrei & Heino 2011), yet little is known about how variation in the maturation threshold influences fitness. In **Chapter 5** I investigate how variation in the maturation threshold of the five *D. magna* clones from Chapter 2 influenced fitness. Estimates of fitness are often dependent on the environment in which they are measured (Dieckmann 1997; Heino et al. 1998; Brommer 2000), therefore three fitness metrics are assessed, including the rate of population increase, *r*; the population carrying capacity, *K*; and the selection coefficient, *s*, following direct competition between genotypes.

1.4 *Daphnia* as a model system

Daphnia (Branchiopoda : Cladocera : Daphniidae) are a diverse genus of small (0.3-5.9 mm) filter feeding zooplankton (Lynch 1980; Fryer 1991), and keystone species in many lentic ecosystems (Shapiro & Wright 1984; Elser et al. 1988). *Daphnia* generally feed on unicellular algae and tend to reproduce by cyclic parthenogenesis, producing large numbers of genetically identical female offspring through apomixis when conditions are favourable (Green 1956), and male offspring and haploid eggs in poor conditions, eventually resulting in a diapausing ephippial stage (Fryer 1991; Ebert 2005). The desiccation tolerant ephippia can remain dormant in 'seed banks' for many years but under the right conditions will break diapauses to produce viable offspring (Decaestecker et al. 2007). This reproductive strategy allows both effective dispersal between lentic bodies, and rapid colonisation of new environments, often leading to their dominance as zooplankton in many lentic systems (Fryer 1991).

Daphnia have a long and rich history as a study organism in evolutionary ecology dating back more than 250 years (Fryer 1991), and covering a variety of fields as diverse as ecotoxicology (Baird et al. 1989a; Lilius et al. 1995), host-parasite biology (Ebert 1995, 2005), ecosystem functioning (Elser et al. 1988; Sarnelle & Knapp 2005), and more recently ecological genomics (Colbourne et al. 2011a) and epigenetics (Harris et al. 2012). *Daphnia* are well suited to many laboratory studies because of their rapid generation times, allowing multi-generation experiments to be carried out with ease. Furthermore, one aspect of their biology that makes them a particularly interesting model organism is the plasticity of their phenotype. Fittingly, it was plasticity in *Daphnia cuculatta* which inspired Woltereck (1909) to coin the term 'reaction norm', and although the concept lay dormant in the literature for

many years, reaction norms in *Daphnia* are yet again illustrating the importance of phenotypic plasticity in the evolutionary process (Tollrian 1993, 1995; Agrawal et al. 1999; Agrawal 2001; Hammill et al. 2008).

1.4.1 Daphnia *as a model system for studying maturation*

Daphnia are suitable for the study of maturation for a number of reasons. As mentioned previously, a maturation threshold has already been identified in *D. magna* (Ebert 1991, 1992, 1994; Mc Kee & Ebert 1996). Furthermore, the developmental changes associated with this threshold have received some attention: for example, it is known that the first commitment to reproduction occurs two instars before eggs are produced (previtellogensis; Zaffagnini 1987), and provisioning of these eggs with resources (vitellogenesis) occurs in the subsequent instar (Bradley et al. 1991). Because their carapace is transparent it is easy to observe vitellogenesis, and in some cases pre-vitellogenesis, which can act as reliable indicators that the maturation process has been initiated.

Parthenogenetic reproduction in *Daphnia* allows one to rear genotypically identical individuals across a range of environments and investigate the evolution of reaction norms: any differences in reaction norms between genotypes are indicative of heritable variation. As well as being an excellent system in which to study genetically inherited development, parthenogenetic *Daphnia* lend themselves to the study of non-genetic inheritance, as parental effects are now recognised to be an important aspect of *Daphnia* life history (LaMontagne & McCauley 2001; Alekseev & Lampert 2004; Sakwińska 2004; Stjernman & Little 2011). The clonality of *Daphnia* also makes it suitable for experimental evolution or competition analyses. Multiple individuals representing two or more distinct genotypes can be introduced to a common environment, and changes in the genotype frequency provide an accurate measure of fitness for that environment. Previous competition experiments have focused on identifying the importance of parasite resistance and the costs that it entails (Little et al. 2002; Capaul & Ebert 2003), or differences in microevolutionary responses following differential selection (e.g. Van Doorslaer et al. 2009). The approach can also be used to investigate the fitness consequences of variation in traits such as the maturation threshold.

Finally since the inception of this thesis, the *D. pulex* genome has been published (Colbourne et al. 2011a). The growing genetic resources available for *Daphnia* (wFleaBase; Colbourne et al. 2005), including annotation of the *D. pulex* genome, provide invaluable resources for the study of gene expression (Hannas & LeBlanc 2010; David et al. 2011;

Jeyasingh et al. 2011). These resources can be applied to study the underlying developmental changes associated with maturation.

Chapter 2

How to measure maturation: a comparison of probabilistic methods used to test for genotypic variation and plasticity in the decision to mature

2.1 Introduction

The age and size at which an organism matures are key life-history traits influencing fitness (Bernardo 1993; Roff 2001; Berner & Blanckenhorn 2007). Age and size upon reaching maturity are remarkably plastic, and maturation reaction norms are commonly used to describe the response of these traits to environmental variation (Stearns & Koella 1986; Perrin & Rubin 1990; Berrigan & Charnov 1994; Olsen et al. 2004; Plaistow et al. 2004; Beckerman et al. 2010). There is also increasing awareness of the importance of underlying ontogenetic processes in shaping these reaction norms (Reznick 1990; Johnson & Porter 2001; Wolf et al. 2001; West-Eberhard 2003; Berner & Blanckenhorn 2007). Maturation is rarely a simple transition from juvenile to adult, but rather a process consisting of a number of co-ordinated and potentially heritable endocrinological and neurophysiological changes, controlling the allocation of resources to growth, maintenance and reproductive function (Bernardo 1993; Stern & Emlen 1999; Nijhout 2003). The limited studies carried out to date (Boorse & Denver 2004; Davidowitz & Nijhout 2004; Mirth & Riddiford 2007; Nijhout 2008) suggest that understanding the proximate causes of maturation is essential to explaining variation in the age and size at which individuals achieve maturity (Marshall & Browman 2007).

2.1.1 Mechanisms underlying age and size at maturity

Wilbur and Collins (1973) first suggested that individuals must achieve a minimum size (a size threshold) before they are able to initiate ontogenetic transitions. Since then, evidence that organisms must reach a minimal size or state before maturing or metamorphosing has been found in biennial plants (Werner 1975; Klinkhamer et al. 1987; Wesselingh & Klinkhamer 1996), crustaceans (Ebert 1992, 1994), acarids (Plaistow et al. 2004), insects (Nijhout & Williams 1974a; Bradshaw & Johnson 1995; Davidowitz et al. 2003; Etilé &

Despland 2008), fish (Policansky 1983; Reznick 1990) and amphibians (Travis 1984; Denver 1997; Morey & Reznick 2000), suggesting that these thresholds are ubiquitous. Studies demonstrating that thresholds can vary among populations (McKenzie et al. 1983; De Moed et al. 1999; Piché et al. 2008; Skilbrei & Heino 2011) and closely related species (Morey & Reznick 2000) further suggest that variation in the position or severity of thresholds is important in shaping the evolution of reaction norms for age and size upon reaching maturity. However, sufficient knowledge of how underlying ontogenetic processes are translated into observed plasticity is not usually available and our understanding of the evolution of age and size upon reaching maturity relies heavily on more phenomenological descriptions of patterns (although for an exception refer to work carried out on the tobacco hornworm moth *Manduca sexta*: Nijhout 2003; Davidowitz & Nijhout 2004; Nijhout et al. 2010) with determinants of the onset of maturation more often assumed than tested for (Ebert 1994; Plaistow et al. 2004; Etilé & Despland 2008; Kuparinen et al. 2008). Quantification of determinants across individuals within a population, rather than at the level of the genotype (e.g. Klinkhamer et al. 1987; De Moed et al. 1999; Morey & Reznick 2000; Engelhard & Heino 2004; Olsen et al. 2004; Mollet et al. 2007; Etilé & Despland 2008) is defensible when data per genotype are difficult to collect (Dieckmann & Heino 2007), but limits our ability to explore their evolutionary potential (Berner & Blanckenhorn 2007; but see Wesselingh & de Jong 1995; Wesselingh & Klinkhamer 1996). Finally, it is still unclear how genetic variation and environmental sensitivity in maturation processes should be quantified and qualified (Van Dooren et al. 2005; Dieckmann & Heino 2007; Kraak 2007; Kuparinen et al. 2008; Uusi-Heikkilä et al. 2011). Adopting the correct methodology is likely to be important, for example, when attempting to disentangle phenotypic plasticity from genetic adaptation to harvesting of fish stocks (Rijnsdorp 1993; Grift et al. 2003; Engelhard & Heino 2004; Ernande et al. 2004; Olsen et al. 2004).

2.1.2 Characterising maturation using probabilistic maturation reaction norms (PMRNs)

The optimality models first used to study reaction norms for age and size at maturity (Stearns & Koella 1986; Berrigan & Koella 1994; Sibly & Atkinson 1994) assumed that maturation occurs as a deterministic process but in reality the timing of maturation may be influenced by many factors, some of which will invariably be stochastic (Bernardo 1993) and which affect the likelihood of maturing at a given age and size. Therefore individuals with comparable juvenile growth trajectories may still differ in the size and age at which they mature (Ebert 1991; Morita & Morita 2002). In order to deal with stochasticity in maturation schedules, the response variable becomes a probability of maturing (Heino et al.

2002; Dieckmann & Heino 2007). The concept of probabilistic maturation reaction norms (PMRNs) based on logistic regression, a class of generalized linear model (GLM), was developed by Heino et al (2002) to describe the probability of maturing as a function of age and size. PMRNs are extensively used to model data from fish stocks with time as a discrete variable, and the probability of maturing at a given size-at-age is assessed for each age class (Grift et al. 2003; Engelhard & Heino 2004; Olsen et al. 2004). However, there is increasing awareness that factors other than age and size may influence the decision to mature, including recent growth history (Morita & Fukuwaka 2006), condition (Mollet et al. 2007; Uusi-Heikkilä et al. 2011) or other physiological features (Van Dooren et al. 2005). Furthermore, because the exact age or size at which an individual matures during the interval between sampling points is often not observed (interval censoring), this approach to PMRNs may be problematic if individuals maturing at unobserved ages within a given time interval are pooled and given the same age at maturity. Although complementary methodologies based on demographic data have been devised to bypass this problem (Barot et al. 2004) and produce results comparable to the traditional PMRN concept (Pérez-Rodríguez et al. 2009), they may still suffer from bias when observation intervals vary in length and therefore in the risk of an individual maturing (Van Dooren et al. 2005). One solution to overcoming these biases in interval censored data is to use time-to-event analysis, focussing on instantaneous rates of maturing (Van Dooren et al. 2005; Kuparinen et al. 2008). Alternatively, logistic regression can be modified to represent such maturation rate models (Lindsey & Ryan 1998; Collett 2003), notably by including an 'offset' that corrects for interval length variation, although this approach has yet to be applied to studies of PMRNs.

If maturation events are stochastic processes, then time-to-event analysis appears to be a natural candidate to describe them. This approach is appealing because it focuses on conditional probabilities, i.e. the probability of maturing given that it has yet to happen. Specifically, maturation is modelled as a process determined by an instantaneous rate of maturing which can depend on the developmental histories of individuals. A maturation status is obtained when the rate of maturation is integrated over an appropriate timescale. This may be the time interval between observations but in some cases it is advantageous to integrate over a timescale other than time/age if it characterizes the operational history of the maturation process better (Duchesne & Lawless 2000). The instantaneous rate of maturing can also depend on a number of maturation determinants, including age and/or size, as well as other explanatory variables. The fit of different maturation rates and timescales can be compared through hypothesis testing rather than being assumed (Van Dooren et al. 2005), such that a comparison of different types of integration can give evidence of certain maturation mechanisms. Though 'rate models' have been successfully used to model the

maturation process (Van Dooren et al. 2005; Kuparinen et al. 2008), they can be time-consuming and difficult to fit (Van Dooren et al. 2005) unless numerous assumptions about the underlying maturation rate and timescale are made such that standard approaches of survival analysis can be applied (Kuparinen et al. 2008). Approximations of rate models through the use of standard GLMs with an offset term are considerably easier to fit, and still allow the importance of different rate effects (covariates in a standard GLM) and timescales (the offset) to be determined. However, they necessarily require certain assumptions to be made about maturation rates, including constancy of maturation rates within growth intervals, and a comparison of alternative timescales for the maturation process using GLMs has yet to be carried out.

Another possibility to consider is that probabilistic maturation processes do not operate continually with a certain rate. For example, when the maturation decision is taken within a certain time window of fixed length, a 'sensitive period' at a moult for example, it makes no sense to integrate rates over the entire length of time between observations. In this case, and when the intervals between observations are longer than the maturation time window, a GLM without an offset should fit the data better, and the model should not be interpreted as representing a rate. Maturation is then better understood as a probabilistic switch that can be flipped at certain instances. When the dependence of the maturation rate on a determinant rises from zero to a very high value, the switch process resembles a threshold and may even be considered non-probabilistic.

Models for probabilistic reaction norms can thus either represent a process with a rate or not. The second possibility can be modelled using GLMs without offset, while rate models can either be fitted using different methods of survival analysis (Kuparinen et al. 2008), integrated parametric maturation rate models (Van Dooren et al. 2005) or using GLMs with an offset. Comparison of deviances and Akaike information criteria (AIC) values can reveal which alternative explains the data best; yet, no study has compared the applicability of the different approaches to modelling the maturation process, or tested the validity of the assumptions made using a GLM approach. This problem is addressed by comparing fits of maturation rate models and GLMs with and without offsets for maturation data collected from 5 clones of *Daphnia magna* Straus and 5 of *D. pulex* Leydig. Other methods of survival analysis, such as those suggested by Kuparinen et al. (2008), were not applied since these do not allow the comparison of alternative time scales.

2.1.3 Measuring variation in the maturation thresholds of Daphnia

In *Daphnia*, the importance of size as a maturation determinant or status has been demonstrated previously (Green 1956; Lynch 1989; Ebert 1991), and descriptive models incorporating a size threshold have been used to explain variation in age and size upon reaching maturity in *D. magna* (Ebert 1992, 1994). Due to its parthenogenetic reproduction, *Daphnia* represents a particularly useful organism for the study of maturation reaction norms, as phenotypic effects of environmental variation can be investigated in genotypically identical individuals. The full extent of plasticity can thus be revealed and comparisons between genotypes (clones) can be drawn. However, the effect of threshold variation on maturation reaction norms has only previously been carried out for a maximum of 2 clones from the same population (Ebert 1994). Moreover, the existence of an exclusively size-dependent threshold was assumed rather than being explicitly tested for, leaving the role of age in the maturation process unclear (Morita & Fukuwaka 2006). As well as comparing the fit of maturation rate models and GLMs with and without offsets for *Daphnia* maturation data, the roles that age and size play in shaping PMRNs is investigated, and it is determined whether these roles vary across a range of individual growth rates, and whether maturation differs between the two species. Finally, clonal variation in PMRNs within each species is examined, and the implications this may have for the evolution of age and size upon reaching maturity are discussed.

2.2 Materials and methods

2.2.1 Experimental animals

Five laboratory clones of both *Daphnia magna* and *D. pulex* were used in this study. Clones originated from a variety of geographic locations across Europe. *D. magna* clone DKN 1-3 came from Kniphagen, Ostholstein, Germany (54°10’36”N 10°48’24”E); clone Ness1 from Ness, Cheshire, U.K. (53°16’16”N, 3°2’47”W); clone H01 from Bogarzo-to, Kiskunsági-Nemzeti Park, Hungary (46°48’N 19°08’E); and clones B5 and B7 both originated from Weston Park, Sheffield, U.K. (53°38’20”N 1° 49’07”W). *D. pulex* clones Cyril, Chardonnay, and Carlos originated from Crabtree pond, Sheffield, U.K. (53°24’17”N, 1°27’25”W), while Boris came from another pond in Sheffield, U.K. (53°24’18”N, 1°27’27”W). Bierbeek was collected from Bierbeek, Belgium (50°49’60”N, 4°46”0”E). All clones were cultured and experiments were carried out at 21 ± 1°C with a 14:10 light:dark photoperiod. *Daphnia* were maintained individually in 150 ml of hard artificial pond water media (OECD 1984)

enriched with a standard organic extract (Baird et al. 1989b). *Daphnia* were fed *Chlorella vulgaris* Beijerinck (quantified by haemocytometer) on a daily basis and media was totally replaced every other day. Clones were acclimated for a minimum of 3 generations under *ad libitum* food rations of 200 cells μl^{-1} day^{-1}. Experimental animals were obtained from the third clutch.

2.2.2 Experimental design

For each clone 64 - 80 neonates were isolated from 3 - 5 mothers (from the same maternal cohort). These were randomly assigned to one of the following eight food rations: for *D. magna* 200, 133, 89, 59, 40, 26, 18 and 12 cells μl^{-1}, and for *D. pulex* 89, 59, 40, 26, 18, 12, 8 and 5 cells μl^{-1}. Rations differed between species because *D. pulex* is known to have a lower incipient limiting concentration of food (Porter et al. 1982), and a preliminary study suggested that, prior to reproduction, this limit occurred below 89 cells μl^{-1} (personal observation). This variation in ration generates a wide variety of growth trajectories and resultant ages and sizes at the onset of maturity. All individuals were checked every day and photographed after moulting at all instars up to the deposition of eggs in the brood chamber. Body size was estimated as the distance from the top of the head to the base of the tail spine and measured from photos using the image analysis software ImageJ (Rasband 1997). Experiments were staggered over a 16-week period due to the amount of work involved in conditioning and assaying clones.

2.2.3 Maturation indicators in Daphnia

Daphnia are not constrained by a fixed number of juvenile instars (Green 1956), but once the maturation process is initiated, they commonly achieve maturity within 3 instars (Bradley et al. 1991). In the first of these instars, nurse cells begin to differentiate into oocytes. The first clearly visible sign of maturation is during the subsequent instar when oocytes are provisioned with yolk, resulting in the enlargement and darkening of the ovaries, and maturity is achieved when eggs are deposited in the brood chamber in the following instar (Bradley et al. 1991; Ebert 1997). These key developmental instars have previously been referred to as IM-1 (oocyte formation), IM-2 (oocyte provisioning) and IM-3 (deposition of eggs in the brood chamber) (Bradley et al. 1991; Enserink et al. 1995; Barata & Baird 1998), a system of classification adopted within this chapter. Any of these maturation ‘indicators’ can be used to model PMRNs, though it is likely that those based on

IM-1 will most accurately describe the role of age and size in initiating maturation (Davidowitz & Nijhout 2004; Wright 2007; Tobin et al. 2010), while those based on IM-3 may be useful in understanding the trade-off between growth and reproduction. PMRNs based on all three maturation indicators were investigated, to describe how effects of age and size changed over the course of the maturation process. In each analysis, developmental histories were censored; individual ages and sizes of the instars following an event for the indicator were not included, as models would then predict the probability of being mature, rather than becoming mature.

2.2.4. Statistical analyses I: Maturation rate models and their GLM approximations

A full and general explanation of how to derive and utilise maturation rate models and their GLM approximations can be found in the methods section of Harney et al. (2012), however, these statistical analyses sections provide an overview of how these approaches were applied to the maturation data of *D. magna* and *D. pulex*. Maturation rate models (Van Dooren et al. 2005) allow one to investigate the determinants of maturation and choose between different ways of obtaining the maturation status variable from instantaneous rates. Typical of such time-to-event models is that the probability that an individual matures within a certain interval (given that it has not done so before that) is equal to $1 - \exp(-S)$ where S is the total change in maturation status: the maturation rate integrated over the interval duration on the chosen operational time scale. This study focused on age or size integration and maturation rates that were dependent on age and/or size. Time-to-event data can also be modelled by means of more standard binomial generalized linear models (Lindsey & Ryan 1998; Collett 2003). In the case of the proposed maturation rate models, where rates can be integrated over age or size and where different time-dependent covariates can exist, two types of binomial GLM can be interpreted as approximations of maturation rate models.

The complementary log-log (cloglog) and logit-link binomial GLMs are able to approximate rate models by including the logarithm of the interval duration as an offset (Lindsey & Ryan 1998; Collett 2003). The cloglog GLM assumes that maturation rates change gradually with age and size, and the logit-link GLM further assumes that the product of the maturation rate and interval length is relatively small. Assuming that maturation rates increase linearly, the age and size of the interval midpoint are used; however, in cases where maturation rates are increasing non-linearly and not very gradually, GLMs which assess rates at interval start- or endpoints might fit data better.

The conclusion is that when these binomial GLMs are fitted to maturation data, the results are interpreted as representing maturation rate models. These GLMs can be compared to maturation rate models with different specific parametric shapes of the rate, as described by Van Dooren et al. (2005), or to other binomial GLMs which are not approximations of rate models. The latter may be appropriate if maturation is not occurring on the basis of a maturation status variable that increases continually. Using data containing variable interval lengths enables one to reject the possibility of a maturation process with rates.

2.2.5 Statistical analyses II: Fitting the models

Maturation rate models and GLMs were fitted separately to both the *D. magna* and *D. pulex* data sets, for all three maturation indicators (IM-1, IM-2, and IM-3). Initially, maximal models containing all explanatory variables and including pair-wise interactions between categorical explanatory variables and covariates were compared. Maturation rates were fitted with analytically integrated Weibull, Gompertz, and generalised functions (Sparling et al. 2006); and age and size were fitted as covariates (except with the generalised function, where only one covariate can be integrated). Furthermore, clone identity was included as a categorical variable. Categorical variables can influence the maturation rate through interactions with age and/or size or through effects on shape parameters. Food ration was not included in analyses, as its purpose was to generate variation in growth trajectories. Maturation rates were integrated over size or age. GLMs with either cloglog or logit link functions were also fitted to the data. Age, size or age and size were included as covariates, using either age/size interval start-, mid- or endpoints, and with values either untransformed or log-transformed, and clone was included as a categorical variable. GLMs were fitted with either an offset, i.e., log age or log size difference per interval, or not. All models discussed so far can be fitted using Maximum Likelihood (ML) methods. Likelihood ratio tests can thus be used to compare nested models and AIC or likelihood comparisons can be used to compare non-nested models.

2.2.6 Statistical analyses III: Predicted reaction norms

Traditionally, ages and sizes at which there is a 50% probability to mature within an age interval of fixed length are plotted as reaction norms (Heino et al. 2002; Beckerman et al. 2010). However, these ages and sizes then change when a different age interval is assumed, which can be problematic for maturation rate models and GLM approximations. An

alternative is to simulate growth curves and track the probability that an individual with that growth curve would have matured already. For a range of simulated growth curves it is then possible to calculate probabilities per growth curve, and the 50^{th} percentiles constituting the PMRN can be approximated or interpolated (Van Dooren et al. 2005). All statistical analyses and generation of PMRNs were carried out using R (R Development Core Team 2011). R packages *rmutil* and *numderiv* were used to create maturation rate models, and *arm* was used in plotting PMRNs.

2.3 Results

2.3.1 Comparison of different modelling approaches

Comparison of AIC values between rate models and GLMs reveals that maturation was best modelled using GLMs with logit-link functions. This was true for both species, and for all three maturation indicators (Table 2.1). GLMs with offsets yielded lower AIC values when considering IM-1 in *D. pulex*, IM-2 in *D. magna* and IM-3 in both species (Table 2.1). In the cases of, IM-1 (*D. pulex*) and IM-2 (*D. magna*), size offsets were preferred to age offsets, i.e. increases in size were more important than increases in age in determining changes in maturation status; in these models size is acting as an operational timescale. Conversely, when modelling the data with IM-3 (both species), age offsets were preferred, i.e. increases in age were more important in determining maturation status changes. Thus in four out of six cases, GLMs with offsets were preferred, suggesting that in *Daphnia*, maturation is likely to be a process with a rate, especially later during development. However, in two cases GLMs without offsets provided a better fit to the data. Inspection of the data revealed that the range of age and size interval variation was smallest for *D. magna* IM-1, which could explain why the model without an offset was preferred there. Also, the number of intervals increased between models when later maturation indicators were used, which is expected to increase the power to discriminate alternative models, while the proportion of observations where maturation probabilities between 0.1 and 0.9 were predicted decreased, which tends to decrease discrimination power. Not selecting a model with an offset might therefore be due to a lack of statistical power. However, for both species, the range of sizes for which models with an offset predicted intermediate maturation probabilities seemed narrowest for IM-1, indicating that earlier on in development, maturation may be more analogous to a probabilistic switch. Appendix Tables A2.1 and A2.2 show AIC values of models with different combinations of offsets and covariates (GLMs) and different combinations of timescales and rate effects (maturation rate models).

Table 2.1. A comparison of rate models and GLMs with and without offsets for both species of *Daphnia*, across all three maturation indicators. Lowest AIC values for each species and indicator combination are highlighted in boldface type and the number of parameters in the model (No. para.) is provided. GLMs always have lower AIC values than rate models, although the presence of an offset did not always reduce AICs in models using maturation indicators IM-1 and IM-2. In both species similar models are preferred when considering a given maturation indicator.

Model Type	GLM offset	Description	AIC	No. para.
D. pulex IM-1				
rate	-	Generalised function, size integration, size rate effects	438.95	11
GLM	Size	resp ~ offset(ln(size)) + (clone) * (ln(age ends) + ln(size ends))	**427.63**	15
GLM	No	resp ~ (clone) * (ln(age ends) + ln(size ends))	434.85	15
D. pulex IM-2				
rate	-	Weibull function, size integration, age and size rate effects	352.37	15
GLM	Size	resp ~ offset(ln(size)) + (clone) *((age ends) + (size ends))	350.23	15
GLM	No	resp ~ (clone) * ((age ends) + (size ends))	**346.34**	15
D. pulex IM-3				
rate	-	Weibull function, age integration, age and size rate effects	331.04	15
GLM	Age	resp ~ offset(ln(age)) + (clone) * (ln(age mids) + ln(size mids))	**317.90**	15
GLM	No	resp ~ (clone) * (ln(age mids) + ln(size mids))	327.82	15
D. magna IM-1				
rate	-	Generalised function, size integration, size rate effects	311.92	11
GLM	Size	resp ~ offset(ln(size)) + (clone) * (ln(age ends) + ln(size ends))	288.61	15
GLM	No	resp ~ (clone) * (ln(age ends) + ln(size ends))	**284.58**	15
D. magna IM-2				
rate	-	Weibull function, size integration, age and size rate effects	261.72	15
GLM	Size	resp ~ offset(ln(size)) + (clone) * (ln(age mids) + ln(size mids))	**246.77**	15
GLM	No	resp ~ (clone) * ((age ends) + (size ends))	247.90	15
D. magna IM-3				
rate	-	Weibull function, age integration, age and size rate effects	236.26	15
GLM	Age	resp ~ offset(ln(age)) + (clone) * (ln(age mids) + ln(size mids))	**204.33**	15
GLM	No	resp ~ (clone) * (ln(age mids) + ln(size mids))	216.08	15

2.3.2 Choice of maturation determinants

In both species and for all maturation indicators the best fitting GLMs included both age and size as covariates. Model simplification was carried out and in all cases the minimum models retained the *clone:age* interaction but not the *clone:size* interaction. For both species, interval endpoints were preferred when using IM-1 as the maturation indicator, whilst interval midpoints were preferred with IM-3. For IM-2, maturation was modelled using interval endpoints for *D. pulex* and interval midpoints for *D. magna*. Interval start points were never preferred. Models with log-transformed age and size were always preferred to those with untransformed values except in the case of *D. pulex*, IM-2.

Aside from these minor differences, the best fitting models for a given maturation indicator were similar in both species (Table 2.1). However, plotting PMRNs based on predicted values from these GLMs reveals within- and between-species differences. Clonal variation in age effects (the *clone:age* interaction) are present in the PMRNs for IM-1 in both *D. magna* (Fig. 2.1) and *D. pulex* (Fig. 2.2). Certain clones initiate maturation at smaller sizes at younger versus older ages, resulting in positively sloped PMRNs (e.g. H01, Fig. 2.1A; Carlos, Fig. 2.2A), whilst others do the opposite, resulting in negatively sloped PMRNs (e.g. B7, Fig. 2.1E; Cyril, Fig. 2.2E). Some clones appear to have maturation thresholds that are at a fixed size (e.g. B5, Fig. 2.1C; Chardonnay, Fig. 2.2C). There is greater variation in age effects in *D. magna* (Fig. 2.1) than *D. pulex* (Fig. 2.2), and consequently the relationship between age and size upon reaching maturity (IM-3, represented by coloured points in Figures 2.1 & 2.2) appears to be more variable in *D. magna*.

2.4 Discussion

Maturation is increasingly recognised as an important heritable developmental trait underpinning the plastic response of a genotype to its environment (Berner & Blanckenhorn 2007; Nijhout et al. 2010). However, our understanding of how maturation phenotypes evolve is still hindered by the debate regarding the best way to quantify and compare maturation reaction norms for age and size upon reaching maturity (Heino et al. 2002; Van Dooren et al. 2005; Dieckmann & Heino 2007; Heino & Dieckmann 2008; Kuparinen et al. 2008; Uusi-Heikkilä et al. 2011). In *Daphnia*, GLM approximations of rate models often provide the best fit to maturation data. A comparison of models containing different combinations of age and size suggests that size is the most important maturation determinant, but that age also plays a role in the maturation process. This was true for some

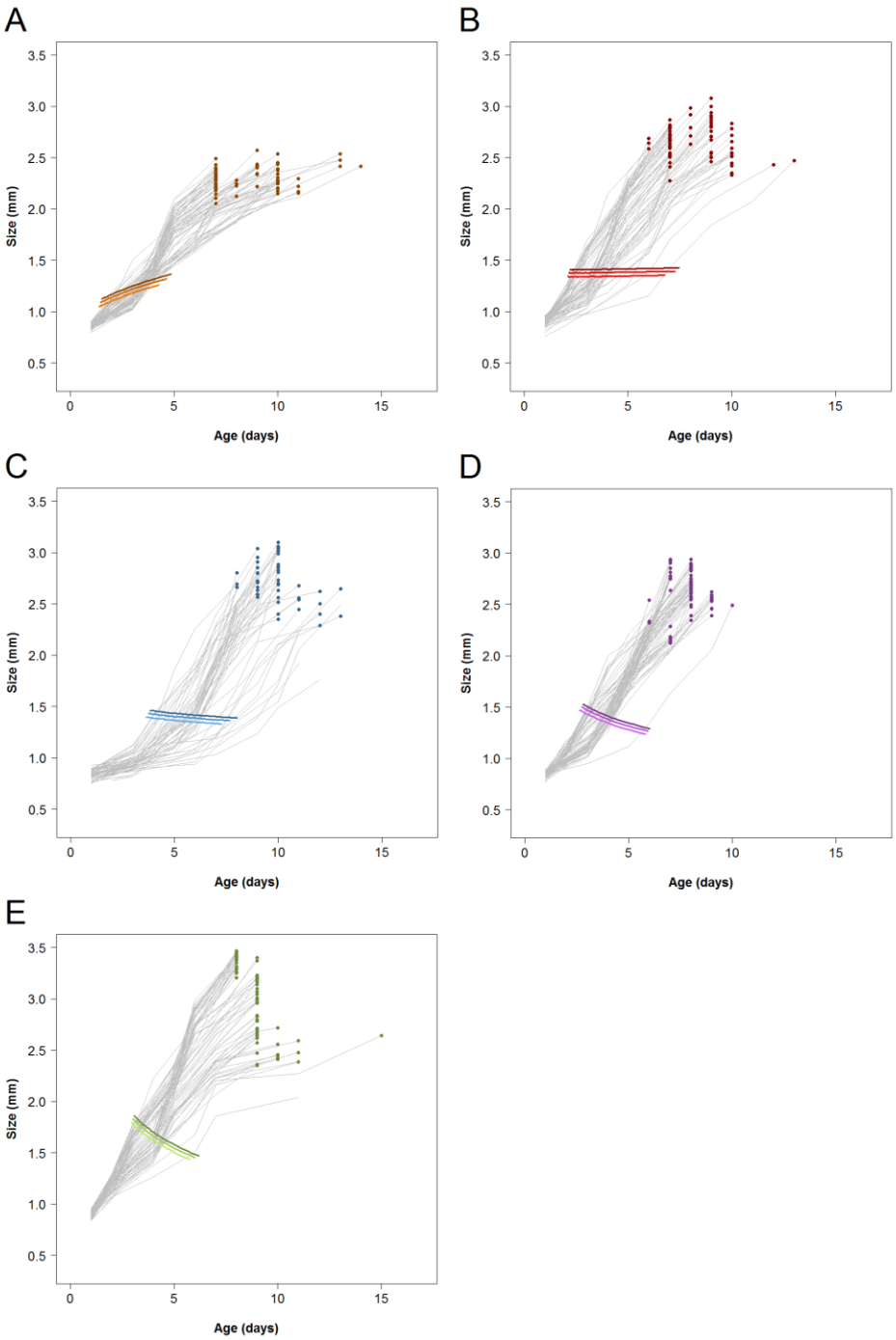

Figure 2.1. PMRNs and their consequences for age and size at maturity in 5 clones of *Daphnia magna*: (A) H01, (B) DKN1-3, (C), B5, (D) Ness1, and (E) B7. Light grey lines are individual growth trajectories; coloured circles are age and size upon reaching maturity (IM-3) and increasingly dark coloured lines represent 25, 50 and 75% probabilistic maturation reaction norms for IM-1 based on the best fitting GLM (no offset, age and size covariates, clone:age interaction). PMRNs vary between clones in terms of both threshold size and the importance of age in determining threshold shape. Variation in PMRNs has consequences for age and size at maturity. Clones with negatively sloped PMRNs such as B7 (E) reach maturity at a broader range of sizes and/or narrower range of ages, compared to clones with positively sloped PMRNs, such as H01 (A).

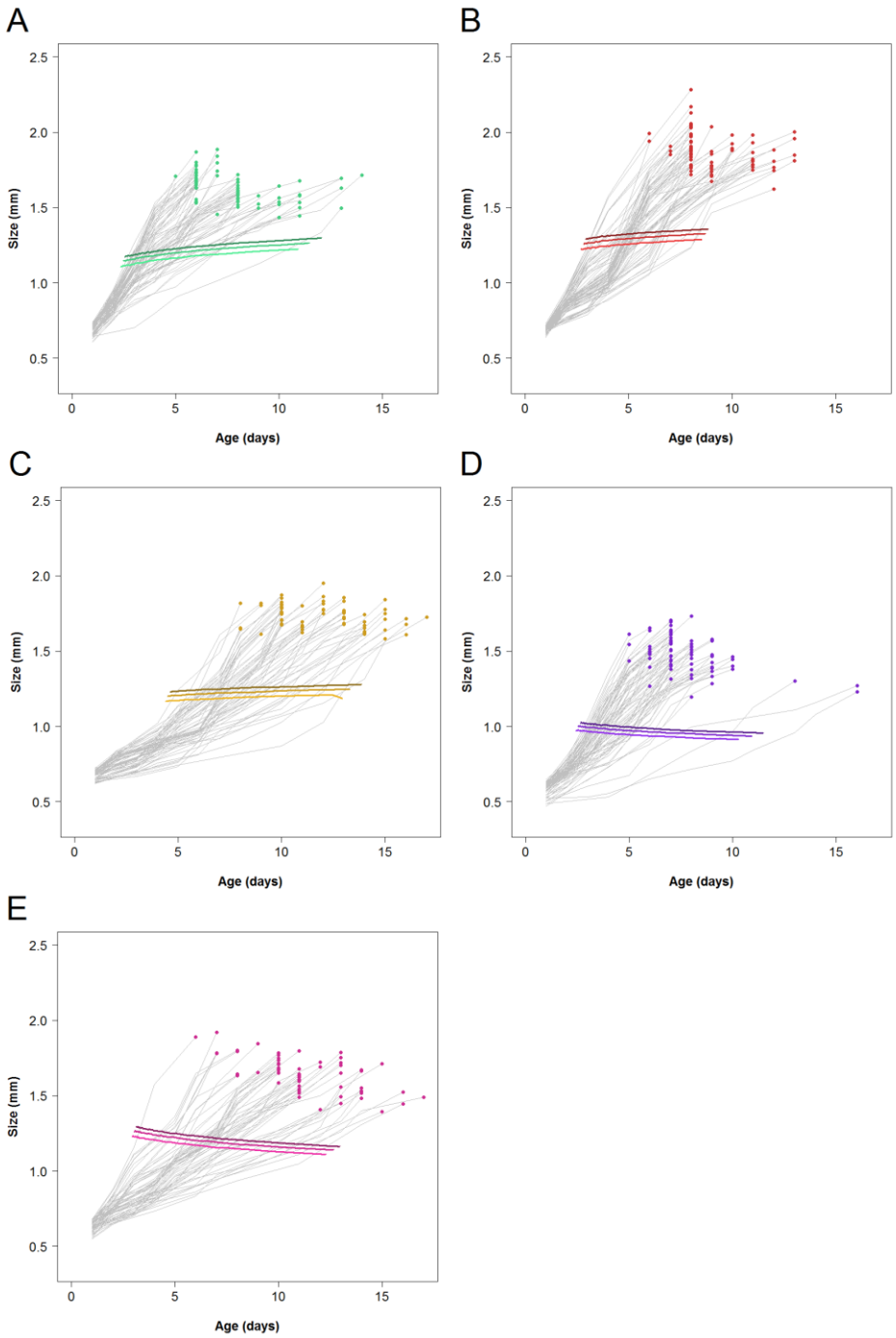

Figure 2.2. PMRNs and their consequences for age and size at maturity in 5 clones of *Daphnia pulex*: (A) Carlos, (B) Boris, (C) Chardonnay, (D) Bierbeek, and (E) Cyril. Light grey lines are individual growth trajectories; coloured circles are age and size upon reaching maturity (IM-3) and increasingly dark coloured lines represent 25, 50 and 75% probabilistic maturation reaction norms for IM-1 based on the best fitting GLM approximation of a rate model (size offset, age and size covariates, clone:age interaction). As with *D. magna*, PMRNs vary between clones in both threshold size and the importance of age. However these differences and subsequently clonal variation in age and size at primiparity are less pronounced in *D. pulex* than in *D. magna*.

clones more than others, demonstrating variation in the position and nature of PMRNs at the level of the genotype.

2.4.1 Statistically modelling maturation

Logit-link GLMs fitted the data better than rate models, suggesting that the additional assumptions involved in these models versus rate models and cloglog GLMs are generally valid and that the nonlinear functional dependence on age and size implicit in the logit-link fits the data better than the functional forms implied by the cloglog link or rate models. This could be because the maturation rate follows a step-like function indicative of a strong size threshold and relatively deterministic maturation, given the maturation determinants that were selected. Since the shape of maturation rate functions have yet to be examined in other systems, it is difficult to comment on the generality of these findings. If maturation rate functions are not step-like in other systems, maturation may be better modelled by the functions contained within rate models, and model comparison will remain an important step in quantifying and comparing maturation phenotypes.

In general, most GLM models were improved by the inclusion of an offset term, indicating that maturation is generally more analogous to a rate than a switch. However some models based on earlier maturation indicators were not improved by the inclusion of an offset. This may be due to a lack of statistical power, or because the time window for maturation is restricted to a fixed interval length, but could also be indicative of stage-specific switches rather than a continually changing maturation status. However, the importance of correcting for interval bias is highlighted by the fact that analyses of maturation using the latest possible indicator of maturation (IM-3), that most closely resembles the sorts of indicators used in other studies (e.g. Plaistow et al. 2004; Kuparinen et al. 2008; Uusi-Heikkilä et al. 2011), were improved by including age interval offsets. Even when the offset does not improve the fit of the model, the corresponding models with an offset should be inspected and presented to assess the strength of evidence for a switch-like process. Furthermore, lacking a discussion of offset effects, the majority of studies that utilise GLMs to predict PMRNs (Grift et al. 2003; Engelhard & Heino 2004; Olsen et al. 2004; Mollet et al. 2007; Beckerman et al. 2010) cannot investigate potentially insightful alternative time scales.

2.4.2 Clonal variation in maturation determinants

Although evidence of between- (Piché et al. 2008) and within- (Skilbrei & Heino 2011) population variation in PMRNs is emerging, few studies are able to compare maturation thresholds of different genotypes. Using parthenogenetic organisms such as *Daphnia* allows us to demonstrate within- and between-population genotypic variation in the position and nature of maturation thresholds and may improve our understanding of how maturation decisions evolve and influence the evolution of age and size at the onset of maturity. Clonal variation in the position of the threshold has previously been shown to differ for two clones from the same population (Ebert 1994). However, unlike previous studies (Ebert 1992, 1994), this study found that maturation thresholds in *Daphnia* varied across a range of growth trajectories and that in some clones the decision to mature depended on both size and age. The strength of this effect was itself variable between different clones and was less apparent in *D. pulex* than *D. magna* (Figures 2.1 & 2.2), although it is unclear whether differences between the two species are due to the narrow geographic origin of the *D. pulex* clones used in this study, a consequence of constrained threshold feeding and incipient feeding concentrations in smaller species (Porter et al. 1982; Gliwicz 1990; Dudycha & Lynch 2005), or reflective of different evolutionary responses to predation (Brooks & Dodson 1965; Lynch 1977; Hart & Bychek 2010).

The finding that age can be an important maturation determinant in *Daphnia* demonstrates that the fixed size thresholds previously assumed in studies of maturation thresholds in *Daphnia magna* (Ebert 1992, 1994, 1997) are an over-simplification. Under a fixed size threshold model, maturation thresholds (and subsequent sizes and ages upon reaching maturity) can only evolve through upward or downward shifts in threshold size. The extreme L-shaped reaction norm predicted by Day and Rowe (2002), and previously observed in some studies (Plaistow et al. 2004), is assumed to be the result of growth plasticity, such that fast growing individuals overshoot the threshold more than slow growing individuals. The PMRN approach adopted in this study explicitly corrects for such growth bias, yet there is still curvature in some PMRNs at the earliest stages of maturation (see Figures 2.1 & 2.2). This suggests that negatively-sloped reaction norms in age and size at the completion of maturation may be generated by the shape of the maturation threshold itself. Organisms that are able to include age (or a correlate of age) as a maturation determinant may be able to reduce the size at which they mature in order to maintain their development rate, or alternatively to maintain or even increase size at maturation at the expense of increasing their development time (Morita & Fukuwaka 2006). Variation in the extremes of these two strategies can be seen in the *D. magna* data by comparing the clone B7 (Fig. 2.1E; green points), which varies more in body size than development time upon reach maturity, with

clone H01 (Fig. 2.1A; orange points), which varies more in development time than body size. Such patterns have previously been predicted by life-history theory (Wilbur & Collins 1973; Stearns & Koella 1986) but the proximate mechanisms underpinning these responses are generally not understood.

2.4.3 Choice of maturation determinants and maturation indicators

If age can have an effect on the decision to mature, it raises the question: 'what other factors can influence this decision?' It is well understood that maturation itself involves the co-ordination of a number of endocrinological and neurophysiological processes that control changing patterns of resource allocation to growth, maintenance and reproductive function (Bernardo 1993; Stern & Emlen 1999; Nijhout 2003). Indeed, the development of the PMRN approach was a response to the realisation that just measuring size and age may not be sufficient for predicting maturation decisions (Morita & Fukuwaka 2006). Having said that, it appears that in *Daphnia*, provided there is good data on the age and size of individuals throughout their life, age and size alone can be used to accurately predict PMRNs. This is reflected in the fact that the 25, 50, and 75% probability contours are always very closely associated with each other (Fig. 2.1; Fig. 2.2) and suggests that *Daphnia* may be a useful and relatively simple model in which to investigate the evolutionary ecology of maturation thresholds. This is especially true since the transparent cuticle of daphniids allows us to observe the progress of the maturation process (IM-1 to IM-3) in a manner often not possible in other systems.

PMRNs based on early maturation indicators should provide the best description of which factors are involved in the maturation decision, yet in many studies maturation is only scored at the end of the maturation process, marked by the appearance of secondary sexual characters or offspring. This is a problem when there is a lag between the initiation of the maturation process and its conclusion (Wright 2007), because the allocation of resources to reproduction can alter the growth curve (Day & Taylor 1997), and because further maturation might be a simple matter of time (Davidowitz & Nijhout 2004), blurring the effects of maturation determinants that led to the decision to mature. Thus if individuals are scored as immature after they have initiated maturation but before they display any evidence of maturation (i.e. at some point during the maturation process), one could expect stronger age effects since later size increases are less relevant for maturation. Such an effect can be observed in this study, where PMRNs estimated using IM-3 are more L-shaped than those estimated using IM-1 (Fig. 2.3; Appendix Figs. A2.1 and A2.2), and feature age integration (Table 2.1; Appendix Tables A2.1 and A2.2). This finding highlights the importance of

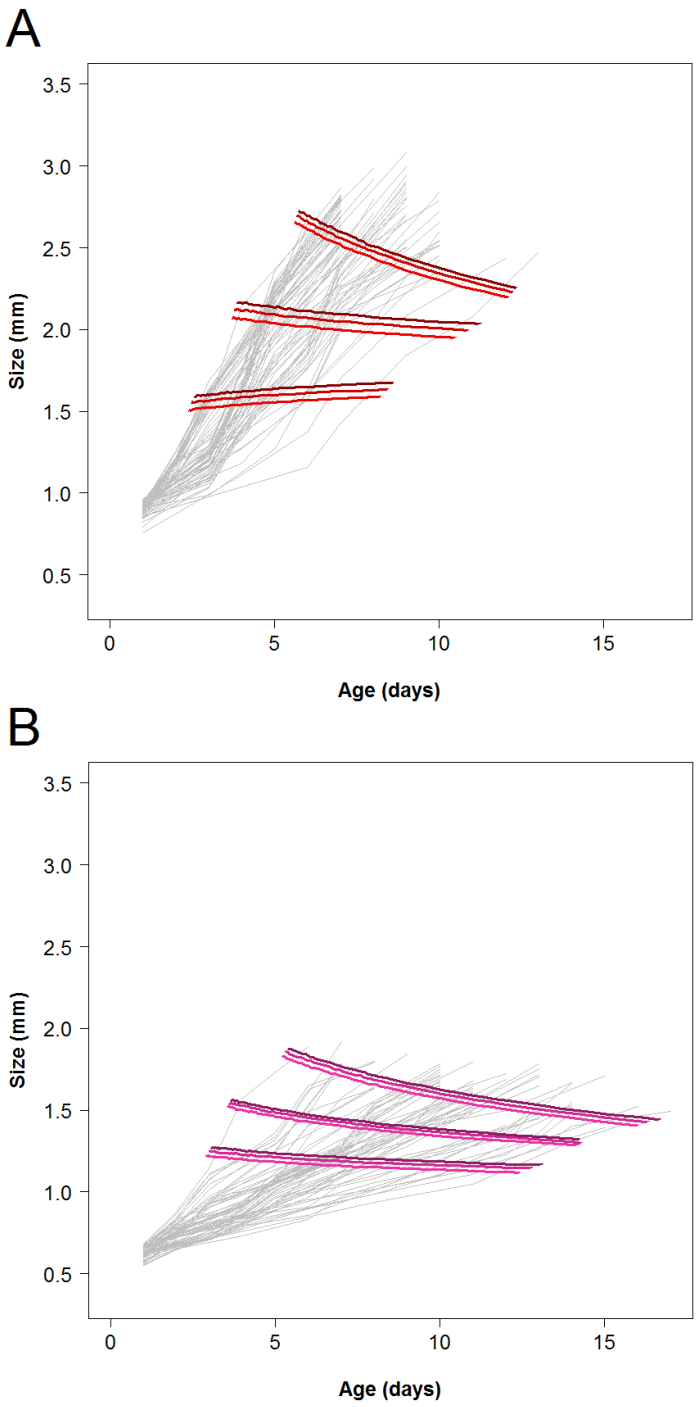

Figure 2.3. PMRNs for 3 different maturation indicators IM-1, IM-2 and IM-3 in 2 species of *Daphnia*. (A) *D. magna* clone DKN1-3 and (B) *D. pulex* clone Cyril. PMRNs for all three maturation indicators have been generated using the model: *response ~ offset(log(size)) + (clone) * (log(age ends) + log(size ends)).* Differences between PMRNs highlight the effects of growth during maturation. In both species, later maturation indicators have PMRNs with more pronounced L-shapes. Prior to the maturation threshold at IM-1 growth differences do not influence the shape of the PMRN. After reaching IM-1, however, individuals with high growth rates achieve IM-2 and IM-3 at larger sizes, and over a wider range of sizes but narrower range of ages than individuals with low growth rates. This pattern is true for all clones.

using traits at the beginning of maturation rather than the end (Tobin et al. 2010). Approaches such as measuring changes in hormone titres or patterns of gene expression may allow more accurate estimation of when the maturation process begins.

2.4.4 Developmental plasticity in maturation

The proximate mechanisms operating in *Daphnia* must be able to explain the strong effect of size on the decision to mature (Ebert 1994), the finding that in some clones older individuals mature at smaller sizes, and the fact that individuals can sometimes exceed the typical 'threshold body size' without maturing when resources are particularly scarce (Enserink et al. 1995). One possible mechanism that may explain these findings is a minimum 'state' or 'condition' below which maturation is unviable. Assuming the level of stored energy reserves individuals can possess is constrained by their body size, this would explain the strong influence of body size on maturation decisions in *Daphnia* (Ebert 1992, 1994, 1997) and is in accordance with the more switch-like nature of the maturation process at IM-1. The apparent age-dependence observed in the PMRNs of some clones could arise if individuals growing in resource-poor environments increase the proportion of resources allocated to storage at the expense of growth (Nogueira et al. 2004). In this way, slower growing individuals could potentially exceed a threshold state at a smaller body size. Plasticity in resource allocation strategy could explain why individuals that are big enough to mature but that experience extremely resource poor environments are constrained from maturing (Enserink et al. 1995).

Ultimately, even subtle differences in how maturation decisions are made draw attention to the fact that the proximate mechanisms leading to developmental plasticity in maturation schedules are often poorly understood (Berner & Blanckenhorn 2007). Whilst factors underpinning the decision to mature will inevitably vary across taxa (Nijhout 2008), in-depth investigation in a few key species, like the spadefoot toad, *Spea hammondii* (Denver 1997; Denver et al. 1998; Boorse & Denver 2004) and the tobacco hornworm moth *Manduca sexta* (Nijhout 2003; Davidowitz & Nijhout 2004; Nijhout et al. 2010) is helping us to understand how maturation phenotypes are assembled during the course of development, from initial maturation decisions through to the completion of the maturation process, and how different environmental variables influence this process. Deconstructing the maturation phenotype in this way will be critical to understanding which parts of the process can evolve and which parts are simply the product of environmental fluctuation and constraints.

2.5 Conclusion

In order to understand how the important life history traits of age and size at the onset of maturity evolve, one must further investigate the underlying ontogenetic processes that produce these phenotypes. Statistically modelling the maturation process using PMRNs can help elucidate the importance of age, size or other maturation determinants in maturation decisions. This study compared the utility of three different approaches to PMRNs: maturation rate models, GLM approximations of maturation rate models and GLMs. In *Daphnia*, GLM approximations of maturation rate models often provided a better fit to the data and suggest that maturation, particularly later on during development, is best modelled as a process with a rate. Because *Daphnia* are clonal, PMRNs also reveal how maturation decisions differ between genotypes. These results suggest that in *Daphnia*, maturation thresholds are variable across growth environments and between genotypes, and therefore may play an important role in the evolution of age and size at the onset of maturity.

Chapter 3

Parental effects on maturation in *Daphnia magna*: an ontogenetic perspective

3.1 Introduction

The maturation threshold is an important developmental trait that helps to shape age and size at maturity. In the previous chapter, maturation thresholds were shown to be plastic in response to variation in resources, and to vary between different genotypes (Chapter 2; Harney et al. 2012). In this chapter another potential source of variation in maturation thresholds is considered: parental effects. The non-genetic inheritance of the parental phenotype or environment can adjust the expression of numerous traits in offspring (Mousseau & Fox 1998b; Marshall & Uller 2007), including the age and size at which they mature (Marshall et al. 2003; Beckerman et al. 2006; Plaistow et al. 2006; Hafer et al. 2011). How parents induce transgenerational phenotypic plasticity in age and size at maturity is often unclear, because their appearance during development is ignored; however, there is increasing evidence that developmental traits are altered by parental effects (e.g. Allen et al. 2008; Schwander et al. 2008; Harvey & Orbidans 2011; Wolf et al. 2011). In this chapter the expression of parental effects throughout ontogeny is investigated, from initial size, via growth and maturation, into adulthood. These effects are quantified in multiple environments and genotypes to explore their evolutionary potential.

3.1.1 Parental effects result in phenotypic variation

Parental effects occur when the phenotype or environment of a parent influences the phenotype of its offspring (Mousseau & Fox 1998b). The resultant transgenerational phenotypic plasticity can have important evolutionary consequences, facilitating selection or maintaining genetic variance for numerous traits (Kirkpatrick & Lande 1989; Mousseau & Fox 1998a; Pal 1998). Adaptive 'anticipatory' parental effects may be selected for when parents can accurately predict future environments and alter the offspring's phenotype to increase parental and offspring fitness (Jablonka et al. 1995; Marshall & Uller 2007; Badyaev & Uller 2009). Parental effects on egg and birth size are perhaps the most well documented examples of transgenerational phenotypic plasticity (Chambers & Leggett 1996;

Fox & Mousseau 1996; Bernardo 1996b; Mousseau & Fox 1998b; Räsänen et al. 2005). Parental effects have also been demonstrated in a wide variety of adult traits including morphology (Kaplan & Phillips 2006); resistance to predators (Agrawal et al. 1999; Agrawal 2001), parasites (Little et al. 2003), heavy metal pollution (Bossuyt & Janssen 2003; Bossuyt et al. 2005) and pesticides (Brausch & Smith 2009); dispersal propensity (Mousseau & Dingle 1991; Duckworth 2009) and foraging behaviour (Segers & Taborsky 2011); mode of reproduction (LaMontagne & McCauley 2001); local competition (Plaistow et al. 2007) and demography (Galloway & Etterson 2007; Donohue 2009); and age and size at maturity (Marshall et al. 2003; Beckerman et al. 2006; Plaistow et al. 2006; Hafer et al. 2011). Subsequently parental effects are often identified as an outcome of natural selection, influencing individual traits and providing a link between an environmental signal experienced by the parent and a discrete or static phenotypic response in the offspring (Rossiter 1996; Wolf et al. 1998; Mousseau & Fox 1998b).

3.1.2 A dynamic role for parental effects

In reality, however, parental effects are part of a dynamic developmental process integrating transferred parental resources into offspring phenotypes throughout ontogeny (Marshall & Uller 2007; Badyaev & Uller 2009), and have context-dependent outcomes that are influenced or modified by the environment they are expressed in, i.e. the offspring environment (Rossiter 1998; Plaistow et al. 2006; Marshall & Uller 2007; Räsänen & Kruuk 2007). Subsequently the expression of parental effects often extends beyond simple differences in egg or birth size (Laugen et al. 2002; Harvey & Orbidans 2011), may involve conflict between the parent and offspring (Livnat et al. 2005; Uller 2008), and can influence multiple developmental processes in different ways (Bonduriansky & Head 2007; Marshall & Uller 2007; Cahan et al. 2011), leading to phenotypic outcomes that may not be anticipated given a static view of parental effects.

A clearer understanding of how parental effects generate functional phenotypic forms is achieved by investigating the ontogenetic origins of parental effects (Dufty Jr et al. 2002; Groothuis & Schwabl 2008). Evidence of parental effects on growth (Alekseev & Lampert 2004; Monteith et al. 2009; Frost et al. 2010; Wolf et al. 2011; Salinas & Munch 2012), dispersal potential of larvae (Allen et al. 2008; Burgess & Marshall 2011), diapause decisions (McWatters & Saunders 1998; Scharf et al. 2010) and developmental switches (Schwander et al. 2008; Michimae et al. 2009; Harvey & Orbidans 2011) support the notion that parental effects influence multiple aspects of development (Kaplan & Phillips 2006;

Marshall & Uller 2007; Burgess & Marshall 2011). One developmental change experienced by virtually all multicellular organisms is maturation. The decision to mature, or maturation threshold (Day & Rowe 2002; Harney et al. 2012), is a promising candidate for explaining how parents produce transgenerational plasticity in age and size at maturity (e.g. Marshall et al. 2003; Beckerman et al. 2006), but parental effects on maturation thresholds have yet to be tested.

3.1.3 Evolutionary consequences of parental effects

As well as generating diverse phenotypes, the expression of parental effects during development may also have important evolutionary consequences (True et al. 2004; Badyaev 2005, 2008, 2011). Recent studies have found that parental effects can be highly variable between genotypes (Mitchell & Read 2005; Stjernman & Little 2011) and populations (Tschirren et al. 2009; Scharf et al. 2010), but the extent to which parental effects on maturation are variable between genotypes is unknown. Given that parental effects on age and size at maturity have been shown to influence population dynamics at ecological timescales (Plaistow et al. 2006; Benton et al. 2008; Hafer et al. 2011), it is possible that transgenerational plasticity in maturation is also important for understanding the dynamics of parental effects over evolutionary timescales. Indeed there is a growing consensus that natural selection, developmental variation and non-genetic inheritance, including parental effects, must be incorporated into a broader evolutionary framework to better explain the relationship between genotype and phenotype (Badyaev & Uller 2009; Bonduriansky & Day 2009; Badyaev 2011; Day & Bonduriansky 2011). Taking an ontogenetic approach that connects differences in initial size, through altered patterns of growth and maturation, to resultant adult phenotypes is necessary to understand the appearance of diverse and persistent parental effects (Marshall et al. 2003; Sakwińska 2004; Wolf et al. 2011) and their fitness consequences (Marshall & Uller 2007; Badyaev & Uller 2009). Furthermore, investigation of genotypic variation in these effects will improve our understanding of the relationships between non-genetic inheritance, developmental plasticity, phenotypic variation and selection (Galloway et al. 2009; Badyaev 2011).

3.1.4 Investigation of parental effects throughout ontogeny in Daphnia magna

In this chapter the role of context-dependent parental effects on ontogeny in three different genotypes of *Daphnia magna* was investigated. Previous studies have shown that parental

effects in *Daphnia* influence resting egg production (Alekseev & Lampert 2001; LaMontagne & McCauley 2001), size at birth (Tessier & Consolatti 1991; Glazier 1992), growth and age at maturity (Alekseev & Lampert 2004), survival (Lynch & Ennis 1983; Gliwicz & Guisande 1992), heavy metal pollution resistance (Bossuyt & Janssen 2003; Bossuyt et al. 2005) and parasite resistance (Mitchell & Read 2005; Frost et al. 2010; Stjernman & Little 2011). However, the persistence of parental effects from birth to adulthood is rarely considered (but see Alekseev & Lampert 2004; Sakwińska 2004; Alekseev & Lajus 2009), and the extent to which parental effects vary between clones from different populations is unknown. Furthermore, parental effects on the maturation threshold have never before been investigated. Investigating parental effects in different ontogenetic traits, including the maturation threshold, and the consequences of this developmental plasticity for adults reveals the mechanisms that parents can use to alter offspring phenotypes. In addition, comparing their expression between different offspring environments and clonal genotypes helps elucidate how parental effects evolve and influence broader evolutionary processes (Badyaev & Uller 2009; Badyaev 2011).

3.2 Materials and methods

3.2.1 Experimental design

Growth, development and life-history traits were measured in three laboratory clones of *Daphnia magna*: DKN 1-3, Ness1, and B5. Clone origins and the conditions for their general maintenance can be found in Chapter 2.2.1. A summary of the experimental design is shown in Fig. 3.1. In order to create parental effects, three individuals from each clonal genotype were assigned to one of two parental effect (PE) treatments for three generations. Animals in the high parental effect (HPE) treatment received a daily food ration of 200 cells μl^{-1} of *Chlorella vulgaris*, while those in the low parental effect (LPE) treatment received a daily food ration of 40 cells μl^{-1} *C. vulgaris*. Individuals from the third clutch of each generation used to set up the next. For the third (parental) generation, the number of individuals in each clone/PE combination was increased from three to ten, and experimental animals were obtained from the neonates produced in the third clutch of five of these parents. In the experimental generation each clone/PE combination consisted of 40 neonates. In order to investigate whether the environment experienced by the offspring altered the expression of parental effects (context-dependence), neonates were randomly assigned to one of the following four food rations: 133, 59, 26, and 12 cells μl^{-1}. These will be referred to as high (H), medium-high (MH), medium-low (ML) and low (L) respectively.

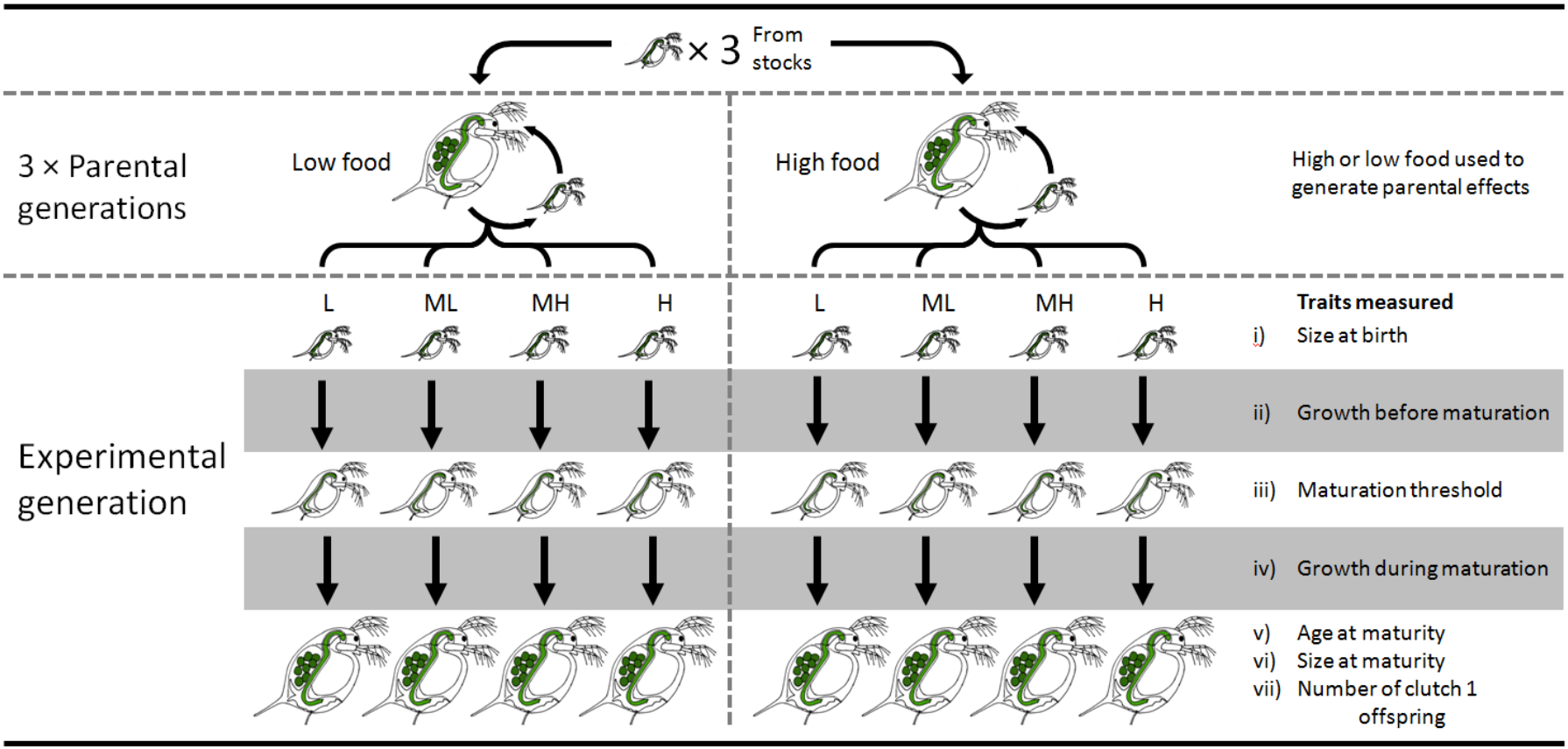

Figure. 3.1. Experimental design. For each of the three clonal genotypes, parental effects were generated by maintaining individuals at high or low food conditions for three generations. In the 4th generation, individuals from both parental conditions were randomly allocated to one of four current food environments: low (L), medium-low (ML), medium-high (MH) and high (H). Four offspring developmental traits (i-iv) and three offspring adult traits (v-vii) were recorded.

This variation in resource availability also generates a variety of growth trajectories, allowing the estimation of probabilistic maturation reaction norms (Chapter 2; Harney et al. 2012). All individuals were checked every day and photographed after moulting for all instars up to the deposition of eggs in the brood chamber, which was considered to be the point at which they matured. Body size was measured according to the methodology detailed in Chapter 2.2.2, and all offspring released in the primiparous instar were counted. In total parental effects on four developmental and three adult traits were recorded: i) neonate size; ii) growth before the initiation of maturation; iii) the maturation threshold; iv) growth during the maturation process; v) age at maturation; vi) size at maturation; and vii) number of offspring in the first clutch (Fig. 3.1). Experiments were conducted over an 8 week period. Clones Ness1 and B5 were assayed simultaneously, followed by DKN1-3, and LPE assays were set up one or two weeks after HPE assays, due to their slower development.

3.2.2 Statistical analyses

Separate statistical models were fitted to each of the seven traits. Parental effects on neonate size were investigated by fitting a linear model containing the factors PE (two levels: HPE and LPE) and clone (three levels: DKN1-3, Ness1 and B5). Growth differences were tested for using methods that corrected for absolute differences in size, using the size at the beginning of an instar (log(size-t)) to explain size at the beginning of the next instar (log(size-t+1)) (Rees et al. 2010). Separate analyses were run for growth in the instars prior to the initiation of maturation, and growth during the two instars of the maturation process (Fig. 3.1; traits ii & iv respectively). Linear mixed effect models were fitted to size-t+1 data, with parental environment, clone and current food (four levels: H, MH, ML and L) as categorical explanatory variables, log(size-t) as a covariate, and individual as a random effect to account for repeated measures. Based on the results of Chapter 2, the maturation threshold was estimated to occur two instars prior to the appearance of eggs in the brood chamber, and modelled using the methodology described in the previous chapter, the only difference being the inclusion of PE and its interactions with clone, age and size as additional terms in the models (Chapter 2; Harney et al. 2012). The age and size at which individuals completed maturation were fitted to separate linear models containing the factors PE, clone and current food. The number of Clutch-1 offspring was not normally distributed, therefore a GLM with Poisson errors was used to model parental effects on this trait as a function of the factors PE, clone and current food; furthermore, because size at maturity often strongly influences first clutch size in *D. magna* (Glazier 1992; Barata & Baird 1998), size at maturity was included as a covariate. Except in the case of PMRNs, full models

containing all interactions were fitted to the data. In all cases, backward stepwise term deletion based on AIC was then used to remove non-significant interactions and terms. *F*-values are presented for linear models and likelihood ratio values (LRT) are presented for GLMs and linear mixed effect models. All statistical analyses were carried out using R (R Development Core Team 2011). R packages *rmutil* and *numderiv* were used to create maturation rate models, *lme4* was used for mixed effects models, and *MASS*, *arm* and *Hmisc* were used to prepare plots.

3.3 Results

3.3.1 Neonate size

Results for all traits are summarised in Table 3.1. Parental effects (PE) influenced neonate size in a clone-specific fashion (PE:clone, $F = 90.514$, df = 2, $p < 0.001$). In clones DKN1-3 and Ness1, neonates produced by LPE mothers were larger than those produced by HPE mothers, and this effect was stronger in Ness1 (Fig. 3.2).

3.3.2 Juvenile growth

Parental effects on pre-threshold growth differed between clones, and were context-dependent (PE:food:clone, LRT = 28.488, df = 6, p <0.001) and influenced by size-*t* (PE:clone:log(size-*t*), LRT = 11.711, df = 2, p = 0.003). PE influenced size-corrected growth of clones DKN1-3 and B5 but not Ness1 (effects on the highest and lowest current foods are shown in Fig. 3.3; effects for all foods in Appendix Figures A3.1, A3.2 and A3.3). The effect of PE in DKN1-3 was context-dependent: HPE individuals had higher size-corrected growth than LPE individuals in high current food, but lower size-corrected growth than LPE individuals in low current food. In clone B5 the effect of PE was not context-dependent: for all current food levels, HPE individuals initially had higher size-corrected growth, but by the time they reached the maturation threshold this difference had disappeared.

3.3.3 Maturation threshold

The maturation threshold was responsive to parental effects. The model with the lowest AIC was a GLM that utilised a logit-link function and contained age and size as covariates

Table 3.1. Summary of results. Parental effects (PE) influenced all developmental and adult traits investigated through their interactions with clone, current environment (food) and size. Traits were analysed using a number of different class of linear model, including general linear model, generalised linear model and linear mixed effects model. Backwards stepwise term deletion was carried out to remove non-significant terms, and Akaike Information criteria (AIC) are provided to show the effect of term removal. AIC of models retaining these terms are in 'none' rows.

Factors influencing trait	df	AIC	Test statistic	*p*
Developmental traits				
Size at birth			*F*	
PE:clone	2	-1469.70	90.514	< 0.0001
none		-1599.70		
Growth before maturation			LRT	
PE:food:clone	6	-2771.80	28.488	< 0.0001
PE:clone:size-*t*	2	-2764.10	11.711	0.0028
none		-2755.30		
Maturation threshold			LRT	
PE: clone	2	156.14	20.873	< 0.0001
clone:ln (age)	2	168.04	32.782	< 0.0001
PE:ln (size)	1	157.87	20.608	< 0.0001
none		139.26		
Growth during maturation			LRT	
PE:food:clone:size-*t*	6	-2013.50	21.232	0.0017
none		-2004.30*		
Adult traits				
Age at maturity			*F*	
PE:food:clone	6	-1010.60	17.599	< 0.0001
none		-1093.10		
Size at maturity			*F*	
PE:food:clone	6	-930.90	11.765	< 0.0001
none		-986.50		
Offspring in first clutch			LRT	
food	3	828.53	57.363	< 0.0001
Size at maturity	1	830.70	55.534	< 0.0001
PE:clone	2	780.15	6.981	0.0305
none		777.17		

* AIC of full model for growth during maturation is higher, however, the likelihood ratio test suggested retaining the 4-way interaction.

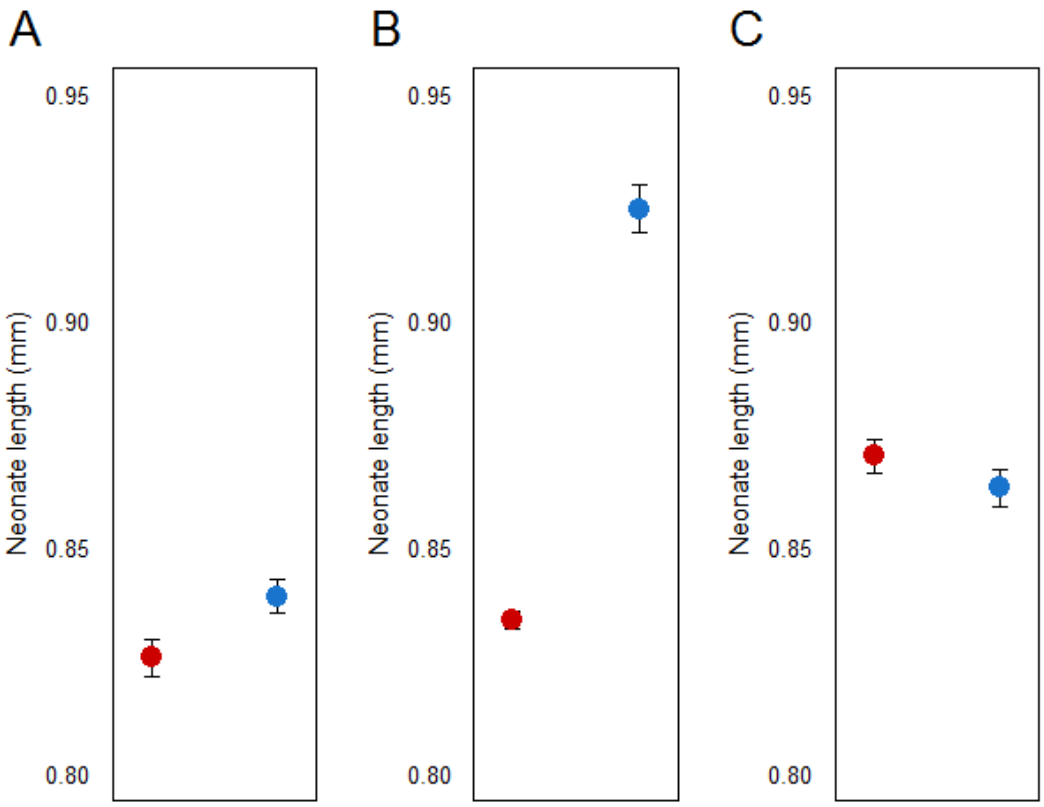

Figure 3.2. Parental effects on mean (± standard error) neonate size: A) DKN1-3; B) Ness1; and C) B5. Red circles are neonates from HPE mothers, blue circles are neonates from LPE mothers.

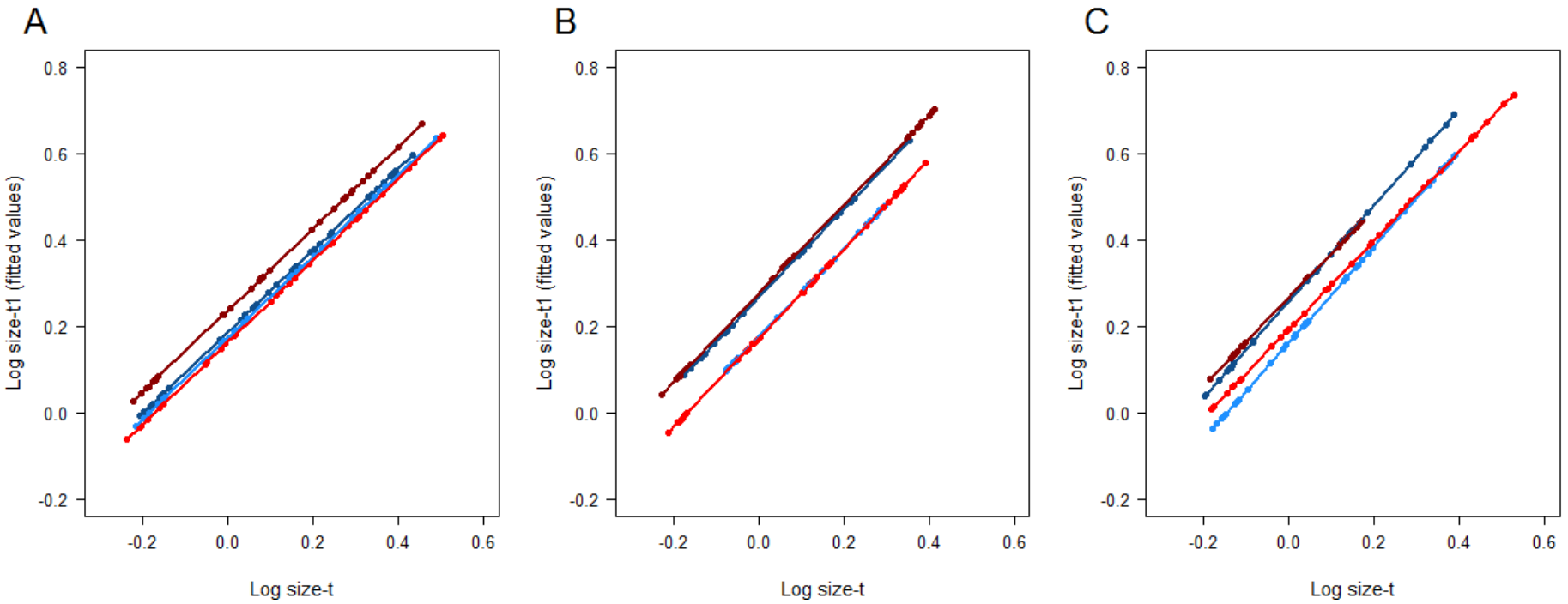

Figure 3.3. Parental effects on size-corrected growth before the maturation threshold: A) DKN1-3; B) Ness1; and C) B5. Logged values of size-*t* are plotted against fitted values of logged size-*t*+1, with model fits overlaid to aid visualisation. For the purposes of clarity just results for the highest (H) and lowest (L) current food are included, however the effects of all treatments can be seen in Appendix figures A3.1, A3. 2 and A3.3. Dark red lines represent HPE-H individuals, pale red lines HPE-L, dark blue lines LPE-H and pale blue lines LPE-L.

(modelled as logged interval ends), clone and PE as factors, and an age offset (Table 3.2). Model simplification resulted in a minimal model that contained all main effects (clone, PE, age and size) and the interactions clone:PE, clone:age and size:PE (clone:PE, LRT = 20.873, df = 2, $p < 0.001$; clone:age, LRT = 32.782, df = 2, $p < 0.001$; size:PE, LRT = 20.608, df = 1, $p < 0.001$). Figure 3.4 shows parental effects on the maturation thresholds of the 3 clonal genotypes. Their influence on the maturation threshold of clone DKN1-3 appears to be small, potentially as a result of the narrow range of growth trajectories exhibited by LPE individuals (Fig. 3.4A). Parental effects influenced maturation decisions in clones Ness1 and B5 in more obvious ways. Generally, LPE individuals initiated maturation at smaller sizes than HPE individuals, and furthermore this threshold size was less influenced by age (Fig. 3.4B and 3.4C).

3.3.4 Growth during maturation

In the interval between the maturation threshold and reaching maturity, parental effects continued to influence size-corrected growth. The four-way interaction between PE, clone, food and size-*t* was retained, (PE:clone:food:log (size-*t*), LRT = 21.232, df = 6, $p = 0.002$), suggesting that the effects of size on growth varied according to parental effects, current environment and the clonal genotype. In clone DKN1-3 the interaction between PE and size-*t* suggests that whether LPE individuals displayed higher size-corrected growth than HPE individuals depended on their current size, as well as their current environment (effects for highest and lowest food in Fig. 3.5A, effects for all foods in Appendix Figure A3.4). On the other hand, in clones Ness1 and B5, parental effects had a more consistent context-dependent effect on size-corrected growth: LPE individuals had higher size-corrected growth than HPE individuals at the highest current food level, but lower size-corrected growth at the two lowest current food levels (Fig. 3.5B and 3.5C; Appendix Figures A3.5 and A3.6).

3.3.5 Adult traits

Age at maturity

Parental effects influenced age at maturity in a context-dependent manner that varied between clones (PE:clone:food, $F = 22.453$, df = 6, $p < 0.001$). In clone DKN1-3 this effect was highly context dependent: in the two highest current food environments, HPE individuals reached maturity earlier than LPE individuals, while in the two lowest current food environments LPE individuals reached maturity earlier (Fig. 3.6A). In clone Ness1

Table 3.2. A comparison of GLMs with and without offsets and maturation rate models for the maturation threshold in *Daphnia magna*, including both clone, PE and their interaction as factors. GLMs are grouped by offset (none, size, age) and the best fitting (lowest AIC) model with age only, size only and age and size covariates is reported. Maturation rate models with all combinations of age and size integration and age and size rate effects are reported for the Weibull function, which provided the best fit to the data. AIC and the number of parameters in the model (No. para.) are also provided. The best fitting model is highlighted in boldface type.

Model Type	GLM offset	Description	Link function	AIC	No. Para.
GLM	No	resp ~ (clone * PE) * (ln(age ends) + ln(size ends))	logit	145.40	18
GLM	No	resp ~ (clone * PE) * (ln(size ends))	logit	173.27	12
GLM	No	resp ~ (clone * PE) * (ln(age starts))	logit	429.51	12
GLM	Size	resp ~ offset(ln(size)) + (clone * PE) * (ln(age ends) + ln(size ends))	logit	148.41	18
GLM	Size	resp ~ offset(ln(size)) + (clone * PE) * (ln(size mids))	cloglog	171.75	12
GLM	Size	resp ~ offset(ln(size)) + (clone * PE) * (ln(age starts))	logit	384.40	12
GLM	**Age**	**resp ~ offset(ln(age)) + (clone * PE) * (ln(age ends) + ln(size ends))**	**logit**	**143.04**	**18**
GLM	Age	resp ~ offset(ln(age)) + (clone * PE) * (ln(size ends))	cloglog	168.90	12
GLM	Age	resp ~ offset(ln(age)) + (clone * PE) * (ln(age starts))	logit	435.41	12
Rate	-	Weibull function, size integration, age and size rate effects	-	162.99	18
Rate	-	Weibull function, age integration, age and size rate effects	-	163.40	18
Rate	-	Weibull function, age integration, size rate effects	-	168.05	12
Rate	-	Weibull function, size integration, size rate effects	-	173.69	12
Rate	-	Weibull function, size integration, age rate effects	-	398.16	12
Rate	-	Weibull function, age integration, age rate effects	-	504.36	12

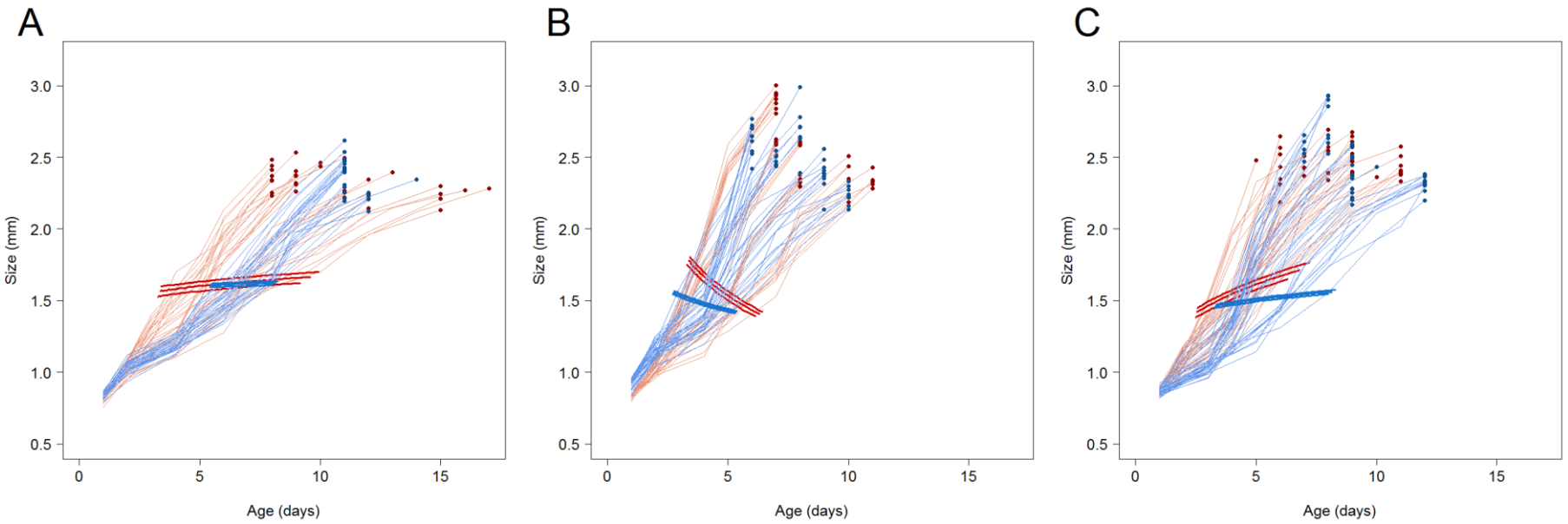

Figure 3.4. Parental effects on probabilistic maturation reaction norms: A) DKN1-3; B) Ness1; and C) B5. Pale red lines represent growth trajectories of HPE individuals, culminating in red circles upon reaching maturity. The three red lines intersecting growth trajectories represent 25, 50 and 75% PMRNs from the best fitting model. Blue lines and circles are growth and maturation data of LPE individuals, and their 25, 50 and 75% PMRNs.

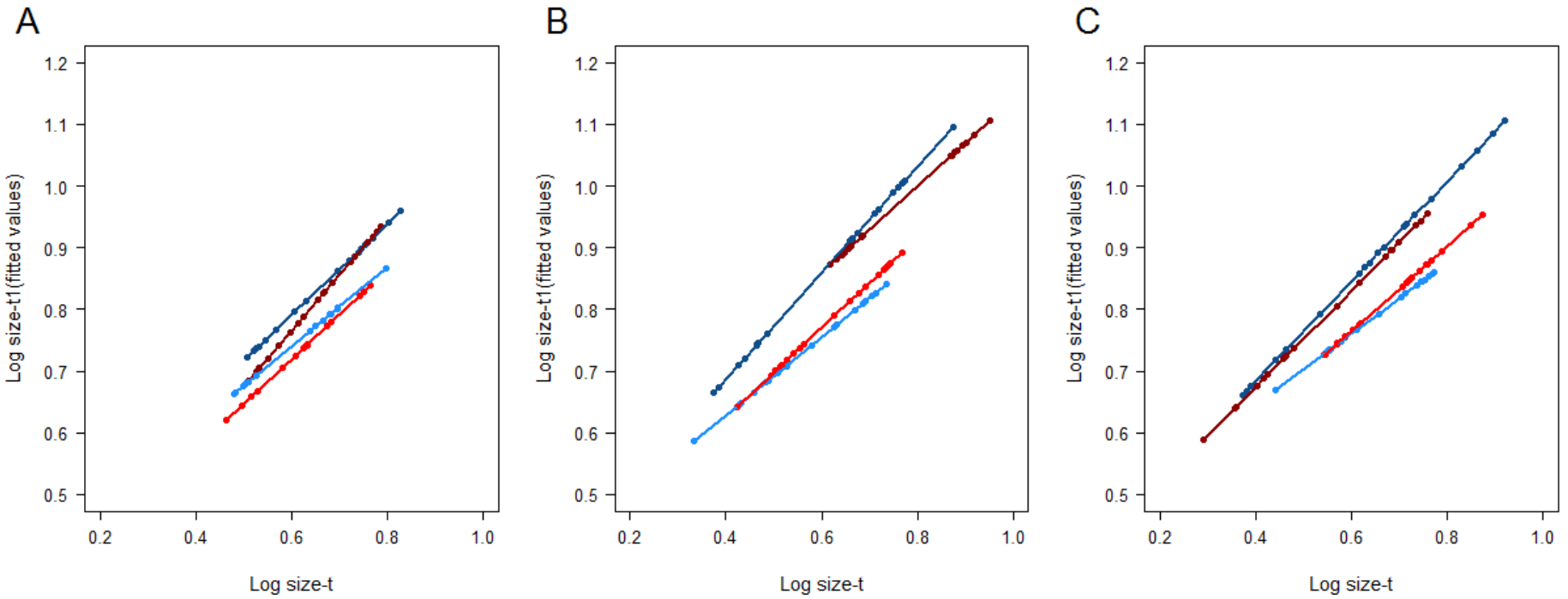

Figure 3.5. Parental effects on size-corrected growth during the maturation process: A) DKN1-3; B) Ness1; and C) B5. Logged values of size-*t* are plotted against fitted values of logged size-*t*+1, with model fits overlaid to aid visualisation. Dark red lines represent HPE-H individuals, pale red lines HPE-L, dark blue lines LPE-H and pale blue lines LPE-L. See Appendix Figures A3.4, A3.5 and A3.6 for parental effects on all four current foods.

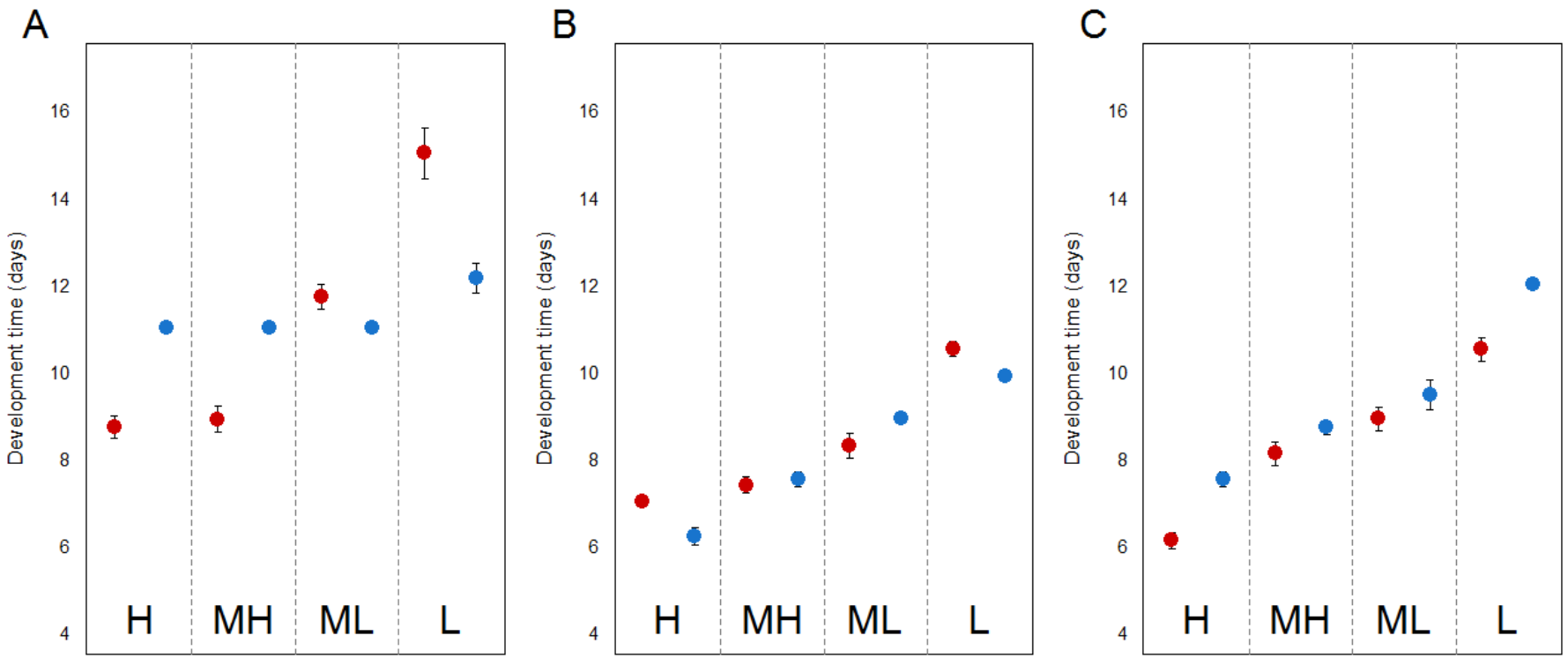

Figure 3.6. Parental effects on age at maturity (± Standard error): A) DKN1-3; B) Ness1; and C) B5. Red circles are neonates from HPE mothers, blue circles are neonates from LPE mothers.

parental effects on age at maturity were more subtle. LPE individuals matured earlier in H and L current foods, but this effect was not present in MH current food, and was reversed in ML current food (Fig. 3.6B). Parental effects in B5 caused HPE individuals to mature earlier, irrespective of the current food environment (Fig. 3.6C).

Size at maturity

Parental effects also influenced size at maturity in a context-dependent manner that varied between clones (PE:clone:food, $F = 11.765$, df = 6, $p < 0.001$). In clone DKN1-3 this effect was weakly context-dependent (Fig. 3.7A). LPE individuals were generally larger at maturity than HPE individuals, but this effect disappeared under lowest current rations. In clone Ness1 parental effects on size at maturity mirrored those on age at maturity: LPE individuals were smaller in H and L current foods, but this effect was not present in MH current food and reversed in ML current food (Fig. 3.7B). In clone B5, LPE individuals were bigger than HPE individuals in H current food but smaller than them in ML and L current foods (Fig. 3.7C).

Number of offspring in first clutch

Parental effects influenced the number of offspring in the first clutch in a genotype-dependent manner (PE:clone, LRT = 6.981, df = 2, $p = 0.03$) over and above differences caused by size. However, size at maturity was of far greater importance in determining the number of offspring in the first clutch (size at maturity, LRT = 55.534, df = 1, $p < 0.001$). A comparison of Figure 3.7 and Figure 3.8 reveals how closely number of offspring in the first clutch is related to size at maturity. Only in clone Ness1 (Fig. 3.7B and 3.8B) does there appear to be a difference between parental effects on size and number of offspring in the first clutch.

3.4 Discussion

Variation in parental food availability generated parental effects that persisted from birth through to the production of offspring in *Daphnia magna*. Whilst many studies have identified parental effects on birth size (Bernardo 1996b; Mousseau & Fox 1998a, 1998b; Räsänen et al. 2005) and age and size at maturity (e.g. Beckerman et al. 2006; Hafer et al. 2011), this study adopted an ontogenetic approach that explicitly considered the expression of these effects during the course of development, and their influence on early growth, the decision to mature, and growth during the maturation process. By investigating how parental effects influenced development it was possible to identify the mechanisms that resulted in

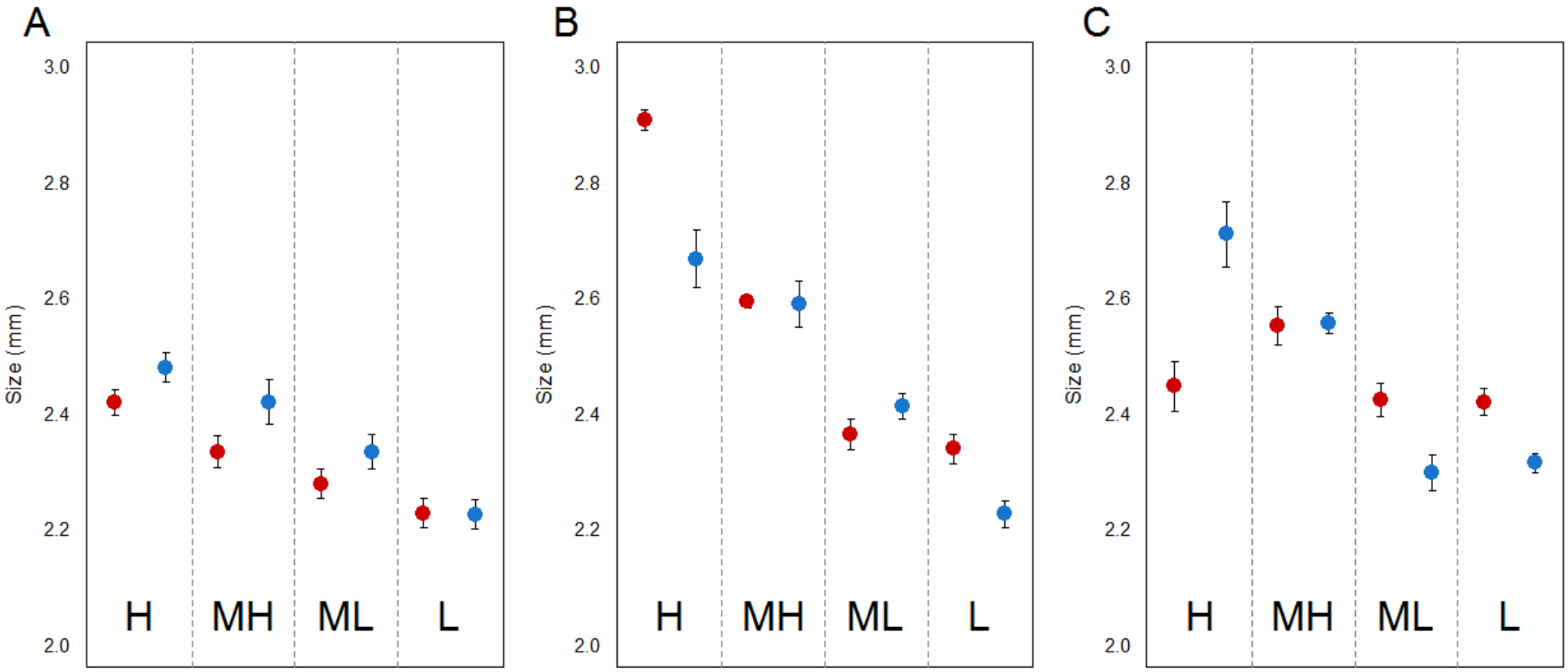

Figure 3.7. Parental effects on size at maturity (± standard error): A) DKN1-3; B) Ness1; and C) B5. Red circles are neonates from HPE mothers, blue circles are neonates from LPE mothers.

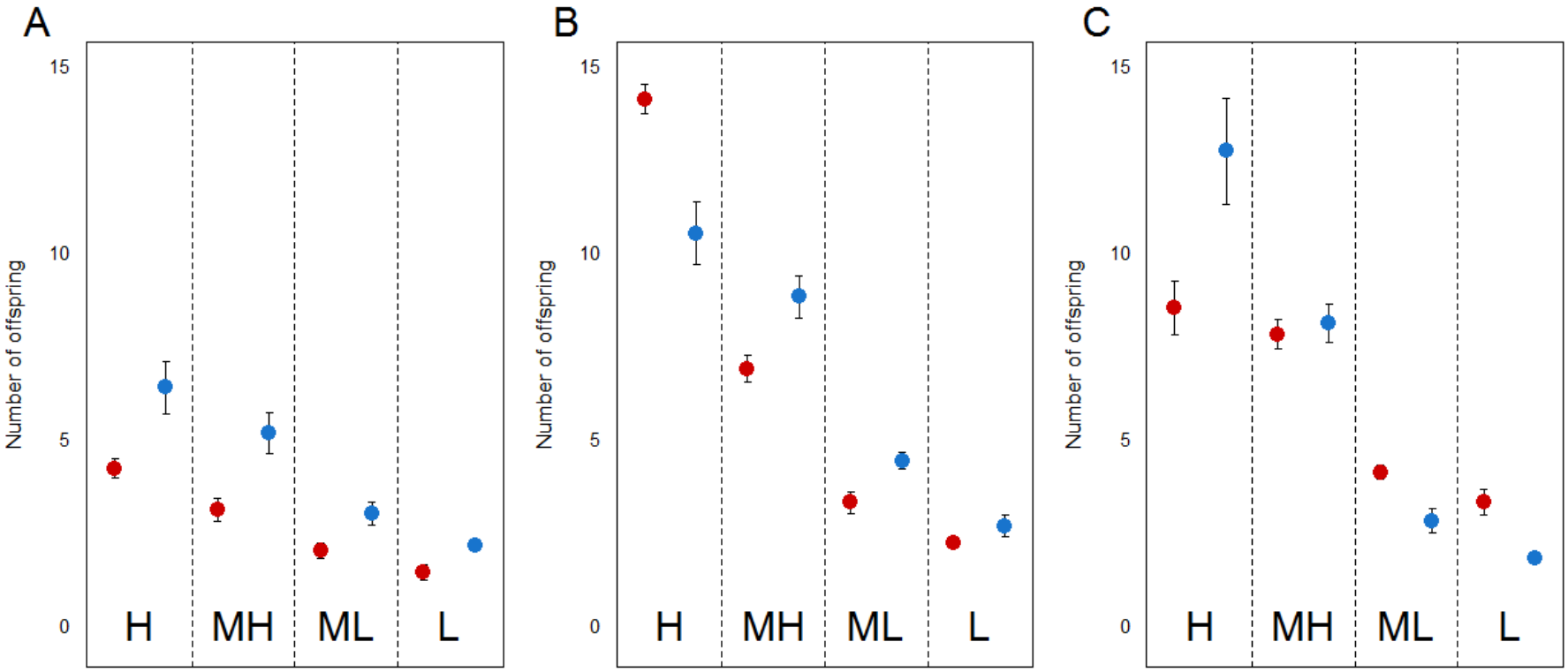

Figure 3.8. Parental effects on number of offspring in the first clutch (± standard error): A) DKN1-3; B) Ness1; and C) B5. Red circles are neonates from HPE mothers, blue circles are neonates from LPE mothers.

parental effects on adult traits, beyond simple differences in birth size. This ontogenetic approach also revealed that parental effects on development (and subsequent adult traits) were context-dependent, and each of the three genotypes under investigation differed in the expression of these effects.

3.4.1 Parental effects on neonate size did not predict the consequences for adults

One of the most frequently observed parental effects is variation in offspring size at birth (e.g. Bernardo 1996a; Mousseau & Fox 1998a, 1998b; Räsänen et al. 2005). However, the persistence of these effects through development and into adulthood is less well understood, and in *Daphnia*, many studies fail to investigate the persistence of parental effects beyond neonate size (but see Alekseev & Lampert 2004; Sakwińska 2004; Alekseev & Lajus 2009). The results from this chapter suggest that, in *D. magna*, parental effects on size at birth are unable to explain much of the variation later on in development. In clone B5, there were no parental effects on birth size (Fig. 3.2A), but significant parental effects on growth (Fig. 3.3C) and maturation (Fig. 3.4C). Furthermore, effects that may appear to be similar during development, such as the smaller sized PMRNs of Ness1 and B5 (Fig. 3.4B & C), were not the result of similar difference in neonate size, as parental effects produced large differences in neonate size for Ness1 (Fig. 3.2B), in comparison to the lack of effect in B5 (Fig. 3.2C). These results highlight the importance of adopting an ontogenetic approach, as parental effects in *D. magna* extend beyond differences in size at birth. The mechanisms that are responsible for these effects, however, are unknown. Persistent parental effects may arise through variation in a manifold cytoplasmic factors (Mousseau & Fox 1998b; Groothuis & Schwabl 2008), epigenetic inheritance of gene expression (Beldade et al. 2011; Wolf et al. 2011), or a combination of both (Badyaev & Uller 2009; Bonduriansky & Day 2009), but investigating their contributions towards parental effects requires a more thorough examination of the genetic and physiological changes that underpin developmental changes, including maturation.

3.4.2 Parental effects influence multiple developmental traits

The ontogenetic approach adopted by this study revealed that parental effects on adult traits are actually a composite of parental effects on multiple developmental traits. For example, in clone DKN1-3, LPE neonates were larger than HPE neonates (Fig. 3.2A). Context-dependent parental effects on growth conferred higher growth rates when parent and

offspring environments matched (Fig. 3.3A), but the PMRN did not differ between parental effect treatments (Fig. 3.4A). Growth of LPE individuals during the maturation process was marginally better than HPE growth (Fig. 3.5A). The result is that, in this clone, LPE adults were quicker to develop under low food, but slower under high food (as a consequence of context-dependent early growth; Fig. 3.6A), and LPE individuals were generally a little larger at maturity (as a result of similar thresholds and improved growth during maturation; Fig. 3.7A). The results of this study support the idea that parental effects influence a diverse array of developmental traits in offspring (Bonduriansky & Head 2007; Cahan et al. 2011), and that complex interactions between genetic, environmental and parental effects are a factor of normal development (Galloway et al. 2009; Yanagi & Tuda 2010; Pascoal et al. 2012). The identification of variation in the expression of parental effects between the three *D. magna* genotypes suggests that trans-generational plasticity may be adaptive, but in this study the three genotypes derived from three different distant populations. Studies of within-population parasite susceptibility in *D. magna* have already revealed that parental effects may differ substantially in their consequences (Mitchell & Read 2005; Stjernman & Little 2011), suggesting that the variation observed in this chapter could be reflective of variation at smaller spatial scales. Variation in parental effects is predicted to arise in response to environmental variation in which these genotypes develop. Recurrent environments experienced by organisms subsequently shape their developmental trajectories, which can be inherited both genetically and non-genetically (West-Eberhard 2003; Badyaev 2005, 2008), and may have profound consequences for the our understanding of the relationship between genotype and phenotype (Badyaev 2011).

Studying the impact of genotype × parental effect interactions on the maturation threshold may be particularly important for understanding how functional adult phenotypes are produced. In the previous chapter (Chapter 2; Harney et al. 2012), probabilistic maturation reaction norms (PMRNs) for the maturation thresholds were shown to be phenotypically plastic with respect to size across a resource gradient. This study supports these findings and also identifies transgenerational phenotypic plasticity in the PMRN of *D. magna* as a result of variation in parental resources. In both Ness1 (Fig. 3.4B) and B5 (Fig. 3.4C), parents from the LPE treatment produced offspring that tended to initiate maturation at a smaller size. Badyaev (2008) suggested that developmental threshold traits were likely targets for the action of parental effects. The observation of parental effects on the maturation threshold in this study suggests that the decision to mature is one such target and adds to the growing body of evidence showing parental effects that act on developmental switches (Schwander et al. 2008; Michimae et al. 2009; Harvey & Orbidans 2011).

3.4.3 Parental effects on development produce diverse adult phenotypes

Interactions between context-dependent parental effects on multiple developmental traits resulted in diverse adult phenotypes between the genotypes that could not have been predicted without the explicit consideration of ontogeny. In some cases these context-dependent parental effects on development produced adult phenotypes that were consistent with predictions based on matching offspring phenotypes to parental environments (Uller 2008). For example, in clone DKN1-3, offspring from HPE mothers grew better than those from LPE mothers in H and MH current food, while offspring from LPE mothers grew better than HPE mothers in L current food (Fig. 3.3A; Appendix Fig. A3.1). Subsequently, HPE individuals matured at an earlier age than LPE individuals in H current food, and the pattern was reversed in L current food (Fig. 3.6A). LPE individuals of clone Ness1 used an alternative mechanism to reduce age at maturity. By initiating maturation at smaller sizes than HPE individuals (Fig. 3.4B), they were able to mature earlier than HPE individuals (Fig. 3.6B). In other cases, the phenotypic outcomes of parental effects did not appear to match the parental environment. LPE individuals of clone B5 initiated maturation at smaller sizes than HPE individuals (Fig. 3.4C), but consistently took longer to mature (Fig. 3.6C), and matured at a smaller sizes in all but the highest food environment (Fig. 3.7C).

A further level of complexity may be introduced through effects on clutch size. Parental effects on the number of offspring in the first clutch were primarily driven by parental effects on size, in line with previous results (Glazier 1992; Barata & Baird 1998). However, there was also evidence that parental effects altered clutch size independently of body size in clone Ness1, where LPE individuals produced more offspring than HPE individuals at MH and L current foods, despite being similar sizes or smaller (Fig. 3.7B and Fig. 3.8B). Inclusion of neonate size, and clutch size and neonate size of subsequent offspring may uncover further complexity in how parental effects shape adult phenotypes of their offspring broods (Livnat et al. 2005; Plaistow et al. 2007).

The smorgasbord of phenotypes produced by context-dependent parental effects highlights the importance of using an ontogenetic approach that considers the developmental origins of these phenotypes, and serves as a reminder that parental effects can serve a variety of functions. Although anticipatory parental effects are expected to arise when parent and offspring environments match (Räsänen et al. 2005; Galloway & Etterson 2007; Marshall & Uller 2007; Burgess & Marshall 2011), transgenerational plasticity does not always alter offspring phenotypes to maximise fitness. Parental effects that seem adaptive in one trait or at one point in ontogeny may be maladaptive in another trait or at another time (Vijendravarma et al. 2009), or in another genotype or population (Marshall & Uller 2007).

Additionally, parent-offspring conflicts can result in parental effects that are suboptimal for offspring (Bernardo 1996a; Mayhew 2001; Kurdíková et al. 2011) as mothers balance the costs of current and future reproduction to maximise their own fitness, not their offspring's (Uller 2008). Furthermore, nutritional stress (Vijendravarma et al. 2009; Frost et al. 2010) and physiological constraints (Marshall & Uller 2007; Uller et al. 2007) can both produce parental effects that do not appear to benefit offspring.

3.5 Conclusions

In conclusion, this study identified persistent parental effects on several aspects of development in *D. magna*, including the maturation threshold, even in cases when there were no obvious parental effects on neonate size. Interactions between parental effects on multiple traits during ontogeny resulted in diverse adult phenotypes that could not be predicted by differences in birth size. The strength and nature of the parental effects observed was dependent on the current environment and differed between the three genotypes under investigation. These findings strengthen the case for incorporating parental effects into a broader evolutionary framework, where non-genetically inherited patterns of development provide a mechanism for adaptation to recurrent environments.

Chapter 4

Gene expression analyses of *Daphnia pulex* maturation

4.1 Introduction

Plasticity in age and size at maturity arises because the maturation process involves a number of environmentally dependent developmental processes acting over time (Bernardo 1993; West-Eberhard 2003; Berner & Blanckenhorn 2007). In this thesis, reaction norms for the maturation threshold have revealed phenotypic plasticity with respect to resource availability both within- (Chapter 2; Harney et al. 2012) and between-generations (Chapter 3). It is possible that the physiological changes associated with the maturation decision of *Daphnia* are occurring earlier in development than has previously been suggested (Zaffagnini 1987), and the observed plasticity is the result of a trade-off between growth and reproduction (Day & Taylor 1997; Berner & Blanckenhorn 2007), but the proximate mechanisms that underlie the threshold have received little attention. More generally, environmental influences on development of arthropods are known to be diverse (Nijhout 2008), and identifying the causes of developmental plasticity in maturation requires improved understanding of the underlying physiology (D'Amico et al. 2001; Dufty Jr et al. 2002), including the preceding transcriptional changes (e.g. White et al. 1999; Heyland et al. 2011). Microarrays can be used to investigate genome-wide transcriptional changes (Gracey & Cossins 2003), including those associated with maturation. They may also provide further information about whether maturation processes are the result of discrete changes in gene expression or gradual changes reflective of a maturation rate (Harney et al. 2012).

4.1.1 Plasticity in developmental physiology

The interactions between the developing phenotype and its environment can result in diverse reaction norms for age and size at maturity (Nijhout 2008). For example, among the holometabolous insects (Endopterygota), the larvae of the tobacco hornworm moth *Manduca sexta* must reach a critical weight or size threshold before they are able to pupate (Nijhout & Williams 1974a), and there is a negative relationship between age and size at pupation as food availability (and growth) declines. The fruit fly *Drosophila melanogaster* must also reach a critical weight (De Moed et al. 1999), but individuals tend to pupate at similar sizes

irrespective of food availability. In contrast, larvae of the dung beetle *Onthophagus taurus* do not cease growth and initiate metamorphosis until their food supply is depleted, resulting in a positive relationship between age and size at maturity as food availability increases (Shafiei et al. 2001).

The physiological changes associated with these patterns of developmental plasticity have been the subject of a number of studies in both *M. sexta* (Nijhout & Williams 1974b; D'Amico et al. 2001; Davidowitz et al. 2003; Davidowitz & Nijhout 2004) and *D. melanogaster* (Colombani et al. 2005; Mirth et al. 2005; Mirth & Riddiford 2007; Layalle et al. 2008). In *M. sexta* the juvenilising factor, juvenile hormone (JH), prevents the secretion of ecdysone and pupation (Nijhout 1994). Cessation of JH secretion is associated with the critical weight (Davidowitz et al. 2003), and the time dependent clearance of JH by the catabolic enzyme JH-esterase leads to the fixed terminal growth period before the release of ecdysone, which causes pupation (Nijhout & Williams 1974b; Davidowitz & Nijhout 2004). In *D. melanogaster* the role of JH is less clear, but it is known that the release of ecdysone is moderated by the nutrient responsive target of rapamycin (TOR) pathway, and nutrient deprived individuals take longer to release sufficient ecdysone to trigger pupation (Layalle et al. 2008). Endocrine factors related to ecdysone and JH are thought to be responsible for the developmental changes associated with maturation and metamorphosis in all crustaceans and insects (Borst & Laufer 1990; Nijhout 1994; Laufer & Biggers 2001), but differences between *D. melanogaster* and *M. sexta* highlight that their environmentally dependent expression is likely to be the result of adaptation to specific life histories or environments (Nijhout 2008).

Outside of these model systems, the physiology underlying maturation processes is less well understood. The application of modern genomic techniques, such as microarrays, to questions of comparative physiology (Gracey & Cossins 2003) allows the investigation of the gene expression changes that precipitate developmental changes (White et al. 1999) and will be of particular importance in understanding adaptive developmental plasticity (Beldade et al. 2011). Comparison of transcriptome profiles have provided insight into the developmental changes of numerous non-model taxa (e.g. Scharf et al. 2005; Azumi et al. 2007; Williams et al. 2009; Heyland et al. 2011; Li et al. 2011), but have not been applied specifically to the investigation of maturation.

4.1.2 Maturation determinants in Daphnia

Although *Daphnia* species have been popular model organisms with evolutionary ecologists for many years, the mechanisms that control maturation in *Daphnia* are not fully appreciated. The publication of the *Daphnia pulex* genome (Colbourne et al. 2011a) now provides invaluable genomic resources that can be used in conjunction with oligonucleotide microarrays to help answer these questions. In *Daphnia*, maturation appears to be strongly influenced by size, suggesting a maturation threshold similar to the critical weights of *M. sexta* and *D. melanogaster*, but also to be a process with a rate, rather than discrete switch (Chapter 2; Chapter 3; Harney et al. 2012). The physiological changes associated with maturation in *Daphnia* have been characterised by cytological observations (Rossi 1980; Zaffagnini & Zeni 1986; Zaffagnini 1987) and this pattern of development (IM-1 to IM-3; see Chapter 2, section 2.2.3) is widely accepted (Bradley et al. 1991; Ebert 1994; Barata et al. 2001). There is, however, evidence that this model of maturation is too simplistic, as maturation in *Daphnia* is phenotypically plastic (Beckerman et al. 2010; Harney et al. 2012). Moreover, unlike insects, in which the relationship between moulting and development is often highly canalised (Esperk et al. 2007), *Daphnia* are not constrained by a predetermined number of moults (Barata et al. 2001) and in adverse conditions will extend the maturation phase beyond two moult cycles (Enserink et al. 1995; E. Harney pers. obs.). Therefore it is still unclear whether the cytological changes associated with maturation (Rossi 1980; Zaffagnini & Zeni 1986; Zaffagnini 1987) are the result of a threshold change that occurs two instars prior to the deposition of eggs in the brood chamber, or a maturation rate that is influenced by other factors.

4.1.3 Investigation of Daphnia *maturation using microarrays*

In this study a microarray approach was used to study differential gene expression prior to and during maturation in *D. pulex*. Using immature individuals in the two instars prior to previtellogenesis, previtellogenic (IM-1) and vitellogenic (IM-2) individuals will reveal changes in gene expression associated with the cytological changes that have previously been reported (Zaffagnini 1987), as well as the identity of the genes and pathways involved in the maturation process. The rate at which transcripts increase or decrease can also reveal whether these gene expression changes are the result of a distinct maturation threshold or a more gradual change indicative of a maturation rate (Chapter 2; Harney et al. 2012).

4.2 Materials and methods

4.2.1 Clone and developmental stage selection

All animals used in this study were of the same laboratory-reared clone of *D. pulex*, Cyril, that originated from a pond in Sheffield, UK (53°24'17"N, 1°27'25"W). Clone Cyril was selected due to its use in a previous microarray study (Plaistow et al, *in prep*). Data obtained in Chapter 2 suggested that, in Cyril, the modal number of instars to reach maturity was 6. Therefore, in order to gain an understanding of the ontogenetic changes that *D. pulex* undergo during maturation, it was decided to sample individuals at instars 2, 3, 4 and 5. In general, these would correspond to the two instars prior to the maturation threshold, as well as the two instars after the threshold (Fig. 4.1). In the previous chapters and much of the established literature, these instars are referred to as: the instar prior to IM-0, IM-0, IM-1 and IM-2 (Bradley et al. 1991; Enserink et al. 1995; Barata & Baird 1998). For simplicity, they will be referred to as developmental stages (DS)-1, DS-2, DS-3 and DS-4 in this chapter. With the exception of DS-4, when the ovaries are clearly visible (Zaffagnini 1987; McCauley et al. 1990b), these stages are not associated with obvious phenotypic changes. Therefore to provide a *post hoc* indication of how accurate the estimation of developmental stage was, the number of individuals that had ovaries in the fourth and fifth instars was recorded at the time they were collected.

4.2.2 Culturing conditions

To minimise the role of parental effects on differential gene expression, all experimental animals were acclimated to experimental conditions for three generations prior to the experimental generation. Throughout acclimation, individuals from the third clutch of each acclimated generation were used to set up the subsequent generation. *D. pulex* were cultured at 21 ± 1°C with a 14:10 light:dark photoperiod. Individual *Daphnia* were maintained under standard conditions (Chapter 2) and fed a diet of 89 cells μl^{-1} *Chlorella vulgaris* on a daily basis, in line with the highest food for *D. pulex* in Chapter 2.

In order to produce enough RNA for amplification and subsequent hybridisation, multiple individuals within a developmental stage sample were pooled. Trial RNA extractions with four replicates of 10, 20 and 30 second instar individuals were carried out to assess the likely number of individuals needed to produce useable quantities of RNA. The results from this trial (Section 4.3.2) informed the decision to aim for 40 second instar, 32 third instar, 24 fourth instar and 24 fifth instar individuals in each sample. Because such large numbers of

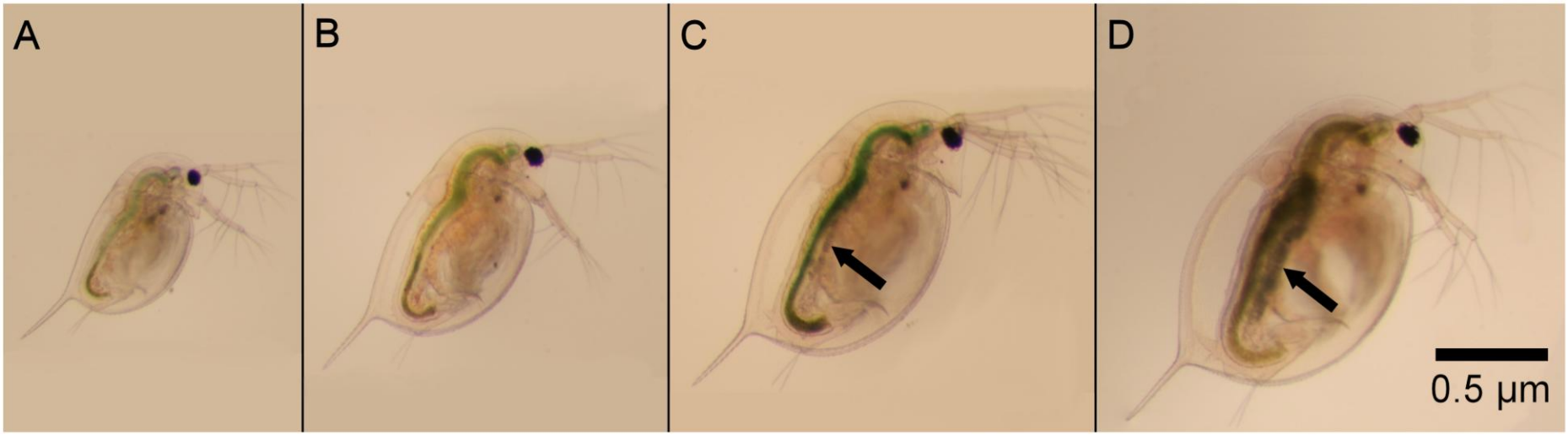

Figure 4.1. *Daphnia pulex* phenotypes throughout maturation, corresponding to instars 2-5 under experimental conditions. During DS-1 (A) and DS-2 (B), individuals do not undergo observable phenotypic changes. During DS-3 (C), individuals begin 'previtellogenesis' (Zaffagnini 1987), which in some cases results in the appearance of the ovaries as faint grey/green lines parallel to the gut (arrow in panel C). However, it is not until DS-4 (D) that vitellogenesis causes the ovaries to accumulate large quantities of vitellogenins, visible as a dark grey/green mass adjacent to the gut (arrow in panel D).

individuals had to be pooled for each RNA sample, samples were generated through four staggered cohorts over a one month period (Fig. 4.2). Within each experimental cohort, there were two concurrent sub-cohorts that shared a great grandmother, but had different grandmothers and were the offspring of eight mothers in the third and final acclimated generation. Experimental sub-cohorts were set up from the third clutch in the 1st and 3rd cohorts, but due to low numbers, experimental sub-cohorts in the 2nd and 4th cohort were set up from the fifth clutch. In all sub-cohorts neonates from the eight mothers were mixed and randomly assigned a DS in which to be harvested. Figure 4.2 shows the number of individuals in all pooled samples that were used in the microarray, and the pairings for hybridisation (see section 4.2.3, penultimate paragraph).

4.2.3 Molecular preparation and microarray experimental design

Once all the individuals belonging to a DS within a sub-cohort reached their designated instar, the presence/absence of ovaries was recorded. They were then added to a watch glass and excess artificial pond water was removed. Animals were sacrificed by adding 500 µl TRIzol to the watch glass. The animals and TRIzol were then transferred to a 1.5 ml microfuge tube and stored at -80°C until all samples had been collected. RNA extractions in TRIzol were completed according to manufacturer's instructions (Life Technologies 1999), and samples were bound, washed and eluted in 30 µl of RNase-free water using an Ambion *Purelink RNA mini kit* (Ambion 2009).

Thirty samples were generated following RNA extraction. Yield and integrity were assessed with an Agilent *2100 bioanalyzer* on Caliper *RNA 6000 nano labchips* (Agilent 2001). Extracting RNA from more samples than required for the 12 × 135K array ensured that lower quality samples were not taken through to the amplification stage. Unfortunately, rapid development in the 2nd cohort (see section 4.3.1) prevented a fully dye balanced interwoven loop design from being selected for the microarray experiment. Using only samples of high RNA integrity that could be hybridised with other samples from the same sub-cohort and cohort limited the choices available for a suitable experimental design. Our design incorporated 22 samples (four DS-1 and DS-3 samples, seven DS-2 and DS-4) in 11 hybrid pairs on 11 arrays.

Amplification and labelling of RNA was achieved using Ambion's *Amino Allyl MessageAmp II aRNA amplification kit* (Ambion 2008). The process involved the generation of cDNA, synthesis of antisense RNA and dye coupling with either Cy3 or Cy5. To confirm that RNA had been successfully amplified and labelled, concentrations of RNA

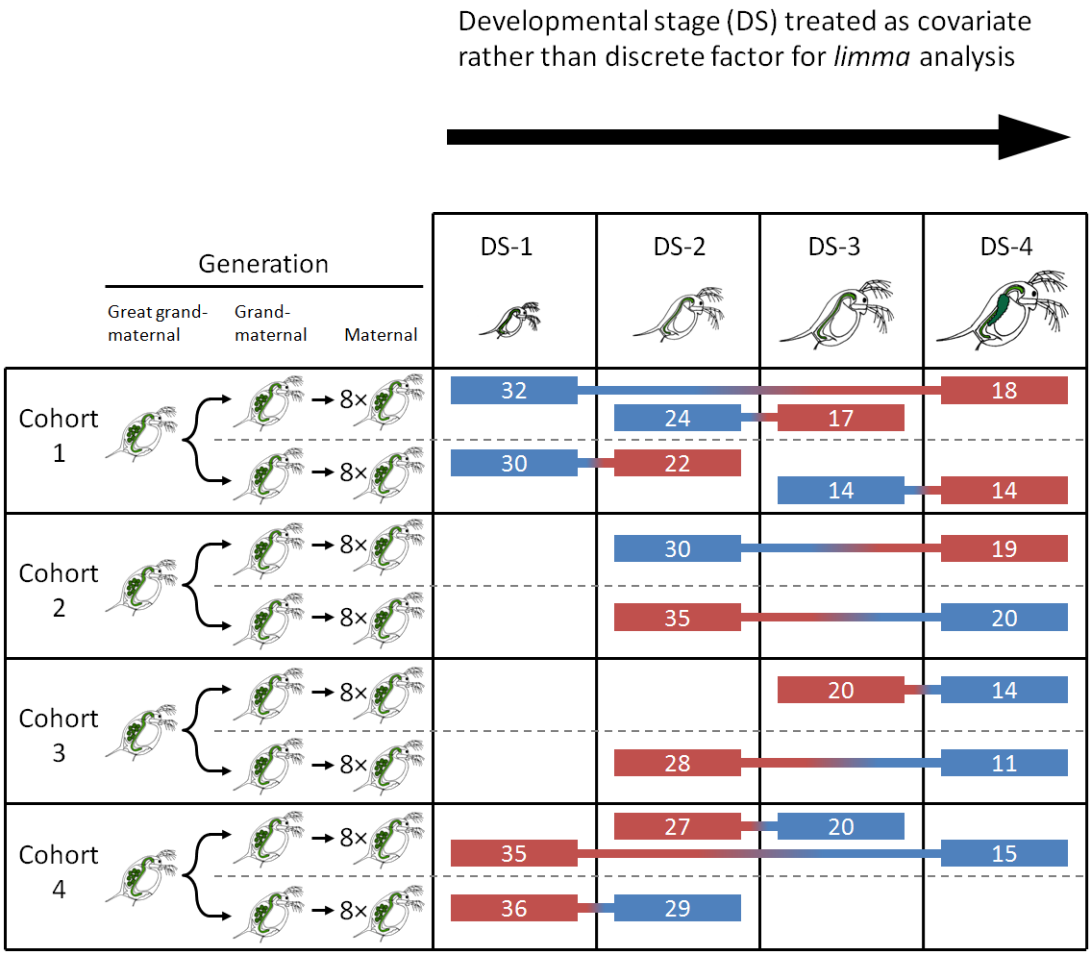

Figure 4.2. Experimental design showing numbers of individuals in pooled samples for each developmental stage within each cohort (shared great grandmother) and sub-cohort (shared grandmother), and hybrid pairs (see section 4.2.3, penultimate paragraph). Red squares were samples dyed with Cy3; blue squares were dyed with Cy5. Thick gradated lines show which samples were hybridised together. Samples were always hybridised with another from the same sub-cohort. Blank squares represent samples that were not used due to low RNA yields. The design is unbalanced and there are only 11 hybrid pairs due to the rapid development of the 2nd cohort. These individuals reached DS-4 in their fourth rather than their fifth instar (see Table 4.1). Subsequently there were no DS-1 individuals in this cohort and the intended hybrid pairs could not be made. Pooled samples also contained slightly fewer individuals than intended, due to the maternal generation producing smaller clutches than anticipated (see 4.2.2).

and dye were quantified using a Nanodrop ND-1000 Spectrophotometer. Dye incorporation was analysed according manufacturer's instructions (Ambion 2008) and ranged from 28-80 dye molecules per 1000 nucleotides for the 24 samples (slightly outside the expected range of 30-60 dye molecules per 1000 nt).

Pairs of samples to be hybridised together were then combined. For each hybrid pair, 1 µg RNA of each of the pair was added to a clean microfuge tube, and the total volume was made up to 9 µl with RNase-free water. One µl of Ambion *Fragmentation Buffer* was added to each hybrid sample, and samples were thoroughly mixed and incubated at 70°C for 15 minutes, before adding 1 µl Ambion *Stop Solution*. Samples were then vacuum-dried in the dark until just dry (approximately 15 minutes).

The study used a fifth generation NimbleGen 12 × 135K array (Roche NimbleGen Inc., USA) designed by the *Daphnia* Genomics Consortium (DGC, Centre for Genomics and Bioinformatics, USA). Within each of the identical 12 sub-arrays, most exonic regions were represented by two or three probes, resulting in a total of 35665 experimentally validated genes. The remaining probes consisted of transcriptionally active and neighbouring regions that have yet to be described (Colbourne et al. 2011b). Hybridisation to the Nimblegen array and subsequent washing were carried out according to manufacturer's instructions (NimbleGen 2009, ch. 4). Our two-colour array was then scanned at a resolution of 2 µm using Agilent's G2565CA Microarray Scanner System (Agilent 2010). Data was extracted from scanned images using NimbleScan software (Roche NimbleGen 2010) as per manufacturer's instructions (NimbleGen 2009, ch. 6).

4.2.4 Statistics and bioinformatics

All statistical analyses were carried out in R (R Development Core Team 2011). Intensity data for each probe were normalised within and between arrays using the *Limma* package (Smyth & Speed 2003) and an intesitiy score for each feature (exons, transcriptionally active regions and neighbours) was obtained by averaging the intensitiy scores across probes of the same sequence. Differential expression throughout ontogeny was also assessed using linear models and empirical Bayesian statistics within *Limma* (Smyth 2004) and using false discovery rates based on the Benjamini-Hochberg method (Benjamini & Hochberg 1995). The microarray design matrix treated DS as a continuous variable, highlighting genes that were generally up-regulated or down-regulated throughout ontogeny rather than identifying those that were differentially expressed between any two given developmental stages.

Identities and functions of differentially expressed genes were inferred by searching gene numbers on the gene pages of wFleaBase (www.wfleabase.org) and with the *D. pulex* taxonomic identifier 6669 in the UniProt Protein Knowledgebase (The UniProt Consortium 2012). Further functional information about putative proteins was obtained by carrying out BLASTs in UniProt of amino acid sequences obtained from wFleaBase. UniProt accession numbers of similar proteins in *D. pulex* and of proteins characterised in other arthropods were retrieved and percentage identity and E-values recorded. Gene Ontology (GO) terms for each gene/gene product from the UniProt Knowledgebase were recorded, and in addition a single higher order GO term that encompassed the majority of the lower order terms was assigned to each gene/gene product (The Gene Ontology Consortium 2000).

The packages *Biobase* and *ggplots* were used to create a heatmap and corresponding dendrogram displaying expression for all hybrid pairs, and clustering of co-expressed genes. A second limma analysis using a design matrix with DS as a discontinuous factor yielded separate coefficients for DS-1, DS-2, DS-3 and DS-4. Coefficients estimated from this analysis but based on genes identified in the original limma analysis were used to graphically illustrate changing patterns of expression throughout ontogeny.

4.3 Results

4.3.1 Preliminary: Estimation of developmental stages

The estimation of developmental stage was robust in cohorts 1, 3, and 4. Very few of the individuals harvested in the fourth instar had ovaries (9/111), whilst most individuals harvested in the fifth instar did have ovaries (82/84). In cohort 2, however, this was not the case. The majority of individuals harvested in the fourth instar had ovaries (34/39), and all individuals harvested in the fifth instar had eggs (30/30). The results are summarised in Table 4.1. Therefore the second, third, fourth and fifth instar corresponded to DS-1, DS-2, DS-3 and DS-4 in cohorts 1, 3 and 4, but to DS-2, DS-3, DS-4 and ‘DS-5’ in cohort 2. Subsequently, samples from the fifth instar in cohort 2 could not be considered for use in the microarray.

4.3.2 Preliminary: RNA trial extractions

Trial RNA extractions on groups of 10, 20 and 30 second instar individuals found that only RNA extractions of 30 individuals could produce more than 1µg of total RNA (Table 4.2).

Table 4.1. Numbers of individuals from each stream and cohort that had developed ovaries and eggs in the fourth and fifth instars. Boldfaced numbers highlight the developmental stage that the majority of individuals were on in an instar. Based on results from Chapter 2, the majority of individuals of *D. pulex* clone Cyril were expected to develop ovaries in the fifth instar. For the 1st, 3rd and 4th cohorts this was the case. The 2nd cohort, however, developed ovaries an instar earlier. This difference in development altered the design of the microarray experiment but was accounted for in statistical analyses.

	Fourth instar				Fifth instar			
Stream	Pre-ovary	Ovaries	Eggs	Total	Pre-ovary	Ovaries	Eggs	Total
G1 gm1	**17**	0	0	17	0	**18**	0	18
G1 gm2	**14**	0	0	14	1	**13**	0	14
G2 gm1	4	**15**	0	19	0	0	**15**	15
G2 gm2	1	**19**	0	20	0	0	**15**	15
G3 gm1	**18**	2	0	20	0	**14**	0	14
G3 gm2	**19**	1	0	20	0	**11**	0	11
G4 gm1	**20**	0	0	20	1	**14**	0	15
G4 gm2	**14**	6	0	20	0	**11**	0	11

Table 4.2. Total RNA yield from pooled samples of second instar *D. pulex* clone Cyril. Pooled samples of 10, 20 and 30 were each replicated four times. Second instar corresponded to the earliest developmental stage (DS-1) that was sampled. RNA concentration was measured using a Nanodrop ND-1000 Spectrophotometer.

No. of individuals in pooled sample	Sample ID	RNA concentration (ng $\mu l^{-1)}$	Total RNA in 30 µl elution (µg)
10	10.1	10.0	0.30
	10.2	5.0	0.15
	10.3	8.1	0.24
	10.4	8.3	0.25
20	20.1	13.8	0.41
	20.2	14.6	0.44
	20.3	13.8	0.41
	20.4	8.8	0.26
30	30.1	43.4	1.30
	30.2	46.8	1.40
	30.3	17.4	0.52
	30.4	13.6	0.41

Ambion's *Amino Allyl MessageAmp II aRNA amplification kit* recommends having a minimum of 0.1µg, and ideally 1µg of total RNA before proceeding with amplification and labelling. Therefore pooled samples of at least 30 individuals would be required to generate the recommended RNA in DS-1. Later developmental stages are larger and therefore require fewer individuals to yield suitable quantities of RNA.

4.3.3 Differential gene expression

A total of 264 probes were significantly differentially expressed in the microarray (Benjamini-Hochberg adjusted FDR < 0.05). Once average intensity scores for each feature had been calculated (exonic regions were generally represented by two or three probes), this dropped to 59 differentially expressed features (BH adjusted FDR < 0.05) of which only five had log fold scores above 1. Of these 59 features, 45 were exonic, 11 were neighbouring regions, and 3 were transcriptionally active regions. In total, 31 exonic regions were up-regulated during the course of development, while 14 were down-regulated.

4.3.4 Gene descriptions and functions

Many *D. pulex* genes were either uncharacterised or had been assigned rather general functions (wFleaBase), or were labelled as uncharacterised putative proteins (UniProt). In these cases, and in cases where UniProt accession numbers were absent, protein BLASTs were carried out on amino acid sequences from wFleaBase. Protein BLASTs against the UniProt Knowledgebase revealed that, of the seven genes that did not have UniProt IDs, six were extremely similar to other *D. pulex* genes (E-values < 1.0E-50; Appendix Table A4.1), and only one (DAPPU 299589) had an E-value greater than 1.0E-5 (Appendix Table A4.2).

Protein BLASTs were carried out for the majority of differentially expressed genes, as only 12 of 45 *D. pulex* gene products in UniProt had been characterised. In all but one case (DAPPU 110469), protein BLASTs yielded a named arthropod protein. Nine of these were proteins of unknown function, but the remaining 23 proteins featured functions in the UniProt database. Genes that were up-regulated and down-regulated during development, inferred from *D. pulex* annotation or protein BLASTs, are reported in Tables 4.3 and 4.4 respectively. Tables 4.3 and 4.4 provide a single higher order GO term with which the gene is associated, and all GO terms from the UniProt Knowledgebase are described in Appendix Tables A4.3 (increased expression later in development) and A4.4 (decreased expression later in development).

Table 4.3. *Daphnia pulex* genes with significant increases in differential expression during the course of development. Log fold changes of gene expression and significance from continuous contrast limma are reported. Where gene products were inferred directly from *D. pulex* annotation, UniProt and organism entries are highlighted in boldface. Blue text indicates annotation of the exact gene, red text indicates that a protein BLAST of the amino acid sequence resulted in the identification of a near-identical *D. pulex* gene and gene-product. Where no information about gene products was available, protein BLASTs were performed and the nearest (lowest E-value) characterised arthropod protein is reported. With two exceptions (DAPPU 303836 and 263168) E-values are lower than 1.0E-5. A single biological process (or in the absence of a process, a molecular function or cellular component) based on the highest order Gene Ontology (GO) term is reported (see Appendix Table A4.3).

DAPPU gene no.	Log fold change	P-value	UniProt ID	Organism	Percentage identity	E-value	Description	Process / function
308303	0.338	2.37E-05	C1BNJ5	*Caligus rogercresseyi*	44.0	1.00E-43	Peflin	Calcium ion binding
222925	0.631	3.25E-05	**E9G757**	***Daphnia pulex***	-	-	Putative cyclin B, copy D	Cell cycle / cell division
60476	0.371	6.92E-06	Q333R2	*Drosophila sechellia*	38.0	2.00E-72	Alpha 1,3-fucosyltransferase	Fucosylation
303879	0.422	5.26E-07	Q29DG0	*Drosophila pseudoobscura*	42.0	5.00E-23	UPF0389 protein GA21628	Integral to membrane
226075	1.779	1.43E-06	**E9GVW7**	***Daphnia pulex***	96.0	0	Vitellogenin fused with superoxide dismutase	Lipid transport
308693	0.654	1.92E-05	D4N2J9	*Paracyclopina nana*	21.0	2.00E-87	Vitellogenin-2	Lipid transport
226068	1.173	4.11E-06	Q1JUB1	*Daphnia magna*	52.0	0	Vitellogenin fused with superoxide dismutase	Lipid transport / oxidation-reduction process
299677	0.801	3.33E-07	**E9HZI6**	***Daphnia pulex***	-	-	Vitellogenin fused with superoxide dismutase	Lipid transport / oxidation-reduction process
226761	0.644	9.19E-07	B0WP11	*Culex quinquefasciatus*	33.0	2.00E-83	Asparagine synthetase	Metabolic process
100140	0.360	3.21E-06	E2BIM6	*Harpegnathos saltator*	61.0	4.00E-85	Pre-mRNA cleavage complex II protein Clp1	mRNA processing
220880	0.518	2.69E-06	B4P0Y7	*Drosophila yakuba*	29.0	6.00E-10	Geminin	Negative regulation of DNA replication
43440	0.526	5.53E-06	**E9FUS8**	***Daphnia pulex***	-	-	Histone H3	Nucleosome assembly
43804	0.535	9.11E-07	B4K413	*Drosophila grimshawi*	98.0	1.00E-51	Histone H3	Nucleosome assembly

Table 4.3 continued.

DAPPU gene no.	Log fold change	P-value	UniProt ID	Organism	Percentage identity	E-value	Description	Process / function
43863	0.507	2.72E-07	**E9FUS8**	***Daphnia pulex***	-	-	Histone H3	Nucleosome assembly
235586	0.524	7.96E-06	**E9FUS8**	***Daphnia pulex***	-	-	Histone H3	Nucleosome assembly
235631	0.674	3.56E-05	**E9FUS9**	***Daphnia pulex***	-	-	Histone H4	Nucleosome assembly
235802	0.478	1.75E-05	**E9FUS8**	***Daphnia pulex***	-	-	Histone H3	Nucleosome assembly
255862	0.536	2.63E-07	**E9FUS8**	***Daphnia pulex***	-	-	Histone H3	Nucleosome assembly
312260	0.765	1.01E-06	**E9FUS9**	***Daphnia pulex***	-	-	Histone H4	Nucleosome assembly
305707	0.392	1.98E-06	**E9FXL5**	***Daphnia pulex***	100.0	0	Alpha-carbonic anhydrase	One-carbon metabolic process
39705	0.372	9.23E-06	F4WDB8	*Acromyrmex echinatior*	46.0	9.00E-93	S-phase kinase-associated protein 2	Phosphorylation
203760	0.400	2.52E-05	G0ZJA2	*Cherax quadricarinatus*	48.0	7.00E-17	Ubiquitin	Protein binding
303836	0.643	6.53E-07	Q29GT5	*Drosophila pseudoobscura*	26.0	3.80E-01	GA15557, part of the PP2C family	Protein dephosphorylation
46545	0.437	1.72E-06	E0VSK2	*Pediculus humanus*	90.0	7.00E-74	Protein C-ets-1-B, putative	Regulation of transcription
304575	0.540	5.22E-06	E2B862	*Harpegnathos saltator*	73.0	2.00E-61	U6 snRNA-associated Sm-like protein LSm1	RNA processing
91889	0.463	2.02E-05	E0W3W7	*Pediculus humanus*	27.0	2.00E-07	Gem-associated protein, putative	Spliceosomal complex assembly
229368	0.356	9.46E-06	F4WIP9	*Acromyrmex echinatior*	41.0	3.00E-43	INO80 complex subunit E	Transcription
304661	0.678	1.62E-05	Q9U943	*Locusta migratoria*	23.0	0	Apolipophorins	Transport
110469	0.651	5.43E-08	-	-	-	-	-	-
263168	0.660	1.62E-05	B4K9R3	*Drosophila mojavensis*	24.0	9.30E-01	GI24314	-
306151	0.539	3.74E-05	**E9GVT8**	***Daphnia pulex***	100.0	1.00E-130	Glycolipid-transport protein	-

Table 4.4. *Daphnia pulex* genes with significant decreases in differential expression during the course of development. Log fold changes of gene expression and significance from continuous contrast limma are reported. No genes or gene-products were inferred directly from *D. pulex* annotation. Furthermore, the majority of genes (8/14) had no known function and in four cases (DAPPU 220921, 328621, 327378 and 312710) the nearest characterised arthropod proteins had E-values greater than 1.0E-5. A single biological process (or in the absence of a process, a molecular function or cellular component) based on the highest order GO term is reported (see Appendix Table A4.4).

DAPPU gene no.	Log fold change	P-value	UniProt ID	Organism	Percentage identity	E-value	Description	Process / function
107198	-0.423	3.83E-05	F4W8S0	*Acromyrmex echinatior*	62.0	1.00E-160	Septin-4	Cell cycle
227396	-0.408	2.71E-05	E2ARN0	*Camponotus floridanus*	39.0	1.00E-68	Putative RNA exonuclease NEF-sp	Exonuclease activity
219379	-0.509	2.06E-05	Q9XYN0	*Schistocerca gregaria*	65.0	0	Innexin 1	Ion transport
305501	-0.442	3.53E-05	E5L878	*Boophilus microplus*	57.0	1.00E-81	Glutathione S-transferase	Metabolic process
305713	-0.260	3.83E-05	P29981	*Blaberus discoidalis*	42.0	1.00E-135	Cytochrome P450 4C1	Oxidation-reduction process
304176	-0.386	2.16E-05	Q1HPW4	*Bombyx mori*	67.0	1.00E-169	Eukaryotic translation initiation factor 3 subunit I	Translation
112957	-0.743	2.11E-05	B4QMT8	*Drosophila simulans*	63.0	5.00E-64	GD12468	-
220921	-1.033	1.25E-09	B4GVT5	*Drosophila persimilis*	27.0	1.30E-01	GL14716	-
228103	-0.396	1.82E-06	A0ND72	*Anopheles gambiae*	38.0	3.00E-12	AGAP002973-PA	-
250400	-0.357	3.05E-05	B4M0F5	*Drosophila virilis*	65.0	1.00E-133	GJ24647	-
299589	-0.678	6.10E-08	B4PHB6	*Drosophila yakuba*	55.0	1.00E-76	GE21946	-
312710	-0.973	3.84E-05	CG4702	*Drosophila melanogaster*	32.0	2.00E-04	CG4702	-
327378	-0.403	3.07E-05	Q71DB3	*Drosophila yakuba*	24.0	3.90E-01	CG9568	-
328621	-0.418	1.08E-05	B3M1V5	*Drosophila ananassae*	40.0	3.00E-04	GF17870	-

4.3.5 Co-expression of genes

The major branches of the hierarchical cluster analysis correspond to the regions that were up-regulated or down-regulated during development (Fig. 4.3). Within these major clusters, minor clusters with similar patterns of co-expression emerge. Some of these clusters have unifying biological features. For example, all six H3 histone genes fall within the same cluster, along with a gene that is likely to code for geminin (involved in DNA replication). Figure 4.3 shows biological functions or processes associated with these minor clusters. Amongst the genes up-regulated during development were several lipid transport genes. Three of these were vitellogenin fused with superoxide dismutase (VTG/SOD) genes, of which two (DAPPU 226068 & 226075) formed a sister group to all other up-regulated genes. The remaining VTG/SOD gene formed a cluster with another VTG gene and the gene for apolipophorins (another lipid transport protein), along with genes for the two H4 histones and a cyclin gene.

The majority of these clusters show gradual linear decreases (Fig. 4.4A) or increases (Fig. 4.4B) in expression during the course of development. However, there is a steep increase in expression of the two VTG/SOD genes (DAPPU 226068 & 226075) between DS-2 and DS-3 (Fig. 4.4C). The other cluster featuring lipid-transport proteins shows a similar, though less dramatic, increase in expression between DS-2 and DS-3 (Fig. 4.4C). VTG/SOD genes DAPPU 226068 and 226075 were also the only characterised genes with log fold changes greater than one (Table 4.3).

4.4 Discussion

Microarray analyses were used to characterise genes that were differentially expressed during the maturation process in *D. pulex*. In total 45 genes were significantly differentially expressed over the course of ontogeny. Of these, 31 showed increasing levels of expression as development progressed, while 14 showed decreasing levels of expression through ontogeny. Among those showing higher levels of expression later in development were genes for a number of lipid-transport proteins (six), H3 and H4 histones (eight) and several involved with RNA processing or transcription (five). On the other hand, genes showing lower levels of expression later in development did not appear to have unifying functions. The majority of the 45 differentially expressed genes appeared to change expression in a continual manner from one developmental stage to the next. However, five of the genes associated with lipid-transport, along with two co-expressed H4 histone genes and a co-

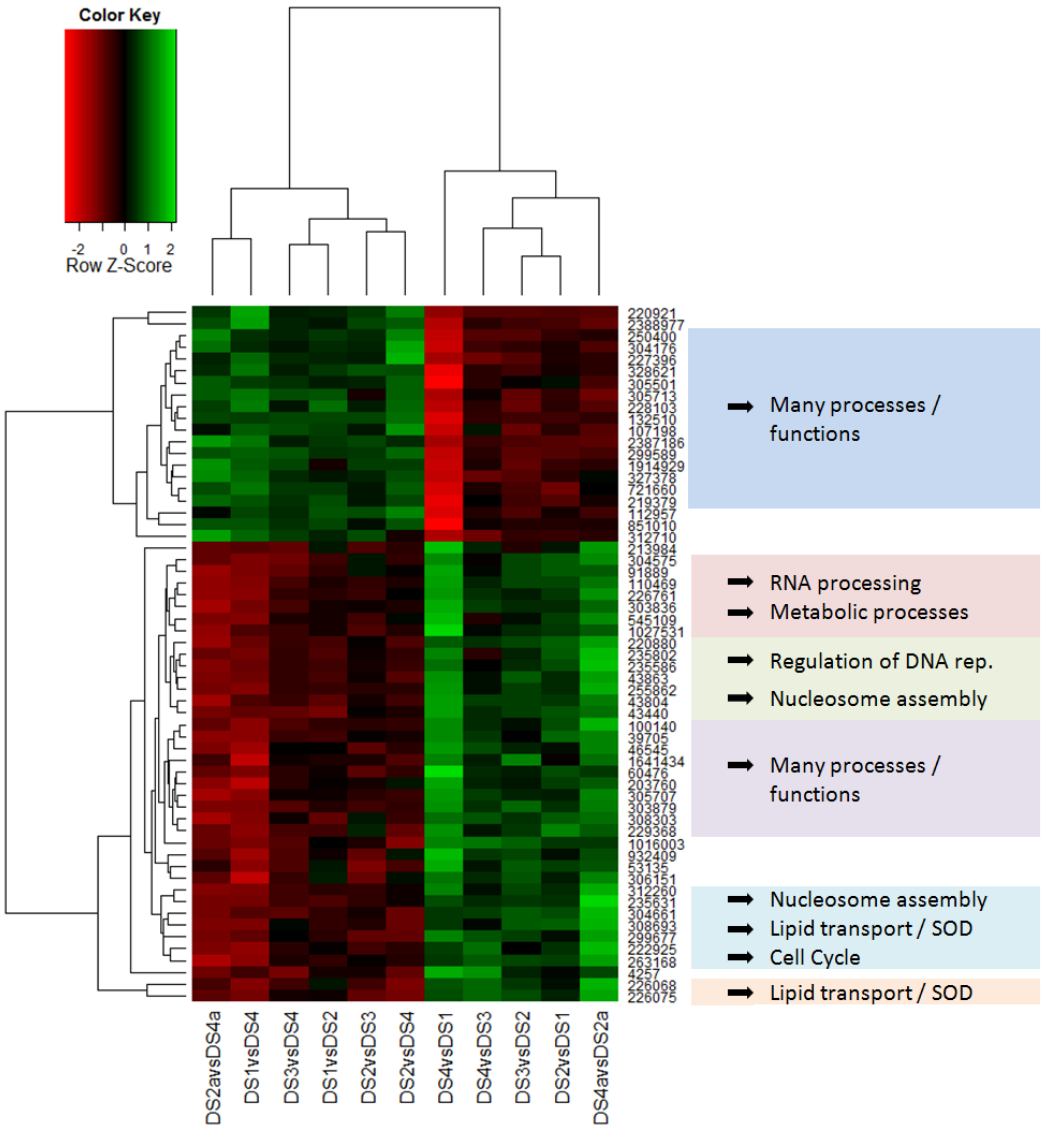

Figure 4.3. Hierarchical clustering analysis and log fold changes in gene expression for regions with significant levels of differential expression identified by limma with developmental stage as a continuous variable. All 59 differentially expressed regions are shown. Codes along the bottom margin distinguish between arrays. For example 'DS1vsDS4' shows levels of gene expression in an array comparing DS-1 to DS-4. The 'a' in 'DS2avsDS4a' and 'DS4avsDS2a' denotes the comparison between samples in the 2nd cohort that developed in an earlier instar. Between arrays, the main branches separate hybrid pairs where the earlier developmental sample was dyed with Cy3 (left-hand 6) from those where it was dyed with Cy5 (right-hand 5). Thus the major branching that occurs between features corresponds to regions down-regulated during development (upper 20 rows) and regions up-regulated during development (bottom 39 rows). Within these clusters levels of co-expression are broadly similar, but lower-order clusters are highlighted by boxes on the right. Where genes within a cluster fell into three or fewer categories of biological process or molecular function, these categories are reported.

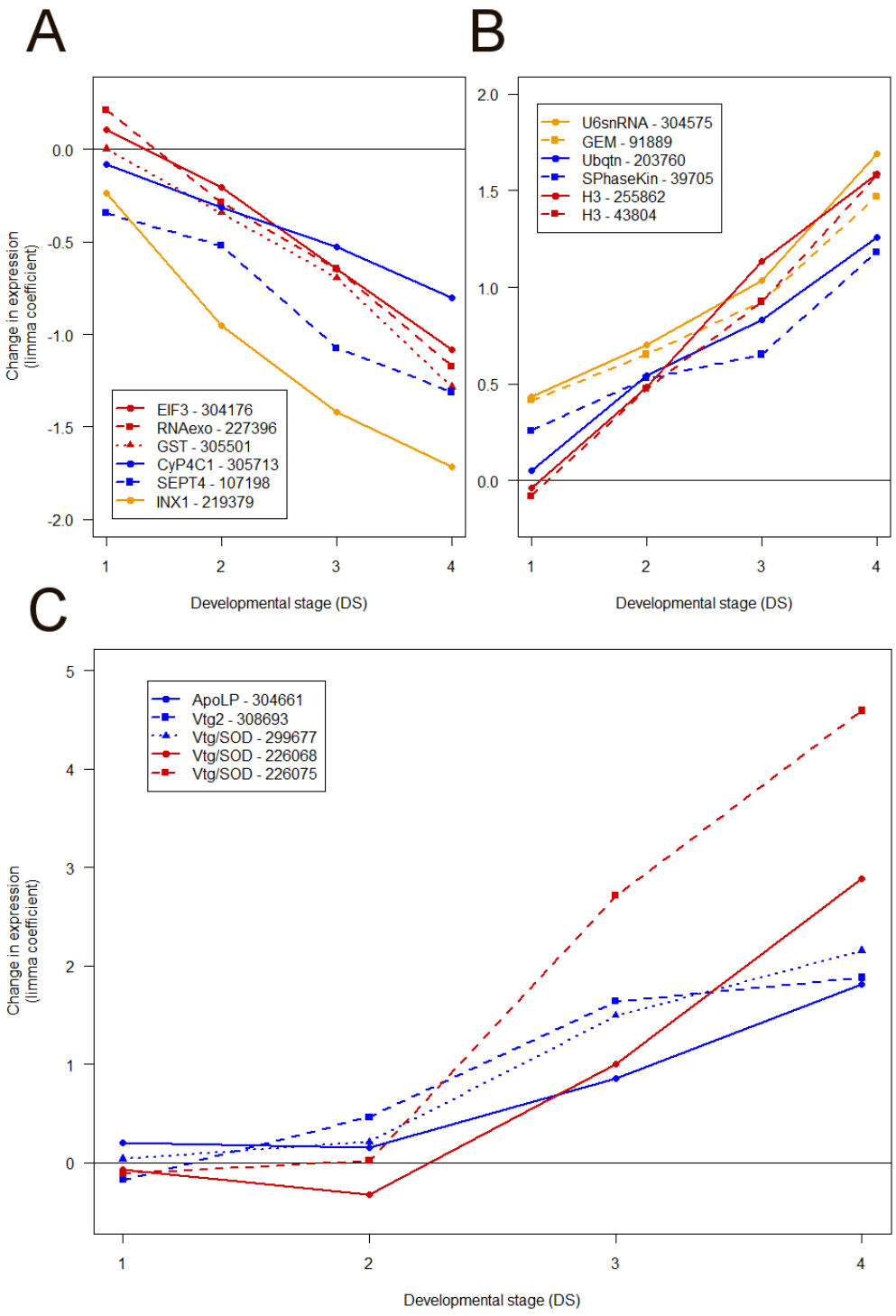

Figure 4.4. Changes in expression of selected genes. Lines of the same colour indicate genes that displayed similar levels of co-expression according to cluster analysis (see Fig. 4.3). A) and B) show patterns of expression for a representative subset of down-regulated and up-regulated genes, respectively. Changes in expression of these genes appear continuous. C) shows expression of lipid transport genes. Up-regulation of most of these genes appears to increase steeply between DS-2 and DS-3, particularly in two of the 'vitellogenin fused with superoxide dismutase' genes (DAPPU 226068 and 226075). Descriptions of up-regulated and down-regulated genes can be found in Tables 4.3 and 4.4 respectively.

expressed putative Cyclin B gene showed a marked increase in expression between DS-2 and DS-3, suggesting that there may be a threshold change in the expression of certain genes during maturation in *D. pulex*.

4.4.1 Increased expression of vitellogenin is inconsistent with a maturation threshold

The most striking increases in gene expression between DS-2 and DS-3 were observed in a pair of vitellogenin fused with superoxide-dismutase (VTG/SOD; Fig. 4.4C) genes. Vitellogenins are glycolipoprotein precursors of the vitellins that serve as yolk proteins for developing embryos in oviparous species (Hagedorn & Kunkel 1979; Wahli et al. 1981). Vitellogenins are known to play an important role in the maturation of numerous crustacean species (Okumura et al. 2007; Phiriyangkul et al. 2007; Subramoniam 2010), including daphniids (Hannas et al. 2011). Previous cytological studies of *Daphnia* have suggested that IM-2 (DS-4 in this study) is the developmental stage primarily associated with vitellogenesis in *D. magna* (Zaffagnini & Zeni 1986; Zaffagnini 1987; Fig. 4.1D), but this experiment suggests that expression of some vitellogenin transcripts increases two instars earlier between DS-1 and DS-2, and that expression of all vitellogenin transcripts increases suddenly between DS-2 and DS-3. The sudden increase in VTG expression between DS-2 and DS-3 (Fig. 4.4C) coincides with the cytological changes associated with maturation previously observed at this transition (Zaffagnini 1987) and is consistent with the proposed maturation threshold at this point in development (Bradley et al. 1991; Ebert 1994; Barata et al. 2001). The results of this microarray suggest, however, that the 'previtellogenic' stage (DS-3) is also associated with increased expression of VTG (not just differentiation of nurse cells into oocytes), and that vitellogenin is therefore produced by tissues other than the ovaries (Phiriyangkul et al. 2007). Furthermore, the expression of VTG transcripts even earlier in development suggests that the maturation process may be initiated even earlier than DS-3, and that VTGs may serve additional functions to the provisioning of oocytes with vitellin. Recent evidence suggests that arthropod vitellogenin may be expressed constitutively (Kang et al. 2008) and could play an important role in numerous processes beyond ovarian maturation (Havukainen et al. 2011), including the induction of polyphenisms in social insects (Scharf et al. 2005; Azevedo et al. 2011), somatic maintenance (Münch & Amdam 2010) and immune function (Amdam et al. 2004). It is possible that increased expression of certain VTG genes is not just a consequence of maturation but part of a more complex process controlling it; however, determining the precise role of different vitellogenins on reproductive maturation is beyond the scope of this study. Another intriguing result that merits further investigation is the role of super-oxide

dismutases (SODs) in the maturation processes of arthropods. The presence of SOD-fused forms of vitellogenin has been previously been recorded in both *D. magna* (Kato et al. 2004; Tokishita et al. 2006) and *D. pulex* (Schwerin et al. 2009), although it is unclear whether the fusion of VTG and SOD is adaptive. The co-expression of non-fused forms of these genes in other arthropods (Corona et al. 2007; Münch & Amdam 2010; Brady et al. 2012) suggests that SOD may play an important role during vitellogenesis or embryogenesis.

4.4.2 Expression of genes related to developmental hormones

Moulting, maturation and metamorphosis in crustaceans is largely controlled through the actions of the hormone methyl farnesoate (MF) and steroid 20-ecdysone (20-E) (Borst & Laufer 1990; Chang et al. 1993; Laufer et al. 1993; Chang & Mykles 2011). The biosynthesis of MF (Bellés et al. 2005) and 20-E (Gilbert et al. 2002) is well understood in arthropods, yet in this study, differential expression of enzymes in these pathways was not observed, despite the important role that MF and 20-E play in development and vitellogenesis (Borst & Laufer 1990; Laufer & Biggers 2001; Subramoniam 2010). These hormones are however, tightly linked to moult cycles as well as development (McWilliam & Phillips 2007). Individuals within a given sample were not synchronised within moult cycles, and probably came from a wide range of stages within the moult cycle. Given the precise timing of MF and 20-E synthesis required for successful moulting in crustaceans (Chang & Mykles 2011), it is possible that differential expression of biosynthetic enzymes was not detected by this microarray. One gene, an ortholog of cytochrome P450 4C1, was observed to display differential expression and has a potential role in the endocrine cycle. Arthropod cytochrome P450 4 (Cyp4) genes are an extremely diverse but poorly understood clade within the cytochrome P450 family (Feyereisen 2006) and as many as 38 different Cyp4 genes may exist in *D. pulex* (Baldwin et al. 2009). Many proteins within this group are thought to be involved in the regulation of the insect and crustacean neuroendocrine systems (Mykles 2011). In insects Cyp4C7 is a JH suppressant (Sutherland et al. 1998) and other Cyp4 protiens are down-regulated in response to ecdysone-agonists (Davies et al. 2006), whilst in crustaceans Cyp4C15 is involved in ecdysteroid synthesis (Dauphin-Villemant et al. 1999; Aragon et al. 2002). The decline in Cyp4C7 during vitellogenesis observed in the cockroach *Diploptera punctata* (Sutherland et al. 1998) is in accordance with the finding in this study that Cyp4C1 (DAPPU 305713) declined during the maturation process. It is difficult to speculate on the exact role of Cyp4C1, however, given the wide range of roles that Cytochrome P450 genes from all 4 major families (Cyp2, Cyp3, Cyp4 and mitochondrial Cyp) may play in the neuroendocrine processes of arthropods (e.g. Helvig et

al. 2004; Dam et al. 2008; Hansen et al. 2008; Rewitz & Gilbert 2008) and the limited annotation available for much of the *D. pulex* genome. Nevertheless, DAPPU 305713 represents a promising candidate for further investigation of the neuroendocrine control of maturation in *D. pulex*.

4.4.3 Histone genes show increasing levels of expression throughout development

Another group of genes that showed differential expression in later developmental stages were the histone genes, including six H3 histones and two H4 histones. Histones form the protein component of chromatin, and are involved in the packaging of DNA within the chromosome. Histone H3 and H4, along with H2A and H2B are the so-called core histones. DNA is wrapped around octamers containing two of each of these proteins, resulting in the basic nucleosome structure. Histones are required for chromosome replication during mitosis and there are a couple of reasons why expression may increase during maturation. One possibility is increasing levels of endoreduplication; the process of genome replication without nuclear or cellular division (Ullah et al. 2009). Endoreduplication results in endopolyploidy, and has been observed across a broad range of taxa (Lee et al. 2009; De Veylder et al. 2011). Endoreduplication is thought to be an essential part of oogenesis, and endopolyploid nurse cells and ovarian follicles of *D. melanogaster* (Hammond & Laird 1985) may facilitate increasing metabolic activity (Edgar & Orr-Weaver 2001), providing adequate resources for developing oocytes (Bastock & St Johnston 2008; Lee et al. 2009). Endopolyploidy in *Daphnia* may serve a similar function, with the identification of endopolyploidy nurse cells (Beaton & Hebert 1989) and the observation of general increases in endopolyploidy during the course of ontogeny (Korpelainen et al. 1997). In this study, endopolyploidy during maturation could be the mechanism responsible for increasing levels of vitellogenin expression during DS-3 and DS-4 as nutrients are synthesised for the developing oocytes (Bastock & St Johnston 2008).

While endoreduplication is one possible reason for elevated histone expression, this interpretation merits caution. For example, it is unclear why Histones H3 and H4 should show elevated expression, while the other core histones of the nucleosome, H2A and H2B, should not. Furthermore the elevated expression of geminin (putative function for DAPPU 220880) and cyclin B (putative function for DAPPU 222925) is somewhat puzzling, as they are thought to be expressed at lower levels in cells undergoing endoreduplication (Narbonne-Reveau et al. 2008; Lee et al. 2009). An alternative, though not mutually exclusive, reason for increasing histone expression during maturation could be the accumulation of histone transcripts within the nurse cells and oocytes during oogenesis, as

has been observed in *D. melanogaster* (Ruddell & Jacobs-Lorena 1985; Walker & Bownes 1998). It is thought that accumulation of histone transcripts in the oocytes facilitates rapid cell division during embryogenesis, and may act as a mechanism for epigenetic inheritance of gene expression (Marzluff et al. 2008). Both of these possibilities remain speculative, and furthermore, it is unclear why differential expression of histone genes was not a feature in a recent comparison of neonate and adult *D. magna* (David et al. 2011). The continual increase in expression of H3 genes during ontogeny (Fig. 4.4B) suggest that these changes are not the result of a switch or threshold between DS-2 and DS-3, as is the case with vitellogenin genes, but are a consequence of a change initiated even earlier in the developmental process.

4.4.4 Timing of developmental switches

There was no evidence to suggest distinct changes in gene expression occurring between DS-3 and DS-4, which implies that the phenotypic changes associated with increased ovary provisioning are not the result of developmental switches, but consequences of the maturation process. With the exception of increased expression of vitellogenin and lipid transport genes between DS-2 and DS-3, there was little evidence for the presence of a discrete maturation threshold, and most changes in gene expression were more suggestive of a maturation rate (Chapter 2; Harney et al. 2012). Vitellogenin clearly plays an important role in reproductive maturation, but it is more likely to be a phenotypic consequence of maturation processes than a switch associated with the maturation threshold. The same is likely to be true for the majority of differentially expressed genes in this study and it remains unclear whether maturation involves the early transient expression of key developmental genes or their constitutive expression during the maturation process. Future studies of development in *Daphnia* prior to and during maturation may benefit from comparisons between groups of individuals at the same period within the moult cycle, as precisely timed synthesis and metabolism of neuroendocrine signals play an important role in the development of arthropods (Gupta 1990; Davidowitz & Nijhout 2004; McWilliam & Phillips 2007; Chang & Mykles 2011).

4.5 Conclusion

This microarray study identified several significant transcriptional changes that take place during maturation of *D. pulex*. Of clear importance is the expression of vitellogenin, which is 'switched on' between DS-2 and DS-3, a result in line with previous cytological (Zaffagnini & Zeni 1986; Zaffagnini 1987) and life-history (Bradley et al. 1991; Ebert 1992, 1994) studies of *Daphnia* maturation. Investment in vitellogenin during maturation thus appears to be the result of a maturation threshold initiated following the moult in DS-2. However, aside from two histone H4 genes and cyclin B, no other genes showed this pattern of expression during development. Although there are no clear changes in gene expression that coincide with the sudden increase in vitellogenin expression, it could be related to reduced expression of a Cyp4C1 ortholog, potentially serving in a hormone-metabolising role, or as a result of increasing endopolyploidy. Both these potential mechanisms merit further investigation and will improve our understanding of the physiological processes that shape developing *Daphnia*. The changes in gene expression observed in this study suggest that the decision to mature is more analogous to a process with a rate (Chapter 2; Harney et al. 2012) than a discrete threshold.

Chapter 5

Fitness consequences of variation in the maturation threshold of *Daphnia*

5.1 Introduction

The maturation threshold is an important developmental trait that is closely linked to the evolution of body size (Nijhout 2008; Nijhout et al. 2010). Maturation thresholds of *Daphnia* are genotypically variable traits (Chapter 2; Harney et al. 2012) suggesting that they have a heritable basis. This thesis has also shown that the interactions between thresholds and genotypically variable growth rates can result in variable reaction norms for age and size at maturity (Chapter 3). Heritable variation in thresholds suggest that they can evolve (D'Amico et al. 2001) and may serve an adaptive function, but few studies have investigated the consequences of variation in maturation decisions, despite the importance of these underlying proximate mechanisms in determining age and size at maturity (Berner & Blanckenhorn 2007). In this chapter the relationship between fitness and threshold was investigated using three different measures: the rate of population increase, (*r*); carrying capacity, (*K*); and selection coefficients from competition experiments (*s*). Both *r* and *K* are traditional metrics based on established life history theory (Pianka 1970; Stearns 1977), but *s* provides a more realistic indicator of fitness in competition under frequency and density-dependent competition (Kawecki et al. 2012). Comparing the relationship between the maturation threshold and different fitness measures for multiple genotypes may provide clues about the evolution of body size more generally.

5.1.1 The evolution of body size

The evolution of body size, or size at maturity, is a fundamental puzzle for life history theory (Blanckenhorn 2000). Size at maturity is typically correlated with fitness (Roff 2002), but an increase in size usually comes at the cost of increasing development time, or age at maturity (Roff 2000), as selection for one appears to be in opposition to selection on the other (Schluter et al. 1991). Furthermore age and size at maturity are both phenotypically plastic traits that are likely to vary in different environments and along different growth

trajectories (Stearns & Koella 1986). The key to explaining the trade-offs between age and size at maturity is to consider the underlying mechanisms that produce these phenotypes (Davidowitz et al. 2005; Berner & Blanckenhorn 2007). For example, in the tobacco hornworm moth, *Manduca sexta*, evolutionary change in adult body size is contingent on variation in three developmental traits, including the critical weight, i.e. the maturation threshold (D'Amico et al. 2001). The critical weight of *M. sexta* varies between populations (Nijhout et al. 2010), and evidence of a heritable basis to maturation thresholds in other organisms, such as the Atlantic salmon *Salmo salar* (Piché et al. 2008; Skilbrei & Heino 2011) suggests that maturation thresholds can evolve. Investigating how developmental traits such as the maturation threshold respond to selection pressures is essential to understanding the evolution of reaction norms for age and size at maturity.

The consequences of variation in body size have been the subject of considerable study in zooplankton, including several species of *Daphnia* (Brooks & Dodson 1965; Hall et al. 1976; Jones & Jeppesen 2007; Hart & Bychek 2010). The size efficiency hypothesis (SEH) proposed by Brooks and Dodson (1965) suggested that zooplankton body size was caught between the two selective pressures of predation (Brooks & Dodson 1965; Galbraith Jr 1967; Hart & Bychek 2010), and competition. The influence of predation on body size is well understood. Visual predators such as fish select for smaller body-size (Galbraith Jr 1967; Tessier et al. 1992), while gape-limited predators such as the phantom-midge larvae *Chaoborus* select for larger body size (Beckerman et al. 2007). The presence of predators often induces plastic variation in body size (Stibor & Lüning 1994; Stibor & Müller-Navarra 2000), suggesting that developmental traits are responding to this selective pressure (Beckerman et al. 2010).

In the absence of predators, the SEH predicts that competition for resources will favour larger individuals, due to reduced costs of metabolism (Threlkeld 1976) and subsequent ability to maintain stable populations under lower resource levels (Tilman 1982), but the importance of body size in determining competitive ability of cladocerans and zooplankton is still a contentious issue (Hart & Bychek 2010). Some studies comparing cladoceran species of different sizes have found support for a competitive advantage of large size (Gliwicz 1990; Achenbach & Lampert 1997; Kreutzer & Lampert 1999; Dawidowicz & Wielanier 2004), but others suggest that large species only enjoy this advantage when resources are plentiful (Romanovsky & Feniova 1985; Tessier & Goulden 1987; Tessier & Woodruff 2002; Pereira & Gonçalves 2008). The limited studies comparing genotypes of the same species have found only weak evidence that larger genotypes are more competitive (Tessier et al. 2000; Pereira & Gonçalves 2008). The relationship between competitive ability and body size may be contingent on other factors, such as temperature (Giebelhausen

& Lampert 2001; Rinke & Petzoldt 2003), or competitive ability may be more closely related to age at first reproduction than size (Van Doorslaer et al. 2009). To date, however, no study has looked at how the developmental basis of body size, i.e. the decision to mature, relates to fitness and competitive ability.

5.1.2 Difficulties in quantifying fitness

Understanding how maturation decisions evolve is complicated by the presence of plasticity in the decision to mature (Chapter 2; Chapter 3; Harney et al. 2012), as the size at which individuals initiate maturation is dependent on the environment. Furthermore the density of competitors plays a role in determining fitness (Mueller 1997), and, genotypic variation in plastic maturation decisions suggests that the frequency of different conspecific competitors could also influence fitness (Heino et al. 1998). Consequently, under these conditions the relationship between the maturation threshold and fitness is likely to be dynamic (Ernande & Dieckmann 2004) and difficult to quantify with a single metric.

In density-dependent populations under frequency-dependent selection, using traditional fitness measures such as the instantaneous *per capita* rate of population increase (*r*), or the net reproductive rate (R_0) is problematic. Whilst both of these parameters are intuitively linked to differential reproductive success and are relatively easy to measure, they make important underlying assumptions that limit their generality. Values of *r* assume that mortality and fecundity remain constant and are thus density-independent, while R_0 assumes that differences in generation time are unimportant (Brommer 2000). MacArthur (1962) explored population density effects in his examination of natural selection, and proposed that the carrying capacity (*K*), of a population could be used as measure of fitness, but difficulties remained in reconciling density-dependent and density-independent measures of fitness (Pianka 1970; Stearns 1976).

The framework for a unifying fitness concept was created with the application of game theory to evolutionary biology (Maynard-Smith & Price 1973), where the fitness of a phenotype is its frequency within the population. The phenotype with the highest fitness can be thought of as having an ‘evolutionary stable strategy’ or ESS (Maynard-Smith & Price 1973; Maynard-Smith 1982), where the ESS is defined as the strategy (*sensu* phenotype) that cannot be invaded by mutant strategies. The application of the ESS to ecological theory and the recognition that fitness is in constant dynamic feedback with the environment led to the development of invasion criteria (Metz et al. 1992; Ferrière & Gatto 1995) and adaptive dynamic models (Dieckmann & Law 1996; Dieckmann 1997; Heino et al. 1997; Roff 2008).

This approach explicitly considers how population density and frequency-dependent selection influence the relationship between organism and environment. Sophisticated adaptive dynamics models now enable the investigation of evolutionary stable life history strategies in response to changing environments (Childs et al. 2003, 2011; Ozgul et al. 2010, 2012).

5.1.3 Invasion and competition among asexual organisms

Parameterising ecological adaptive dynamic models is often difficult (Childs et al. 2011). When considering the ESS concept of fitness in asexual organisms, an alternative approach is to directly compete different genotypes against each other over multiple generations in microcosm experiments (Bell 1990, 1997; Kawecki et al. 2012). Over successive generations, selection will lead to genotypes with higher fitness replacing those with lower fitness (Capaul & Ebert 2003; Kawecki & Ebert 2004). Changes in the frequency of genotypes during competition experiments or experimental evolution can be used to derive selection coefficients (*s*) that describe the relative success of each genotype in a population compared to the others (Salathé & Ebert 2003; Chevin 2011), providing an inclusive measure of fitness for that environment. Competition experiments which also quantify genotype-specific fitness through metrics such as *r*, R_0 and/or *K*, have found discrepancies between competitive ability and such traditional fitness values (Vanni 1987; Capaul & Ebert 2003), highlighting the importance of considering the density-, frequency-, and state-dependence of fitness. Competition experiments are also a useful tool for comparing the effects of different environmental pressures on fitness; in different environments different genotypes may prevail (Capaul & Ebert 2003; Koskella & Lively 2009) or the rate of change in frequency may differ (Stomp et al. 2008).

Daphnia are an ideal organism for competition experiments, because of their parthenogenetic reproduction. Previous experimental evolution and competition experiments have investigated whether different genotypes of *Daphnia* differ in their parasite resistance (Capaul & Ebert 2003; Zbinden et al. 2008), or if parasite resistance reduces competiveness (Little et al. 2002). Other experiments have tested costs of inbreeding (Salathé & Ebert 2003) or charted microevolutionary responses to temperature (Van Doorslaer et al. 2009, 2010), fish predation (Orsini et al. 2012) and anthropogenic stressors such as pesticides (Jansen et al. 2011). A number of different methodologies can be used to compete genotypes against one another, including small scale paired experiments against ‘tester’ clones (Little et al. 2002; Salathé & Ebert 2003) and large scale competition in semi-natural environments involving hundreds of genotypes followed by life history assays in isolation (Van Doorslaer

et al. 2009). Alternatively, changes in the frequency of multiple genotypes can be monitored if the competitors can be distinguished by polymorphic microsatellite markers or allozymes (Little et al. 2002). A small subset of individuals can then be removed periodically from competing populations, and genotyped to monitor changes in clone frequency and determine values of *s*.

5.1.4 Relating fitness to the maturation threshold

In this study the fitness consequences of the maturation threshold were investigated using five clones of *Daphnia magna*. Fitness was quantified by measuring values of *r* and *K* in isolation, and by carrying out mixed clone competition experiments under stable or disturbed population dynamics, from which values of *s* were derived. Values of *r*, *K* and *s* were related, by covariation or correlation, to PMRN sizes obtained in Chapter 2. Because the PMRN was phenotypically plastic, two size values were used, corresponding to early and late maturation. Investigating the relationship between threshold and fitness under such a broad range of conditions (density- and frequency-independent, and density and frequency-dependent) may expose reasons for the maintenance of heritable variation in this important developmental trait.

5.2 Materials and Methods

5.2.1 Experimental system

Five laboratory clones of *Daphnia magna* were used in this study. These were clones DKN 1-3, Ness1, H01, B5 and B7, and were the same clones used in Chapter 2 (see Chapter 2.2.1 for geographic origins). *D. magna* were maintained under standard conditions and acclimated for three generations as described previously (Chapter 2). For both experiments, *D. magna* were fed on a diet of 100 cells μl^{-1} *C. vulgaris* every two days. This diet was lower than in previous experiments (Chapter 2; Chapter 3) to reduce the chances of sexual stages (males and ephippia) being produced under crowded conditions (Olmstead & Leblanc 2001).

5.2.2 Estimating r and K

An overview of the experimental design is provided in Figure 5.1. Parthenogenetic rate of increase (*r*) was estimated by regular counts of individuals in expanding populations. For each clone, five neonates were used to found six (Ness1) or eight (DKN1-3, H01, B5 and B7) replicated populations in 450ml jars of media. Jars were checked every day, and once individuals within each population were seen to be reproducing (after 10 and 12 days), populations were counted every two days for 18 days (i.e. up to day 28-30). Populations were also counted once a week for six further weeks to provide an estimate of carrying capacity (*K*). Counts were carried out by slowly straining media through a fine mesh filter and counting individuals on the mesh. The mesh was kept damp at all times to prevent individuals from drying. Media was topped up when counts were carried out, and individuals were transferred to fresh media every two weeks.

5.2.3 Competition trial set up and sampling

Competition trials were carried out in 16 5-litre tanks over a 16-week period. Populations were founded with 84 individuals; 12 (4 adult, 4 adolescents and 4 juvenile) from clones B5, B7 and H01 and 24 (8 adult, 8 adolescents and 8 juvenile) from clones Ness1 and DKN1-3. These clones derived from three (B5, B7 & H01) or six (Ness1 & DKN1-3) monoclonal 5-litre tanks maintained at experimental conditions for 4 months prior to the experiment. Starting numbers were uneven because both Ness1 and DKN1-3 were originally thought to represent two distinct genotypes (but analysis of 14 microsatellite markers revealed this not to be the case, see section 5.2.4).

Competition trials were run for a total of 16 weeks, and each treatment was replicated eight times. Tanks were subject to one of two treatments. In ‘steady’ tanks population density was relatively stable, whilst ‘perturbed’ tanks suffered periodic destabilization. The treatment was imposed through the sampling strategy applied. The number of individuals sampled over the course of the experiment did not differ between treatments, however the steady tanks experienced regular sampling of a small number of individuals (5 individuals per week), while perturbed tanks experienced less frequent sampling of a greater number of individuals (20 individuals at the end of a 4 week period). To assess the effect of treatment on population density, full population counts of all tanks were carried out at the end of the 16-week experiment.

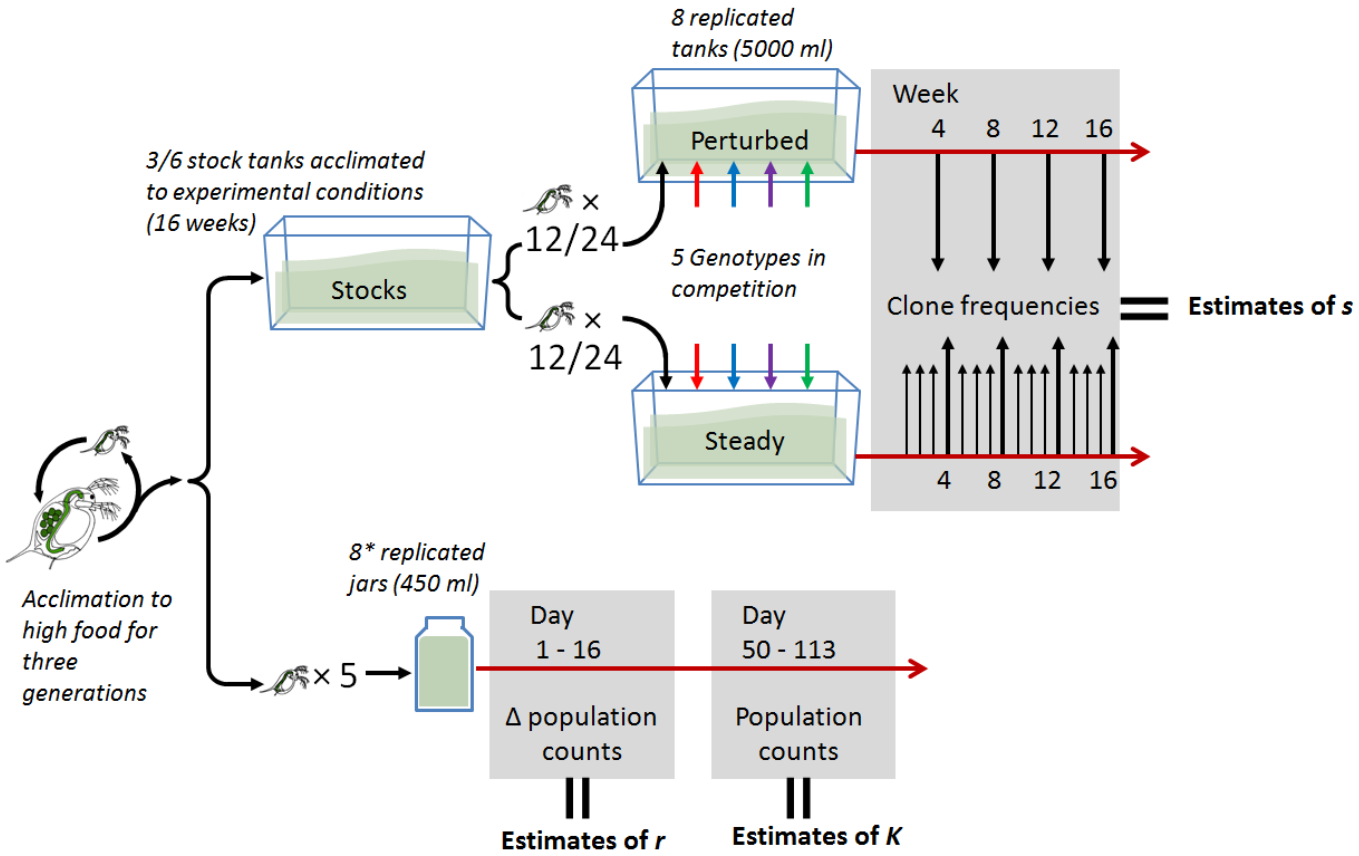

Figure 5.1. Experimental design for estimating *r*, *K* and *s*. For each of the five genotypes, estimates of *r* and *K* were obtained by regularly recording population sizes in eight* 450 ml jars (* except Ness1: only six jars). Values of *s* were estimated from direct competition. Following a period of acclimation, 12 (B5, B7 and H01) or 24 (Ness1 and DKN1-3) individuals of each and every genotype were introduced to 5-Litre tanks under steady or perturbed conditions (eight replicates of each) and competed for 16 weeks. Estimates of *s* for each clone in each tank were obtained from changes in clone frequency.

Tanks contained 5 litres of artificial pond water media and were rotated back-to-front and their position within the CT-room was altered on feeding days to reduce position effects. Plastic lids were placed on the tanks to reduce evaporation, and tanks were topped up with media on a weekly basis. All individuals within a population were transferred to clean tanks with fresh media every 4 weeks. Individuals were transferred by straining media from old tanks through the fine mesh filter and then immersing the mesh in the fresh media.

To ensure random sampling, tanks were stirred for 20 seconds and the fine mesh filter was passed through the tank. The filter was upended and *Daphnia* were removed and placed in a watch-glass. Stirring, filtering and removal was repeated until five (steady treatment) or twenty (perturbed treatment) individuals were in the watch-glass. To reduce sampling bias individuals on the mesh were systematically selected from the left-hand edge, moving right. Individuals caught in the filter but not required were immediately placed back in their tank. Once all individuals were isolated on the watch-glass, excess media was removed and *Daphnia* were transferred to an individual 1.5 ml micro-centrifuge tube. Samples were frozen at -80°C until DNA extraction

5.2.4 Competition trial genotyping

DNA extractions of individual *Daphnia* were carried out using Chelex ® resin (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) and proteinase-K (pK). Stock chelex solution was made by mixing 5g of Chelex powder in 100 ml DN-ase free H_2O and autoclaving, and pK aliquots were created by dissolving 20 mg pK in 1 ml H_2O. A mastermix of 50µl chelex and 1µl pK per *Daphnia* was made up, and 50 µl dispensed into each micro-centrifuge tube containing an individual *D. pulex*. Samples were crushed with a sterile pestle and incubated in a water bath at 56°C for three hours. Samples were then digested at 90°C for 15 minutes and put on ice. Once cooled, they were centrifuged for 1 minute, and the supernatant was used as the template for subsequent polymerase chain reaction (PCR).

Genotypic differences were assessed by polymorphic microsatellite markers. Initially 14 *D. magna* markers were tested. Markers 3617, 4276, 7001, 8344, 8397, 8693, 8608 are described in Colson et al. (2009), while markers DMA11, DMA12, DMA14, A055, B114, B222 and B231 are described in Routtu et al. (2010). Markers DMA12 and A055 were selected for use in genotyping, as they were polymorphic for all five clones, and produced consistent results during testing. Two markers were used to provide redundancy should one fail at the PCR or genotyping stage. Forward primers of these markers were fluorescently labelled with 6-FAM and NED (DMA12) or PET and VIC (A055). Using four different

fluorescent primers allowed both markers of two samples to be genotyped simultaneously. Template and primer were amplified using PCR; each reaction consisted of 1µl of template DNA, 0.3µl of fluorescently-labelled forward, and 0.3µl of reverse primers (both at 20pmol μl^{-1}), 1.0µl of bovine serum albumin (10 mg ml^{-1}), 5.0µl of 2×Biomix^{TM} Red (Bioline Reagents Ltd., London, UK) and 2.4µl of RN-ase free H_2O, for a total volume of 10µl. After initial denaturation at 94°C for 180 seconds (s), reactions were cycled 35 times through 94°C for 30s, 56°C for 30s and 72°C for 45s, with a final extension at 72°C for 300s. To confirm that the PCR had been successful, electrophoresis of every eighth sample was carried out on 1.5% agarose gel with ethidium bromide using Hyperladder^{TM} I (Bioline Reagents Ltd., London, UK) as a DNA standard and positive control.

Fluorescently labelled fragments were detected using the Applied Biosystems 3130*xl* Genetic Analyzer. A mixture of 0.5µl PCR products was dispensed into a 96-well optical reaction plate; the mixture contained four PCR products from two DNA samples; one DNA sample labelled with 6-FAM (DMA12) and PET (A055), and one with NED (DMA12) and VIC (A055). Samples were stabilised with 9.5µl formamide containing the size standard GS500-LIZ. Samples were denatured at 94°C for 180s before being run through an Applied Biosystems 3130*xl* Genetic Analyzer. Fragment lengths were interpreted using GeneMapper v4.0 (Applied Biosystems Inc) and a clonal identity was assigned to each fragment.

5.2.5 Statistical Analyses I: estimating r *and* K

To test for clonal differences in rate of population increase (*r*), a linear mixed effects model was fitted to the data for logged population sizes up to day 16. The model contained the covariate time, clone and their interaction as fixed effects and jar identity as a random variable. Individual values of *r* for each jar were required for covariation analysis with the threshold. In order to achieve this, a second model was fitted to the data for population sizes up to day 16. This model was much like the first, but included jar identity as a categorical explanatory variable, and *r* values were estimated from slope coefficients (i.e. the log(time) × identity interactions) from this model.

To assess whether jars had reached a stable population size and test for clonal differences in *K*, a linear mixed effects model with time, clone and their interaction as fixed effects and jar identity as a random variable was fitted to the data for population sizes from day 50 to 113. Models were simplified through term deletion and model comparison was used to assess the importance of individual terms. As with *r*, individual values of *K* for each jar were required for covariation analysis with the threshold. A second linear model was fitted to population

size data from day 50 to 113, with jar identity as the sole explanatory variable. Intercept values for each jar provided estimates of *K*.

5.2.6 Statistical Analyses II: selection coefficients

The effect of treatment on population density was investigated using a one-way ANOVA on final population counts. To aid visual and statistical comparison of changes in clone frequency, weekly clone counts for each steady treatment population were pooled at the end of each 4 week period. At the end of each of these 4 week periods, an odds ratio was produced by expressing the number of each clone present as a fraction of the total for that 4 week period. This 'odds ratio' was analysed by using a generalised linear mixed effects model (GLMM) with binomial errors (Crawley 2007). Separate GLMMs were carried out for each clone to investigate whether frequency changed over time and whether there was an effect of treatment. Treatment (a factor with two levels: steady and perturbed), time (a covariate) and their interaction were included as fixed effects, and tank identity was included as a random variable. Models were simplified through term deletion and model comparison was used to assess the importance of treatment through its interaction with time.

Selection coefficients (*s*) were calculated for each clone within each tank as the sum of the differences in logged clone frequencies (p) over the number of time points (up to four) in which clones were observed (modified from Chevin 2011):

$$s = \sum_{i=1}^{4} \frac{\Delta \ln(p_i)}{\Delta \mathrm{t}}$$

Problems estimating *s* arise if clone frequencies drop to zero. Therefore, if clones were not observed for 2 or more consecutive time points up to and including the final time point at 16 weeks, they were assigned an arbitrary value of p = 0.01 for the first time point in which they were absent, and subsequent time points were ignored. If clones were observed in all but the final time point, they were assigned a value of p = 0.01 and all time points were included in calculation of selection coefficients.

For each clone, a linear mixed effects model with treatment, time and their interaction as fixed effects and tank as a random effect was fitted to the odds ratio data for clone

frequency. The presence of a significant interaction was used as evidence of a treatment effect on clone frequency.

5.2.7 Statistical Analyses III: relating maturation thresholds to fitness indicators

Values of *r* and *K* for each jar and values of *s* for each clone in each tank were related to two clone specific maturation threshold sizes. These sizes corresponded to thresholds experienced by clones that matured early, under high food, and those that matured late, under low food. Size values were selected from a list of 50th percentile PMRNs sizes from the growth trajectories simulated in Chapter 2. These 'early' and 'late' threshold values were chosen by visually inspecting where simulated threshold sizes coincided with the early and late boundaries of actual growth trajectories (coloured circles in Fig. 5.2; Table 5.1); although extremely slow growing individuals were ignored when selecting late threshold sizes. For *r* and *K*, a linear regression against threshold sizes was carried out. For *s*, data was extremely negatively skewed, therefore values of both the selection coefficient and the threshold were ranked and a Spearman rank correlation was performed. Separate correlations were carried out for selection coefficients from steady and perturbed treatments for both early and late maturation threshold values.

5.3 Results

5.3.1 Estimation of r *and* K

There was a significant difference in mean rate of population increase (*r*) between clones (clone × time interaction; $\chi^2 = 16.17$, df = 4, $p = 0.0028$). Predicted growth curves overlaid on logged population size for replicated populations of each clone are shown in Figure 5.3A-E. Mean population growth rates are also presented (Fig. 5.3F).

There was a significant effect of clone on population size using data collected after 50 days ($\chi^2 = 18.63$, df = 4, $p = 0.0009$). Time was not a significant covariate ($\chi^2 = 2.4028$, df = 1, $p = 0.1211$) suggesting that these populations had reached carrying capacity (*K*). Estimates of *K* for individual replicated populations of each clone are presented in Figure 5.4A-E, and mean values are shown in Figure 5.4F.

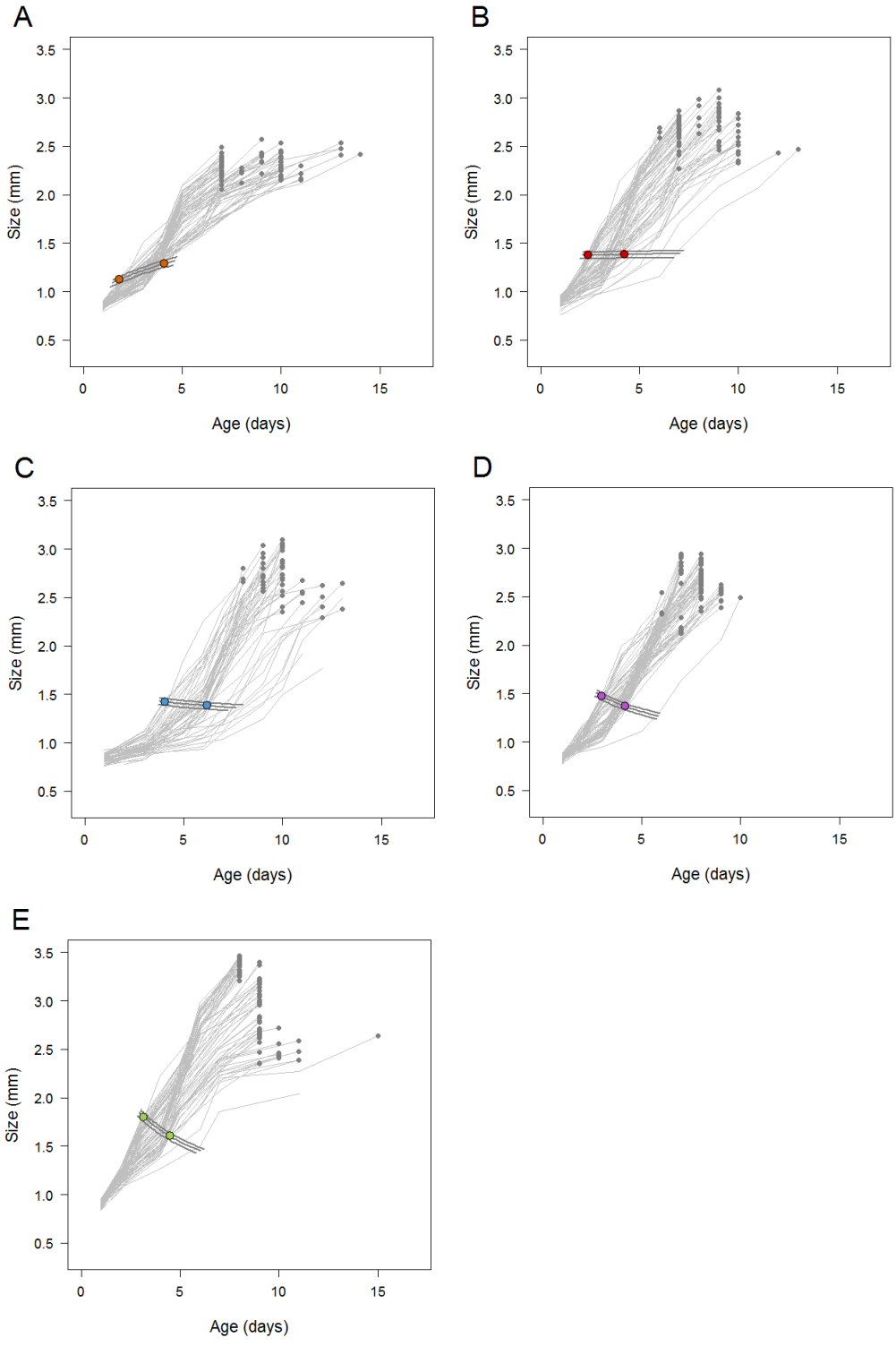

Figure 5.2. Maturation thresholds for five *D. magna* clones: A) H01; B) DKN1-3; C) B5; D) Ness1 and E) B7. For each clone two values of maturation threshold size were selected; 'early' threshold sizes corresponded to those experienced by individuals in high food, while 'late' threshold sizes corresponded to individuals in low food environments. Threshold sizes were selected from a list of 50th percentile PMRNs under simulated growth trajectories (see chapter 2) by visually inspecting where simulated sizes coincided with actual growth trajectories (values given in Table 5.1).

Table. 5.1. Maturation threshold values for *D. magna* clones H0, DKN1-3, B5, Ness1 and B7. For each clone two values of maturation threshold size were selected, corresponding to growth in high food (early) and low food (late) conditions (see section 5.2.6 and Fig. 5.1 for selection criteria of these values).

Clone	Early threshold size (mm)	Late threshold size (mm)
B5	1.425	1.385
B7	1.801	1.604
DKN1-3	1.379	1.383
H01	1.124	1.292
Ness1	1.476	1.368

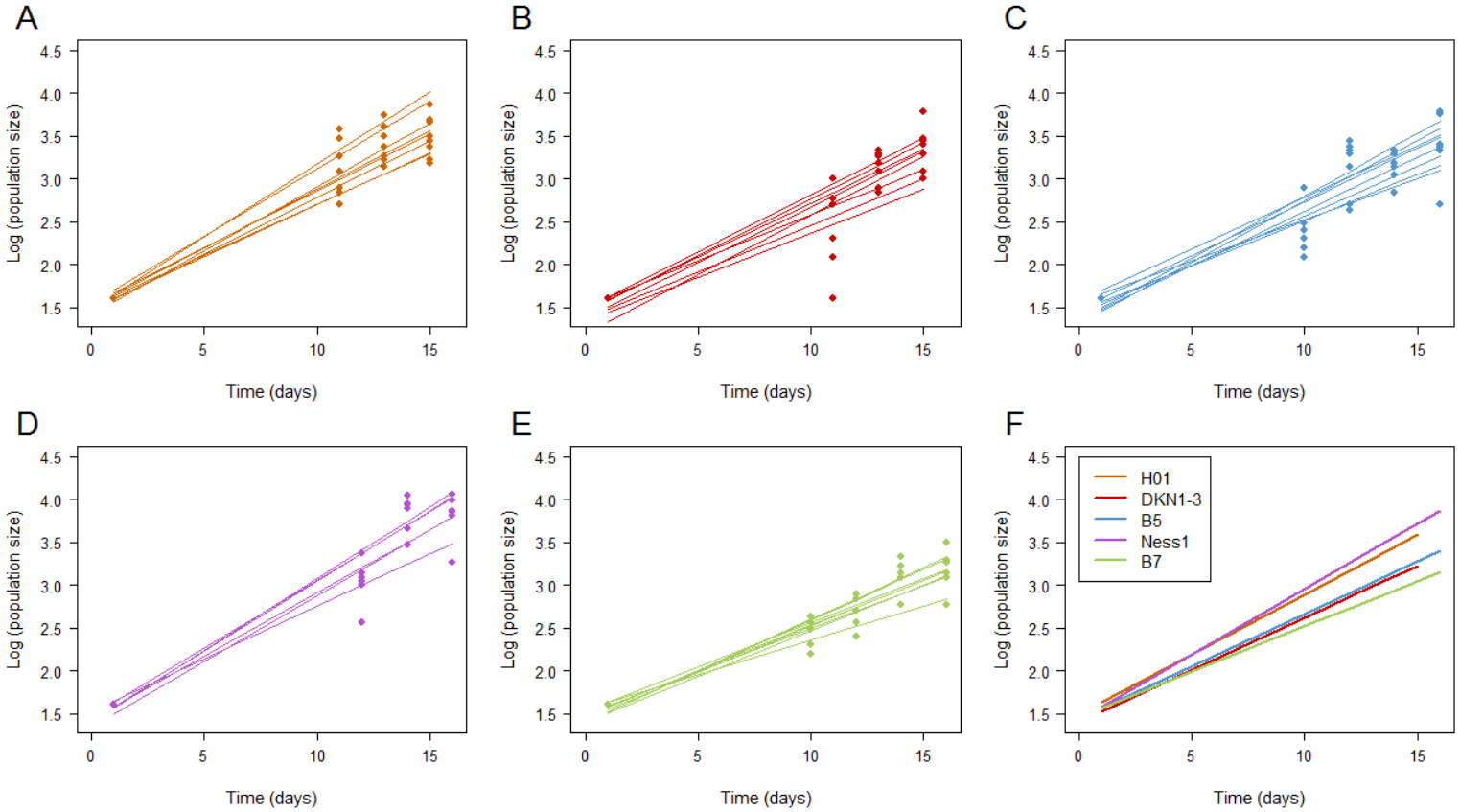

Figure 5.3. Population growth of five *D. magna* clones: A) H01; B) DKN1-3; C) B5; D) Ness1 and E) B7. Replicated populations were founded with 5 neonates (day 1) and fed on a diet of 100 cells μl^{-1} *C. vulgaris* every two days. Populations were counted every two days from the appearance of neonates (day 10,11 or 12). For each population, linear regressions of logged values of population size (up to day 16) against time have been overlaid. Slope coefficients were used as estimates of *r* and were correlated with threshold sizes in subsequent analyses (see section 5.3.3; Fig. 5.7). In F), mean growth curves using data from all populations are presented for all clones.

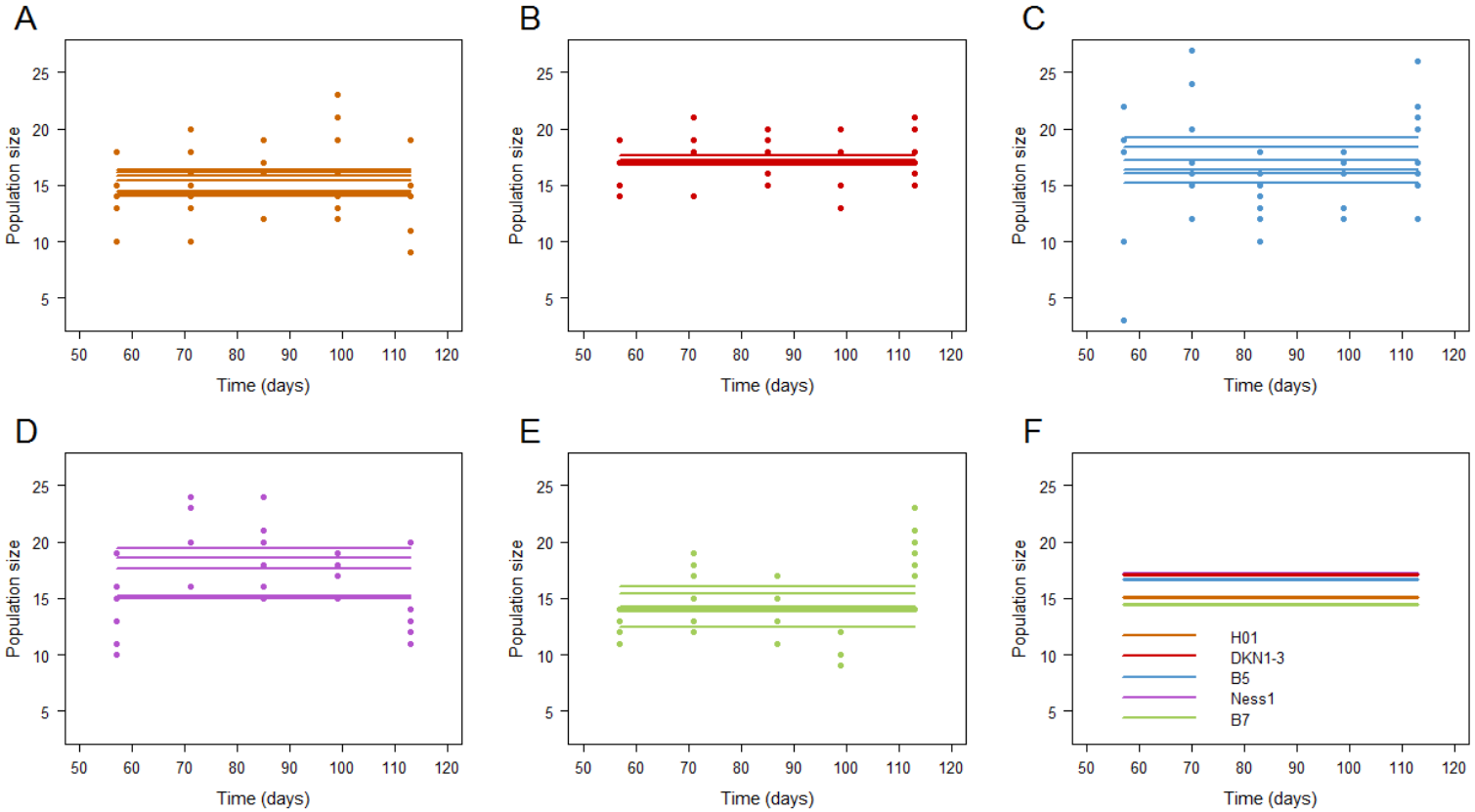

Figure 5.4. Carrying capacity of five *D. magna* clones: A) H01; B) DKN1-3; C) B5; D) Ness1 and E) B7. Populations used to determine rate of population increase (see Fig. 5.3) were allowed to reach carrying capacity. Populations were counted every 2 weeks and counts between day 50 and 120 were used to determine clonal carrying capacity. Intercept values were used as estimates of *K* and correlated with threshold sizes in subsequent analyses (see section 5.3.3; Fig. 5.7). In F), mean population size for each clone using data from all populations.

5.3.2 Competition trials: effect of treatment and changes in clone frequency

There was no effect of treatment on final population counts (F = 0.0872, df = 1, p = 0.7721), suggesting that the treatment did not exert a strong influence on population density. However, during the course of the experiment mean clone frequencies changed in all clones (Fig. 5.5; Fig. 5.6; Appendix Fig. A5.1), and treatment influenced this change in clones DKN1-3 (treatment × time interaction: χ^2 = 5.314, df = 1, p = 0.0212), Ness1 (χ^2 = 18.110, df = 1, p < 0.0001) and B7 (χ^2 = 10.385, df = 1, p = 0.0013), but not clones H01 (χ^2 = 2.862, df = 1, p = 0.0906) or B5 (χ^2 = 0.449, df = 1, p = 0.503). Only clone Ness1 went 'extinct' in all tanks before the end of the experiment: by week 12 in steady treatment tanks and week 8 in perturbed treatment tanks. The mean frequency of clone B5 declined to less than 0.05 by week 16 in both treatments. Mean frequencies of clones H01, DKN1-3 and B7 increased across both treatments. However, as Figure 5.5 and Figure 5.6 show, increases in frequency for these three clones were complex and dynamic, not monotonic.

5.3.3 Relation of fitness traits to the maturation threshold

The rate of population increase (r) was negatively related to both early threshold size (Fig. 5.7A: F = 10.487, df = 1, p = 0.0026) and late maturation size (Fig. 5.7B: F = 17.771, df = 1, p = 0.0002). Clonal genotypes that initiated maturation at smaller sizes were able to produce more offspring over the course of 16 days than those with larger maturation thresholds. K did not relate to early threshold size (Fig. 5.7C: F = 1.126, df = 1, p = 0.2957), but displayed a negative relationship with late threshold size (Fig. 5.7D: F = 4.446, df = 1, p = 0.042). The late maturation threshold (estimated from individuals under low food) is a better predictor of K, and clones with larger thresholds tended to have smaller population sizes. Ranked values of the selection coefficient and early maturation threshold size were negatively correlated in steady treatment tanks (Fig. 5.8A: r_{SP} = -0.4173, p = 0.0074) but selection coefficients did not rank with late threshold size in steady tanks (Fig. 5.8B: r_{SP} = 0.0307 p = 0.8506), or either estimate of maturation threshold size in perturbed tanks (early maturation threshold; Fig. 5.8C: r_{SP} = -0.1683, p = 0.2993; late maturation threshold; Fig. 5.8D: r_{SP} = 0.199, p = 0.2183).

5.4 Discussion

The threshold size in *Daphnia magna* was negatively related to the rate of population increase (r), and the population carrying capacity (K). The presence of a significant negative

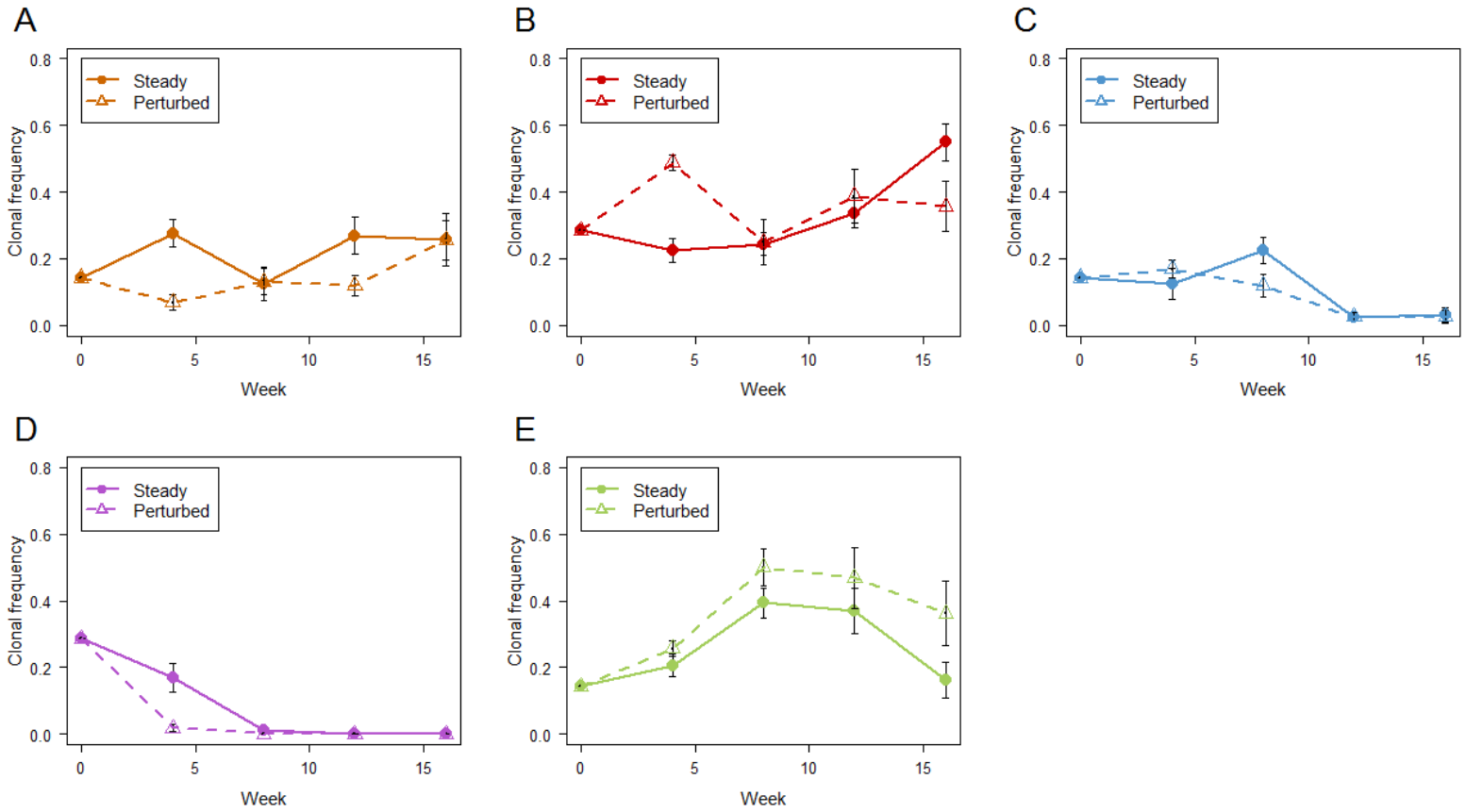

Figure 5.5. Mean changes in clone frequency for five *D. magna* clones in steady and perturbed treatments: A) H01; B) DKN1-3; C) B5; D) Ness1 and E) B7.

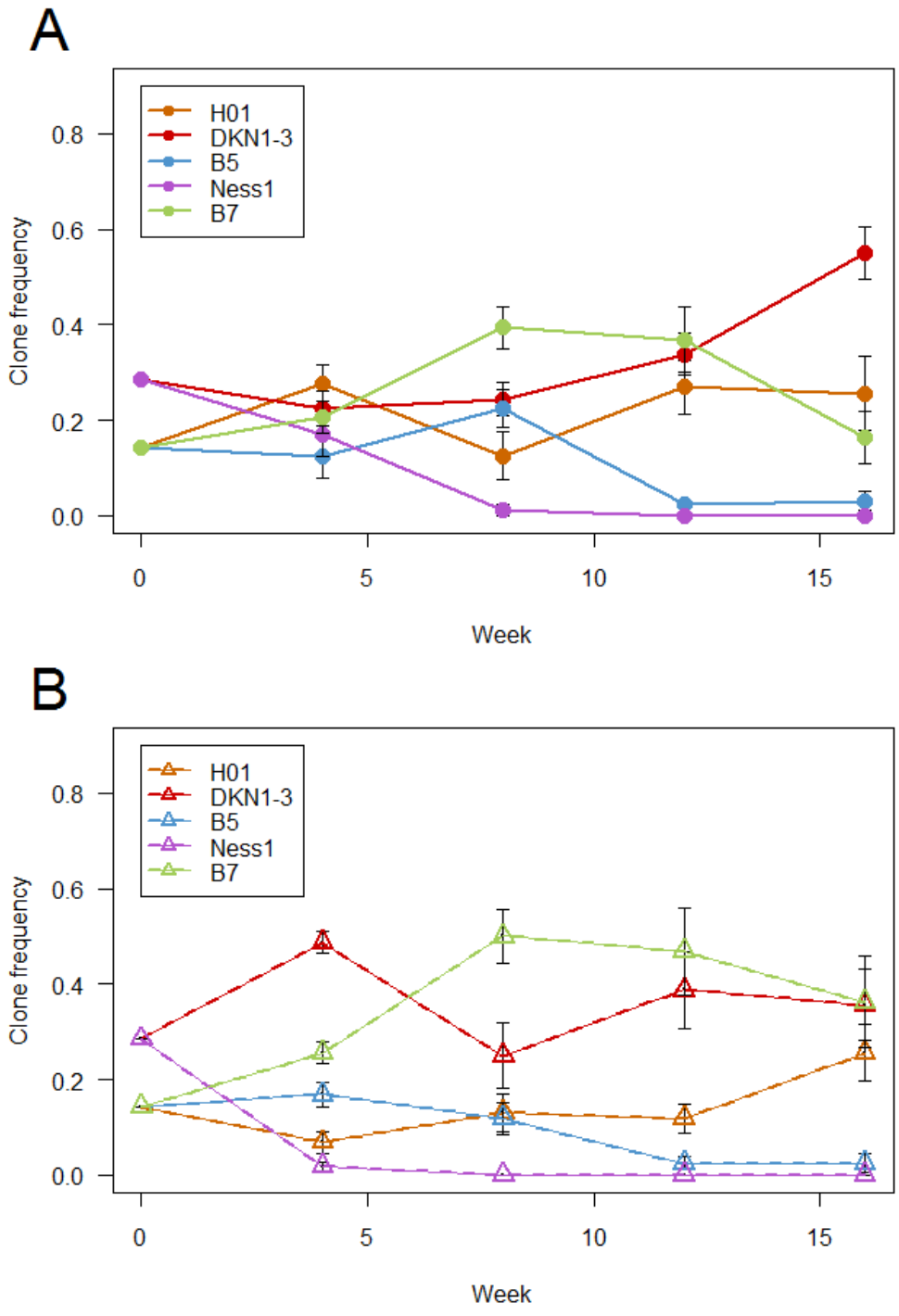

Figure 5.6. Mean changes in clone frequency between A) steady and B) perturbed treatments.

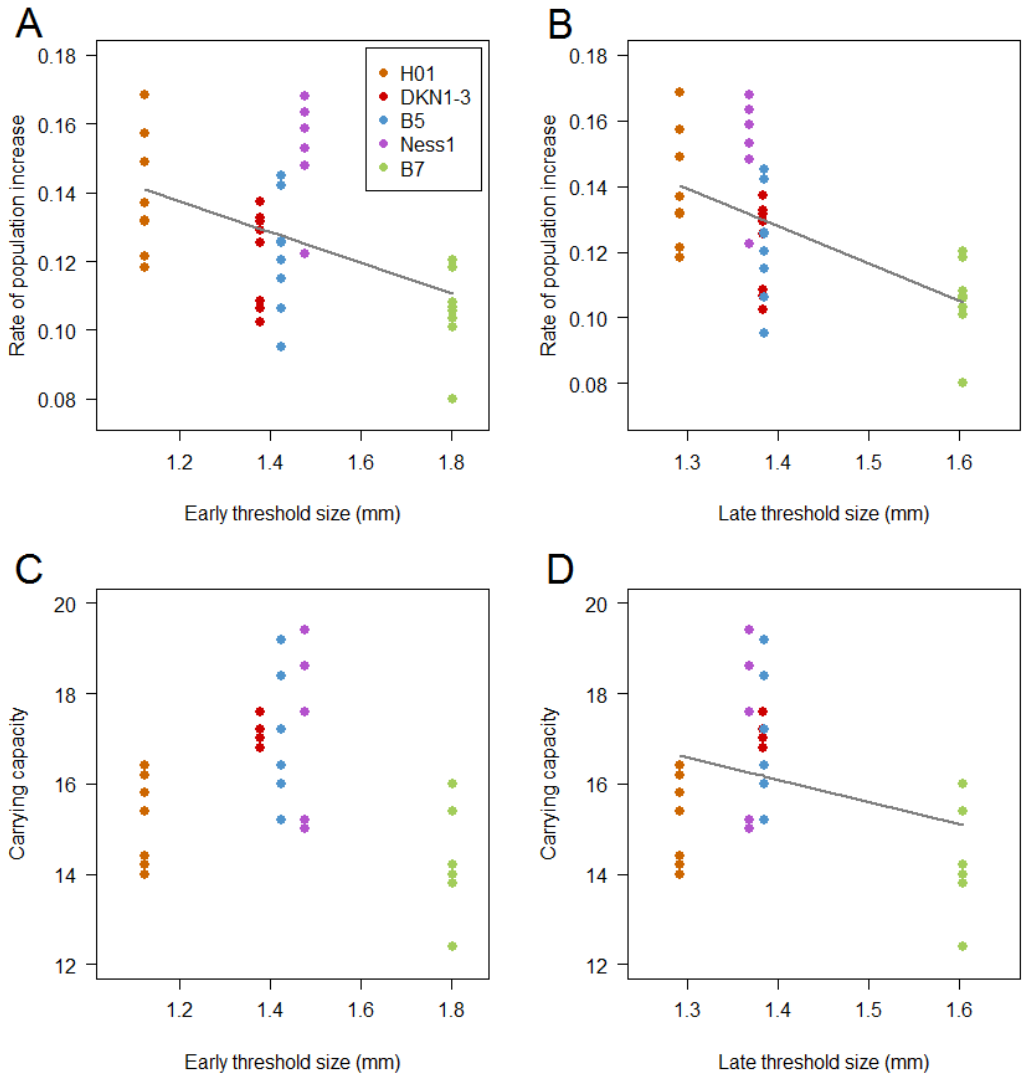

Figure 5.7. Relationships between clone-specific maturation threshold sizes and rate of population increase (A and B) and carrying capacity (C and D). Plots A and C correspond to early maturation threshold sizes, plots B and D to late maturation threshold sizes. Significant correlations in A, B and D are shown by grey lines.

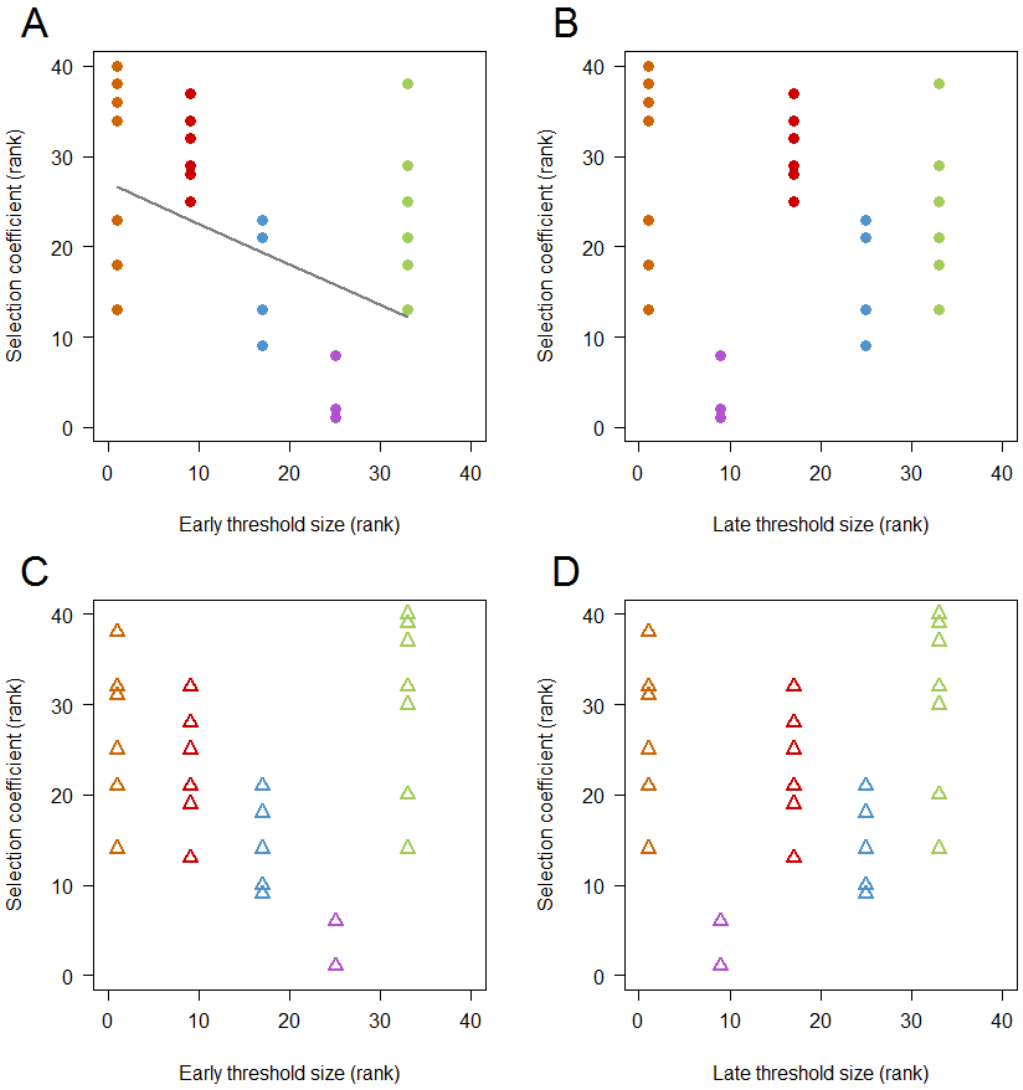

Figure 5.8. Correlations between ranked clone-specific maturation threshold sizes and ranked selection coefficients in steady tanks (A and B; closed circles) and perturbed tanks (C and D; open triangles). Plots A and C correspond to early maturation threshold sizes, plots B and D to late maturation threshold sizes. See Fig. 5.7 for legend relating colours to clones. A significant correlation in A is shown by the grey line in that panel.

relationship between threshold size and *r* suggests that initiating maturation at smaller sizes could be beneficial for clones in density-independent conditions. The negative relationship between threshold size and *K* confirms that genotypes with smaller thresholds also form larger stable populations, but smaller threshold size did not necessarily equate to success in the mixed clone competition experiments. These experiments did produce repeatable results, with two genotypes consistently either disappearing or declining to low frequencies across all 16 tanks; however the outcomes of competition experiments (estimates of the selection coefficients, *s*) did not generally relate to threshold size. There was a significant negative correlation between *s* and early threshold size in steady treatments, suggesting that clones that initiate maturation at smaller sizes may continue to have an advantage under density- and frequency-dependent competition, but this result must be interpreted with caution, as *s* did not correlate with late maturation threshold size in either treatment. The results of these experiments must be interpreted with caution, as only five genotypes were compared, but there is a suggestion that genotypes with smaller maturation thresholds have higher fitness under density-independent conditions. In more competitive environments where density- and frequency-dependent conditions operate, fitness may be better predicted by age and size at maturity than the maturation threshold. In this case, the interactions between threshold size and other factors during development (such as variation in individual growth rate) may determine fitness.

5.4.1 The maturation threshold may be important in density-independent conditions

The fitness trait that had the strongest relationship to maturation threshold size was *r*. The negative covariance between *r* and both early and late maturation threshold size (Fig. 5.7A & Fig. 5.7B) indicates that in density-independent conditions, clones that matured at smaller sizes had higher fitness. Although some previous studies of *Daphnia* have not found a strong relationship between body size and *r* (e.g. Vanni 1987; De Meester 1994), the results here are consistent with classic life history theory, which predicts that *r* will be maximised by maturing at smaller sizes (Pianka 1970; Stearns 1977), and genotypes will increase *r* when population density fluctuates or there is opportunity for rapid population growth. This is likely to be the case in *Daphnia* systems, which have been shown to display a variety of different population dynamics (McCauley et al. 2008), including unstable dynamics with large-amplitude cycles (McCauley et al. 1999). Furthermore, *D. magna* often inhabit ephemeral pools subject to stochastic environmental change and periodic drying (Herbert 1978; Koivisto 1995). Population dynamics with large-amplitude cycles and stochastic

environmental pressures could both result in numerous opportunities for rapid population growth, and this could provide the selective pressure for smaller thresholds.

5.4.2 No evidence for a competitive advantage of being large

There was also negative covariation between *K* and the late threshold, suggesting that larger clones had smaller populations at carrying capacity. This result is intuitive in the sense that a habitat with limited resources will support more individuals if they are smaller; however, larger species require fewer resources per gram body mass for metabolic maintenance (Threlkeld 1976) and could therefore form stable populations under lower resource levels (Tilman 1982), outcompeting smaller forms (Kreutzer & Lampert 1999). The idea that larger forms are more efficient at utilising scarce resources is a central tenet of the size efficiency hypothesis (SEH) in zooplankton (Brooks & Dodson 1965), but few studies have investigated resource-use efficiency from an intra-specific perspective; those that have indicate that body size is not of primary importance (Tessier et al. 2000; Pereira & Gonçalves 2008). The results of the competition experiments in this chapter are in agreement with these studies, and found no evidence for positive correlation between *s* and threshold size (Fig. 5.8). Indeed, the only significant relationship between *s* and threshold size was a negative correlation between *s* and early threshold size in the steady treatments (Fig. 5.8A). This result would suggest that small size is advantageous in competitive environments, contravening the predictions of the SEH, but must be interpreted with caution, as it is expected that maturation thresholds in densely populated tanks will resemble late maturation threshold estimates more than early maturation threshold estimates.

5.4.3 Competition did not reveal strong relationships between threshold and fitness

Overall, results from the competition experiments suggest that clonal differences in resource use efficiency were not closely related to clonal variation in the maturation threshold. Changes in clone frequency were, however, repeatable and broadly consistent across tanks. After 16 weeks of competition between all five clones there was no outright ‘winner’ in either treatment, but two of the clones were ‘losers’, with B5 reduced to low clonal frequencies and Ness1 completely excluded. Clones H01, DKN1-3 and B7 all increased their mean frequency during the course of the experiment. While competition experiments sometimes observe a single genotype that outperforms all others (Capaul & Ebert 2003; Stomp et al. 2008), many studies have found that competition does not result in a clear

winner (Vanni 1987; Little et al. 2002; Koskella & Lively 2009). Running the experiment for longer may have revealed an outright winner, but the presence of dynamic changes in clone frequency, such as the increase and decrease in B7 (Fig. 5.5; Fig. 5.6) suggests that complex frequency-dependent effects may also explain patterns of clonal diversity in competition among these clones.

If maturation threshold size alone is not an important factor in explaining competitiveness, what other factors are important? One possibility is that clones with higher individual growth rates enjoyed a competitive advantage. Although growth rates weren't measured in this study, it is interesting to note that growth of one of the poor competitors, B5 (Fig. 5.2C; data from Chapter 2) is noticeably lower than in other clones (Fig. 5.2). Variation in individual growth rates is an important part of explaining patterns of life history variation (Arendt 1997; Dmitriew 2011). This thesis has already identified genotypic variation in the plasticity of growth rates among *D. magna* clones; the ontogenetic approach adopted in Chapter 3 revealed that age and size at maturity were a product of genotypically and phenotypically variable maturation thresholds and growth rates. Understanding the interactions between growth and developmental traits, such as the maturation threshold, is an essential part of explaining how selection produces adaptive body size and development time (Davidowitz et al. 2005). Furthermore, conflicting selection between these processes may make it difficult to relate these traits directly to fitness, and age and size at maturity may remain better indicators of fitness (Nijhout et al. 2010).

5.4.4 What causes variation in maturation thresholds?

A clear result of the Chapters 2 and 3 of this thesis is that maturation thresholds are variable traits. They are genotypically variable and phenotypically plastic, responding to variation in their own environment and the environment of their parents. Maturation thresholds are expected to play an important role in the evolution of body size (Nijhout et al. 2010). One of the strongest selection pressures on *Daphnia* body size is thought to be competition (Brooks & Dodson 1965; Hall et al. 1976; Threlkeld 1976). Although the maturation threshold size did covary with values of *r* under density-independent conditions, the mixed clone competition trials suggested that threshold size was not a strong predictor of competitive success, and that the relationship between body size and fitness will depend on more than just resource-use efficiency (Pereira & Gonçalves 2008). The interaction between variable maturation thresholds and variable individual growth rates (Chapter 3; Dmitriew 2011) may

be important in relating body size to fitness (Nijhout et al. 2010), but other factors must be also considered.

Predation in particular, is thought to shape adaptive body sizes in *Daphnia* (Galbraith Jr 1967; Tessier et al. 1992; Hart & Bychek 2010). The direction of the selection varies depending on whether the predation is from visual predators or tactile gape-limited predators. Visual predators such as fish select for smaller body-size (Galbraith Jr 1967; Tessier et al. 1992), while gape-limited predators such as the phantom-midge larvae of the genus *Chaoborus* select for larger body size (Spitze 1991). Size selective predation can have a direct effect by selecting for larger or smaller size at maturity (e.g. Spitze 1991), but *Daphnia* can also mediate their response to size selective predation through plastic changes, adjusting their size at maturity to match the presence of predator cues (kairomones) in the environment. Thus in the presence of fish kairomones, *Daphnia* will reduce the size at which they mature (Stibor 1992; Stibor & Müller-Navarra 2000), whilst in the presence of *Chaoborus* kairomones, they will increase size at maturity (Tollrian 1995). Furthermore, although Daphnia may be locally adapted to the predation pressure they most frequently experience (Boersma et al. 1999), genotypes are often able to maintain responses to both these cues (Weider & Pijanowska 1993; Stibor & Lüning 1994; Beckerman et al. 2010). These plastic life history responses are created by decoupling growth and development (Beckerman et al. 2007). One mechanism by which size at maturity is increased or decreased in response to kairomones is through plasticity in maturation, as shown by the concurrent manipulation of food and predation by Beckerman et al. (2010), although it unclear in this experiment whether plasticity was as a result of plasticity in the maturation threshold or in growth. It seems likely that under different selective pressures, variation in both the maturation threshold size and plasticity in the threshold may be important for generating adaptive reaction norms for age and size at maturity.

5.5.5 Extensions of this study should incorporate ecological realism

The patterns this study found between the maturation threshold size and fitness are interesting, but must be interpreted with some caution. Only five clones were used in this study, a tiny number compared to the genetic diversity that is likely to exist in many ponds (Van Doorslaer et al. 2009). Furthermore, little is known of the ecology of these clones, but if they originate from diverse ecological backgrounds with variable selective pressures, differences in maturation thresholds may not reflect normal levels of variation in this trait. If this is the case, it is perhaps not surprising that maturation threshold size alone is not

indicative of fitness. A logical extension of the approach taken here is to compete clones with known life history traits from within a population against each other, and to relate fitness to multiple traits throughout ontogeny, from maturation threshold and individual growth rate through to age and size at maturity. Within population variation in life history traits can be substantial (De Meester & Weider 1999; Michels & De Meester 2004), and is likely to be underpinned by corresponding variation in developmental traits including the maturation threshold (Chapter 2; Harney et al. 2012). Such a comparison could thus reveal the fitness consequences of variation in the maturation process among naturally co-occurring genotypes. Furthermore, if the environment of the clones is well understood, naturally occurring conditions and ecologically realistic perturbations can be recreated.

5.5 Conclusion

This study found a negative correlation between the rate of population increase, *r*, and the size at which maturation was initiated. This suggests that in expanding populations, genotypes that initiate maturation at a smaller size are at an advantage. Under density-dependent and frequency-dependent competition, however, the outcome of competition trials may be better predicted by age and size at maturity than underlying traits such as threshold size or individual growth rate. The study did not impose a strong selective pressure on the populations of *D. magna*, but two clones were clearly at a competitive disadvantage, while three others increased their frequencies within populations. The discrepancy between traditional fitness measures and measures of clonal success from competition trials highlight the importance of using frequency- and density-dependent fitness measures and suggest that frequency-dependent dynamics may be important in determining clonal fitness.

Chapter 6

General discussion

Maturation is an important ontogenetic transition for many organisms, marking the transition from a period of larval or juvenile growth to the attainment of reproductive function (Bernardo 1993). However, maturation is not a simple switch from one life stage to the next, but is a complex and developmentally plastic process. One of the key underlying mechanisms is the maturation threshold, which sets a minimum state, usually size, for maturation (Wilbur & Collins 1973; Day & Rowe 2002). There is increasing awareness that the evolution of age and size at maturity is dependent on underlying traits, such as the maturation threshold (Davidowitz et al. 2005; Nijhout et al. 2010). The aim of this thesis was to understand how maturation thresholds evolve and influence subsequent reaction norms for age and size at maturity, using *Daphnia* as a model organism. A comprehensive approach to modelling maturation revealed that *Daphnia magna* Straus and *D. pulex* Leydig had maturation thresholds that more closely resembled a process with a rate, than a discrete switch. Thresholds varied between genotypes, both between- and within-populations (Chapter 2), and the environment (Chapter 2) and parental environment (Chapter 3) were found to modify the expression of the maturation threshold in a genotype-dependent manner. Parental effects influenced multiple traits during development, and context-dependent and genotype-specific outcomes resulted in a variety of adult phenotypes (Chapter 3). The proximate causes and timing of the maturation threshold were investigated in a microarray study comparing gene expression in *D. pulex* (Chapter 4), which identified a marked increase in expression of lipid transport proteins where the first investment in reproduction is predicted to occur (Zaffagnini 1987; Bradley et al. 1991). However, most patterns of gene expression suggested that maturation was more similar to a rate than a switch, corroborating the results of the PMRNs (Chapter 2; Chapter 3). Furthermore, a candidate for endocrine control of the threshold and a potential mechanism for epigenetic inheritance across generations were identified. The fitness consequences of variation in the maturation threshold were then investigated using traditional metrics such as the rate of population increase (r) and carrying capacity (K), and by direct competition (Chapter 5). Genotypes with small thresholds generally had higher values of r, but threshold size did not predict competitive success in mixed clone tanks, suggesting fitness in these artificial conditions is not determined solely by the size at which maturation begins. Using a multidisciplinary approach to improve our understanding of the maturation threshold has revealed that this is a

dynamic aspect of development that is responsive to the environment and potentially able to shape patterns of evolution in age and size at maturity.

6.1 What are maturation thresholds?

Maturation or ontogenetic progression often depends on reaching a minimum size threshold. Following Wilbur and Collins' (1973) seminal paper identifying maturation thresholds, numerous empirical studies described the presence of thresholds in a broad range of taxa. Day and Rowe (2002) formalised the Wilbur and Collins hypothesis using optimality models, and provided a framework for studying developmental thresholds and plasticity in age and size at maturity. However, few studies have investigated phenotypic plasticity in thresholds across environmental gradients, or the extent to which thresholds vary between genotypes.

Chapter 2 found that the threshold size for maturation in both *D. magna* and *D. pulex* was plastic across a resource gradient. This result contrasts with previous investigations of the maturation threshold in *D. magna* (Ebert 1992, 1994), but is in accordance with the plastic critical weight (analogous to a threshold) of the tobacco hornworm moth *Manduca sexta* (Davidowitz et al. 2003, 2004). Furthermore, the maturation threshold was also plastic in response to transgenerational food availability (Chapter 3). Clearly the maturation threshold is able to respond to a number of environmental signals, which begs the question: what other factors influence the maturation threshold? Plasticity in age and size at maturity has been observed in response to predation (e.g. Crowl & Covich 1990; Beckerman et al. 2010), and time of year (Nylin et al. 1989; Johansson et al. 2001) and plasticity in the underlying threshold is one possible explanation for this pattern.

Reaction norms for age and size at maturity that are underpinned by plastic thresholds may have important implications for the study of changing maturation schedules in fish stocks. The application of probabilistic maturation reaction norms (PMRNs) to fisheries data has been proposed as a tool to help disentangle phenotypic plasticity and genetic adaptation to harvesting (Heino et al. 2002; Dieckmann & Heino 2007; Heino & Dieckmann 2008). Many studies have subsequently determined that evolutionary change was the driving cause of phenotypic change. If, however, there is phenotypic plasticity in underlying maturation thresholds, interpretation of changing maturation schedules is more difficult. Furthermore, traditional methods for estimating PMRNs may suffer from bias if intervals between observations vary, or if maturation is more analogous to a rate than a switch (Van Dooren et

al. 2005). Further exploration of the evolutionary potential of these important traits is dependent on having appropriate methodological tools available to measure them.

6.2 How to model maturation

Reaction norms for age and size at maturity were initially modelled using optimality approaches (Stearns & Crandall 1981; Stearns & Koella 1986; Berrigan & Koella 1994; Sibly & Atkinson 1994), but these approaches do not account for the mechanisms underlying maturation, or the stochasticity that inevitably arises from development in heterogeneous environments. PMRNs based on logistic regression of age, size and other variables against probability of maturing were developed to interpret stochastic data from fisheries (Heino et al. 2002; Morita & Fukuwaka 2006; Dieckmann & Heino 2007) and disentangle the effects of genetic change and phenotypic plasticity on reduced size at maturity (Grift et al. 2003; Engelhard & Heino 2004; Olsen et al. 2004; Mollet et al. 2007; Heino & Dieckmann 2008). These models may, however, suffer from interval bias if the time between observations varies. In Chapter 2 this bias was accounted for by including an 'offset' (Lindsey & Ryan 1998; Collett 2003), correcting for interval length variation. A further limitation of the classic PMRN is that it does not consider how the underlying developmental changes may be involved in changing maturation schedules (Van Dooren et al. 2005; Marshall & Browman 2007; Wright 2007; Tobin et al. 2010).

In both Chapter 2, and Chapter 3, GLMs with offsets generally fitted the data better than the traditional PMRNs, suggesting that accounting for interval bias may be vital to successfully modelling maturation. In Chapter 2, analyses using the latest possible indicator of maturation (IM-3; the deposition of eggs in the brood chamber) were always improved by including age interval offsets. This indicator most closely resembles those used in other studies (e.g. Plaistow et al. 2004; Kuparinen et al. 2008; Uusi-Heikkilä et al. 2011), highlighting the problem faced by PMRNs that do not account for interval bias. The results of both Chapters 2 & 3 suggest that reaction norms for age and size at maturity may well be underpinned by plasticity in developmental thresholds and growth responses. These results could have profound implications for our understanding of evolution in fisheries if the difficulty of distinguishing genetic from plastic change is compounded by plastic responses during early development. Furthermore, little consideration has been afforded by fisheries scientists to the role of trans-generational plasticity in driving phenotypic change in maturation schedules; yet, parental effects on development can produce important phenotypic consequences for adults (Chapter 3). Clearly further efforts are required to

understand the proximate causes of maturation in fish, and the influence of parental effects on maturation. When physiological changes are not outwardly apparent, the causes and timing of maturation may be studied using a microarray (Chapter 4). Studying the role of parental effects on fisheries may require applying comprehensive modelling approaches, such as the those advanced here (Chapter 3), to model fish systems (Uusi-Heikkilä et al. 2011) over multiple generations. This could reveal whether trans-generational effects are in part responsible for the apparent long-term changes in commercial fish stock life histories.

More generally, the PMRN approach adopted in this thesis can be used to investigate maturation trends in numerous organisms. In *Daphnia*, it appears that maturation can be accurately predicted using age and size, but the generality of these findings in other systems remains to be seen; age and size, after all, are just proxies for underlying processes (Van Dooren et al. 2005; Marshall & Browman 2007; Heino & Dieckmann 2008). The methodology presented in Chapter 2, however, is a flexible one that can incorporate numerous factors or covariates. As such, it can be thought of as a framework for testing the importance of various factors that shape maturation in diverse biological systems.

6.3 The nature and timing of maturation decisions

Maturation is modelled as a stochastic process because the underlying developmental and physiological processes are often poorly understood. One system where ontogenetic decisions are well understood, however, is that of *M. sexta*. In this species, extensive endocrinological studies have revealed that body size and development time are controlled by a critical weight (developmental weight), the growth rate, and the interval between achieving critical weight and pupating (D'Amico et al. 2001; Nijhout 2003; Davidowitz & Nijhout 2004; Davidowitz et al. 2004). Knowledge of how these traits interact during development is essential to understanding the evolution of body size and development time in this species (Davidowitz et al. 2005; Nijhout et al. 2010).

As this thesis has shown, the maturation threshold of *Daphnia* can be phenotypically plastic within- (Chapter 2) and between generations (Chapter 3), and statistical analysis using PMRNs suggests that it resembles a process more than a discrete switch. In order to understand the nature of the process underlying the maturation threshold, microarray analyses can be applied to investigate changes in gene expression that precipitate or accompany the decision to mature. The microarray study in Chapter 4 found that changes in gene expression of maturing *D. pulex* were generally continuous, rather than abrupt. These

findings echo those of the PMRN analyses in Chapters 2 and 3: that the maturation threshold is more similar to a process with a rate than a discrete switch. Expression of one group of genes did, however, increase suddenly during the maturation process. An increase in vitellogenin (VTG) and other lipid-transport genes coincided with the predicted maturation threshold instar, IM-1 (Zaffagnini 1987; Bradley et al. 1991). Given its probable role in providing nutrients to oocytes, VTG may represent the first significant cost of maturation to developing *D. pulex*. The relatively sharp increase in expression of VTG is probably the reason for the increasing effects of growth plasticity following IM-1, as resource allocation decisions are exposed under resource limitation. This more discrete change in gene expression of VTG may also explain why the threshold is best modelled by GLMs with a logit-link. The nonlinear functional dependence on age and size implicit in the logit-link fits the data better if maturation has a step-like function, which may be the case if VTG production increases following the maturation decision.

The microarray study also identified a gene that may serve a regulatory role (Cytochrome P450 4C1), and an increase in the histones H3 and H4. One possible reason for the increase in H3 and H4 is that these proteins facilitate rapid cell division during embryogenesis. Furthermore, this accumulation of histones in the developing embryo may act as a mechanism for the epigenetic inheritance of gene expression (Marzluff et al. 2008). Currently these are speculations that require validation; however, *Daphnia* represents an excellent model for studying the changes in gene expression that underlie maturation, particularly in light of the recent publication of its genome (Colbourne et al. 2011a). Furthermore, recent molecular phylogenetic analysis suggests that the class to which *Daphnia* belong, the Branchiopoda, is more closely related to the Hexapoda than to other crustaceans (Meusemann et al. 2010; Rota-Stabelli et al. 2011; von Reumont et al. 2012). If this phylogeny is correct, comparisons of maturation processes in *Daphnia* and insects could be highly informative of conserved mechanisms among Hexapods.

Understanding the proximate mechanisms that influence maturation decisions also has serious applications for human health. With improvements in healthcare and nutrition, age and size at maturity of human populations is shifting too (Frisch & Revelle 1970; Frisch 1978; Parent et al. 2003). In many western societies menarche(the first instance of female menstruation) is taking place at increasingly early ages, a trend that has been linked to improvements in early nutrition (Ahmed et al. 2009), both at very early postnatal ages (Ong et al. 2009) and during later childhood (Dunger et al. 2006). Reduced age at menarche is of interest to many medical researchers because of corresponding increases in chronic adulthood diseases such as type 2 diabetes (Eriksson et al. 1999; Ong et al. 2004) and cancer (Hamilton & Mack 2003), and may have social implications because of the broadening

dichotomy between earlier sexual maturity and delayed psychosocial maturity in many modern societies (Gluckman & Hanson 2006). Medical physiology is revealing the underlying mechanisms of puberty in humans (Daftary & Gore 2005; Veldhuis et al. 2006; Thankamony et al. 2012), however describing the evolution of this trend is difficult, with most statistical methods reliant on twin data (e.g. Treloar & Martin 1990; Meyer et al. 1991; Eaves & Erkanli 2003; van den Berg et al. 2006). The modelling approach outlined in Chapter 2 and Harney et al. (2012) could be used on data that is much more freely available, perhaps using earlier markers for maturation, such as the onset of adrenarche (Ong et al. 2004; Thankamony et al. 2012). With increasingly comprehensive records available to medical statisticians, it would be interesting to investigate the plasticity of sexual maturity in humans, model the evolution of reduced age at menarche in different human populations at different times, and to investigate the potential for transgenerational effects (Dunger et al. 2006) to alter its evolution.

6.4 Can maturation thresholds evolve?

Maturation thresholds for size have been observed in a wide variety of taxa, including biennial plants (Werner 1975; Klinkhamer et al. 1987; Wesselingh & Klinkhamer 1996), acarids (Plaistow et al. 2004), crustaceans (Ebert 1992, 1994; Twombly 1996), insects (Nijhout & Williams 1974a; Bradshaw & Johnson 1995; De Moed et al. 1999; Juliano et al. 2004; Etilé & Despland 2008), fish (Policansky 1983; Reznick 1990) and amphibians (Travis 1984; Denver 1997; Morey & Reznick 2000), but few studies have investigated the extent to which thresholds are variable and can evolve (but see Nijhout et al. 2010). Chapter 2 found significant genotypic variation in the maturation thresholds of both *D. magna* and *D. pulex*. This result suggests that developmental thresholds are not just physiological constraints but adaptive traits that can evolve. Using a parthenogenetic organism such as *Daphnia* overcomes the limitations of studying maturation in sexual organisms and provides the first evidence of a genotype-specific PMRN. The genotypes used in this thesis originated from a wide variety of geographic locations, and environmental variation was imposed through resource availability. A logical extension then is to apply the PMRN and ontogenetic approaches developed here to clones with specific ecologies across numerous environmental gradients. This approach is an essential step in understanding how extrinsic factors such as predation, temperature, inter-specific competition, and periodic drying influence the expression of the maturation threshold. Furthermore, because *Daphnia* species leave dormant egg banks in the lake sediment, it is possible to use resurrection ecology

(Decaestecker et al. 2007) to hatch *Daphnia* resting eggs many years old and investigate whether phenotypic responses to selective pressures have changed over time. Genome scans of resting eggs have shown that selective pressures such as predation and changes in land use result in selection at the level of the genome (Orsini et al. 2012); adopting a PMRN approach to age and size at maturity in clones that have experienced such pressures could help in understanding how this genetic differentiation is manifested in developing phenotypes.

Mc Kee & Ebert (1996) suggested that the adaptive significance of the size threshold in *D. magna* was as a mechanism to reduce variation in size at maturity. Reduced variation in size at maturity may be adaptive in *Daphnia* if maturing at the wrong size carries high costs, perhaps from size-selective predation (Beckerman et al. 2010; Hart & Bychek 2010). However, given the plasticity of the threshold observed in this thesis (Chapter 2; Chapter 3), it is interesting to speculate that plastic maturation thresholds might also generate adaptive plasticity in age and size at maturity, rather than simply buffering development against environmental heterogeneity. Indeed, a combination of both of these factors could explain why the maturation threshold of *D. magna* is more plastic than that of *D. pulex*. The ponds that *D. magna* inhabit tend to be more ephemeral and subject to stronger environmental fluctuation, and this species is often absent from lakes with fish predators, due to its large size (Herbert 1978). *D. pulex*, though still large by cladoceran standards, may be small enough to escape much fish predation, and inhabits permanent water bodies that exhibit less environmental stochasticity. It is possible that plasticity in the maturation threshold allows *D. magna* to withstand the stochastic environments of ephemeral pools (Koivisto 1995), while a fixed maturation threshold in *D. pulex* may act as a buffer against environmental heterogeneity (Ebert 1997) and ensure maturation occurs at sizes too large for invertebrate predators but too small for fish (Beckerman et al. 2010). Testing these predictions, and distinguishing threshold size plasticity from resource allocation constraints (Dudycha & Lynch 2005) could be achieved by investigating threshold plasticity of multiple genotypes in both species from a range of environments that vary in their heterogeneity.

More generally, the importance of maturing at the right size in *Daphnia* may explain why the number of instars to maturity can vary (Ebert 1997). Canalising size at the expense of age and number of instars results in reaction norms for age and size at maturity that are 'L-shaped' (Day & Rowe 2002) in a rather loose sense (Chapter 2, Fig. 2.3). However, in organisms which are constrained by a fixed number of instars, such as the soil mite *Sancassania berlesei*, the L-shaped reaction norm predicted by Day and Rowe (2002) is far more apparent (Plaistow et al. 2004). Other intriguing examples of trade-offs between instar number and size exist. Insect species are commonly considered to have a fixed number of

instars, but there are exceptions to this rule (Esperk et al. 2007). The forest tent caterpillar *Malacosoma disstria* may take between five and eight instars to mature (Etilé & Despland 2008), allowing adults that grow in poor conditions to reach a similar size to those in good conditions. In comparison, a fixed number of larval instars, such as five in the autumn moth *Epirrita autumnata* may result in large differences in size, but reduced variation in age (Tammaru 1998), which is likely to be an advantage in a species with a very brief growing season. In the extreme case of the Tenebrionid beetle *Zophobas atratus*, the number of instars may be delayed indefinitely (>20) until crowding cues subside (Quennedey et al. 1995). Clearly the mechanisms that control maturation are able to respond to numerous environmental cues, including nutrition, seasonality and crowding, in order to produce functional, adaptive phenotypes. Although the maturation threshold is a vital aspect of maturation, the importance of genotypically variable and phenotypically plastic growth rates must be considered (Abrams et al. 1996; Dmitriew 2011). As was shown in Chapter 3, both the maturation threshold and growth rate can be genotypically variable and developmentally plastic. If these traits are more evolvable, there is likely to be a corresponding increase in the evolutionary potential of adult phenotypes (Moczek et al. 2011). Further investigation of their responses to selection is required to better understand the evolution of these age and size at maturity reaction norms (Nijhout et al. 2010). Precise information about the timing and nature of development can also be used to inform dynamic optimization techniques that model life history transitions as strategic decisions based on internal and external states (McNamara & Houston 1986; 1996). Successful application of these models could be vital to improving our understanding of the evolution of adaptive phenotypes for age and size at maturity under variable environments (e.g. Childs et al. 2012).

Diverse context-dependent responses of these traits may lead to the evolution of novel adult phenotypes (Moczek 2010; Badyaev 2011; Moczek et al. 2011). If these phenotypes are adaptive they will be preserved and genetic accommodation may lead to their continued expression in a population (West-Eberhard 2003; Badyaev 2009), and in extreme cases may be constitutively expressed, or genetically assimilated into normal development (Schlichting & Smith 2002; West-Eberhard 2003; Badyaev 2005; Braendle & Flatt 2006; Suzuki & Nijhout 2006; Kalinka et al. 2010). The epigenetic or parental inheritance of context-dependent developmental plasticity may be an important mechanism underlying genetic accommodation and genetic assimilation (West-Eberhard 2003; Jablonka & Raz 2009), as evidenced by examples where heritable reaction norms for traits such as pigmentation have their roots in environmental factors such as diet (Badyaev 2007). Thus developmental plasticity is not just a target for natural selection but is involved in the production of adaptive phenotypes (Badyaev 2009), leading to the interdependence of genetic and

environmental effects in phenotypic evolution (Fusco & Minelli 2010). The dynamic interdependence of genes and environment through the process of development suggests that the production of phenotypic variation is involved in a complex feedback between developmental processes, inheritance and natural selection (Fig. 6.1). In order to incorporate these interacting processes, it seems likely that the evolutionary framework of the modern synthesis will have to be expanded (Pigliucci et al. 2006; Müller 2007; Badyaev 2011; Day & Bonduriansky 2011). *Daphnia* appears to be a natural system in which to explore these exciting questions. Developmental plasticity as a result of variation in both past and present environments produces variation in adult phenotypes; genotype specific reaction norms can be quantified, and parent-offspring regressions following sexual crosses can also be studied to better understand the genetic and non-genetic components of heritable developmental plasticity.

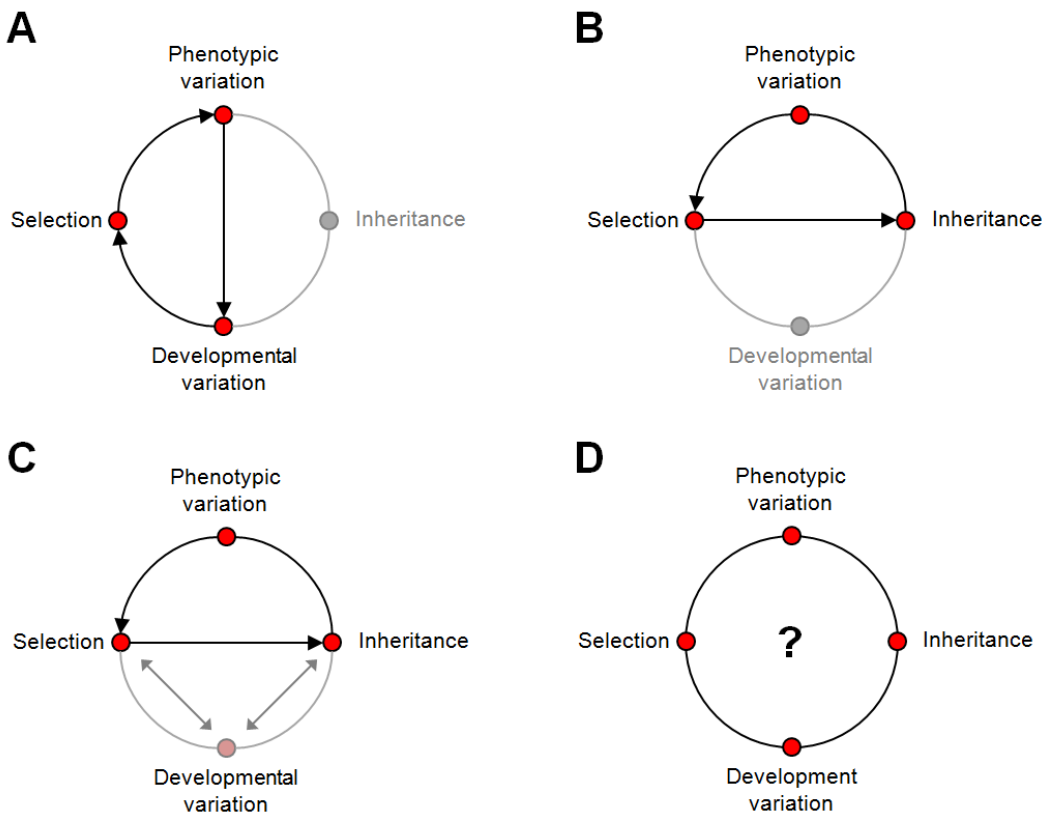

Figure 6.1. Understanding the interactions between phenotypic variation, selection, development and inheritance in the evolutionary framework. A) Darwin establishes the importance of natural selection in the evolution of phenotypes, but there is no mechanism for inheritance; B) The modern synthesis incorporates Mendelian inheritance into the framework, but largely ignores developmental variation; C) Current views in which development may generate variation for selection, and selection may act on developmental variation, or where inheritance is contingent on its expression during development; D) A unified theory linking all these factors has yet to be established. *Adapted from Badyaev, A.V., Origin of the fittest: link between emergent variation and evolutionary change as a critical question in evolutionary biology, Philos. Trans. R. Soc. Lond. B, 2011, 278, 1714, 1921-1929, by permission of the Royal Society.*

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Appendix

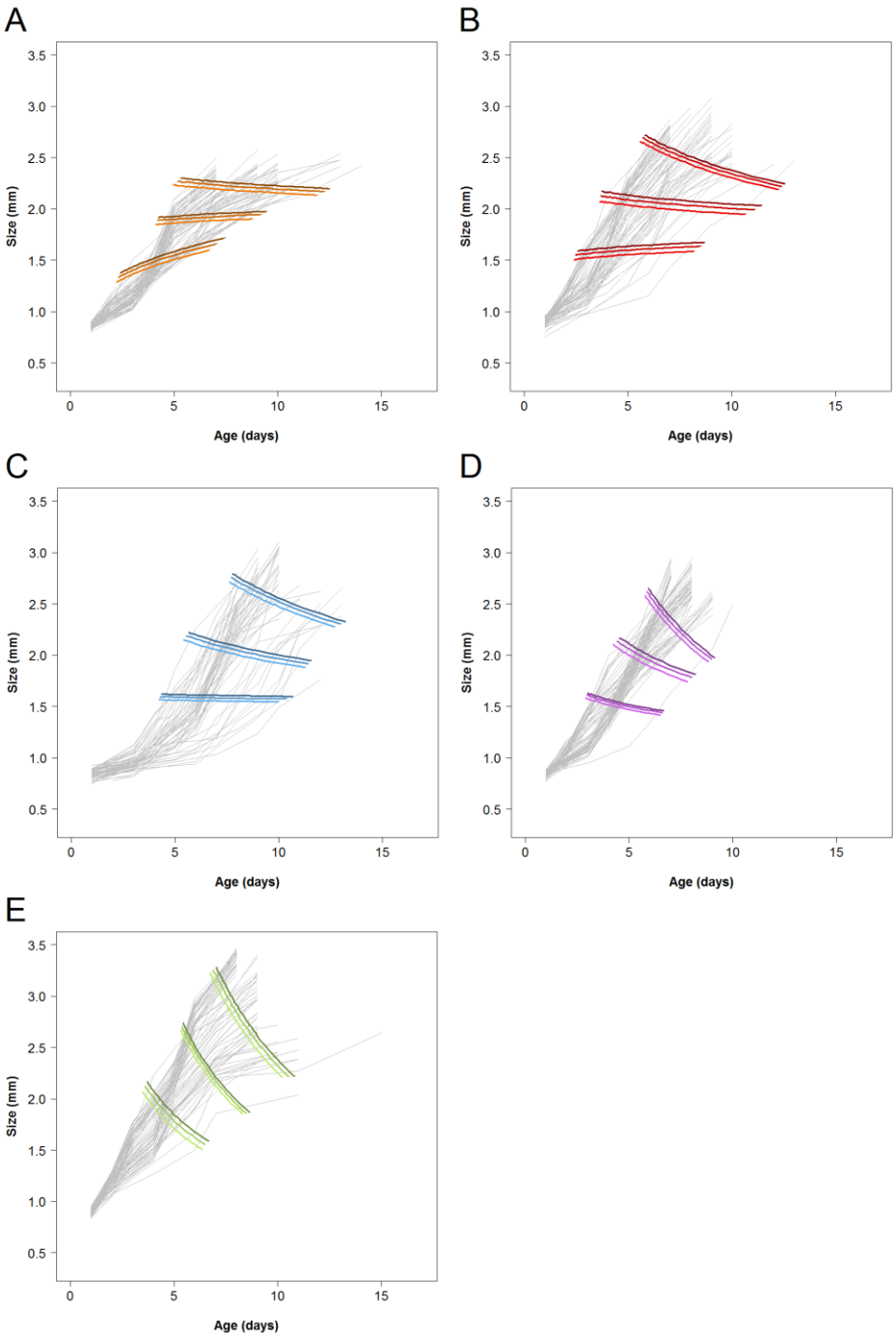

Figure A2.1. PMRNs for 3 different maturation indicators IM-1, IM-2 and IM-3 in 5 clones of *Daphnia magna*: (A) H01, (B) DKN1-3, (C), B5, (D) Ness1, and (E) B7. The model *response ~ offset(log(size)) + (clone) * (log(age ends) + log(size ends))* has been used to generate PMRNs for IM-1 and IM-2, and *response ~ offset(log(age)) + (clone) * (log(age ends) + log(size ends))* was used to generate PMRNs for IM-3. Although these models are not the best fitting in all cases, using models of the same type (GLM approximation of a rate model) based on the same measure of the interval (logged interval ends) highlights the effect of growth between maturation indicators.

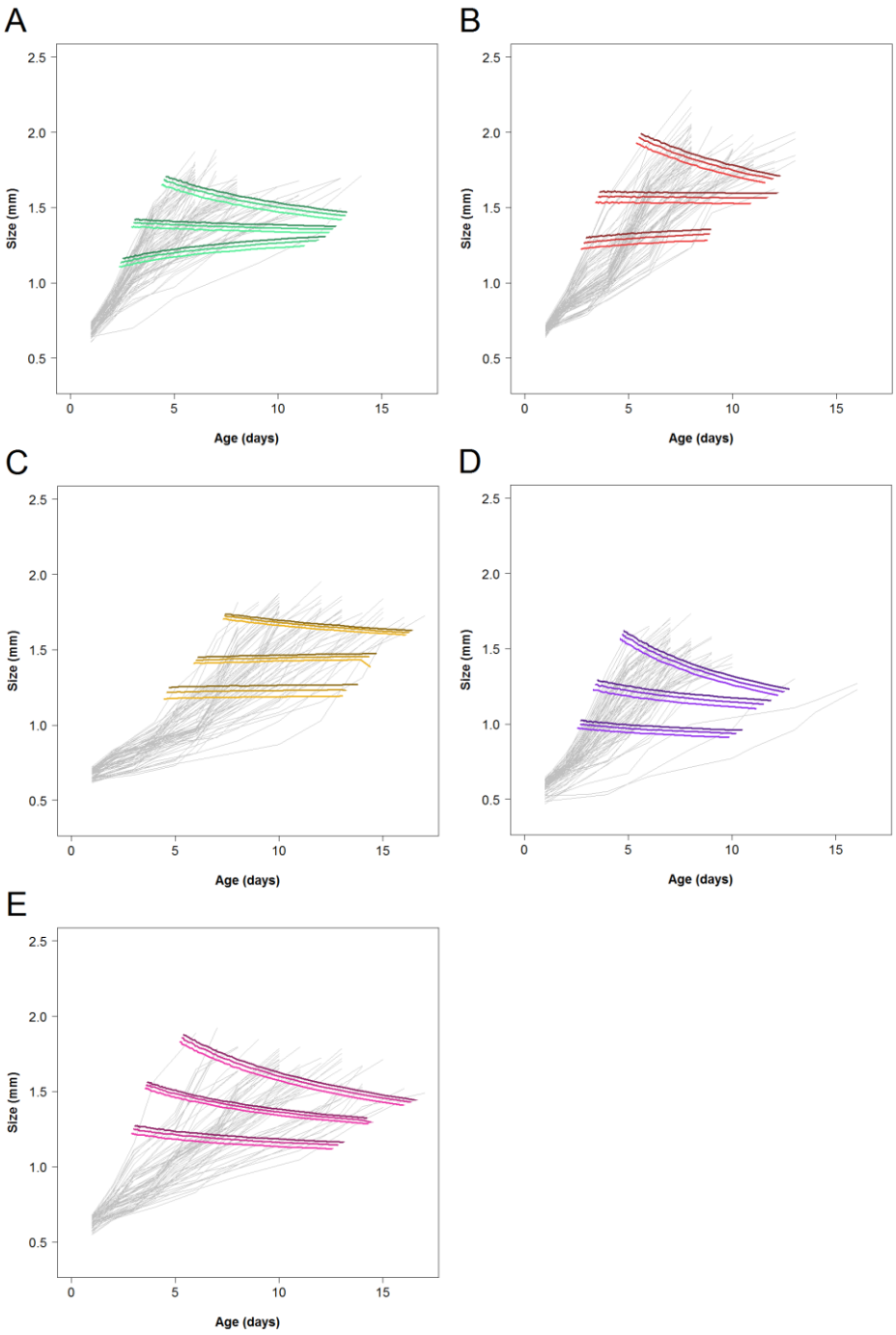

Figure A2.2. PMRNs for 3 different maturation indicators IM-1, IM-2 and IM-3 in 5 clones of *Daphnia pulex*: (A) Carlos, (B) Boris, (C) Chardonnay, (D) Bierbeek, and (E) Cyril. The model *response ~ offset(log(size)) + (clone) * (log(age ends) + log(size ends))* has been used to generate PMRNs for IM-1 and IM-2, and *response ~ offset(log(age)) + (clone) * (log(age ends) + log(size ends))* was used to generate PMRNs for IM-3. Although these models are not the best fitting in all cases, using models of the same type (GLM approximation of a rate model) based on the same measure of the interval (logged interval ends) highlights the effect of growth between maturation indicators.

Table A2.1. A comparison of GLMs with and without offsets and maturation rate models for all three maturation indicators (IM-1, IM-2 and IM-3) for *Daphnia magna*. For each indicator, GLMs are grouped by offset (none, size, age) and the best fitting (lowest AIC) model with age only, size only and age and size covariates is reported. For each indicator, maturation rate models with all combinations of age and size integration and age and size rate effects are reported for the function that provided the best fit to the data. The best fitting model for each indicator is in boldface type and the number of parameters in the model (No. para.) is provided.

Model Type	GLM offset	Description	AIC	No. para.
D. magna IM-1				
GLM	**No**	**resp ~ (clone) * (ln(age ends) + ln(size ends))**	**284.58**	**15**
GLM	No	resp ~ (clone) * (ln(size mids))	300.26	10
GLM	No	resp ~ (clone) * (ln(age mids))	624.46	10
GLM	Size	resp ~ offset(ln(size)) + (clone) * (ln(age ends) + ln(size ends))	288.61	15
GLM	Size	resp ~ offset(ln(size)) + (clone) * (ln(size mids))	298.33	10
GLM	Size	resp ~ offset(ln(size)) + (clone) * (ln(age mids))	564.45	10
GLM	Age	resp ~ offset(ln(age)) + (clone) * (ln(age ends) + ln(size ends))	293.27	15
GLM	Age	resp ~ offset(ln(age)) + (clone) * (ln(size mids))	310.81	10
GLM	Age	resp ~ offset(ln(age)) + (clone) * (ln(age starts))	623.01	10
Rate	-	Generalised function, size integration, size rate effects	311.92	11
Rate	-	Generalised function, age integration, size rate effects	350.89	11
Rate	-	Generalised function, size integration, age rate effects	551.40	11
Rate	-	Generalised function, age integration, age rate effects	696.04	11
D. magna IM-2				
GLM	No	resp ~ (clone) * ((age ends) +(size ends))	247.90	15
GLM	No	resp ~ (clone) *(ln (size mids))	299.82	10
GLM	No	resp ~ (clone) *(ln (age mids))	661.96	10
GLM	**Size**	**resp ~ offset(ln(size)) + (clone) * (ln(age mids) + ln(size mids))**	**246.77**	**15**
GLM	Size	resp ~ offset(ln(size)) + (clone) * (ln(size starts))	300.48	10
GLM	Size	resp ~ offset(ln(size)) + (clone) * (ln(age mids))	609.18	10
GLM	Age	resp ~ offset(ln(age)) + (clone) * (ln(age mids) + ln(size mids))	249.17	15
GLM	Age	resp ~ offset(ln(age)) + (clone) * (ln(size mids))	306.31	10
GLM	Age	resp ~ offset(ln(age)) + (clone) * (ln(age starts))	669.88	10
Rate	-	Weibull function, size integration, age and size rate effects	261.72	15
Rate	-	Weibull function, size integration, size rate effects	331.76	10
Rate	-	Weibull function, age integration, size rate effects	338.13	10
Rate	-	Weibull function, size integration, age rate effects	651.34	10
Rate	-	Weibull function, age integration, age and size rate effects	1686.79	15
Rate	-	Weibull function, age integration, age rate effects	2079.54	10

Table A2.1 continued.

Model Type	GLM offset	Description	AIC	No. para.
D. magna IM-3				
GLM	No	resp ~ (clone) * (ln(age mids) + ln(size mids))	216.08	15
GLM	No	resp ~ (clone) * (ln(size starts))	299.27	10
GLM	No	resp ~ (clone) * (ln(age ends))	632.78	10
GLM	Size	resp ~ offset(ln(size)) + (clone) * (ln(age mids) + ln(size mids))	219.43	15
GLM	Size	resp ~ offset(ln(size)) + (clone) * (ln(size starts))	315.34	10
GLM	Size	resp ~ offset(ln(size)) + (clone) * (ln(age ends))	601.64	10
GLM	**Age**	**resp ~ offset(ln(age)) + (clone) * (ln(age mids) + ln(size mids))**	**204.33**	**15**
GLM	Age	resp ~ offset(ln(age)) + (clone) * (ln(size starts))	270.40	10
GLM	Age	resp ~ offset(ln(age)) + (clone) * (ln(age mids))	618.99	10
Rate	-	Weibull function, age integration, age and size rate effects	236.26	15
Rate	-	Weibull function, size integration, age and size rate effects	264.06	15
Rate	-	Weibull function, size integration, size rate effects	461.78	10
Rate	-	Weibull function, age integration, size rate effects	325.13	10
Rate	-	Weibull function, size integration, age rate effects	678.69	10
Rate	-	Weibull function, age integration, age rate effects	714.98	10

Table A2.2. A comparison of GLMs with and without offsets and maturation rate models for all three maturation indicators (IM-1, IM-2 and IM-3) for *Daphnia pulex*. For each indicator, GLMs are grouped by offset (none, size, age) and the best fitting (lowest AIC) model with age only, size only and age and size covariates is reported. For each indicator, maturation rate models with all combinations of age and size integration and age and size rate effects are reported for the function that provided the best fit to the data. The best fitting model for each indicator is in boldface type and the number of parameters in the model (No. para.) is provided.

Model Type	GLM offset	Description	AIC	No. para.
D. pulex IM-1				
GLM	No	resp ~ (clone) * (log(age ends) + log(size ends))	434.85	15
GLM	No	resp ~ (clone) * (log(size ends))	443.54	10
GLM	No	resp ~ (clone) * (log(age starts))	972.61	10
GLM	**Size**	**resp ~ offset(log(size)) + (clone) * (log(age ends) + log(size ends))**	**427.63**	**15**
GLM	Size	resp ~ offset(log(size)) + (clone) * (log(size ends))	432.23	10
GLM	Size	resp ~ offset(log(size)) + (clone) * (log(age starts))	849.69	10
GLM	Age	resp ~ offset(log(age)) + (clone) * (log(age ends) + log(size ends))	440.15	15
GLM	Age	resp ~ offset(log(age)) + (clone) * (log(size ends))	457.06	10
GLM	Age	resp ~ offset(log(age)) + (clone) * (log(age starts))	979.62	10
Rate	-	Generalised function, size integration, size rate effects	438.95	11
Rate	-	Generalised function, age integration, size rate effects	525.21	11
Rate	-	Generalised function, size integration, age rate effects	815.45	11
Rate	-	Generalised function, age integration, age rate effects	1018.00	11
D. pulex IM-2				
GLM	**No**	**resp ~ (clone) * ((age ends) +(size ends))**	**346.34**	**15**
GLM	No	resp ~ (clone) * (size ends)	361.97	10
GLM	No	resp ~ (clone) *(log (age starts))	1014.46	10
GLM	Size	resp ~ offset(log(size)) + (clone) * ((age ends) + (size ends))	350.23	15
GLM	Size	resp ~ offset(log(size)) + (clone) * (log(size mids))	362.33	10
GLM	Size	resp ~ offset(log(size)) + (clone) * (log(age starts))	916.54	10
GLM	Age	resp ~ offset(log(age)) + (clone) * ((age ends) + (size ends))	355.54	15
GLM	Age	resp ~ offset(log(age)) + (clone) * (size ends)	367.45	10
GLM	Age	resp ~ offset(log(age)) + (clone) * (log(age starts))	1072.18	10
Rate	-	Weibull function, size integration, age and size rate effects	352.37	15
Rate	-	Weibull function, size integration, size rate effects	370.48	10
Rate	-	Weibull function, age integration, age and size rate effects	401.10	15
Rate	-	Weibull function, age integration, size rate effects	403.55	10
Rate	-	Weibull function, size integration, age rate effects	960.43	10
Rate	-	Weibull function, age integration, age rate effects	1218.67	10

Table A2.2 continued.

Model Type	GLM offset	Description	AIC	No. para.
D. pulex IM-3				
GLM	No	resp ~ (clone) * (log(age mids) + log(size mids))	327.82	15
GLM	No	resp ~ (clone) * (log(size starts))	368.84	10
GLM	No	resp ~ (clone) * (log(age ends))	1072.99	10
GLM	Size	resp ~ offset(log(size)) + (clone) * (log(age mids) + log(size mids))	328.46	15
GLM	Size	resp ~ offset(log(size)) + (clone) * (log(size starts))	367.30	10
GLM	Size	resp ~ offset(log(size)) + (clone) * (log(age ends))	1012.42	10
GLM	**Age**	**resp ~ offset(log(age)) + (clone) * (log(age mids) + log(size mids))**	**317.90**	**15**
GLM	Age	resp ~ offset(log(age)) + (clone) * (log(size starts))	358.99	10
GLM	Age	resp ~ offset(log(age)) + (clone) * (log(age starts))	1065.06	10
Rate	-	Weibull function, age integration, age and size rate effects	331.04	15
Rate	-	Weibull function, size integration, age and size rate effects	364.22	15
Rate	-	Weibull function, age integration, size rate effects	391.58	10
Rate	-	Weibull function, size integration, size rate effects	506.15	10
Rate	-	Weibull function, size integration, age rate effects	1048.67	10
Rate	-	Weibull function, age integration, age rate effects	1119.36	10

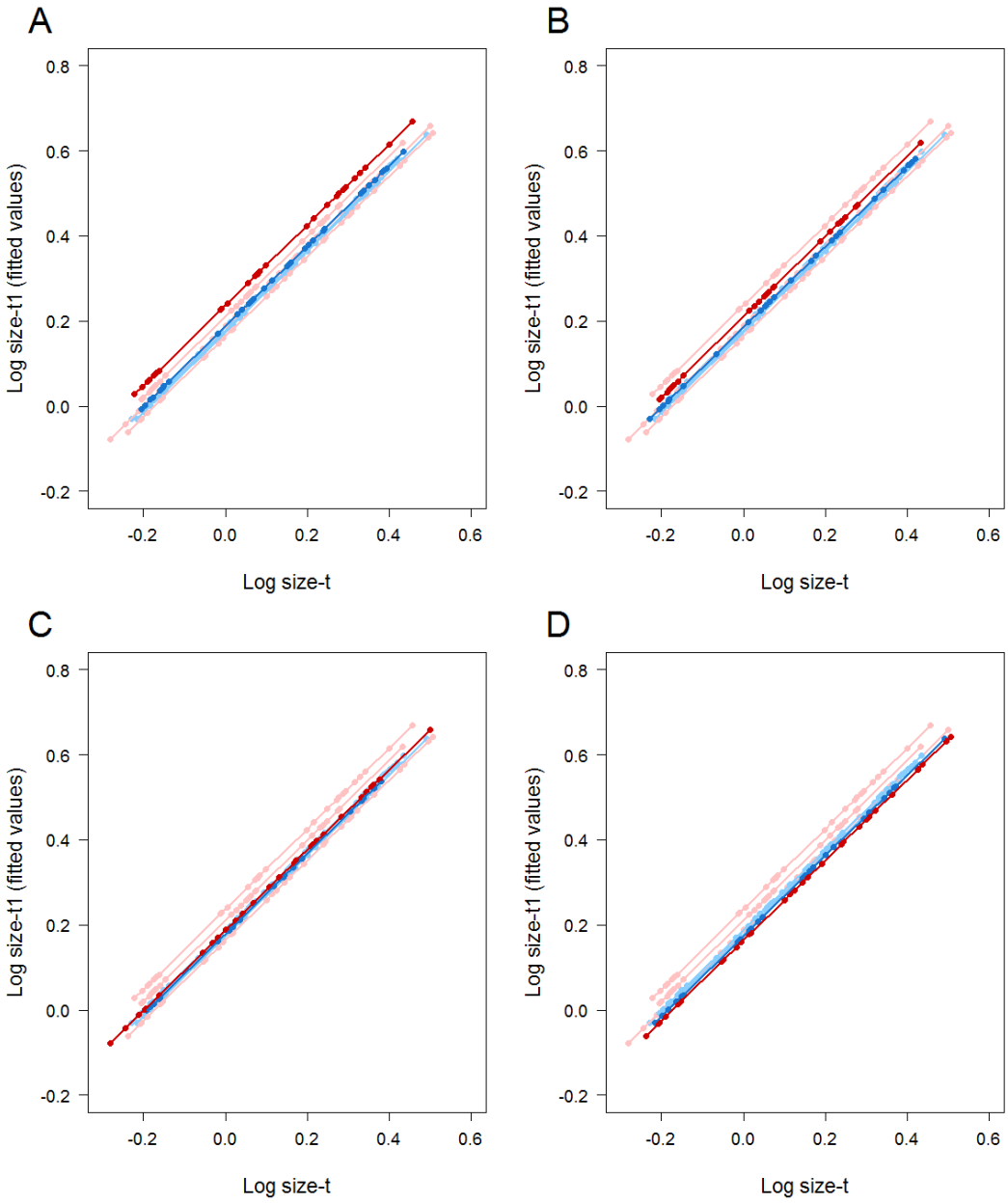

Figure A3.1. Parental effects on size-corrected growth before the maturation threshold in clone DKN1-3 at four offspring food levels: A) High; B) Medium High; C) Medium Low; D) Low. Logged values of size-*t* are plotted against fitted values of logged size-*t*+1, with model fits overlaid to aid visualisation. Red lines represent HPE, blue lines LPE. Size corrected growth of focal food is in bold colours, whilst the remaining 3 foods are plotted in pale colours for reference.

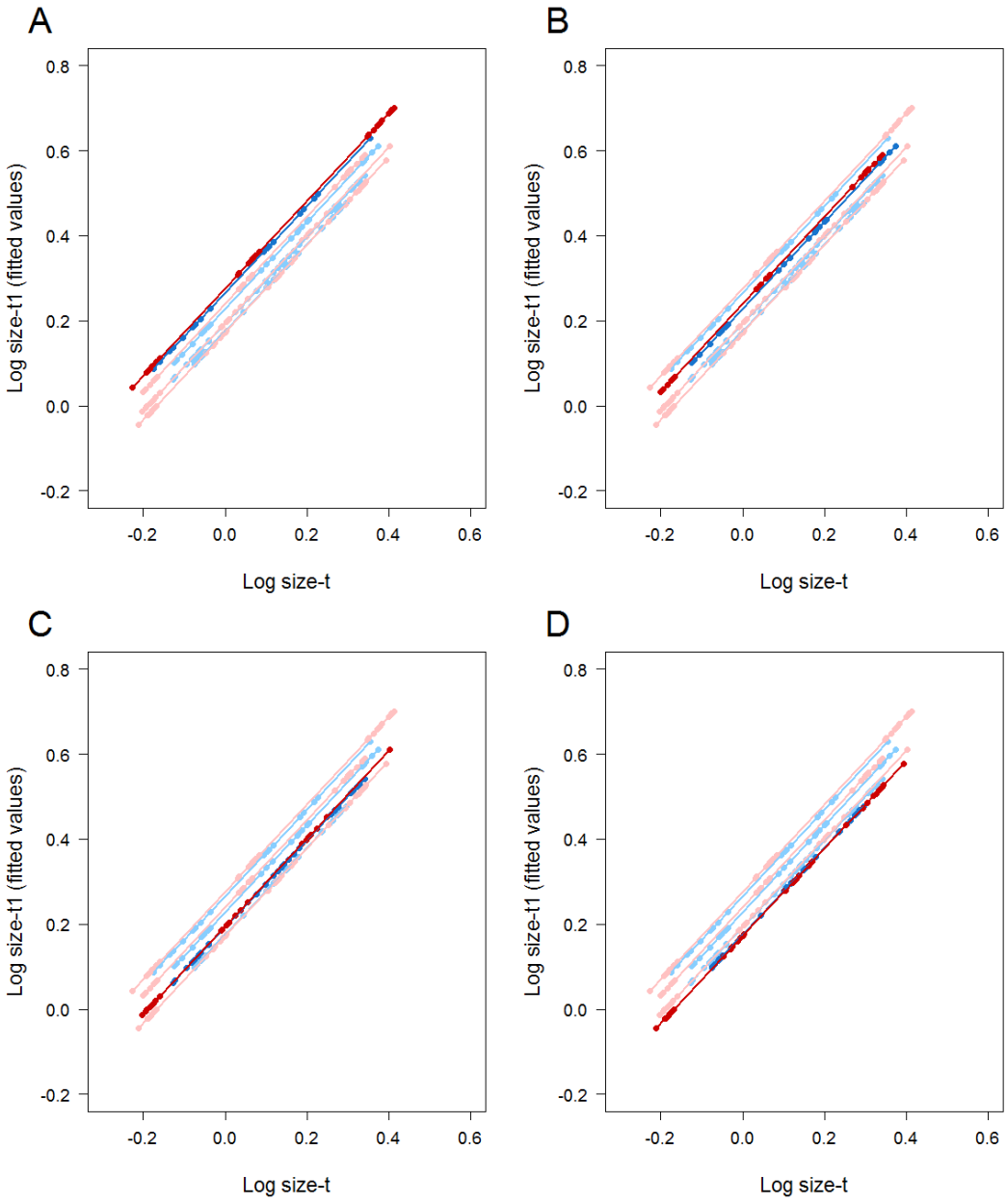

Figure A3.2. Parental effects on size-corrected growth before the maturation threshold in clone Ness1 at four offspring food levels: A) High; B) Medium High; C) Medium Low; D) Low. Logged values of size-*t* are plotted against fitted values of logged size-*t*+1, with model fits overlaid to aid visualisation. Red lines represent HPE, blue lines LPE. Size corrected growth of focal food is in bold colours, whilst the remaining 3 foods are plotted in pale colours for reference.

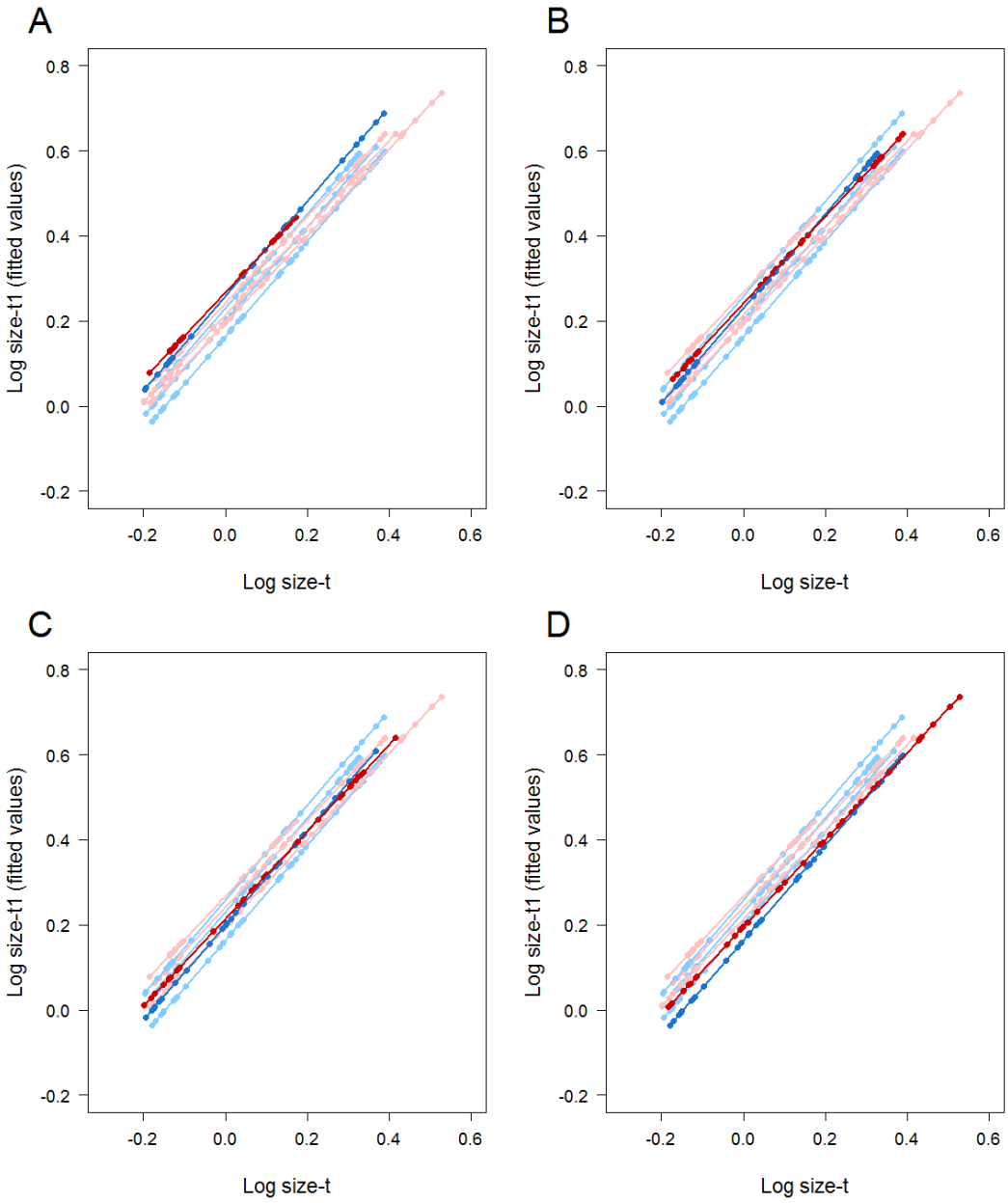

Figure A3.3. Parental effects on size-corrected growth before the maturation threshold in clone B5 at four offspring food levels: A) High; B) Medium High; C) Medium Low; D) Low. Logged values of size-*t* are plotted against fitted values of logged size-*t*+1, with model fits overlaid to aid visualisation. Red lines represent HPE, blue lines LPE. Size corrected growth of focal food is in bold colours, whilst the remaining 3 foods are plotted in pale colours for reference.

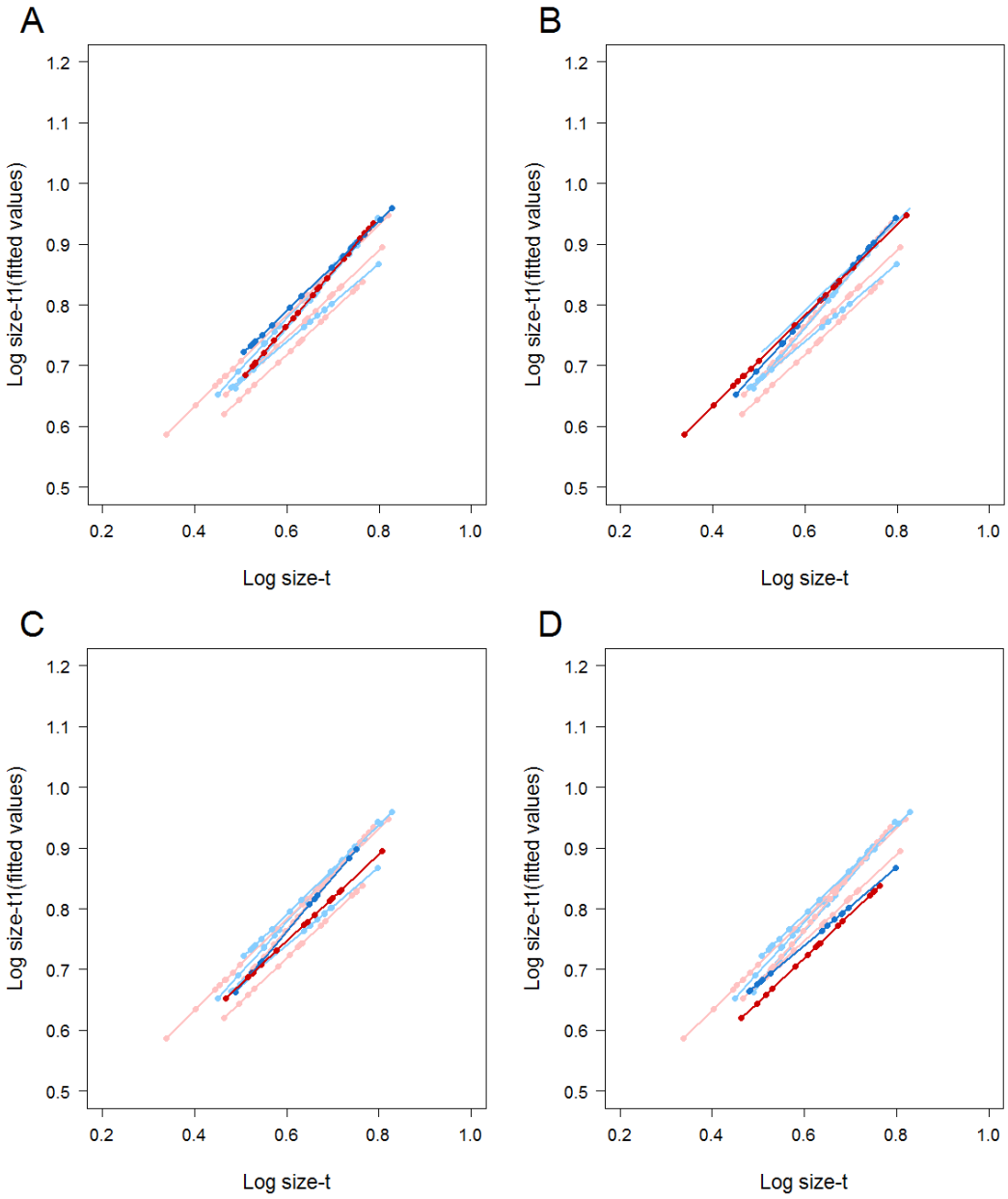

Figure A3.4. Parental effects on size-corrected growth during the maturation process in clone DKN1-3 at four offspring food levels: A) High; B) Medium High; C) Medium Low; D) Low. Logged values of size-*t* are plotted against fitted values of logged size-*t*+1, with model fits overlaid to aid visualisation. Red lines represent HPE, blue lines LPE. Size corrected growth of focal food is in bold colours, whilst the remaining 3 foods are plotted in pale colours for reference.

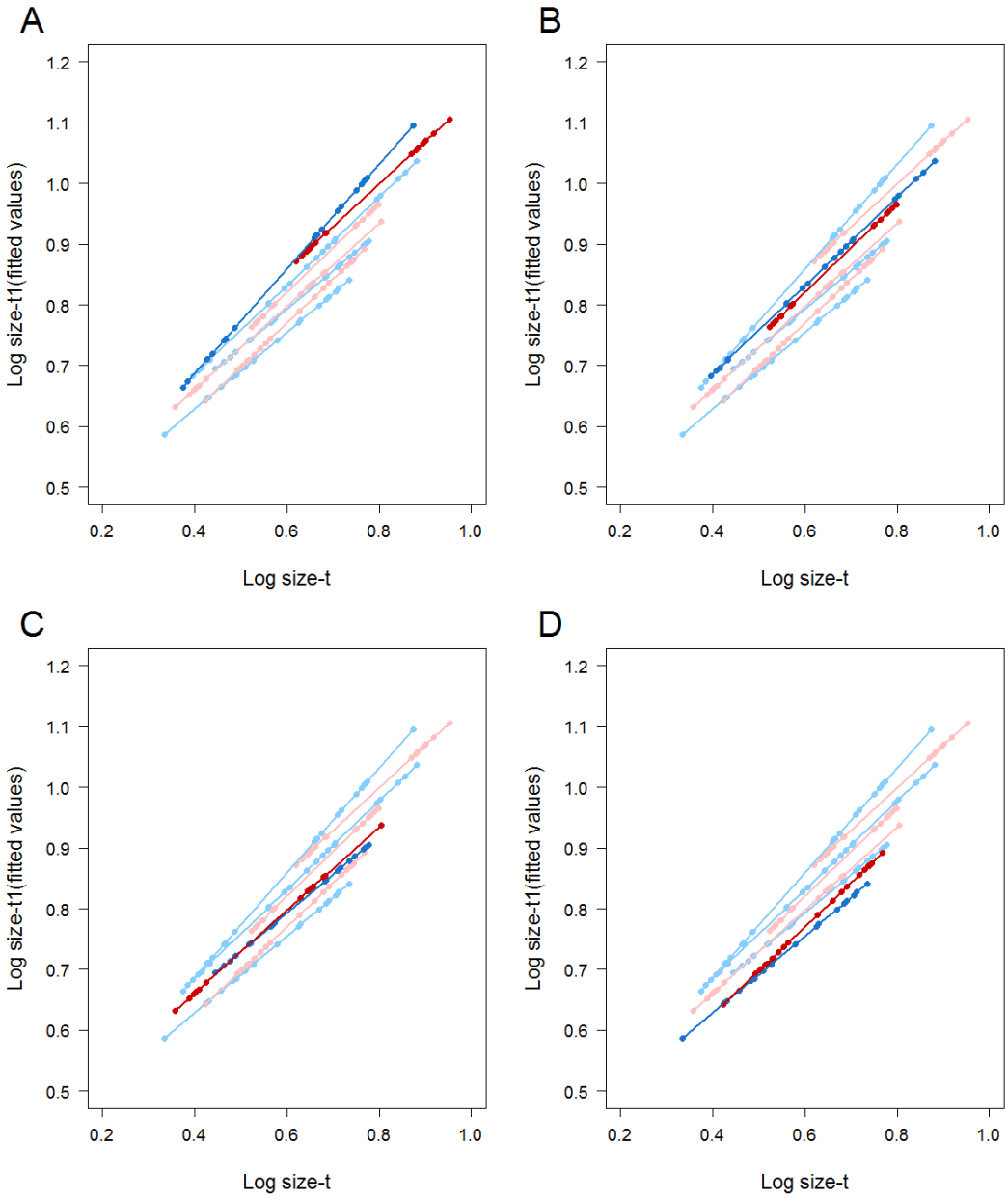

Figure A3.5. Parental effects on size-corrected growth during the maturation process in clone Ness1 at four offspring food levels: A) High; B) Medium High; C) Medium Low; D) Low. Logged values of size-*t* are plotted against fitted values of logged size-*t*+1, with model fits overlaid to aid visualisation. Red lines represent HPE, blue lines LPE. Size corrected growth of focal food is in bold colours, whilst the remaining 3 foods are plotted in pale colours for reference.

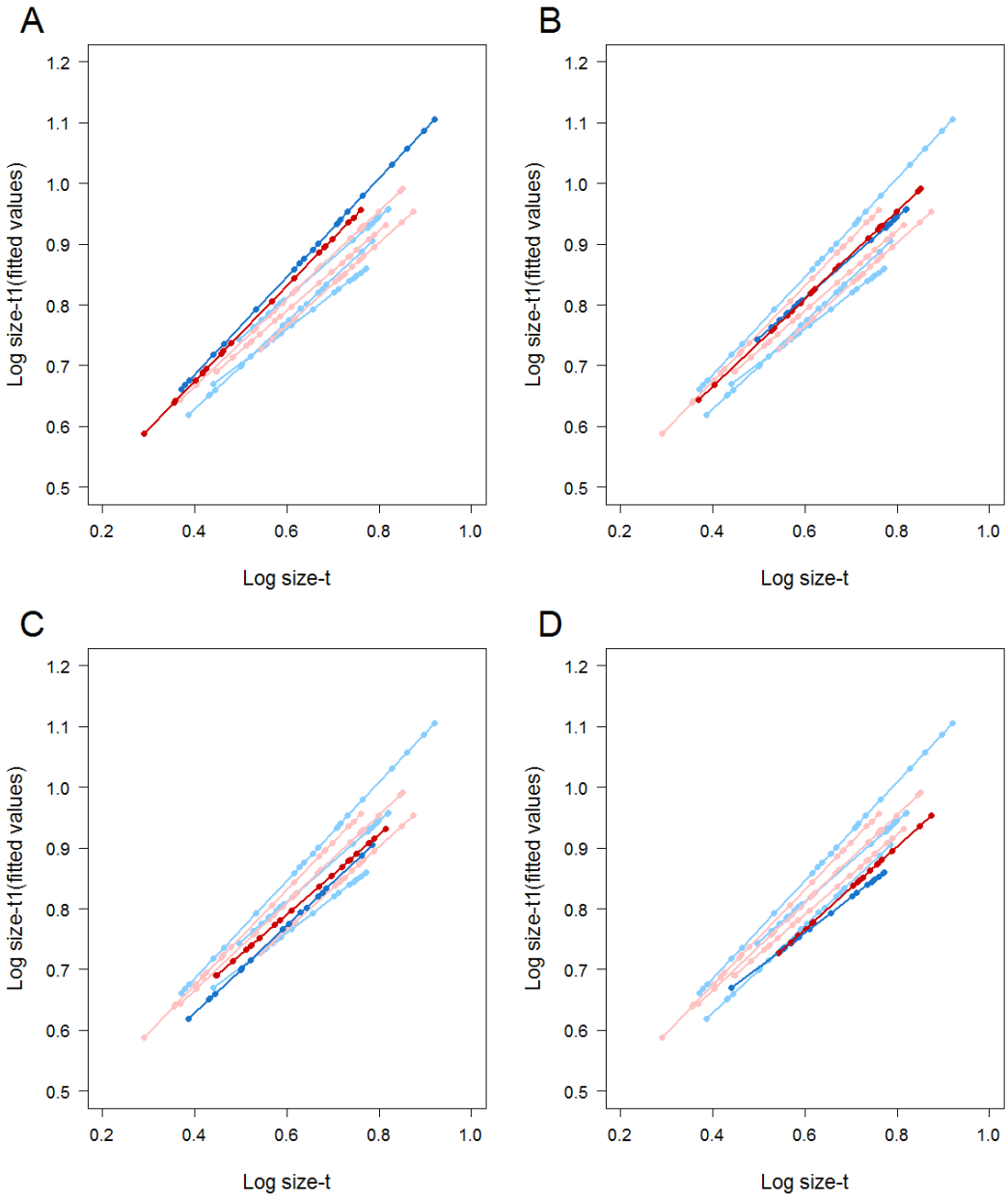

Figure A3.6. Parental effects on size-corrected growth during the maturation process in clone B5 at four offspring food levels: A) High; B) Medium High; C) Medium Low; D) Low. Logged values of size-*t* are plotted against fitted values of logged size-*t*+1, with model fits overlaid to aid visualisation. Red lines represent HPE, blue lines LPE. Size corrected growth of focal food is in bold colours, whilst the remaining 3 foods are plotted in pale colours for reference.

Appendix Table A4.1. *Daphnia pulex* genes with significant increases in differential expression during the course of development. Log fold changes of gene expression and significance from continuous contrast limma are reported. Uniprot IDs and gene-product descriptions from wFleaBase and UniProt are provided where they were available Where wFleaBase gene IDs did not correspond to UniProt protein IDs, protein BLASTs were carried out and the closest *D. pulex* UniProt gene product is reported, with E values and percentage identities (red boldface type).

DAPPU gene no.	Log fold change	P-value	wFleaBase description	UniProt ID	Percentage identity	E-value	UniProt description
39705	0.372	9.23E-06	SCF ubiquitin ligase. Skp2 component	E9FQV6			
43440	0.526	5.53E-06	Histone H3 and H4	E9FUS8			Histone H3
43804	0.535	9.11E-07	Histone H3 and H4	E9G035			
43863	0.507	2.72E-07	Histone H3 and H4	E9FUS8			Histone H3
46545	0.437	1.72E-06	Predicted transcription factor	E9G6P4			
60476	0.371	6.92E-06	Galactoside 3-fucosyltransferase, Fucosyltransferase	E9HAL8			
91889	0.463	2.02E-05		E9FU89			
100140	0.360	3.21E-06		E9G9H9			
110469	0.651	5.43E-08		E9H6C3			
203760	0.400	2.52E-05	Ubiquitin and ubiquitin-like proteins	E9HM22			
220880	0.518	2.69E-06		E9FW53			
222925	0.631	3.25E-05	Cyclin B and related kinase-activating proteins	E9G757			Putative cyclin B, copy D
226068	1.173	4.11E-06		**E9GVW1**	**99.0**	**0**	
226075	1.779	1.43E-06	Predicted lipoprotein	**E9GVW7**	**96.0**	**0**	**Vitellogenin fused with superoxide dismutase**
226761	0.644	9.19E-07	Asparagine synthase	**E9H1C3**	**100.0**	**0**	
229368	0.356	9.46E-06		E9HNI7			
235586	0.524	7.96E-06	Histone H3 and H4	E9FUS8			Histone H3
235631	0.674	3.56E-05	Histone H4	E9FUS9			Histone H4
235802	0.478	1.75E-05	Histone H3 and H4	E9FUS8			Histone H3

Appendix Table A4.1 continued.

DAPPU gene no.	Log fold change	P-value	wFleaBase description	UniProt ID	Percentage identity	E-value	UniProt description
255862	0.536	2.63E-07	Histone H3 and H4	E9FUS8			Histone H3
263168	0.660	1.62E-05		E9HP86			
299677	0.801	3.33E-07	Predicted lipoprotein	E9HZI6			Vitellogenin fused with superoxide dismutase
303836	0.643	6.53E-07		**E9GIW7**	**59.0**	**2.00E-54**	
303879	0.422	5.26E-07		E9GIP2			
304575	0.540	5.22E-06	Small Nuclear ribonucleoprotein splicing factor	E9FVF2			
304661	0.678	1.62E-05	Predicted lipoprotein	E9FVG7			
305707	0.392	1.98E-06	Carbonate dehydratase, Nitrogen metabolism, Carbonic anhydrase	**E9FXL5**	**100.0**	**0**	Alpha-carbonic anhydrase
306151	0.539	3.74E-05	Glycolipid-transport protein	**E9GVT8**	**100.0**	**1.00E-130**	Glycolipid-transport protein
308303	0.338	2.37E-05	Ca^{2+}-binding protein, EF-Hand protein superfamily	E9H790			
308693	0.654	1.92E-05	Predicted lipoprotein	E9H8Q4			Putative uncharacterized protein VTG4
312260	0.765	1.01E-06	Histone H4	E9FUS9			Histone H4

Appendix Table A4.2. *Daphnia pulex* genes with significant decreases in differential expression during the course of development. Log fold changes of gene expression and significance from continuous contrast limma are reported. Uniprot IDs and gene-product descriptions from wFleaBase and UniProt are provided where they were available. Where wFleaBase gene IDs did not correspond to UniProt protein IDs, protein BLASTs were carried out and the closest *D. pulex* UniProt gene product is reported, with E values and percentage identities (red boldface type).

DAPPU gene no.	Log fold change	P-value	wFleaBase description	UniProt ID	Percentage identity	E-value	UniProt Description
107198	-0.423	3.83E-05	Septin family protein (P-loop GTPase)	E9GWA3			
112957	-0.743	2.11E-05		E9HDK6			
219379	-0.509	2.06E-05		E9HSG3			
220921	-1.033	1.25E-09		E9FUA9			
227396	-0.408	2.71E-05	3'-5' exonuclease	E9H6J0			
228103	-0.396	1.82E-06		E9HAZ3			
250400	-0.357	3.05E-05		E9GYH2			
299589	-0.678	6.10E-08		**E9GIY6**	**33.0**	**2.00E-04**	
304176	-0.386	2.16E-05		E9GJP1			
305501	-0.442	3.53E-05	Glutathione transferase, Glutathione metabolism, Glutathione S-transferase	E9FX61			Putative uncharacterized protein DpGSTM4
305713	-0.260	3.83E-05		E9FXR6			
312710	-0.973	3.84E-05		E9G014			
327378	-0.403	3.07E-05		E9HAK7			
328621	-0.418	1.08E-05	Conserved Zn-finger protein	E9HE92			

Appendix Table A4.3. Gene Ontology (GO) terms for all *Daphnia pulex* genes/gene orthologs with significant increases in differential expression during the course of development. Gene-product descriptions derive from UniProt (except DAPPU 306151, which derives from wFleaBase). All biological process, molecular function and cellular component GO terms associated with a gene product were obtained from the European bioinformatics institute (EMBL - EBI) QuickGO database. The highest order biological process (or in the absence of a biological process, molecular function or cellular component) was used as a summary term. Where terms were obtained from *D. pulex* genes, text is boldface and blue (exact gene) or red (similar gene). In three cases (denoted by *,† and ‡; footnote at end of table), genes/gene products had no GO terms associated with them. Here, GO terms were inferred from better characterised model organisms (*Drosophila melanogaster* and *Homo sapiens*). In all three cases the genes in question were associated with RNA processes that are likely to be highly conserved, though GO terms should be interpreted with caution.

				Gene Ontology terms			
DAPPU gene no.	UniProt ID	Organism	Description	Biological Process	Molecular function	Cellular component	Process / function
39705	F4WDB8	*Acromyrmex echinatior*	S-phase kinase-associated protein 2	phosphorylation	kinase activity	-	Phosphorylation
43440	**E9FUS8**	***Daphnia pulex***	Histone H3	nucleosome assembly	DNA binding	nucleosome nucleus chromosome	Nucleosome assembly
43804	B4K413	*Drosophila grimshawi*	Histone H3	nucleosome assembly	DNA binding	nucleosome	Nucleosome assembly
43863	**E9FUS8**	***Daphnia pulex***	Histone H3	nucleosome assembly	DNA binding	nucleosome	Nucleosome assembly
46545	E0VSK2	*Pediculus humanus*	Protein C-ets-1-B, putative	regulation of transcription, DNA-dependent	DNA binding sequence-specific DNA binding transcription factor activity sequence-specific DNA binding	nucleus	Regulation of transcription

Appendix Table A4.3 continued.

DAPPU gene no.	UniProt ID	Organism	Description	Gene Ontology terms: Biological Process	Molecular function	Cellular component	Process / function
60476	Q333R2	*Drosophila sechellia*	Alpha 1,3-fucosyltransferase	protein glycosylation fucosylation	fucosyltransferase activity transferase activity transferase activity, transferring glycosyl groups	Golgi apparatus membrane integral to membrane Golgi cisterna membrane	Fucosylation
91889	E0W3W7	*Pediculus humanus*	Gem-associated protein, putative	spliceosomal complex assembly		nucleus	Spliceosomal complex assembly
100140*	E2BIM6	*Harpegnathos saltator*	Pre-mRNA cleavage complex II protein Clp1	tRNA splicing, via endonucleolytic cleavage and ligation* mRNA processing*	nucleotide binding* ATP binding*	tRNA-intron endonuclease complex* nucleus*	mRNA processing
110469	-	-	-	-	-	-	-
203760	G0ZJA2	*Cherax quadricarinatus*	Ubiquitin	-	protein binding	-	Primary metabolic process
220880	B4P0Y7	*Drosophila yakuba*	Geminin	negative regulation of DNA replication	-	-	Negative regulation of DNA replication
222925	**E9G757**	***Daphnia pulex***	Putative cyclin B, copy D	regulation of cyclin-dependent protein kinase activity cell cycle cell division regulation of cell cycle	protein kinase binding	nucleus	Cell cycle / cell division

Appendix Table A4.3 continued.

DAPPU gene no.	UniProt ID	Organism	Description	Gene Ontology terms: Biological Process	Molecular function	Cellular component	Process / function
226068	Q1JUB1	*Daphnia magna*	Vitellogenin fused with superoxide dismutase	superoxide metabolic process lipid transport oxidation-reduction process	lipid transporter activity metal ion binding	-	Lipid transport / oxidation-reduction process
226075	**E9GVW7**	***Daphnia pulex***	Vitellogenin fused with superoxide dismutase	lipid transport	lipid transporter activity	-	Lipid transport
226761	B0WP11	*Culex quinquefasciatus*	Asparagine synthetase	asparagine biosynthetic process metabolic process	asparagine synthase (glutamine-hydrolyzing) activity	-	Metabolic process
229368†	F4WIP9	*Acromyrmex echinatior*	INO80 complex subunit E	DNA repair† DNA recombination† transcription, DNA-dependent† regulation of transcription, DNA-dependent† response to DNA damage stimulus†	protein binding†	nucleus† INO80 complex†	Transcription
235586	**E9FUS8**	***Daphnia pulex***	Histone H3	nucleosome assembly	DNA binding	nucleosome	Nucleosome assembly
235631	**E9FUS9**	***Daphnia pulex***	Histone H4	nucleosome assembly	DNA binding	nucleosome	Nucleosome assembly
235802	**E9FUS8**	***Daphnia pulex***	Histone H3	nucleosome assembly	DNA binding	nucleosome	Nucleosome assembly
255862	**E9FUS8**	***Daphnia pulex***	Histone H3	nucleosome assembly	DNA binding	nucleosome	Nucleosome assembly

Appendix Table A4.3 continued.

DAPPU gene no.	UniProt ID	Organism	Description	Gene Ontology terms: Biological Process	Gene Ontology terms: Molecular function	Gene Ontology terms: Cellular component	Process / function
263168	B4K9R3	*Drosophila mojavensis*	GI24314	-	-	-	-
299677	**E9HZI6**	***Daphnia pulex***	Vitellogenin fused with superoxide dismutase	superoxide metabolic process lipid transport oxidation-reduction process	lipid transporter activity metal ion binding	-	Lipid transport / oxidation-reduction process
303836	Q29GT5	*Drosophila pseudoobscura*	GA15557, part of the PP2C family	protein dephosphorylation	catalytic activity phosphoprotein phosphatase activity protein serine/threonine phosphatase activity hydrolase activity metal ion binding	protein serine/threonine phosphatase complex	Protein dephosphorylation
303879	Q29DG0	*Drosophila pseudoobscura*	UPF0389 protein GA21628	-	-	integral to membrane membrane	Integral to membrane
304575‡	E2B862	*Harpegnathos saltator*	U6 snRNA-associated Sm-like protein LSm1	nuclear mRNA splicing, via spliceosome‡ RNA processing‡ cytoplasmic mRNA processing body assembly‡	-	spliceosomal complex‡ small nuclear ribonucleoprotein complex‡	RNA processing

Appendix Table A4.3 continued.

DAPPU gene no.	UniProt ID	Organism	Description	Gene Ontology terms			Process / function
				Biological Process	Molecular function	Cellular component	
304661	Q9U943	*Locusta migratoria*	Apolipophorins	Wnt receptor signaling pathway Lipid transport transport	lipid binding lipid transporter activity	extracellular region	Transport
305707	**E9FXL5**	***Daphnia pulex***	Alpha-carbonic anhydrase	one-carbon metabolic process	carbonate dehydratase activity zinc ion binding lyase activity metal ion binding	-	One-carbon metabolic process
306151	**E9GVT8**	***Daphnia pulex***	Glycolipid-transport protein	-	-	-	-
308303	C1BNJ5	*Caligus rogercresseyi*	Peflin	-	calcium ion binding	-	Calcium ion binding
308693	D4N2J9	*Paracyclopina nana*	Vitellogenin-2	lipid transport	lipid transporter activity		Lipid transport
312260	**E9FUS9**	***Daphnia pulex***	Histone H4	nucleosome assembly	DNA binding	nucleosome	Nucleosome assembly

* DAPPU 100140 (E2BIM6): GO terms inferred from *Drosophila melanogaster* Clp1 (Q7K284)

† DAPPU 229368 (F4WIP9) GO terms inferred from *Homo sapiens* INO80E (Q8NBZ0)

‡ DAPPU 304575 (E2B862) GO terms inferred from *D. melanogaster* LSm1 (Q9W2K2)

Appendix Table A4.4. Gene Ontology (GO) terms for all *Daphnia pulex* genes/gene orthologs with significant decreases in differential expression during the course of development. Gene-product descriptions derive from UniProt. All biological process, molecular function and cellular component GO terms associated with a gene product were obtained from the European bioinformatics institute (EMBL - EBI) QuickGO database. The highest order biological process (or in the absence of a biological process, molecular function or cellular component) was used as a summary term.

DAPPU gene no.	UniProt ID	Organism	Description	Gene Ontology terms			Process / function
				Biological Process	Molecular function	Cellular component	
107198	F4W8S0	*Acromyrmex echinatior*	Septin-4	cell cycle	nucleotide binding GTP binding	septin complex	Cell cycle
112957	B4QMT8	*Drosophila simulans*	GD12468	-	-	-	-
219379	Q9XYN0	*Schistocerca gregaria*	Innexin 1	transport ion transport	ion channel activity gap junction channel activity	plasma membrane gap junction membrane integral to membrane cell junction	Ion-transport
220921	B4GVT5	*Drosophila persimilis*	GL14716	-	-	-	-
227396	E2ARN0	*Camponotus floridanus*	Putative RNA exonuclease NEF-sp		nucleic acid binding exonuclease activity	intracellular	Exonuclease activity
228103	A0ND72	*Anopheles gambiae*	AGAP002973-PA	-	-	-	-
250400	B4M0F5	*Drosophila virilis*	GJ24647	-	-	-	-
299589	B4PHB6	*Drosophila yakuba*	GE21946	-	-	-	-

Appendix Table A4.4 continued

DAPPU gene no.	UniProt ID	Organism	Description	Gene Ontology terms			Process / function
				Biological Process	Molecular function	Cellular component	
304176	Q1HPW4	*Bombyx mori*	Eukaryotic translation initiation factor 3 subunit I	translation translational initiation	translation initiation factor activity	cytoplasm	Translation
305501	E5L878	*Boophilus microplus*	Glutathione S-transferase	metabolic process	glutathione transferase activity transferase activity		Metabolic process
305713	P29981	*Blaberus discoidalis*	Cytochrome P450 4C1	oxidation-reduction process	monooxygenase activity iron ion binding electron carrier activity oxidoreductase activity oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen heme binding metal ion binding aromatase activity	endoplasmic reticulum endoplasmic reticulum membrane microsome membrane	Oxido-reduction process
312710	CG4702	*Drosophila melanogaster*	CG4702	-	-	-	-
327378	Q71DB3	*Drosophila yakuba*	CG9568	-	-	-	-
328621	B3M1V5	*Drosophila ananassae*	GF17870	-	-	-	-

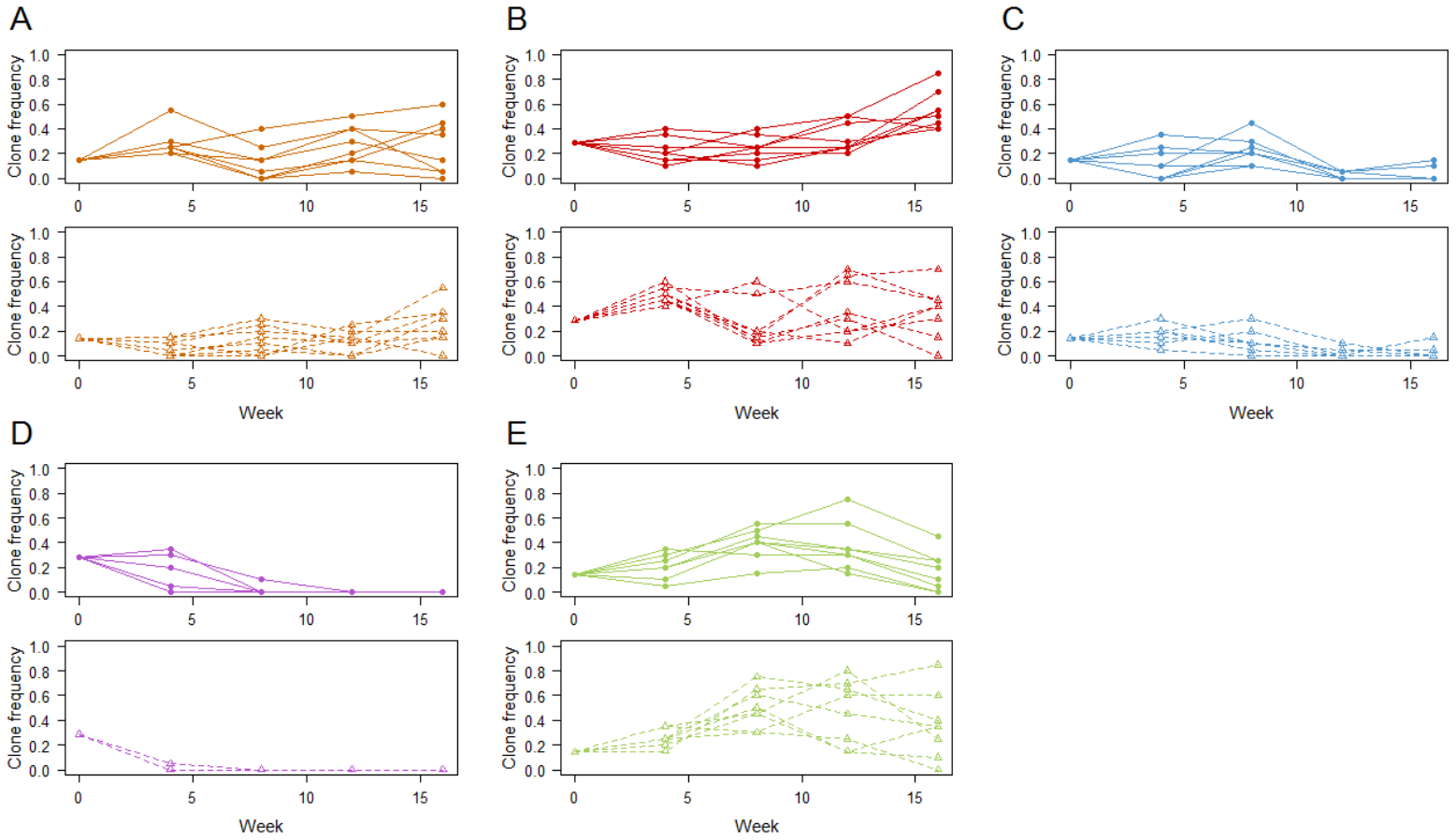

Figure A5.1. Changes in clone frequency for five *D. magna* clones: A) H01; B) DKN1-3; C) B5; D) Ness1 and E) B7. Upper panels (solid lines, closed symbols) correspond to steady treatments, lower panels (dashed lines, open symbols) correspond to perturbed treatments.